Immunoglobulin Heavy Chain Gene Expression in Peripheral Blood B Lymphocytes

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Abstract

cDNA libraries for IgM heavy chain variable regions were prepared from unmanipulated peripheral blood lymphocytes of two healthy people. Partial sequencing of 103 clones revealed V_H gene family use and complete CDR3 and J_H sequences. The libraries differed in the two subjects. In one person's cDNA the V_H5 family was overexpressed and the V_H3 family underexpressed relative to genomic complexity. In the second person's cDNA, V_H3 was most frequently expressed. In both libraries, J_H4 was most frequent. V_H segments of several clones were closely related to those in fetal repertoires. However, there was also evidence of mutation in many cDNAs. Three clones differed from the single nonpolymorphic V_H6 germline gene by 7–13 bases. Clones with several differences from V_H5 germline gene V_H251 were identified. CDR3 segments were highly diverse. J_H portions of several CDR3's differed from germline J_H sequences. 44% of the clones had D_H genes related to the D_{LR} and D_{XP} families, most with differences from germline sequences. In 11 D_{LR2}-related sequences, several base substitutions could not be accounted for by polymorphism. Thus, circulating IgM-producing B cell populations include selected clones, some of which are encoded by variable region gene segments that have mutated from the germline form. (*J. Clin. Invest.* 1992. 89:1331–1343.) Key words: antibody • B cell • cDNA library • diversity • repertoire

Introduction

The extensive diversity of antibody variable regions is due in large measure to the division of germline coding regions into segments, e.g., the V_H, D_H, and J_H segments which together encode the heavy chain variable region (1, 2). Random combinations of the V gene segments give the immune system a vast potential repertoire. In the mouse, for example, the potential repertoire exceeds 10^9, and perhaps 10^{10}, different antigen binding sites (3). But because there are only 10^8 B cells in a mouse, only certain elements of the potential repertoire are represented at any given time in the actual repertoire of the animal. Our understanding of how B cells use the tremendous capacity of the potential repertoire to generate the actual repertoire is limited.

Results of previous studies suggest that the actual, or *expressed,* immunoglobulin repertoire is not simply a random representation of the germline V gene potential. Nonrandom V_H gene utilization is especially marked in the early stages of fetal development in both mice and humans (4–10), in malignant B cells (11, 12), in $CD5^+$ B cells (13, 14), and in autoantibody-forming B cells (15, 16). However, the lack of information about the B cell repertoire in normal adults makes it difficult to assess the significance of the restricted use of immunoglobulin V genes during development and in disease. It is not known, for example, whether the preferential expression of V_H5 and V_H6 gene families early in ontogeny (4) is a peculiarity of fetal B cells, or whether the B cell repertoire of normal adults can also manifest such a bias.

Until recently, investigations of the human B cell repertoire were, for technical reasons, confined to EBV-transformed B cell clones, neoplastic B cells, and a relatively small number of hybridoma-produced monoclonal antibodies (17, 18). In situ hybridization with V_H gene probes can greatly increase the number of B cells that can be surveyed (19, 20), and polymerase chain reaction (PCR)[1]-based analyses have increased the number even more (21–26). Nevertheless, all these methods introduce their own bias. For example, neither the mitogen-stimulated B cells used for most in situ hybridization studies nor EBV-transformed B cells are representative of the entire population (27). cDNA amplification by PCR has allowed analysis of CDR3 sequences of human immunoglobulin cDNA populations (21, 24, 26), and it has been used in mice to estimate the frequency of rearrangement or expression of members of a given gene family (23, 28). But the lack of universal primers that would enable unbiased amplification of all V gene families has limited the scope of the PCR technique for studies of the expressed immunoglobulin repertoire.

We have recently described a sensitive method for amplifying the variable regions of immunoglobulin cDNAs of all V_H families in a diverse mixture of B cells (29). The cDNA is amplified without using primers from variable region sequences, thus avoiding technical bias in the selection of amplified cDNA populations. The representative sampling allowed by the method permits analysis of immunoglobulin genes expressed by unmanipulated B cells, and gives a "snapshot" of the actual immunoglobulin repertoire at a given time. We report here an analysis of 103 unique clones from IgM libraries obtained by this method from two normal healthy adults. The clones were from cDNA libraries prepared from peripheral blood lymphocytes that were not stimulated in vitro, and whose only manipulation was centrifugation through Ficoll-Hypaque.

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1. *Abbreviation used in this paper:* PCR, polymerase chain reaction.

Methods

Preparation of cDNA Libraries from human peripheral blood lymphocytes. cDNA libraries were prepared from peripheral blood lymphocytes as described previously (29). Lymphocytes were centrifuged through a Ficoll-Hypaque medium and washed with PBS; they were not further manipulated before preparation of RNA. Double-stranded cDNA was synthesized from total cellular RNA according to the method of Gubler and Hoffman (30) and blunt-ended with T4 DNA polymerase. The primer for cDNA synthesis was complementary to a sequence within the Cμ1 region (29). Two steps of PCR amplification were performed, as described previously (29). The first step was primed by oligonucleotide linkers attached to the ends of the double-stranded (ds) cDNA. The products were ligated into M13mp19 RF DNA. A second amplification used a downstream-nested Cμ primer and an upstream primer within the M13 vector DNA. The second PCR products were again ligated to M13 RF DNA. This ligation mixture was transformed into DH5α bacteria to form the cDNA library for screening.

Analysis of the libraries. Libraries were screened for hybridization with a degenerate human J_H gene oligonucleotide probe and V_H family-specific oligonucleotide probes (17). M13 plaques were lifted onto GeneScreen membranes, which were then prehybridized, hybridized, and washed as described (31). Inserts in M13 phage were sequenced by chain termination with dideoxynucleoside triphosphates and Sequenase (US Biochemical Co., Cleveland, OH). For full V region analysis, sequencing was performed with two or three different primers, giving large overlaps that verified sequencing accuracy. Sequences were compared to those in the human Genbank database with the FASTA program of the GCG software package. The BESTFIT, LINEUP, and TRANSLATE programs were used for further sequence analysis.

Table I. V_H Gene Family Usage in μcDNA clones

V_H gene family	Library Aμ (n = 54)	Library Tμ (n = 49)	Germline gene complexity*
	%	%	%
1	28	25	33
2	4	4	11
3	24	49	40
4	15	17	12
5	19	6	4
6	5	0	1
‡	5	0	?

* From Berman et al. (32).
‡ V_H genes with < 78% identity to members of V_H1 to V_H6 families.

Results

Amplified IgM cDNA libraries were prepared from RNA of unstimulated peripheral blood lymphocytes from two healthy adult donors (35 and 36 yr old). In both libraries, > 85% of the plaques hybridized with a degenerate J_H oligonucleotide probe. Sequencing of randomly picked J_H-positive clones began from the 5′-end of the Cμ region and continued through the J_H, CDR3 and at least the FR3. Complete V region sequences were obtained in selected cases. The sequence data allowed assignment of V_H families and full analysis of D_H and J_H gene segments. All clones discussed below had unique CDR3 sequences. All but four of the sequences corresponded to functional rearrangements with open reading frames through the V_H, CDR3, and J_H segments. A total of 103 clones from the two libraries were examined.

Use of V_H gene families in the normal adult repertoire. The 54 randomly picked clones of the first normal subject (Aμ) included more V_H1 than V_H3 family genes—28% vs. 24% (Table I). This result was surprising because the V_H3 gene family has the greatest genomic complexity and was the most frequent family detected in studies of expressed V_H genes from 104- and 130-d fetal liver cells (5, 6), in adult peripheral B cells examined by in situ hybridization (19, 20), and in EBV-transformed B cells (17, 33). The higher frequency of V_H1 than V_H3 family genes in the Aμ cDNA library was confirmed by hybridization to plaque lifts with FR3 specific oligonucleotide probes; with this assay, 35% and 25% of 400 J_H-positive clones were members of the V_H1 and V_H3 families, respectively.

Another notable feature of the Aμ library was that the two-member V_H5 family was highly represented (Table I), occurring in 10 (19%) of the 54 sequenced clones. The high representation of this small family was confirmed in two different IgM libraries prepared from the same RNA sample. Results with the two Aμ preparations are combined in Table I. 3 of the 54 Aμ clones were related to the single germline V_H6 gene. The distribution of V_H gene family usage in the Aμ library was at the borderline of being significantly different from that expected from the genomic complexity of the families (32) ($P \sim 0.05$).

In contrast with the Aμ library, the Tμ library was a closer reflection of the genomic complexity of V_H gene families; V_H3 members were most frequent, and the V_H5 family was not prominent (Table I). Statistically, the distribution of V_H gene usage in the Tμ library was not different than expected from the genomic complexity of the gene families ($P > 0.05$). Only 4 of the 103 IgM sequences in both cDNA libraries could be assigned to the V_H2 family. The frequency of expression of genes of the V_H2 family (~ 4%), which is estimated to contain five genes (34), was confirmed by plaque hybridization with a V_H2-specific FR3 oligonucleotide probe (17) (not shown). Our result is consistent with previous observations (19).

A distinct subgroup or a possible new V_H gene family. The V_H segments of three clones (Aμ4.1, Aμ92.1, and Aμ2.2) differed substantially from any known member of the V_H1 to V_H6 families. These three clones were similar to each other in the V_H segment but each used a different D gene, so they were distinct clones. Their V_H region sequences had 78% overall identity with a known V_H1 gene, 20P3 (5), which was the most closely related gene among reported members of the 6 V_H gene families. Their FR1 and FR2 sequences were, in fact, 93% identical to highly conserved V_H1 gene sequences. However, they had only 67% identity with any known V_H1 sequence in CDR2 and FR3 (Fig. 1). These three V_H sequences had closest overall identity (96%) with that of a previously described autoantibody with dual rheumatoid factor and anti-DNA activity, Ab47, which was considered to be a subgroup of the V_H1 family (18) (Fig. 1). PCR amplification of nonlymphoid genomic DNA was used to test whether related genes were present in the germline or whether these novel sequences may have arisen from a somatic process such as gene conversion (35). A sequence related to the three new clones was indeed found in the nonlymphoid genomic DNA of the donor for the Aμ library (data not shown). One primer for this PCR was in the unique region of the FR3 and the other was in the V_H1-like FR1. This combination of primers amplified a product of appropriate size from genomic DNA. By contrast, a control combination of the

```
                                                                                  1
Aμ2.2                                                   GCAACAGGTGCCCACTCCCAGGTGCAGCTGGT
Aμ4.1                                                   --------------------------------
Aμ92.1                                                  --------------------------------
Ab47                                                                      --------------
20P3                                                    --C-----A-----------------------

Aμ2.2     CCAATCTGGGTCTGAGTTGAAGAAGCCTGGGGCCTCAGTGAAAATTTCCTGCGAGACTTCTTGATACACCTTCACTAGCTA
Aμ4.1     G-----------------------------------------GG-----C--A--G-----G-------------------
Aμ92.1    G-----------------------------------------GG--------A--G-----G-------------------
Ab47      G-----------------------------------------GG--------A--G-----G------------G----C-
20P3      G--G------G-----G-------------------------GG-C------A--G-----G-------------CG----
                                                                                     CDR1

Aμ2.2     TGCTATGAATTGGGTGCGACAGGCCCCTGGACAAGGGCTTGAGTGGATGGGATGGATCAACACCAACACTGGGAGTCCAAC
Aμ4.1     --------------------------------------------------------------------------AC-----
Aμ92.1    --------------------------------------------------------------------------AC-----
Ab47      ------C-----------G-------------------------------------CT----------------AC-----
20P3      CTA----C-C---------------------------------------------------C-T----G---TGGCA---A
            CDR1                                                            CDR2

Aμ2.2     TTATGCCCAGGGCTTCACAGGACGGTTTGTCTTCTCCTTGGACACCTCTGTCAGCACGGCATATCTTCAGATCAGCAGCCT
Aμ4.1     G-----------------------------------------------------------------G--------------
Aμ92.1    G-----------------------------------------------------------------G--------------
Ab47      G-----------------------------------------------------------------A--------------
20P3      C-----A---AAG--TCAG--CA--G-CAC-A-GA--AG------G--CA-------A--C--CA-GG--C-G-----G--
                CDR2

Aμ2.2     AAAGGCTGAGGACACTGCCGTGTATTACTGTGCGAGA
Aμ4.1     -------------------------------------
Aμ92.1    C-----------------------------C----AG
Ab47      ---------A--------------------------G
20P3      G-GAT----C-----G-------------------CG
```

Figure 1. Three novel V_H sequences in the Aμ cDNA library, compared with the sequence of autoantibody Ab47 described previously by Sanz et al. (18) and the germline V_H1 gene 20P3 (5). The Aμ and Tμ sequences in Figs. 1 and 2 have been submitted to Genbank, and have been assigned accession numbers M82889 to M82899.

unique FR3 primer with a V_H3 FR1 primer did not yield an amplification product.

Expressed V_H genes in circulating B cells have mutations in CDR1 and CDR2. For several clones in which partial sequences were very similar to those of known germline V_H genes, sequencing was extended at least through the CDR1. Three clones from the Aμ libraries (Aμ34.2, Aμ46.2, and Aμ51.1) differed from the single, highly conserved germline V_H6 gene by 7, 13, and 7 bases (Fig. 2 *a*). To ensure that these variations were indeed mutations, the germline V_H6 gene of the Aμ donor was cloned and sequenced. It was identical to the published sequence of germline V_H6. In Aμ34.2, five of the seven V_H base substitutions were in CDR2 and 2 were in FR2. The J_H segment had one difference from germline JH5; this change was in the 5′-end, which forms part of CDR3. The D_H segment could not be assigned to a known germline gene.

In Aμ46.2, seven differences from V_H6 were clustered within an 11-base segment in FR3,[2] The other differences were scattered, with two in FR and four in CDR sequences. The CDR3-encoding portion of its J_H gene segment differed by one base from the germline J_H4 sequence. The heavy chain variable region codons of clone Aμ46.2, therefore, contained 12 base substitutions from germline V_H and J_H gene segments; five of those differences were in CDRs. The D segment of this clone differed by three bases from a 17-base portion of a germline D_{N1} gene. The third V_H6-related clone, Aμ51.1, differed by seven bases from the germline V_H6 sequence. One difference was in CDR1, one was in CDR2, and five were in framework regions. The CDR3-encoding region of the J_H segment differed by one base from a germline J_H5 sequence. The D gene segment of Aμ51.1 could not be assigned to a known germline D_H sequence.

Five clones were very closely related to either V_H251 or V_H32, the two functional germline members of the small and minimally polymorphic V_H5 family (Fig. 2, *b* and *c*). Two clones from the Aμ library (Aμ59.1 and Aμ99.1) differed by 15 (Aμ59.1) and 5 (Aμ99.1) positions from V_H251. The substitutions tended to occur in the hypervariable regions; 10 of the 20 substitutions in these two clones were in either the CDR1 or CDR2. The D segment of Aμ59.1 could not be assigned, but that of Aμ99.1 had a six-base sequence identical to part of D_{Q52}. Clone Aμ2.1 in the Aμ library differed at one position (in CDR2) from V_H32, had an unassignable D segment, and had a J_H segment differing by one base in the CDR3-encoding portion from J_H1 (Fig. 2 *c*).

There were fewer differences from V_H5 germline genes among the Tμ library clones. One clone in this library, Tμ16, differed by a single base from V_H251. Its D segment differed by two bases from a 15-base portion of the $D_{XP'1}$ sequence. It had only one "N" base, at the D_HJ junction, and it had an unmutated J_H2 gene. A second Tμ clone, Tμ0, differed from V_H251 at four positions (one in CDR1). Its CDR3 had an 11-base sequence identical to a portion of $D_{XP'1}$, and its J_H6 sequence had one base change, which was in the CDR3-encoding portion of the gene.

cDNA of gene V_H26 (also termed $V_H18/2$ and 30P1 [36]) was represented in two clones selected at random for sequenc-

2. The clustering suggested that these differences may have arisen from a hybridization artifact that can arise when related genes are present (see reference 75). However, the 11-base pair segment involved was not closer to any other V_H family, and the rest of the V_H structure, on both sides of the cluster, had a sequence characteristic of V_H6.

```
                 1
VH6      GGTGTCCTGTCACAGGTACAGCTGCAGCAGTCAGGTCCAGGACTGGTGAAGCCCTCGCAGACCCTCTCACTCACCTGTGCCA
Aμ34.2   ----------------------------------------------------------------------------------
Aμ46.2   -----------------------------------G---------------------------------------------G
Aμ51.1   ---------------------------------------A----------------------------G-------------

VH6      G  V  L  S  Q  V  Q  L  Q  Q  S  G  P  G  L  V  K  P  S  Q  T  L  S  L  T  C  A  I
Aμ34.2   -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
Aμ46.2   -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  V
Aμ51.1   -  -  -  -  -  -  -  -  -  -  -  -  -  R  -  -  -  -  -  -  -  -  -  -  -  -  -  -

VH6      TCTCCGGGGACAGTGTCTCTAGCAACAGTGCTGCTTGGAACTGGATCAGGCAGTCCCCATCGAGAGGCCTTGAGTGGCTGGGAAGGACAT
Aμ34.2   -----------------------------------------------------------C----G-------------------------
Aμ46.2   ----------------------T-------------------------------------------------------------------
Aμ51.1   --------A------------C---------------------------A----------------------------------------

VH6       S  G  D  S  V  S  S  N  S  A  A  W  N  W  I  R  Q  S  P  S  R  G  L  E  W  L  G  R  T  Y
Aμ34.2    -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  P  -  -  -  -  -  -  -  -  -  -
Aμ46.2    -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
Aμ51.1    -  -  -  -  -  -  T  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
                            CDR1                                                         CDR2

VH6      ACTACAGGTCCAAGTGGTATAATGATTATGCAGTATCTGTGAAAAGTCGAATAACCATCAACCCAGACACATCCAAGAACCAGTTCTCCC
Aμ34.2   ------------------T-----G----------------G--G-A-------------------------------------------
Aμ46.2   --C----------------------C-----------------T----------TA--A--TGTT-------------------------
Aμ51.1   -----G------------------------------------------------------------------------------------

VH6       Y  R  S  K  W  Y  N  D  Y  A  V  S  V  K  S  R  I  T  I  N  P  D  T  S  K  N  Q  F  S  L
Aμ34.2    -  -  -  -  -  F  -  G  -  -  -  -  -  E  G  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
Aμ46.2    H  -  -  -  -  -  -  -  -  -  -  -  -  N  -  -  -  I  -  -  V  -  -  -  -  -  -  -  -  -
Aμ51.1    -  G  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
                    CDR2

VH6      TGCAGCTGAACTCTGTGACTCCCGAGGACACGGCTGTGTATTACTGTGCAAGA    N          D            N
Aμ34.2   -----------------------------------------------------              GGGAGAGATGGCTACA      (*)
Aμ46.2   -----------------------------------------------------   GATCCA    TATAGCAtCAagTGG        (DN1)
Aμ51.1   ------------T----------------------------------------         GAGGCGGGGAGGGCCACACAG      (*)
                                                                           CDR3

VH6       Q  L  N  S  V  T  P  E  D  T  A  V  Y  Y  C  A  R
Aμ34.2    -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
Aμ46.2    -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
Aμ51.1    -  -  -  F  -  -  -  -  -  -  -  -  -  -  -  -  -

VH6            JH
Aμ34.2      TcCGACTCC TGGGGCCAgGGAACCCTGGTCACCGTCTCCTCA (JH5)
Aμ46.2    TACgTTGACTcC TGGGGCCAgGGAACCCTGGTCACCGTCTCCTCA (JH4)
Aμ51.1   CTGGTTCGACcCC TGGGGCCtgGGAACCCTGGTCACCGTCTCCTCA (JH5)
           CDR3
```

a

```
          1
VH32      GGAGTCTGTGCCGAAGTGCAGCTGGTGCAGTCCGGAGCAGAGGTGAAAAAGCCCGGGGAGTCTCTGAGGATCTCCTGTAAGG
Aμ2.1     ----------------------------------------------------------------------------------

V32       G  V  C  A  E  V  Q  L  V  Q  S  G  A  E  V  K  K  P  G  E  S  L  R  I  S  C  K  G
Aμ2.1     -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

VH32     GTTCTGGATACAGCTTTACCAGCTACTGGATCAGCTGGGTGCGCCAGATGCCCGGGAAAGGCCTGGAGTGGATGGGGAGGATTGAT
Aμ2.1    --------------------------------------------------------------------------------------

V32       S  G  Y  S  F  T  S  Y  W  I  S  W  V  R  Q  M  P  G  K  G  L  E  W  M  G  R  I  D
Aμ2.1     -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
                         CDR1                                                    CDR2

VH32     CCTAGTGACTCTTATACCAACTACAGCCCGTCCTTCCAAGGCCACGTCACCATCTCAGCTGACAAGTCCATCAGCACTGCCTACCT
Aμ2.1    ----------------------------T---------------------------------------------------------

V32      P  S  D  S  Y  T  N  Y  S  P  S  F  Q  G  H  V  T  I  S  A  D  K  S  I  S  T  A  Y  L
Aμ2.1    -  -  -  -  -  -  -  -  -  L  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
                   CDR2

VH32     GCAGTGGAGCAGCCTGAAGGCCTCGGACACCGCCATGTATTACTGTGCGAGA    N          D         N
Aμ2.1    ----------------------------------------------------  CGGGGCTTCAATGGCCAACTGATTTT  (*)
                                                                     CDR3

V32       Q  W  S  S  L  K  A  S  D  T  A  M  Y  Y  C  A  R
Aμ2.1     -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

                 JH
Aμ2.1    C  TGGGGCCAGGGaACCCTGGTCACCGTCTCCTCA  (JH1)
```

c

Figure 2. The heavy chain variable region cDNA sequences of clones in the Aμ and Tμ libraries containing V_H segments related to (*a*) the single germline V_H6 gene, (*b, opposite page*) the germline V_H5 family gene V_H251, and (*c*) the germline V_H5 family gene V_H32.

```
        1
VH251   GGAGTCTGTGCCGAGGTGCAGCTGGTGCAGTCTGGAGCAGAGGTGAAAAAGCCCGGGGAGTCTCTGAAGATCTCCTGTAAGGGTTCTGGATACAGCTTTACCAG
Aμ59.1            ---------------------------------------------------------G--------------C-----------------G--C
Aμ99.1                                                                                                     ------------C
Tμ16               ---------------------------------------------------------------------------------------------
Tμ0                                                                                                         -----------A

VH251   G V C A E V Q L V Q S G A E V K K P G E S L K I S C K G S G Y S F T S
Aμ59.1          - - - - - - - - - - - - - - - - - - R - - - - A - - - - - S T
Aμ99.1                                                                - - - T
Tμ16            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Tμ0                                                                   - - - N
                                                                          CDR1

VH251   CTACTGGATCGGCTGGGTGCGCCAGATGCCCGGGAAAGGCCTGGAGTGGATGGGGATCATCTATCCTGGTGACTCTGATACCAGATACAGCCCGTCCTTCCAA
Aμ59.1  ----------------T------------------------------AC---------G--C-----C---------------AC------------------
Aμ99.1  -----------C--------------------------------------------------T-------------------------------------G--
Tμ16    -------------------------------------------------------------------------------------------------------
Tμ0     -----------------------------------------C-------G-----------------------------------------------------

VH251   Y W I G W V R Q M P G K G L E W M G I I Y P G D S D T R Y S P S F Q
Aμ59.1  - - - - - L - - - - - - - - - Y - - - V H - R - - - - N - - - - - -
Aμ99.1  - - - A - - - - - - - - - - - - - - - - F - - - - - - - - - - - - E
Tμ16    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Tμ0     - - - - - - - - - - - - - - - - V - - - - - - - - - - - - - - - - -
         CDR1                                     CDR2

VH251   GGCCAGGTCACCATCTCAGCCGACAAGTCCATCAGCACCGCCTACCTGCAGTGGAGCAGCCTGAAGGCCTCGGACACCGCCATGTATTACTGTGCGAGA
Aμ59.1  ------------T-------------------------------------------------------------------------C-T----------
Aμ99.1  -------------------T-------------------------------------------------------------------------------
Tμ16    ----------------------------------------------------------------------------A----------------------
Tμ0     -----C--------------------------------------------------------A-------------A----------------------

VH251   G Q V T I S A D K S I S T A Y L Q W S S L K A S D T A M Y Y C A R
Aμ59.1  - - - - F - - - - - - - - - - - - - - - - - - - - - - - - F - - -
Aμ99.1  - - - - - - V - - - - - - - - - - - - - - - - - - - - - - - - - -
Tμ16    - - - - - - - - - - - - - - - - - - - - - - - - - N - - - - - - -
Tμ0     - H - - - - - - - - - - - - - - - - - - - - - - - N - - - - - - -

VH251        N               D                N
Aμ59.1            CTCGGGTGGCCGGGCCAGGAACAATACC              (*)
Aμ99.1  TTTGCGACTC          CTGGGG                C        (DQ52)
Tμ16                    GGTaCGGGGCaTTAT           G        (DXP'1)
Tμ0     CTTTCTT            TATGGTTCGGG            AGGAC    (DXP'1)
                           CDR3

                             JH
Aμ59.1                  ACTTTGACTgg   TGGGGCCAgGGAACCCTGGTCACCGTCTCCTCA   (JH4)
Aμ99.1          CTACTACTACGGTATACGTC   TGGGGcaAAGGGACCACGGTCACCGTCTCCTCA   (JH6)
Tμ16                  GGTACTTCGATCTC   TGGGGCCGTGGCACCCTGGTCACTGTCTCCTCA   (JH2)
Tμ0     TACTACTaAtTACTACGGTATGGACGTC   TGGGGGCAAGGGACCACGGTCACCGTCTCCTCA   (JH6)
            CDR3
                                                                                  b
```

Figure 2 (Continued)

ing. It was identified by hybridization of membrane lifts with CDR1 and CDR2 oligonucleotide probes. Two clones isolated on this basis differed by one and three bases from the $V_H18/2$ gene (not shown); $V_H18/2$ is a highly conserved gene (36).

Biased use and diversity of the J_H4 gene. The J_H4 gene was overrepresented in the cDNA libraries of both subjects. It occurred in 48% of all sequenced clones (Fig. 3). Use of other J_H genes differed in the two subjects. For example, J_H5 occurred in 24% of the Aμ sequences and 13% of the Tμ sequences. The J_H1 and J_H2 families were present infrequently, an observation also made by others (19, 21).

Considerable diversity was found in the 5′ end of the 49 sequenced J_H4 gene segments (Fig. 4). This region of the gene contributes to the CDR3 of the heavy chain. The diversity arose both from variation in the point of J_H4 joining to the rest of the CDR3 and from nucleotide differences from the germline sequence. Apart from a previously recognized polymorphic G/A variation (37), 15/28 Aμ clones and 11/21 Tμ clones contained substitutions in J_H4, ranging from one to three in number, that cannot be accounted for by polymorphism. Most (81%) of the substitutions were clustered in the CDR3-coding region of the gene and most led to amino acid substitutions (Fig. 4 *b*).

Heterogeneity of D_H regions. The D_H segment sequences in both libraries were highly diverse (Figs. 5–7). Parts of some CDR3 sequences, ranging in length from 6 to 26 bases, were identical to portions of known germline D_H gene segments; such germline sequences were found in 10/54 Aμ clones (18%) and in 16/49 Tμ clones (30%). The D_H region sequences of 12 other Aμ clones (21%) and 9 other Tμ clones (19%), from 15 to 29 bases long, had only one or two differences from germline D_H genes. The remaining 56 clones had more substantial differ-

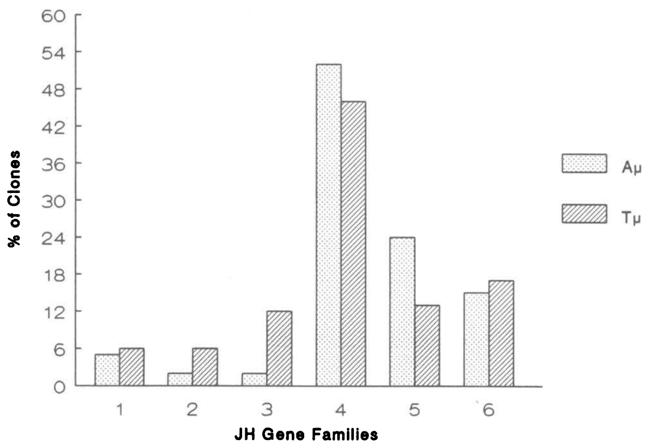

Figure 3. Preferential usage of J_H4 genes in the 54 Aμ and 49 Tμ clones of the cDNA libraries. J_H gene use was assigned on the basis of the complete J_H sequence for each clone.

ences from known germline D_H sequences (Fig. 5 *a*). 21 (Fig. 6) were not assigned to known D_H genes because they had either no identifiable sequence identity or, in some cases, because they had < 75% identity with a known D_H gene.

Direct comparisons between observed and germline sequences were possible with the D_{LR} and D_{XP} families, for which the expected germline members are known (38, 39), and with the single D_{Q52} gene (40). The majority of the expressed members of these families in both Cμ libraries contained differences, ranging from one to five bases, from germline sequences (Fig. 5). All assignable clones used only part of the germline D_H gene segments, and N insertions were observed in all of them (Figs. 5 and 7). The average length of N at the V_H-D_H junctions was 5.7 and at the D_H-J_H junctions was 4.7 bases. Among clones with long N regions, eight CDR3 sequences might be accounted for by D-D or D-DIR fusion (Fig. 7).

D_H gene usage was not random. In the combined libraries, the D_{LR} and D_{XP} gene families were used with high frequency; these two families accounted for 54% of the assignable clones (44% of all clones). Sequences related to the $D_{LR}2$ gene alone were present in 11 clones; sequences related to D_K4 and D_N1 also occurred at high frequency (Fig. 5). The D_{Q52} gene segment, which is overrepresented in human fetal liver (5), was present only once in the Aμ library and twice in the Tμ library.

Discussion

The sampling procedure. We have used a sensitive cDNA/PCR cloning method to examine usage of Ig heavy chain variable region genes in peripheral blood B cells of two normal adult donors. The procedure uses no variable region primers and, therefore, does not itself bias the V gene sampling (29). Moreover, since the lymphocytes were not stimulated in vitro, the results provide an insight into the V gene repertoire of circulating B cells in their native state at the time blood is drawn.

We do not know whether all B cells synthesize enough mRNA for a cell to be scored in this analysis. In humans, many of the circulating human B cells appear to be resting cells. Only 0.1–1% of peripheral blood mononuclear cells synthesize mRNA at levels that can be detected by *in situ* hybridization (19, 41). PCR has a high sensitivity and probably samples a larger population than is detected by in situ hybridization.

The inherent error in PCR-based sequencing. The total number of nucleotides in the fully sequenced J_H4, V_H5, and V_H6 genes was 4,065, among which there were 96 differences from germline sequences, for a rate of ~ 24 bases per 1,000 (4×10^{-4} per nucleotide incorporated in the two PCR steps of 30 cycles each). That frequency of base substitutions is much higher than the error rate of the PCR technique, which is ~ 5×10^{-5} per nucleotide incorporated, both in the reported experience of others (25, 42, 43) and in our own experience. For example, several clones in different libraries from one individual that we studied had identical CDR3 sequences and were probably multiple copies of a single cDNA. The substitution frequency among those sequences was ~ 1/300 bases (2×10^{-5} per nucleotide incorporated), a level at which PCR error could not be distinguished from clonal divergence.

V_H gene family usage. Previous studies of V_H gene family usage by circulating B cells from human adults, carried out by in situ hybridization with V_H family-specific oligonucleotide probes, have drawn different conclusions, perhaps because of variations in technique and in sampling procedures. In the experiments of Guigou et al. (19), there were differences among individuals, but an average pattern of V_H gene family expression by unstimulated cells could be defined. V_H3 family genes were the most frequently expressed, and V_H gene family usage correlated roughly with their genomic complexity. Zouali and Thèze (20) averaged results of protein A–stimulated B cells from eight adults. They observed that the V_H gene families were not represented in a random way; the V_H1 family was under-represented, whereas the V_H3 family was overrepresented relative to genomic complexity.

The results of our study emphasize that there are indeed differences among single samples from different normal individuals, as Guigou et al. (19) found. In the cDNA library of one subject (Aμ), a nonrandom representation was seen, with disproportionate representation of V_H5, and fewer than expected V_H3 gene family members. The Aμ library, which also contained 3 V_H6 members, resembles, in its overall composition, a fetal Cμ library (5, 6). Further study will be required to determine whether that pattern is stable for the donor of the Aμ lymphocytes. It is possible that the nonrandom V_H distribution in this library reflects an unknown, recent immunizing stimulus. V_H gene usage in the cDNA library from the Tμ donor, by contrast, more closely paralleled the genomic complexity of the families; however, that single library does not exclude a continuously changing pattern of V_H gene usage.

The three novel sequences, with FR1 and FR2 sequences characteristic of V_H1 genes and unique CDR2 and FR3 sequences, along with Ab47 (18), may represent a distinct subgroup of the V_H1 family, as suggested by Sanz et al. (18), or a new V_H gene family. A closely related combination of sequences exists in the germline DNA, as shown by PCR amplification. This subset of genes may have arisen from a gene conversion or recombination in evolutionary time rather than as a somatic event. The clones in this group have 78% overall sequence identity with the mouse immunoglobulin gene V_H9.

The normal V_H gene repertoire contains genes used by fetuses and for autoantibodies. Table II summarizes the findings in seven clones (sequenced at least from CDR1 to the end of FR3) with 97% or more identity to members of the set of V_H

a

	CDR3	
JH4	TACTTTGACTAC	TGGGGCCAAGGAACCCTGGTCACCGTCTCCTCA
Aμ4.1	-----------	--------G------------------------
Aμ49.1	--------GG	--------G------------------------
Aμ52.1	----	--------G------------------------
Aμ59.1	----------G	--------G------------------------
Aμ61.1	-------T-	--------G------------------------
Aμ70.1	----T-	--------G------------------------
Aμ90.1	---------C-	--------G----G-------------------
Aμ92.1	---------	--------G------------------------
Aμ94.1	---------	--------G------------------------
Aμ95.1	--G-G----	--------G------------------------
Aμ96.1	-A-------	--------G------------------------
Aμ100.1	-----------	--------G---G--------------------
Aμ2.2	-----	--------G------------------------
Aμ29.2	---------	--------G------------------------
Aμ3.2	--------	--------G------------------------
Aμ31.2	----------	--------G------------------------
Aμ37.2	-C-G--------	--------G------------------------
Aμ39.2	----------	--------G------------------------
Aμ4.2	------------	--------G------------------------
Aμ40.2	-C--------	--------G------------------------
Aμ42.2	----A-------	--------G------------------------
Aμ44.2	--A--A---	--------G------------------------
Aμ45.2	-C--------	--------G------------------------
Aμ46.2	---G------C-	--------G------------------------
Aμ47.2	--------	--------G------------------------
Aμ52.2	-----	--------G------------------------
Aμ6.2	----------GG	--------G------------------------
Aμ8.2	-------	--------G------------------------
Tμ5	-------	--------G------------------------
Tμ10	----------	--------G------------------------
Tμ17	----------	--------G---G--------------------
Tμ19	-----G--T-	--------G------------------------
Tμ20	-------	--------G------------------------
Tμ21	---------	--------G------------------------
Tμ22	--A-GG---	--------G------------------------
Tμ23	-----------	--------G------------------------
Tμ24	----	--------G------------------------
Tμ29	-------	--------G------------------------
Tμ41	---GA-------	--------G------------------------
Tμ42	------------	--------G------------------------
Tμ61	------	--------G-------A----------------
Tμ74	-----	--------G------------------------
Tμ75	--T---------	--------G--G---------------------
Tμ76	--------	--------G---------A--------------
Tμ84	----	--------G------------------------
Tμ87	------CT-	--------G------------------------
Tμ90	--A--------	--------G---T----------------T---
Tμ98	---C--	--------G------------------------
Tμ100	---C---G----	--------G------------------------

b

	CDR3														
JH4	TACTTTGACTAC				TGGGGCCAAGGAACCCTGGTCACCGTCTCCTCA										
	Y	F	D	Y	W	G	Q	G	T	L	V	T	V	S	S
Aμ4.1		-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aμ49.1		-	-	W	-	-	-	-	-	-	-	-	-	-	-
Aμ52.1				-	-	-	-	-	-	-	-	-	-	-	-
Aμ59.1		-	-	W	-	-	-	-	-	-	-	-	-	-	-
Aμ61.1		-	-	F	-	-	-	-	-	-	-	-	-	-	-
Aμ70.1			-	F	-	-	-	-	-	-	-	-	-	-	-
Aμ90.1		-	-	H	-	-	-	-	S	-	-	-	-	-	-
Aμ92.1		-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aμ94.1		-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aμ95.1		L	G	-	-	-	-	-	-	-	-	-	-	-	-
Aμ96.1		Y	-	-	-	-	-	-	-	-	-	-	-	-	-
Aμ100.1		-	-	-	-	-	-	-	A	-	-	-	-	-	-
Aμ2.2				-	-	-	-	-	-	-	-	-	-	-	-
Aμ29.2		-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aμ3.2			-	-	-	-	-	-	-	-	-	-	-	-	-
Aμ31.2		-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aμ37.2	D	V	-	-	-	-	-	-	-	-	-	-	-	-	-
Aμ39.2		-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aμ4.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aμ40.2		L	-	-	-	-	-	-	-	-	-	-	-	-	-
Aμ42.2	-	Y	-	-	-	-	-	-	-	-	-	-	-	-	-
Aμ44.2		L	E	-	-	-	-	-	-	-	-	-	-	-	-
Aμ45.2		L	-	-	-	-	-	-	-	-	-	-	-	-	-
Aμ46.2	-	V	-	S	-	-	-	-	-	-	-	-	-	-	-
Aμ47.2			-	-	-	-	-	-	-	-	-	-	-	-	-
Aμ52.2				-	-	-	-	-	-	-	-	-	-	-	-
Aμ6.2	-	-	-	W	-	-	-	-	-	-	-	-	-	-	-
Aμ8.2			-	-	-	-	-	-	-	-	-	-	-	-	-
Tμ5			-	-	-	-	-	-	-	-	-	-	-	-	-
Tμ10		-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tμ17		-	-	-	-	-	-	-	A	-	-	-	-	-	-
Tμ19		-	G	F	-	-	-	-	-	-	-	-	-	-	-
Tμ20			-	-	-	-	-	-	-	-	-	-	-	-	-
Tμ21		-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tμ22		L	G	-	-	-	-	-	-	-	-	-	-	-	-
Tμ23		-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tμ24				-	-	-	-	-	-	-	-	-	-	-	-
Tμ29			-	-	-	-	-	-	-	-	-	-	-	-	-
Tμ41	-	D	D	-	-	-	-	-	-	-	-	-	-	-	-
Tμ42	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tμ61			-	-	-	-	-	-	-	Q	-	-	-	-	-
Tμ74				-	-	-	-	-	-	-	-	-	-	-	-
Tμ75	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tμ76			-	-	-	-	-	-	-	-	I	-	-	-	-
Tμ84				-	-	-	-	-	-	-	-	-	-	-	-
Tμ87		-	-	L	-	-	-	-	-	-	-	-	-	-	-
Tμ90		I	-	-	-	-	-	-	S	-	-	-	-	-	-
Tμ98			-	H	-	-	-	-	-	-	-	-	-	-	-
Tμ100	-	L	G	-	-	-	-	-	-	-	-	-	-	-	-

Figure 4. (*a*) J_H4-related base sequences in clones of the Aμ and Tμ cDNA libraries. Most differences from the germline J_H4 gene occur in the portion that contributes to CDR3. (*b*) Translated amino acid sequences of J_H4-related segments of cDNA clones. Most of the base substitutions in the CDR3 portion lead to amino acid substitutions.

genes, such as 58P2, that has been a feature of the immunoglobulin V gene repertoire of fetal B cells. Five of the seven V_H genes represented in these clones are known to be used to form autoantibodies such as rheumatoid factor, cold agglutinins, and anti–DNA and anti–cardiolipin antibodies (V_H251, 21/28, FL2-2, V_H6, and V_H32 (37). To those we can add the three genes closely related to Ab47, a rheumatoid factor/anti–DNA antibody. VH genes with one and three base differences from the germline V_H26, used in anti–DNA autoantibodies, were also identified in the Aμ library.

B cells capable of forming such autoantibodies are highly represented among human–human hybridomas (36), EBV-transformed cells (13, 14, 44), and B-cell malignancies (11, 12). Many of them, like those listed in Table II, use V_H genes that are also expressed by fetal B cells, with few or no mutations from the germline V_H, D_H, or J_H components. These results are compatible with the conclusion, drawn from studies of EBV-transformed B cells, that cDNAs associated with IgM autoantibodies are highly represented in the normal B cell repertoire (44). Some such immunoglobulins, encoded by V_H genes with few mutations, may bind to both autoantigens and foreign antigens such as bacterial polysaccharides (45, 46).

Evidence that circulating B cells have undergone selection. Several lines of evidence, when taken together, strongly suggest that many IgM^+ B cells in the circulating blood are not naive, but instead have undergone selection and clonal expansion. Four aspects of our results support that conclusion: overrepresentation of V region gene families, or of individual V region gene segments; somatic mutation of V genes; the high frequency of replacement substitutions compared to silent mutaa

```
           N                            D                              N

                                        D_A1
                                        TGACTACAGTAACTAC
Aμ106.1    GACCCCCCCA                   -------G-CG-----               TTGGAG
Aμ92.1     T                              ------T-G-----               GCC
Aμ37.2     ACGAGTTT                        ----G--G-----               GTTACTTCCG
Tμ17       ACCCGCTACGG                     ----G--G---
Tμ92       ACACCAGAGA                   --G--------                    TT

                                        D_K1
                                        GTGGATATAGTGGCTACGATTAC
Tμ41.2     GATTT                               --C--A------

                                        D_K4
                                        GTGGATACAGCTATGGTTAC
Aμ4.2      TGTAT                         --A-----A----------
Aμ35.2     ATATT                        -A-------------T-              CCC
Aμ51.2     ATATTGA                         ---CA-------T-              CC
Tμ10       CGAGAGATCT                       ---T---G----               GAGCTACATTTT
Tμ12       TAA                             ----------------
Tμ13       GATACTGAG                      ----------------
Tμ56       TGTCCG                       ------                         CATGAT   1
Tμ84                                       -------------               C

                                        D_M1
                                        GGTATAACTGGAACTAC
Aμ29.2     GGCAAGTC                       -----A---                    CTGGTCT  2
Aμ103.1    TTCGGGCC                          -----C---C-               TGGGGT   3
Tμ75       GA                                --------                  TAGACGGATCCTAC

                                        D_M2
                                        GGTATAACCGGAACCAC
Aμ47.2/r   GCCCGCCTAT                            --------              TGCCCCCATC 4
Tμ24       GAGGCGG                             ------T--               GGTA
Tμ59       ACCGGT                       -C----C-------                 TGAT

                                        D_M5
                                        GGTATAACTGGAACAAC
Tμ82       GGTTTAG                        --G---------                 TTTAC

                                        D_N1
                                        GGGTATAGCAGCAGCTGGTAC
Aμ37.1     GAAGGTGG                     -------------------            TCGGAGT
Aμ81.1     GGG                          --------TG-G----AC--           TT
Aμ85.1     TTGGTG                                 -----------          AAAC
Aμ94.1     GCGCCC                          -------C----------
Aμ31.2     TCCCAAATCC                         -------------            CT
Aμ46.2     GATCCA                          -------T--AG---
Tμ20       ATAACG                                -------------         G
Tμ47       CACG                                  -------------         TTCAGT

                                        D_N4
                                        GAGTATAGCAGCTCGTCC
Aμ98.1     GG                           ----C--------                  TCG
Aμ32.2     GTTCCGACCCGAAAAGGCAAACC      -C---------------              AGGAGTCC
Tμ4        GGAGG                                --------

                                        D_IR2
                                        .....AGCCTCCGGAGCCCCCGCAGAGACCC...
Tμ58       A                                   ---------TTA--A-C-T--   TTGGC

                                        D_IR2
                                        ......AGCCCAGCCCCCCACCCAGGAG......
Tμ61       CGAG                                     ---A-T------       TTGAAG
```

Figure 5. (*a* and *b*) Relationship of CDR3 base sequences of clones in the Aμ and Tμ cDNA libraries to known germline D_H genes. Numerically annotated clones: *1,* Tμ56 may be assigned equally well to D_K4, D_K1, or D_K5; *2,* clone Aμ29.2 may be assigned equally well to D_M1 or D_M5; *3,* clone Aμ103.1 may be assigned equally well to D_M1, D_M2, or D_M5; *4,* clone Aμ47.2/r indicates that the sequence is reversed. Position 20 in $D_{xp'}1$ in *b* has been reported as C (39) or A (38).

tions; and the clustering of nucleotide changes in hypervariable regions. Setting aside the question of preferential utilization of certain V_H genes in pre-B cells—which occurs in fetal life (6, 47, 48)—the biased representation of certain groups of V genes, or the repeated use of individual or highly related V genes in the repertoire point to the effect of ligand selection on the population (23). This was found for V_H gene families in the Aμ library, where V_H5 genes were present out of proportion to their expected frequency.

There are probably more than 30 human D_H genes (37, 38). Thus any individual D_H gene in an unbiased population should have a frequency of less than 1/30 (< 3.3%). Another indica-

b

	N	D	N
		$D_{LR}2$	
		AGGATATTGTAGTGGTGGTAGCTGCTACTCC	
Aμ41.1	CATTCCCC	-C-------A--C---G--	GGGCAAACGG
Aμ78.1	T	------C-----G---	AAAC
Aμ95.1	GGAGAGGGGCGG	-------------------	CTTGGGGTCTTTTGG
Aμ1.2	A	--------------------------	GATCCTCCGCGGGAAG
Aμ3.2	CTGGT	-C--T------------C--A-------A-	AAGGGTT
Aμ8.2		----------A--C------	TCAGGGGAG
Tμ19	AGACGAC	------C------T-	GAGCCTAGATCGT
Tμ22	TGTGAAAGG	-------T-----C-G---	CCCATCTCT
Tμ26	TCCAG	---TC-------------------------	TG
Tμ49	GATACCC	-C------------------------	GAACCG
Tμ98/r	C	---------C-C---	AGCACATC
		$D_{LR}3$	
		AGCATATTGTGGTGGTGATTGCTATTCC	
Tμ86	CTGG	------C--A---C----	CTCCTT
		$D_{LR}4$	
		AAGGATATTGTAGTAGTACCAGCTGCTATGCC	
Aμ22.1	C	-G-----------C-	TGAGCGGGGGG
Aμ91.1	GATGTCGTG	--G-C------------------------	GG
Aμ93.1	GACTCCCT	----A------T-GA------------	
Aμ40.2	CTAAGA	--------G------------	CC
Aμ45.2	CTA	-G--------------------	CC
		D_{Q52}	
		CTAACTGGGGA	
Aμ99.1	TTTGCGACTC	------	C
Tμ76	CCA	---C----	TGGCAGTG
		$D_{XP}1$	
		GTATTACTATGGTTCGGGGCGTTATTATAAC	
Aμ96.1	GGAACGAG	-A-----A---A-----	A
Aμ60.2	GATGGCA	--------------A------	CGATC
Tμ0	CTTTCTT	-----------	AGGAC
Tμ6	CATGGGGA	-------------C-	CA
Tμ16		---A· -----A----	G
Tμ73	C	-T-------------	AAG
Tμ74	GGAGG	----------------A-	CA
Tμ90	C	-------A----C-	TCGG
		$D_{XP}1$	
		GTATTACGATATTTTGACTGGTTATTATAAT	
Aμ44.2	GGCTCCCGAAT	---------------------------	CGGGCTTT
Tμ1	TATTGGTGGGGGC	------------	CCCCCTAAAA
Tμ60	GG	---------------	CCTT
		$D_{XP}2$	
		GTATTATGATTACGTTTGGGGGAGTTATGCTTATACC	
Tμ29	CT	---C-T-------------------CG--	CGG
Tμ91	C	--C---------	CTTCGGACA
		$D_{XP}3$	
		GTATTACTATGATAGTAGTGGTTATTACTAC	
Tμ42	GCAT	-----------A-G---	
Tμ46	GCTC	-----------------------	
Tμ83	CCGAGGCCG	-------------------------G-	GG
Tμ87	CCGC	----------G-GG---	GCATGT
Tμ100	GAGAAAC	----------------------	
		$D_{XP}4$	
		GTATTACGATTTTTGGAGTGGTTATTATACC	
Aμ101.1	G	---C-G----------	C
Aμ10.2	GATCGGCG	-A-----------------------	TA
Tμ21	C	-------------------	CCC

Figure 5 (Continued)

tion of selection in the libraries we tested is the over-representation of the $D_{LR}2$ genes, present in 6 of the 54 Aμ clones (11%) and 5 of the 49 Tμ clones (10%). The assignment of $D_{LR}2$ genes is possible because all five members of the D_{LR} family are known (38, 39).

Gu et al. (23) have analyzed members of a large V_H gene family (J558) expressed by B cells from three unimmunized CB.20 mice. In contrast to populations of pre-B cells, which expressed the ~ 100 J558 genes randomly, populations of mature surface IgM^+ splenic B cells were found to express preferen-

Aμ1.1	CCCCGTGACTTATGGGTCCACGAT
Aμ2.1	CTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
Aμ5.1	GGTTCGACCCCTGGGGCCAGGGAACCCTGGTCACC
Aμ49.1	TGGCCCGGCCAACCCGACTCCTCGCAACAG
Aμ51.1	GAGGCGGGGAGGGCCACACAG
Aμ52.1	GATCCTTTGAAGTCCGCGG
Aμ59.1	CTCGGGTGGCCGGGCCAGGAACAATACC
Aμ61.1	GTTGTAGGCGAAGTAAACTTTGGGAAAGTTGCGTTTT
Aμ70.1	TTCGCCGACGATGATCCCGAG
Aμ73.1	CGGGGCTTCAATGGCCAACTGATTTT
Aμ90.1	ATTTGCGGAATTAAGAACTGGCTCGGCCC
Aμ100.1	CGGGGCTCGGCTGGTACAGGGTA
Aμ6.2	GTATTGTTCCTGGCCCGGCACCCGAG
Aμ34.2	GGGAGAGATGGCTACA
Aμ42.2	TACTTCGCGCAA
Aμ43.2	ACAGACAGGCAGTACGAA
Tμ11	CGAGGGCCAATCACGGTGGTAACTCCGGAGGTGC
Tμ25	GTCGATCCAGGATAACAGTGGCTGAAATGGAC
Tμ50	GCCAAGGACCGGCTG
Tμ69	GAGGACATGG
Tμ70	ATCGTCGAGTCTTTGAGTACC

Figure 6. CDR3 base sequences that could not be assigned to germline D_H gene segments. These cDNAs do not contain a portion with more than 75% identity with known germline D_H genes.

tially certain members of the J558 family. This finding is analogous to ours, above, and to the repeated expression of $V_H18/2$, a member of the V_H3 family, in humans (17). On the basis of their studies in the mouse, Gu et al. (23) proposed that the peripheral B cell population contains many B cells that have undergone ligand selection, perhaps soon after they emerge from the bone marrow. A similar conclusion can be drawn from our studies.

Somatic mutation of V genes is a cardinal manifestation of clonal selection of B cells (49–56). In the case of the V_H gene segments we analyzed, definitive evidence of somatic mutation was found in the case of the 3 V_H6 clones (Fig. 2 *a*). These three clones differed from Aμ's own germline sequence by 7 (Aμ34.2), 13 (Aμ46.2), and 7 bases (Aμ51.1). The Aμ34.2 and Aμ51.1 clones also had evidence of somatic mutation in their CDR3 sequences.

The sequences of the two functional V_H5 genes, V_H251 and V_H32, are remarkably consistent in the human germline (37). The nucleotide sequences of all five examples of V_H5 family genes in both libraries differed from V_H251 and V_H32 by 1–15 bases (Fig. 2 *b*). Given the conservation of V_H251 and V_H32 in the germline, it is highly likely that the variations we observed can be attributed to somatic mutation.

Adding to the evidence from the V_H sequences for somatic mutation is the finding that the CDR3 portions of many J_H4 sequences in the Aμ library differ from germline genes in a way that cannot be explained by polymorphism (Fig. 4). Whereas polymorphic sites, such as the G → A substitution in J_H4, are identical in all clones from the same subject (Fig. 4), somatic mutations of V genes are typically clone-specific. Furthermore, the variations from the J_H4 germline sequence were not random, but clustered at the 5′ end of the gene; of the 42 bases that differ from the germline (not counting the polymorphic G → A substitution), 81% occur at the 5′ end of the gene, in the region that contributes to the CDR3.

A mechanism other than polymorphism is also required to account for the several D_H sequences that are closely related to D_{LR} and D_{XP} (Fig. 5). Even if subject Aμ had polymorphic

```
              N                                   D                             N

                                              D_K1          D_LR3/r
                                          TATAGTGGCTAC TCACCACCAC
Aμ4.1     GC                              ---T-------- ---A---G--               GGGAC

                                              D_XP4         D_N4
                            TATTACGATTTTTGGAGTGGTTATTA CAGCTCGTCC
Aμ2.2     ACA                               ----T-----------------C--- -C-T------               GGGCTGA

                                              D_M5          D_N1
                                             TGGAACAAC GCAGCAGCTGGTAC
Aμ39.2    GGCGGAGGA                          --------- ----TG--------           TAGACG

                                              D_N4          D_XP2
                                      ATAGCAGCTCGTCC     TTTGGGG
Aμ52.2    ACG                         ---A----------GTCAC-------

                                              D_N1          D_IR1
                                    TATAGCAGCAGCTGGTAC GAGGCCCC
Tμ5       GATCA                     --------TG-------- --T-----                 TTTCG

                                              D_N1          D_Q52
                                         GTATAGCAGCAGC CTAACGGGGG
Tμ7       GATCTAACCTCTCT                 ------------- ----------               GTTTTCGGGAGAT

                                              D'_XP1/r  D_N1
                                      ATAATAACGCCCCGAA GGGTATAGCAGCAGCTGGTAC
Tμ15      G                           ---TGC---G------A-A---------TG--------    CGGCG

                                              D_LR4         D_IR2/r
                              TTGTAGTAGTACCAGCTGCTATGC GGCTTGTGGGCG
Tμ23      GT                  ----------------A------- ----G-CA----             AGTAC
```

Figure 7. CDR3 base sequences that may be accounted for by D-D or D-D_{IR} fusions. These include examples in which one of the fused segments is reversed ($D_{LR}3$/r, $D'_{XP}1$/r, and $D_{IR}2$/r).

Table II. cDNAs Closely Related to Germline V_H Genes

Clone No.	Percent identity	Related gene	V_H family	No. of bases sequenced
Tμ16	99.6	VH251	5	292
Tμ59	99.1	M60	2	222
Tμ73	99.1	58P2	4	230
Tμ74*	99.1	21/28	1	229
Tμ49	97.3	FL2-2	1	218
Aμ51.1	97.8	VH6	6	315
Aμ2.1	99.7	VH32	5	303

* Clone Tμ74 has the same N and D_H sequence as the anti-DNA autoantibody 21/28 (see reference 76).

differences from the published $D_{LR}2$ germline sequence in both alleles—an unlikely proposition because a sequence identical to D_{LR2} was found in one clone—that would account for only two of the six Aμ genes related to $D_{LR}2$.

Apart from the evidence compiled from the nucleotide sequences themselves, a cogent argument for the occurrence of somatic mutation in circulating IgM$^+$ B cells is that the majority of the base substitutions we observed were not silent but resulted in a changed amino acid sequence. In studies of V gene sequences of antibodies produced during the secondary response of the mouse to several different classes of antigens, mutations causing amino acid substitutions ("replacement mutations") were found to exceed silent mutations by far, and were characteristically located in the CDRs (50, 52, 53, 57–64). That pattern is striking in the J_H4 gene of the Aμ library. Of the 42 base differences from the germline sequence found among 49 J_H genes (not counting the polymorphic G → A substitution), 5 were silent and 37 were replacement variants; of those 37 replacement variants, 31 (84%) occurred in the 5′ CDR3-coding region of the J_H genes (Fig. 4 *b*).

A similar, but less striking picture emerges from analysis of the seven V_H5 and V_H6 genes. The total number of base variations from germline sequences was 54; of those, 41 were replacement and 13 were silent (of the latter, 10 occurred in the three V_H6 genes). And of the 22 amino acid replacements in the V_H5 genes, 50% occurred in either CDR1 or CDR2. Although framework mutations can affect antibody binding properties (65), mutations in the CDRs, which are largely responsible for the ligand-binding surface of the immunoglobulin molecule, are the principal molecular signs of clonal selection. It is thus highly likely that the mutations we observed are a reflection of the selective effect of a ligand on the circulating B cell population.

These findings, when viewed as a whole, suggest that ligand-selected IgM$^+$ B cells not only circulate in the blood of normal adults but they may comprise a substantial fraction of the B lymphocytes in human blood. They could correspond to long-lived memory cells that have been rescued from programmed cell death by contact with antigen (66). Indeed, it is likely that the B lymphocyte dies soon after it completes its differentiation, as a result of apoptosis, unless it undergoes selection by antigen (67). Our finding of somatic mutation in circulating B cells is of interest because B cells engaged in responses to specific antigens are generally thought to reside in the germinal centers of the spleen and lymph nodes (68). However, it was recently shown that during the secondary immune response of the mouse to horseradish peroxidase, B cells have been found to leave the germinal centers, enter the circulation, and seed the bone marrow where they mature into antibody-producing plasma cells (69). Presumably, those cells underwent at least the initial stages of antigen selection, although the molecular evidence to support that conclusion is presently lacking.

Another noteworthy aspect of our results is that V region genes in a Cμ library showed evidence of somatic mutation. In the experiments of Gu et al. (23), no somatic mutations were found among 44 complete V region sequences in Cμ libraries from young unimmunized mice; Manser and Gefter (70) also found no somatic mutations in naive mice. Even so, it is known that IgM antibodies can be encoded by mutated V region genes (71), and that somatic mutation can be detected very early in the immune response (63), independently of heavy chain class switching (72).

The molecular signs of clonal selection in circulating B cells suggest that the selective ligand was encountered after the pre-B cell stage of differentiation, when the maturing B cell has rearranged its V genes (73) and expressed at least a surface heavy chain. In the steady state, such B cells could represent long-lived circulating memory cells that provide an early defense against microbial reinvasion; or, in some instances they may represent selection of the repertoire by idiotypes or anti-idiotypes (74). In either case, any analysis of V gene repertoires in disease will have to take into account the variations in composition and structure of the normal repertoire.

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