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Clonal Analysis of a Human Antibody Response:

II. Sequences of the V_H Genes of Human IgM, IgG, and IgA to Rabies Virus Reveal Preferential Utilization of V_HIII Segments and Somatic Hypermutation1

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Abstract

The construction of mAb-producing cell lines has been instrumental in dissecting the fine specificities and genetic makeup of murine antibodies to exogenous and self Ag. The analysis of the genetic composition of human antibody responses has been hampered by the difficulty in generating human mAb of predetermined class and specificity. Using B lymphocytes from three healthy subjects vaccinated with inactivated rabies virus vaccine, we generated nine human mAb binding to rabies virus and analyzed the genes encoding their V_H regions. Six mAb (five IgG1 and one IgA1) were monoreactive and displayed high affinities for rabies virus Ag. The remaining three mAb (IgM) were polyreactive and displayed lower affinities for rabies virus Ag. Seven mAb (3 IgG1, the IgA1, and the three IgM) utilized V_H gene segments of the V_HIII family. The remaining two IgG1 mAb utilized gene segments of the V_HI and V_HIV families. Of the seven expressed V_HIII family genes, three were similar to the germline V_H26c gene, two to the germline 22-2B gene, one to the germline H11 gene, and one to the germline 8-1B gene. The expressed V_HI and V_HIV genes displayed sequences similar to those of the germline hv1263 and V71-4 genes, respectively. The V_H genes of all but one mAb (mAb55) resembled those that are predominantly expressed by $C\mu^+$ clones in human fetal liver libraries. When compared with known germline sequences, the V_H genes of the rabies virus-binding mAb displayed variable numbers of nucleotide differences. That such differences resulted from a process of somatic hypermutation was formally demonstrated (by analyzing DNA from polymorphonuclear neutrophil of the same subject whose B lymphocytes were used for the mAb generation) in the case of the V_H gene of the high affinity (anti-rabies virus glycoprotein) IgG1 mAb57 that has been shown to efficiently neutralize the virus in vitro and in vivo. The distribution, mainly within the complementarity determining regions, and the high replacement-to-silent ratio of the mutations, were consistent

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with the hypothesis that the mAb57-producing cell clone underwent a process of Ag-driven affinity maturation through clonal selection. The D gene segments of the rabies virus-selected mAb were heterogeneous and, in most cases, flanked by significant N segment additions. The J_H segment utilization was unbalanced and reminiscent of those of the adult and fetus. Four mAb utilized J_H4, two J_H6, two J_H3, and one J_H5; no mAb utilized J_H1 or J_H2 genes. The present data suggest that the adult human Ig V gene assortment expressed as the result of selection by a proteinic mosaic Ag is more restricted than previously assumed and resembles that of the putatively unselected adult B cell repertoire and the unselected $C\mu^+$ cell repertoire of the fetus. They also document somatic Ig V gene hypermutation in human B cells producing high affinity antibodies.

Thorough knowledge of the clonal composition of specific murine antibody responses has been gained through the immunochemical and genetic analyses of mAb generated from animals injected with conjugated haptens, including 2-phenyl oxazolone (1, 2), phosphorylcholine (3–5), arsonate (6, 7), and NP[6] (8–10), or infected with viruses, such as influenza virus (11–14). These studies have been made possible by the systematic application of the somatic cell hybridization technology introduced by Kohler and Milstein (15). Analysis of mAb-producing cell lines generated at different stages of the antibody responses established that: 1) dependent on the nature of the Ag, the dominant B cell clonotypes recruited in the primary response can “mature” throughout the secondary response or can be substituted with newly recruited and different clonotypes (1, 3, 7, 9); and 2) somatic hypermutation of V genes, particularly within the CDR, constitutes a powerful mechanism to finely tune antibody specificity by increasing affinity of the Ag-binding site (1–5, 7–14, 16, 17).

Because of the lack of similar human B cell technology, the cellular and molecular mechanisms underlying the antibody response in mice are merely inferred to be operative in humans. Recent progress, however, in the generation of human mAb-producing cell lines (18, 19) has allowed some insight into the clonal bases of the human antibody responses to self and exogenous Ag (20–29). For instance, we quantitated the circulating B cells committed to the production of antibodies to rabies virus and analyzed their phenotypes in healthy humans before and after vaccination with inactivated virus vaccine (25). Using EBV-transformed human B cells in concert with somatic cell hybridization techniques, we established cell lines secreting IgM, IgG, or IgA mAb to rabies virus, including mAb57, which efficiently neutralizes the virus in vitro and in vivo (25, 30).

In the present studies, we analyzed the V_H genes utilized by these IgM, IgG, and IgA mAb to rabies virus. In addition, we analyzed the configuration with respect to somatic mutations of the gene encoding the V_H segment of the virus-neutralizing IgG1 mAb57 by cloning and sequencing the corresponding germline V_H gene from PMN DNA of the subject used as a source of B cells for the generation of this mAb. The selection of the V_H genes by the low and high affinity mAb to rabies virus reflected that of the early and adult B cell repertoires. The nature and distribution of the somatic mutations in the virus-neutralizing mAb57

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[6]Abbreviations used in this paper: NP, 4-hydroxyl-3-nitrophenyl acetyl; CDR, complementarity determining region; FR, framework region; R:S, replacement to silent mutation ratio; PMN, polymorphonuclear neutrophil; PCR, polymerase chain reaction

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suggests that an Ag-driven affinity maturation process underlies the human high affinity response to exogenous Ag.

Materials and Methods

Generation of mAb-secreting cell lines

PBMC from subjects immunized with inactivated rabies virus vaccine were isolated, depleted of T cells, and infected with EBV (18–21). EBV-transformed B cells were selected for production of IgM, IgA, or IgG to rabies virus Ag by sequential subculturing. Selected EBV-transformed B cell blasts were stabilized by fusion with F3B6 cells, an Ig nonsecretor human-mouse heterohybridoma (18–21).

Cloning and sequencing of expressed Ig V_H genes

mRNA was isolated from the established hybrid cell lines using the Fast Track mRNA isolation kit (Invitrogen, La Jolla, CA) according to the manufacturer's protocol. cDNA was synthesized from 5 μg of mRNA by a modified Gubler-Hoffman method (31). cDNA was complemented with *Not*I-*Eco*RI adaptors (Pharmacia LKB Biotechnology, Uppsala, Sweden) and ligated into the *Eco*RI site of λgt11 phage vector using T4 ligase. A cDNA library was constructed for each mAb-producing cell line (26). Each cDNA library was screened by filter hybridization using ^{32}P-labeled DNA probes to the Ig V_H and C regions (one probe for each V_H gene family and one for each Ig class) of each mAb (26). In each case, after the second or third plating and screening, multiple plaques hybridizing with both the V_H and C region probes were isolated. Each was suspended in 50 μl of distilled water and boiled for 15 min. The boiled phage suspension (10 μl) was subjected to PCR amplification using the forward and the reverse λgt11 primers (New England Biolab, Beverly, MA) and *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT). Twenty-five cycles of amplification were performed. Each cycle consisted of a denaturing, an annealing, and an extension step at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min, respectively. The PCR-amplified material was fractionated on a 1% agarose gel containing 1 μg/ml ethidium bromide, and the DNA band of appropriate size was excised. The DNA fragment was purified, digested with *Eco*RI, and ligated into pUC18 vector, which was used to transform competent DH5α cells. Single colonies were amplified in culture, and the plasmid DNA was purified using Qiagen-Pack columns (Qiagen, Inc., Studio City, CA). Dideoxy sequencing was performed using double stranded plasmid DNA and the *Taq* sequencing kit (Promega, Madison, WI). Each mAb V_H gene sequence was generated from the analysis of multiple independent clones, originally derived from at least three viral plaques. Differences in nucleotide sequences among the recombinant clones generated from the same mAb-producing cell line were observed in few cases (frequency, approximately 0.002/base). Such variants were excluded from the sequence analysis.

PCR amplification of genomic V_H segments from PMN DNA and hybridoma DNA

Genomic DNA was extracted from peripheral blood PMN isolated from the subject used as the source of B cells for the generation of the mAb57-producing cell line. Genomic DNA was also extracted from the hybridoma cell line producing mAb57. DNA (100 ng) was supplemented with the appropriate 5′ and 3′ primers (10 pmol each). PCR was performed in

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a 50-μl reaction volume using *Taq* DNA polymerase under denaturing, annealing, and extension conditions similar to those described above, but for 30 cycles. The oligonucleotide primers used were as follows: 1) HI-6 encompassing a leader sequence (5′ CTGGAGGTTCCTCTTTGTGGT 3′) (residues −47 to −27 of cDNA) shared by the expressed mAb57 V_H gene and its closest published germline V_H gene, hv1263 (see *Results*); 2) HI-7 consisting of a sequence [5′ GCCGTGTCATCAGATCTCAGG 3′] complementary to the FR3 (residues 255 to 275) of the expressed mAb57 V_H gene differing by a single base (A instead of C at position 9 of HI-7) from the hv1263 complementary sequence; and 3) 57CR1 consisting of a sequence [5′ CAACAGGTATACTGTCAACTG 3′] encompassing the 3′ end of FR1, the whole CDR1, and the 5′ region of FR2 (residues 87 to 107) of the expressed mAb57 V_H gene. This sequence differs in five bases from that of hv1263. No sequence identical or highly homologous to 57CR1 was found in a database search. Precautions against cross-contamination of amplified material were taken according to the recommendations by Kwok et al. (32). To analyze the amplified V_H DNA, the PCR products were fractionated on a 1.2% agarose gel containing 1 μg/ml of ethidium bromide. DNA was transferred to a filter membrane (Gene Screen, New England Nuclear Research Products, Boston, MA) and hybridized at 48°C, under the conditions recommended by manufacturer, with the 57CR1 oligonucleotide probe previously ^{32}P-labeled using polynucleotide kinase (Promega, Madison, WI). After hybridization, the filter was washed twice with 2× SSC/0.5% SDS at room temperature for 30 min and twice with 1× SSC/0.5% SDS at 52°C for 30 min. The membrane was then exposed on Kodak XAR film (Eastman Kodak, Rochester, NY).

Cloning and sequencing of the germline V_H segment that gave rise to the expressed mAb57 V_H gene

The PCR-amplified material from PMN DNA using the HI-6 and HI-7 primers was used for cloning of the germline V_H gene segment that putatively gave rise to the expressed mAb57 V_H gene. The PCR-amplified DNA fragment was ligated into the pCR1000 plasmid vector (Invitrogen), and the recombinant vector used to transform competent INVαF′ cells (Invitrogen) according to the manufacturer's protocol. Transformed cells were plated on Luria-Bertani agar containing 50 μg/ml kanamycin. The recombinant clones were selected according to the length of insert and sequenced as described above.

Analysis of DNA sequence data

DNA sequencing data were analyzed using the software package of the Genetics Computer Group of the University of Wisconsin, Release 6, and a model 6000-410 VAX computer (Digital Equipment Corp., Marlboro, MA). Homology searches of the expressed V_H genes were performed using the GenBank database and the sequences published by Berman et al. (33).

Statistical analysis of V_H gene utilization

The frequencies of V_H family gene utilization were analyzed using the exact binomial distribution test. The complexity of each germline gene family was defined as reported in Table II.

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Results

Generation of rabies virus-binding mAb

Table I shows the nine mAb-producing cell lines, three IgM, five IgG1, and an IgA1 we generated from three unrelated healthy subjects immunized with inactivated rabies vaccine. The source, H chain isotypes, L chain types, and Ag-binding properties of the five mAb from subjects B and the three mAb from subject C have been reported previously (25). The IgG1 mAb107-producing cell line was generated for the purpose of these investigations using B cells from subject D and purified rabies virus glycoprotein as a selecting Ag. All five IgG1 (mAb53, mAb56, mAb57, mAb58, and mAb107) and the IgA1 (mAb105) mAb were monoreactive and displayed high affinities for rabies virus components (dissociation constant, 5.0×10^{-9} to 1.1×10^{-10} g/μl). The three IgM mAb (mAb52, mAb59, and mAb55) were polyreactive and displayed lower affinities for rabies virus Ag (dissociation constant, 1.0 to 1.2×10^{-6}g/μl).

V_H segments of the rabies virus-binding mAb

Figure 1 shows the nucleotide and deduced amino acid sequences of the V_H segments of the nine mAb to rabies virus. The differences in nucleotide and predicted amino acid sequences when compared with the closest known germline V_H genes are summarized in Table I. The mAb59 (IgM), mAb53 (IgG1), and mAb107 (IgG1) V_H gene sequences displayed the highest degree of similarity (94.2 to 98%) to the germline V_H26c gene (34), which is identical to the 30pl V_H gene expressed in fetal liver (35). The nucleotide differences were mainly in the CDR, and many of them resulted in amino acid differences. The mAb52 (IgM) and mAb105 (IgA1) V_H gene sequences displayed the highest degree of identity with the germline 22-2B gene (33). Nucleotide differences between mAb52 V_H and 22-2B genes were mostly in the leader, the FR1, the CDR1, and the first part of FR2 (19 of 23). No amino acid differences were detected in the second half of FR2, the CDR2, or the FR3 (Fig. 1). In contrast, the nucleotide differences between the mAb105 V_H and 22-2B genes were concentrated within the CDR2 and the FR3 (11 of 14). The three nucleotide differences in the FR1 and FR2 resulted in only one amino acid difference. Thus, the V_H nucleotide and amino acid sequences of mAb52 and mAb105 were virtually identical to the 22-2B gene in their second and first halves, respectively. The mAb55 (IgM) V_H gene sequence was 96.9 and 98.4% identical to the germline H11 (36) and 52G25 (H. Ikematsu, et al., manuscript in preparation) genes, respectively. The deduced amino acid sequence of the mAb55 V_H region showed only two differences compared with that of the 52G25 gene. The mAb56 (IgG1) V_H gene sequence displayed the highest degree of similarity to the germline 8-1B gene (33), the sequence of which is 98% identical with the 60p2 V_H segment, a gene expressed in fetal liver (35). A variation in the deduced amino acid sequence of 60p2 from that of 8-1B was shared by the mAb56 V_H deduced amino acid sequence, and only three differences were found between the mAb56 and 60p2 deduced amino acid sequences. The mAb58 (IgG1) V_H gene sequence displayed the highest degree of identity when compared with the germline gene V71-4, a member of the V_HIV family (37). The germline gene V71-4 is virtually identical (only two base differences) with the expressed 58p2 segment found in fetal liver (35). The degree of similarity between the mAb58 V_H gene sequence and that of V71-4 was relatively low (92.1%). The nucleotide and amino acid differences were distributed

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throughout the V_H segment. mAb57 is an IgG1 with a high affinity for rabies virus glycoprotein and virus-neutralizing activity in vivo and in vitro (25, 30). The mAb57 V_H gene sequence was 94.6% identical with the germline hv1263 gene (38). The hv1263 gene is more than 94% identical with the "fetal" 51p1 (35). Comparison of the deduced amino acid sequences of hv1263 and mAb57 V_H genes revealed that the vast majority of the differences were concentrated within the CDR.

D segments of the rabies virus-binding mAb

The sequences of the expressed D segments were compared with those of the published germline D and DIR segments (39–43) using the "FASTA" program (44). Some sequence similarities between the expressed and germline D genes were found in most mAb (Table I). The structure of the D segments of mAb52, mAb53, mAb57, mAb105, and, perhaps, mAb56 and mAb107 may be explained by the conventional V_H-D-J_H rearrangement mechanisms complemented by N segment additions (Fig. 2*A*). The D segments of mAb59 and mAb55 were relatively long (55 and 66 nucleotides, respectively), although the stretches of identity to D21-9 and DXP'1 accounted for only 33 and 11% of their length, respectively (Fig. 2*A*). The remaining portions of these expressed D genes could not be accounted for by any known germline D segment sequences. The mAb58 D segment displayed a stretch of similarity to the complementary sequence of DXP4 and may have, therefore, resulted from an inverted D joining (45).

J_H segments of the rabies virus-binding mAb

The expressed J_H gene sequences were compared with those of the available human germline J_H segments (Fig. 2, *A* and *B*). All four expressed J_H4 segment sequences displayed an identical nucleotide variation from the germline J_H4 gene sequence originally reported by Ravetch et al. (39). This variation has been previously reported in other expressed Ig genes (26, 35, 46) and is consistent with the prototypic J_H4 sequence proposed by Yamada et al. (47). Two mAb, mAb52 and mAb55, utilized J_H6 genes containing an identical variation from germline J_H6 gene originally reported by Ravetch et al. (39). As in the case of the J_H4 gene sequence, these two sequences agreed with the prototypic J_H6 sequences reported by Yamada et al. (47). Finally, two antibodies, mAb58 and mAb107, utilized J_H3 genes and mAb57 a J_H5 gene.

Configuration of the mAb CDR3 regions

The predicted amino acid sequences of the D-J_H segments of the nine mAb are depicted in Figure 2*B*. Each sequence is divided into CDR3 and FR4 stretches according to the method of Kabat et al. (48). The expressed CDR3 sequences were highly divergent and of highly variable lengths, ranging from 8 to 30 amino acids. The expressed FR4 sequences were invariable in length and displayed little diversity.

Somatic mutations in the expressed mAb57 V_H gene

Because of the high Ag-binding affinity of mAb57 and of the distribution of nucleotide differences when compared with the hv1263 gene, we hypothesized that this expressed V_H gene consisted of a somatically mutated form of the hv1263 or a hv1263-like gene. PCR

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amplifications were performed using selected oligonucleotide primers and the genomic DNA from autologous PMN or DNA from the mAb57-producing hybridoma cells. The sense primer, corresponding to the CDR1 of the mAb57 (57CR1), and differing in five nucleotides from the hv1263 gene, was used in conjunction with the antisense HI-7 primer, encompassing a stretch of FR3 sequence identical in the expressed mAb57 and the genomic hv1263 V_H gene. The two combined primers amplified DNA from the hybridoma, but not from autologous PMN. The molecular size of the amplified product was consistent with that of the sequence spanning residues 87 to 275 (CDR1-FR3 portion) of the V_H gene (Fig. 3*A*). This suggested that the expressed V_H gene was somatically mutated. Utilization of the same antisense primer (HI-7) and the sense HI-6 primer encompassing a stretch of leader sequence, which was identical in the expressed mAb57 V_H gene and in the hv1263 and related germline genes, resulted in V_H gene amplification using DNA from both PMN and hybridoma cells. The amplified products were ~400 bp in size and consistent with the number of residues intervening between the HI-6 and HI-7 sequences in the mAb57 V_H gene (Fig. 3*B*). This demonstrated that the failure to amplify any DNA from autologous PMN DNA in the first experiment was not due to flaws inherent to the DNA preparation. Southern blot analysis showed that the ~400-bp amplification product from the hybridoma, but not PMN, DNA hybridized with the ^{32}P-labeled 57CR1 oligonucleotide probe (Fig. 3*C*). Thus, these experiments suggested that the expressed mAb57 V_H gene constitutes a somatically mutated form of a germline hv1263-like gene.

To analyze the autologous germline V_H gene that putatively gave rise to the expressed mAb57 V_H gene, the ~400-bp DNA amplified from PMN DNA (using the HI-6 and the HI-7 primers) was cloned. Twelve independent clones were sequenced: 1) Two clones contained an identical V_H gene, termed 57GTA8. Throughout the overlapping area, the sequence of the 57GTA8 gene displayed 96.5%, about 99%, and absolute identity with those of the mAb57 V_H gene, the germline hv1263 gene (Fig. 1*A*), and the 51p1 V_H gene expressed in fetal liver, respectively. 2) Three clones contained V_H genes whose sequences (data not shown) differed in only 1 or 2 bases from that of the 57GTA8 gene and displayed less than 96% identity with that of the mAb57 V_H gene. 3) The remaining seven clones contained V_H genes whose sequences (data not shown) differed from one other and the first five and displayed less than 93% identity with that of the mAb57 V_H gene. Throughout the overlapping area, the 57GTA8 gene sequence shared 5 of the 14 the nucleotide differences found between the sequences of the mAb57 V_H and hv1263 genes, supporting the hypothesis that 57GTA8 is the germline gene that gave rise to the mutated mAb57 V_H gene. Of the 9 base differences displayed by this gene when compared with the germline 57GTA8 gene, six were in the CDR1 and CDR2 and one was immediately adjacent to the CDR1 (Fig. 1*A*). These seven nucleotide differences resulted in six amino acid replacements (Fig. 1*B*), yielding a R:S ratio of 6:1. The two nucleotide changes in the FR3 resulted in one amino acid replacement, yielding a R:S ratio of 1:1. The new polarity and charge conferred to the mutated V_H region compared with those of the deduced amino acid sequence of the corresponding germline V_H gene are summarized in Table III.

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Discussion

We analyzed the genes encoding the V_H regions of nine human mAb we generated, by selection for binding to rabies virus Ag, using B cells from three healthy subjects who had been immunized with inactivated rabies virus vaccine. We found the following: 1) Seven mAb utilized genes of the V_HIII family. In particular genes similar or identical to two members of this family, V_H26c and 22-2B, were used five times. 2) Most of the V_H genes utilized by both the low and high affinity mAb were similar to those found to be predominantly expressed in fetal liver and in the putatively unselected adult human B cell repertoire. 3) By isolating and sequencing of the autologous germline segment that gave rise to the expressed gene, we formally proved that the gene encoding the V_H region of the high affinity virus-neutralizing mAb57 displayed a number of somatic point mutations distributed in a fashion and with features characteristic of those resulting from a process of Ag-driven affinity maturation through somatic hypermutation and selection.

In the mouse, the V_H genes of families proximal to D and J_H loci are preferentially rearranged at early stages of life (49–53). The simplest interpretation of this phenomenon is that the recombinatorial machinery “tracks” upstream from the D-J_H complex to recruit V_H segments (49). Later in the life of the animal, V_H gene expression is thought to normalize, that is, V_H genes of all different families are expressed at frequencies proportional to the complexity of each family and its representation in the haploid genome (stochastic expression) (54–56). Hypothesized mechanisms for the normalization of V_H gene usage in adult animals include programmed changes in the generation of the primary repertoire and selection forces that operate subsequent to the generation of B cells in the primary lymphoid organs.

In humans, preferential expression of V_HV and V_HVI genes, located most proximal to the D and J_H loci on chromosome 14, have been reported at a very early stage of fetal life (day 50) (57). However, later in gestation (days 104 and 130), the V_HVI gene is expressed by only 8% of the total human liver $C\mu^+$ cDNA clones (35, 46), and instead, genes of the V_HIII family are predominantly expressed (58% of all $C\mu^+$ cDNA clones) (Table II), in particular, only three to six of a total of about 30 members (35, 46, 58). These include 30pl, 60p2, and FL13-45, the equivalents of the germline V_H26c, 8-1B, and 22-2B genes, respectively. A very limited number of V_H genes belonging to families other than V_HIII are also expressed recurrently in the fetal liver, including 51pl and 58p2, the equivalents of the germline hv 1263 and V71-4 genes, respectively (35, 46). Although without knowledge of the complete repertoire of V_H genes the identification of the germline origin V_H cDNA sequences is still tentative, it is intriguing that among the nine rabies virus-selected mAb, 30pl-like genes were used three times (mAb53, mAb59, and mAb107), FL13-45-like genes were used twice (mAb52 and mAb105), and 60p2-, 58p2-, and 51pl-like genes were each used once (mAb56, mAb58, and mAb57, respectively) (Table I). Thus, a high frequency of expression of V_HIII family genes at large and/or selected V_HIII genes may be a general feature of not only the fetal but also the adult B cell repertoire. This speculation would be further supported by the significant overutilization of these V_H genes by: 1) the putatively unselected B PBL (59–61) (Table II); 2) the antibodies produced in response to *Haemophilus influenzae* type b of polysaccharide, an Ag different in nature from those on rabies virus (63) (Table II); 3) a

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large sample of leukemic B cells (59, 64) (Table II); and 4) autoantibodies to various self Ag generated in healthy subjects and autoimmune patients (65–71) (Kasaian et al. and Ikematsu et al., manuscripts in preparation).

High frequency of expression of V_HIII genes throughout ontogeny may reflect a pivotal role of this V_H family in phylogeny, at least in vertebrates (72). Both birds and rabbits have multiple V_H genes, but all are V_HIII-like. V_HIII-like elements might have been positively selected during phylogeny in response to Ag shared by common pathogens. The present experiments corroborate this view and suggest that the "functional" human V_H gene repertoire is smaller than assumed previously. As hypothesized by Chen (73), it is plausible that at least some of the most frequently expressed V_H genes display recognition signal-like sequences that make them inherently more recruitable for rearrangement. The mechanisms of such putatively inherent high susceptibility to rearrangement are now under investigation in our laboratory.

Positive selection of cell clones displaying somatic point mutations that yield a net increase in binding strength of the surface receptor for Ag has been shown to be the primary mechanism underlying the affinity maturation process in the course of a specific antibody response. Somatic point mutations appear in antibody V_H and V_L segments during the late stages of the primary response and accumulate at higher frequency throughout the secondary response (1–5, 7–17). They distribute preferentially within the CDR of the V_H and/or V_L chains, inasmuch as these segments play a primary role in Ag capture. For example, the antibodies predominant in the secondary response to the hapten NP shared identical somatic mutations in the V_H CDR1, namely a W instead of an L in position 33. As shown by site-directed mutagenesis experiments, such W is crucial in NP binding (74). Information concerning the somatically mutated status of Ig V genes in mice of a given inbred strain can be readily derived from comparison of the expressed V gene sequences with the corresponding germline V gene sequences characteristic of the strain. Because of the outbreeding of the human population and the high degree of V gene polymorphism, the formal assessment of the somatically mutated status of an expressed V_H segment requires the identification of the germline gene that gave rise to it. We used this approach to determine that the rabies virus-neutralizing mAb57 V_H gene is significantly point-mutated. Consistent with their Ag-dependent selection (1, 4, 9, 10, 11, 16, 17, 75, 76), the somatic point mutations within coding regions: 1) were highly concentrated in the CDR or immediately adjacent residues (seven of nine) (Fig. 1, *A* and *B*); and 2) displayed a characteristically high R:S mutation ratio. This resulted in the acquisition of a more positive charge by the mAb57 V_H segment when compared with the deduced amino acid sequence of the corresponding germline V_H gene (Table III). A thorough evaluation, however, of the contribution of these changes to the overall properties of the mAb57 Ag-binding site must take into account also the configuration of the V_L segment. The mAb57 V_H CDR and FR R:S mutation ratio values (6:1 and 1:1, respectively) are comparable to those of the V genes of high affinity murine antibodies and autoantibodies (1, 4, 16, 71, 75, 76), and are significantly higher and lower, respectively, than the theoretical R:S value, ~2.9, calculated for somatic mutations occurring randomly in a gene encoding a protein that need not be preserved in structure (77). Whereas high CDR R:S mutation ratios reflect the positive

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selective pressures applied by Ag on the gene products that come in close contact with Ag, low FR R:S mutation ratios reflect the negative pressure for mutant selection applied to structural components that need to be conserved.

It seems likely that, similar to the mAb57 V_H gene, the genes encoding the V_H regions of the monoreactive high affinity mAb53 and mAb107 constitute somatically mutated forms of V_H26c or V_H26c-like genes, although the possibility that they represent the expression of yet-to-be-characterized germline genes cannot be ruled out. A similar consideration applies to the V_H segment of the monoreactive high affinity mAb56 to rabies virus ribonucleoprotein. This segment likely represents a somatically mutated form of the 60p2 gene, the "fetal" expressed homologue to the germline 8-1B gene, as suggested by the distribution within the CDR of two of the three differences displayed by the mAb56 V_H gene when compared with 60p2. Finally, considering the very limited polymorphism of the V_HIV family members (70), the extensive differences between the mAb58 V_H gene and the germline V71-4 segment suggest that the mAb58 V_H segment resulted from the expression of a still-uncharacterized V_HIV gene.

As for the V_H segments of two of the rabies virus-binding polyreactive IgM, mAb59 and mAb55, they may be encoded in (still unidentified) germline V_H26c- and H11-like genes, respectively, or in somatically mutated V_H genes, as a result of selection by Ag, possibly other than those borne on rabies virus. The latter hypothesis would imply that, in an originally polyreactive antibody, positive selection of somatic mutations by a given Ag is not necessarily associated with loss of the antibody ability to bind different and multiple Ag. It would also support the speculation that, in a polyreactive antibody, different Fab structures can mediate the binding to different Ag. As recently suggested by Foote and Milstein (78), a precise evaluation of the contribution of different structures and/or superimposed somatic mutations to the overall Fab site binding strength for a given Ag cannot be based only on the measurement of antibody affinity, which is relevant only at equilibrium, but must include the analysis of the on-rate constant.

The exclusive concentration of the nucleotide differences within the first half of the sequence (nucleotides 1 through 132) of the V_H gene of the third IgM (mAb52) when compared with the 22-2B germline gene, can be consistent with the hypothesis that this expressed gene arose from a recombinatorial event by which the first half of a 22-2B or 22-2B-like gene was substituted with a sequence from a donor germline V_H gene or pseudogene segment. Our preliminary findings suggest that such recombinatorial events (possibly gene conversion) may be frequent during phylogeny and/or ontogeny (H. Ikematsu et al., manuscript in preparation). The clustered nucleotide differences in the second half of the mAb105 V_H gene compared with the germline 22-2B V_H gene sequence suggest that this expressed segment may also represent an example of such a recombinatorial event.

The sequences of the D segments expressed by our panel of mAb were heterogeneous. Their compositions, however, were similar to those of the D gene segments of other human mAb of different classes and specificities, and to those of the D segments expressed by "unselected" adult B PBL (47, 60, 79). The predominant J_H4 and, to a lesser extent, J_H6 gene utilization by the mAb to rabies virus was similar to that of human natural and Ag-

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induced mAb selected in vitro for binding to a variety of self and exogenous Ag (26, 70, 80, 81), as well as to the J_H utilization of the "unselected" adult and fetal B cell repertoires (47, 35, 46, 60, 79). In no case, however, did the rabies virus-selected mAb utilize the J_H-proximal D_HQ52 D gene, which is frequently expressed by $C\mu^+$ cells in fetal life (35, 46). Also, in contrast with the relatively short V_H-D-J_H junction sequences (CDR3) of the fetal B cell repertoire (46), most mAb to rabies virus V_H-D-J_H junction sequences displayed significant N segment additions. This suggests that a developmentally regulated shift in the clonal composition of the human B cell repertoire occurs at some point during ontogeny. Such a shift may be due to the presence of different mechanisms operating in V_H-D-J_H joining and/or terminal segment additions at sequential stages of ontogeny and, perhaps, in different B cell types. Analysis of these mechanisms and underlying enzymatic activities should further our understanding of the developmental dynamics of the human B cell repertoire and, perhaps, lay the basis for targeted immune intervention.

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A.

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                       5'UT           -57    .          Leader        .        .        .
VH26c                gatcaggactgaacagagagaactcaccATGGAGTTTGGGCTGAGCTGGCTTTTTCTTGTGGCTATTTTAAAAGGTGTCCAGTGT  -1
mAb59                                     -61 -------------------------------------------------------------
mAb53                                                   -45 ---------------------------------------------
mAb107                                         -54 ------------------------------------------------------

                .         .         .         .   FR1   .         .         .         .         . CDR1 .
VH26c    GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGCCA 100
mAb59    ----------------------------C-------------------C---------------------------------------A--A--------
mAb53    ------------------------------------------------C--------------------------------T------T--C--------
mAb107   -----------------------------------C-------------------------------------------------------------T--

                .         .  FR2    .         .         .         .  CDR2   .         .         .
VH26c    TGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCG 200
mAb59    ---CT-------------G----------------------------A--------------------TA------T--------------T--------
mAb53    -----------------------------------------------------G---------------A------------------------------
mAb107   ---A------------------------A------------------AG----C---AG-----------------T----------------G------

                .         .         .         .   FR3   .         .         .         .
VH26c    GTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAA 294
mAb59    ------------------------------------------A---------------G-----------------------------------
mAb53    ----------------------------------------------------------------------------------------------
mAb107   --------------------------------T-G----------------T--------------------------T-------------GG

                                          -57    .          Leader        .        .        .
22-2B                                      ATGGAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATTATAAAAGGTGTCCAGTGT  -1
mAb52                                          -48 ------T-----------------------T--G--------------
mAb105                                            -45 ---------------------------------------------

                .         .         .         .   FR1   .         .         .         .         . CDR1 .
22-2B    CAGGTGCAGCTGGTGGAATCTGGGGGAGGCTTGGTCAAGCCTGGAGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTCAGTGACTACTACA 100
mAb52    G----AG----------G-----------------AC--T--------------------------------------------------AGT--TG-A-
mAb105   --A---------------------------------------------------------------G--------------------------------

                .         .  FR2    .         .         .         .  CDR2   .         .         .
22-2B    TGAGCTGGATCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTTTCATACATTAGTAGTAGTGGTAGTACCATATACTACGCAGACTCTGTGAAGGGCCG 200
mAb52    ---A----G----G-----------------T--------------------------------------------------------------------
mAb105   -------------G---------------------------------------------------------G-G---------A-G-------G------

                .         .         .         .   FR3   .         .         .         .
22-2B    ATTCACCATCTCCAGGGACAACGCCAAGAACTCACTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCGAGA 294
mAb52    ---------------A-----------------------------------------------------------T--T---------------
mAb105   -------G----A----------G----------G-------------------------------------------C------------C--

                        5'UT      .  -57    .          Leader        .        .        .
H11                         tgcacacagagaactcaccATGGAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAGTGT  -1
52G25                                         -50 --------T-----------------------------------------
mAb55                                                                   -28 ----------------------------

                .         .         .         .   FR1   .         .         .         .         . CDR1 .
H11      GAGGTGCAGCTGGTGGAGTCCGGGGGAGGCTTAGTTCAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTCAGTAGCTACTGGA 100
52G25    ----------------------------------------------------------------------------------------------------
mAb55    ---------------------------------------------------------------------A----------------------GC------

                .         .  FR2    .         .         .         .  CDR2   .         .         .
H11      TGCACTGGGTCCGCCAAGCTCCAGGGAAGGGGCTGGTGTGGGTCTCACGTATTAATAGTGATGGGAGTAGCACAACGTACGCGGACTCCGTGAAGGGCCG 200
52G25    ---------------------------------------------------------------------------GC-----------------------
mAb55    -------------------------------------------------------G-----------------G-GC-----------------------

                .         .         .         .   FR3   .         .         .         .
H11      ATTCACCATCTCCAGAGACAACGCCAAGAACACGCTGTATCTGCAAATGAACAGTCTGAGAGCCGAGGACACGGCTGTGTATTACTGTGCAAGA 294
52G25    ------------------------------------------------------------------------------T-- 281
mAb55    ----------------------------------T--------------------------------------------------------GTG

                        5'UT             -57    .          Leader        .        .        .
8-1B                                      cATGGAGTTTGCGCTGAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAGTGT  -1
mAb56         -85 gatcagcactgaacacagaggactcac----------TG-----------------------------------------G----

                .         .         .         .   FR1   .         .         .         .         . CDR1 .
8-1B     GAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTCCAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCGTCAGTAGCAACTACA 100
60p2     -----------------------A---------A-------------------------------------------G----------------------
mAb56    --------A--------------A-----G---A-----------TC------------------------------G-------------C--------

                .         .  FR2    .         .         .         .  CDR2   .         .         .
8-1B     TGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGTTATTTATAGCGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCAGATT 200
60P2     -----------------------------------------------------------------------------------------------C----
mAb56    -----------------------------------------------A-G-----------------T---------------------------C----

                .         .         .         .   FR3   .         .         .         .
8-1B     CACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTTCAAATGAACAGCCTGAGAGCCGAGGACACGGCTGTGTATTACTGTGCGAGA 291
60p2     ------------------------------------------------------------------------C------------------
mAb56    ------------------------------A-----------------------------------------C--A-----------C---
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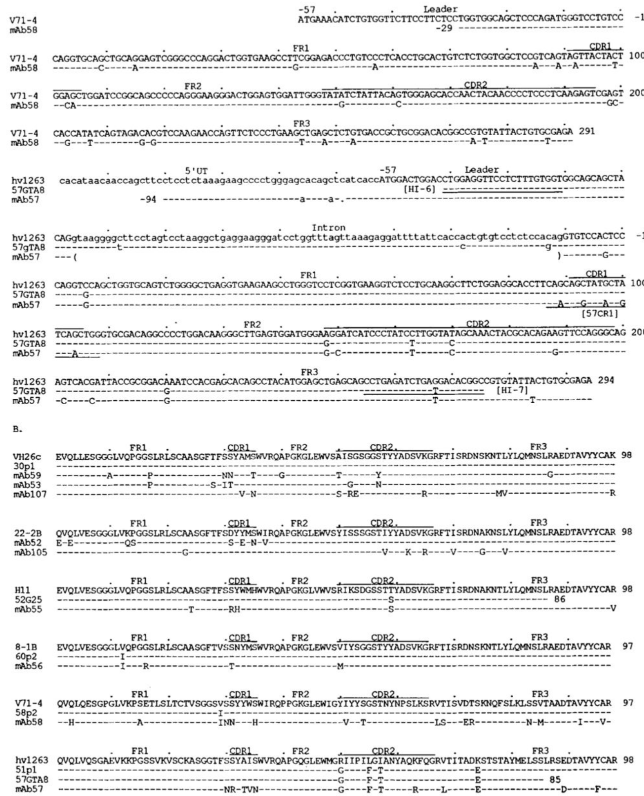

FIGURE 1.

Nucleotide (*A*) and deduced amino acid (*B*) sequences of the V_H genes utilized by the mAb binding to rabies virus. In each cluster, the *top sequence* is given for comparison and represents the published germline V_H gene displaying the highest degree of identity to the expressed V_H genes. The VH26c, 22-2B, H11, and 8-1B V_H genes belong to the V_HIII family. V71-4 and hv1263 are members of the V_HIV and V_HI gene families, respectively. *Dashes* indicate identities. *Solid lines on the top of each cluster* depict CDR. *Small letters* denote 5′ untranslated sequences (UT) and, in the case of hv1263 and 57GTA8, introns. 52G25 is an unpublished germline sequence (see text). 57GTA8 is the germline sequence we amplified from PMN DNA of the subject whose B cells were used for the generation of mAb57. The sequences or complementary sequences of the primers adopted for genomic V_H gene amplification are underlined. 30p1, 60p2, 58p2, and 51p1 are V_H genes expressed in fetal liver (35). Their nucleotide and deduced amino acid sequences are used for comparison. The present sequences are available from EMBL/GenBank/DDBJ under accession numbers L-08082, L-08083, L-08084, L-08085, L-08086, L-08087, L-08088, L-08089 and L-08090.

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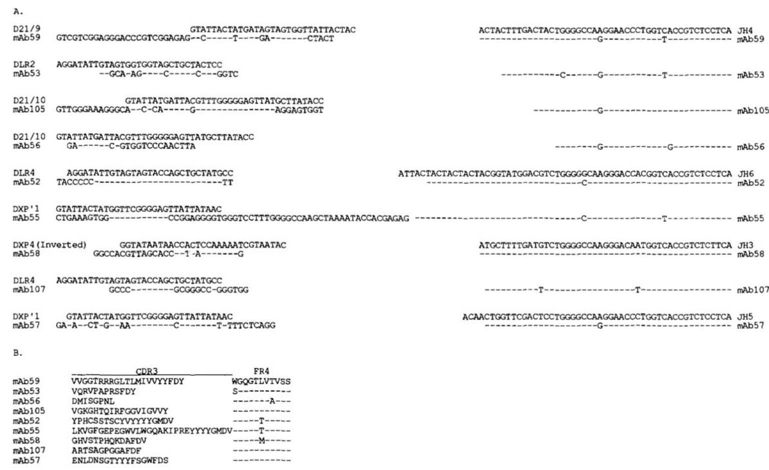

FIGURE 2.

Nucleotide (*A*) and deduced amino acid (*B*) sequences of the D and J_H segments of the mAb binding to rabies virus. Germline D genes are given for comparison. *Dashes* indicate identities. Inverted DXP4 sequence is the reverse strand of the germline DXP4 sequence. The present sequences are available from EMBL/GenBank/DDBJ under accession numbers L-08082 through L-08090.

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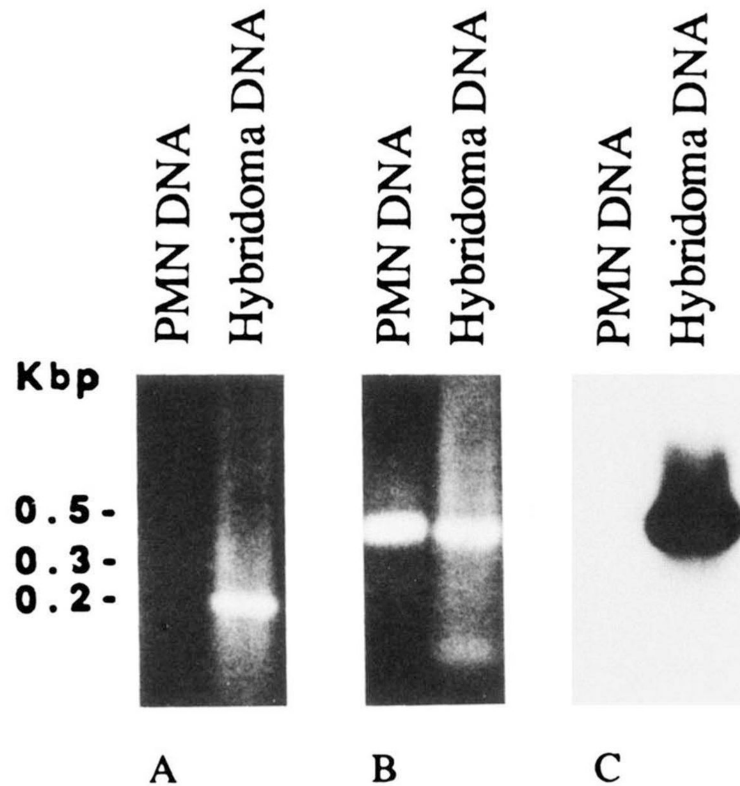

FIGURE 3.

PCR analysis of somatic mutations in the expressed mAb57 V_H gene. *A,* Ethidium bromide staining of amplified DNA fractionated in agarose gel electrophoresis (10 µl of reaction mixture were applied to each lane). Using the CDR1 (57CR1) and FR3 sequence (HI-7) oligonucleotide primers (see *Materials and Methods*), an amplification product of appropriate size (last 3′ portion of the V_H segment, about 200 bp) was obtained by priming DNA from mAb57 B cells (hybridoma DNA) but not the DNA from autologous PMN (PMN DNA). *B,* Ethidium bromide staining of amplified DNA-fractionated in agarose gel electrophoresis (10 µl of reaction mixture were applied to each lane). Using the leader (HI-6) and FR3 (HI-7) sequence oligonucleotide primers (see *Materials and Methods*), amplification products of identical and appropriate size (about 400 bp) were obtained by priming DNA from both mAb57 B cells (hybridoma DNA) and autologous PMN (PMN DNA). *C*, Southern blot hybridization of the PCR products shown in *B* with the ^{32}P-labeled oligonucleotide probe encompassing the CDR1 sequence of the expressed mAb57 V_H gene (see *Materials and Methods*).

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Table I

V_H, D, and J_H gene composition of human mAb to rabies virus

Clone	Sub-ject	H, L Chains	Reac-tivity[a]	Dissociation Constant (g/μl)	V_H Gene Family	Closest V_H Member		% Nucleotide Identity (Amino Acid)[d]	No. of Nucleotide Differences (No. of Amino Acid Differences)							Closest Segment	
						Germline genomic[b]	Fetal expressed[c]		FR1	CDR1	FR2	CDR2	FR3	Total in: CDR	Total in: FR	D[e]	J_H
mAb59	B	μ, λ	Poly [RNP]	1.0×10^{-6}	V_HIII[f]	V_H26c	30p1	95.2 (90.8)	3 (3)	3 (2)	1 (1)	5 (2)	2 (1)	8 (4)	6 (5)	D21/9	J_H4
mAb53	B	γ1, κ	Mono [M]	1.2×10^{-9}	V_HIII	V_H26c	30p1	98.0 (93.9)	3 (3)	1 (1)	0 (0)	2 (2)	0 (0)	3 (3)	3 (3)	DLR2	J_H4
mAb107	D	γ1, λ	Mono [G]	1.0×10^{-9}	V_HIII	V_H26c	30p1	94.2 (90.8)	1 (0)	2 (2)	1 (0)	7 (4)	6 (3)	9 (6)	8 (3)	DLR4	J_H3
mAb52	B	μ, κ	Poly [RNP]	1.7×10^{-6}	V_HIII[f]	22–2B	FL13–45	93.2 (91.8)	7 (4)	7 (3)	3 (1)	0 (0)	3 (0)	7 (3)	13 (5)	DLR4	J_H6
mAb105	C	α1, κ	Mono [RNP]	5.8×10^{-9}	V_HIII[g]	22–2B	FL13–45	95.2 (92.9)	2 (1)	0 (0)	1 (0)	5 (3)	6 (3)	5 (3)	9 (4)	D21/10	J_H4
mAb55	C	μ, κ	Poly [RNP]	1.0×10^{-6}	V_HIII[i]	H11	ND	96.9 (96.9)	1 (1)	2 (2)	0 (0)	4 (1)	4 (1)	6 (3)	5 (2)	DXP'1	J_H6
mAb56	C	γ1[h], λ	Mono [RNP]	6.5×10^{-9}	V_HIII	8–1B	60p2	94.5 (95.9)	7 (2)	1 (1)	0 (0)	3 (1)	5 (0)	4 (2)	12 (2)	D21/10	J_H4
mAb58	B	γ1[h], κ	Mono [G]	5.0×10^{-9}	V_HIV	V71–4	58p2	91.8 (83.5)	6 (4)	4 (2)	0 (0)	2 (2)	12 (8)	6 (4)	18 (12)	DXP4 (inverted)[i]	J_H3
mAb57	B	γ1[h], λ	Mono [G]	1.1×10^{-10}	V_HI	hv1263	51p1	94.6 (86.7)	2 (1)	4 (4)	0 (0)	5 (4)	5 (4)	9 (8)	7 (5)	DXP'1	J_H5

[a]Reactivity: poly, polyreactive; mono, monoreactive; RNP, ribonucleoprotein; M, M protein; G, glycoprotein of rabies virus (25).

[b]The complete sequences of the genomic germlines V_H genes have been reported as follows: VH26c (34), 22–2B, (33), H11 (36), 8–1B (33), V71–4 (37), and hv1263 (38).

[c]For complete sequences of the expressed fetal 30pl, 60p2, 58p2, and 51 pl V_H genes, see Schroeder et al. (35). The expressed fetal FL 13–45 gene has been claimed by Raaphorst et al. (58) to be identical with the genomic 22–2B gene.

[d]Compared with the germline genomic sequences.

[e]D21/9 and D21/10 genes have been described by Buluwela et al. (41), and all other D genes have been reported in Refs. 39, 40, 42, and 43.

[f]Previously reported as V_HIIIb (25). The V_HIIIb denomination has been since abandoned.

[g]Previously reported as V_HVI by Northern blot analysis (25).

[h]mAb 56, 57, and 58 have been previously reported as γ2 by immunochemical analysis (25). The present isotypic attributions are based upon new immunochemical analysis using monoclonal reagents and the nucleotide sequence of the first 5′ portion of Cγ.

[i]Part of the mAb58 D segment consists of the inverted sequence of DXP4.

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Table II

Complexity of the six human V_H gene families and V_H gene expression in the fetal and the adult B cell repertoires, in B cell leukemias, and in the responses to Haemophilus influenzae type b polysaccharide and rabies virus

Origin	V_H Gene Families and Numbers of Members (%)						Total
	V_HI	V_HII	V_HIII	V_HIV	V_HV	V_HVI	
Genomic[a]							
Germline genes	25	5	28	14	3	1	77
Functional and pseudogenes	(32.5)	(6.5)	(36.3)	(18.2)	(3.9)	(1.3)	(100)
Germline genes	13	5	15	14	2	1	50
Functional genes only	(26.0)	(10.0)	(30.0)	(28.0)	(4.0)	(2.0)	(100)
Fetal liver[b]	3*	2	14*	2*	1	2	24
[C μ^+ clones]	(12.5)	(8.3)	(58.3)	(8.3)	(4.2)	(8.3)	(100)
Adult peripheral blood[c]	13***	1**	57***	19	5	2	97
[in vitro Ag nonselected B cells]	(13.4)	(1.0)	(58.8)	(19.6)	(5.1)	(2.1)	(100)
B cell leukemias[d]	16***	7	61*	23	13**	12**	132
	(12.1)	(5.3)	(46.2)	(17.4)	(9.9)	(9.1)	(100)
mAb to *Haemophilus influenzae*	0	0	5**	0	0	0	5
Type b polysaccharide[e]	(0)	(0)	(100)	(0)	(0)	(0)	(100)
mAb to rabies virus[f]	1	0	7**	1	0	0	9
	(11.1)	(0)	(77.8)	(11.1)	(0)	(0)	(100)

[a] The complexity of each V_H family is based on the data by Walter et al. (62) and those reviewed by Pascual and Capra (70). Two different terms of comparison (expected frequencies of expression) are given: (1) total genes (functional and pseudogenes) ($n = 77$); and (2) functional genes only ($n = 50$). The proportion of pseudogenes is tentative and based on the data by Berman et al. (33), and those reviewed by Pascual and Capra (70). Total functional and pseudogenes include those ($n = 76$) reported by Walter et al. (62) plus the functional V_HV gene V_H32 (70). To test whether the frequency of expression of the different V_H genes was stochastic in each sample (e.g., fetal liver C μ^+ clones), statistical analysis (exact binomial distribution test) was performed. In each sample, the distribution of the expressed V_H genes was compared with that expected on the basis of the *total* numbers (functional and pseudo, $n = 77$) (and family complexities) of genomic germline V_H genes;

* $P < 0.05$;

** $P < 0.01$;

*** $P < 0.001$. Comparable p values were calculated when the statistical analysis was performed using the numbers of *functional* ($n = 50$) genes only as expected frequencies of expression.

[b] Frequency of V_H gene expression in fetal liver is based on analysis of C μ^+ cDNA from days 104 and 130 fetuses (35, 46).

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[c] Frequency of V_H gene expression of adult peripheral blood is based on the V_H gene utilization by EBV-transformed B cell lines generated from adult PBMC, in the absence of any *in vitro* selection (59).

[d] Number of B cell leukemia clones is a cumulative value of the data on acute lymphoblastic leukemia and chronic lymphocytic leukemia (59, 64).

[e] Based on the study by Adderson et al. (63).

[f] Present findings.

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Table III

Somatic changes in the mAb57 V_H deduced amino acid sequence when compared with the germline 57CTA8 gene

Region	Nucleotide Mutations			Amino Acid Mutations					
	Total	R	R:S	Residue no.		Germline (57GTA8)			Expressed V_H (mAb57)
FR1	1[a]	1	1:0	30	S	[U][polar, OH][b]	>	N	[U][polar, NH_2]
CDR1	4	4	4:0	31	S	[U][polar, OH]	>	R	[Very basic, NH_2^+]
				33	A	[U][nonpolar]	>	T	[U][polar, OH]
				34	I	[U][nonpolar]	>	V	[U][nonpolar]
				35	S	[U][polar, OH]	>	N	[U][polar, NH_2]
CDR2	2	1	1:1	50	G		>	G	No mutation
				63	K	[Basic, NH_3^+]	>	R	[Very basic, NH_2^+]
Total CDR + FR1	7	6	6:1						
FR3	2	1	1:1	68	V	[U][nonpolar]	>	L	[U][nonpolar]
				69	T		>	T	No mutation

[a] FR1 mutation immediately adjacent to CDR1 (see *Results*).

[b] Amino acid features; U, uncharged; OH or NH_2, polar groups.