Restricted Use of Fetal VH3 Immunoglobulin Genes by Unselected B Cells in the Adult

Predominance of 56p1-like VH Genes in Common Variable Immunodeficiency

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Abstract

The large VH3 family of human immunoglobulin genes is commonly used throughout B cell ontogeny. However, B cells of the fetus and certain autoantibody-producing clones are restricted to a recurrent subset of VH3 genes, and VH3 B cells are deficient in certain immunodeficiency diseases. In this study, we have sequenced a set of rearranged VH3 genes generated by genomic polymerase chain reaction (PCR) from normal adults and those with common variable immunodeficiency (CVI). In both groups, all clones were readily identifiable with the fetal VH3 subset, and were further distinguished by limited DH motifs and exclusive use of JH4. In CVI, the residual population of VH3 B cells were notable for predominant use of 56p1-like VH genes. All clones displayed sequence divergence (including somatic mutation) with evidence of strong selection against complementarity-determining region (CDR) coding change. A survey of other V gene families indicates that human V gene diversity may be restricted in general by germline mechanisms. These findings suggest that the expressed antibody repertoire in the human adult may be much smaller than anticipated, and selected by processes in part distinct from the paradigm of maximal antigen-binding diversity. (*J. Clin. Invest.* 1992. 89:1395–1402.) Key words: B lymphocyte development • antibody repertoire • somatic mutation • ontogeny • autoimmunity

Introduction

Protective immunity depends on a highly diverse antibody repertoire. This need is met by the availability of a large number of germline V, D, and J segments which are independently recombined, junctionally mutated, and assorted (with respect to active heavy and light chain genes) by each B cell clone. These processes provide an enormous potential repertoire of $\sim 10^{11}$ antibodies (1, 2) that meets or greatly exceeds the total B cell population of the individual (10^{11} and 10^8 for human and mouse, respectively) (3). Subsequently, each clone has the capacity for further diversification by the antigen-driven processes of variable region hypermutation in concert with strong

positive biologic selection for favorable coding changes at sites contributing to antigen-binding (complementarity determining region, CDR).[1] In the adult mouse, the B cell pool appears to be formed by full use of these diversification mechanisms (4–10). In humans, these mechanisms are believed to contribute to repertoire formation in a manner analogous to the mouse, based on surveys of V gene family use (11–14) and somatic mutation in malignant B cell clones of follicular lymphoma (15, 16).

Paradoxically, clonal diversity during pre-B cell development and early immune ontogeny is restricted. This insight, initially established in murine studies (4–6), has been confirmed in the human fetal to neonatal period by the predominant use of the small VkIV, VH5, and VH6 gene families, and the selective use of certain DH, VkIII, VH1, and VH3 genes during the fetal to neonatal period (13, 14, 17–19). For example, among the ~ 30 members of the VH3 gene family, only 5 are abundant in fetal B cell cDNA libraries (20p1, 30p1, 38p1, 56p1, 60p2). In fact, certain highly homologous genes human VH3 and murine 7183 genes (e.g., human 30p1 and murine E415; human 56p1 and murine 81X) have a similar developmental restriction. The preferential use of these genes is presumed to reflect favored accessibility to the recombination mechanism due to factors such as chromosomal position, local *cis*-regulatory regions, and B cell subsets (5, 6). It is also notable that compared to B cell clones from adults, those from early ontogeny disproportionately express autoantibodies. Some of these fetal-type clones have drawn attention due to their repetitive occurrence in B cell malignancies associated with autoreactive antibodies.

We have observed that human B cells bearing rearranged VH3 genes are selectively deficient at the germinal center stage, a critical period for antigen-driven memory differentiation (20). Certain immunodeficiencies (HIV infection, common variable immunodeficiency [CVI]) are associated with an overabundance of B cells phenotypically related to germinal center cells, and lymphoid populations from these individuals also display the selective depletion of VH3 B cells (21, 22). These findings prompted us to suggest that the B cell dysfunction in these diseases may have in common a maturational arrest of B cells at the germinal center stage. A paradox raised by these studies was the cause of the physiologic VH3 B cell deficit in germinal center cells, since VH3 B cells are abundant in populations representing both germinal center cell precursors (bone marrow pre-B cells) and descendants (the recirculating pool of blood and mantle zone B cells).

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1. *Abbreviations used in this paper:* CDR, complementarity determining region; CVI, common variable immunodeficiency; FR, framework region; PCR, polymerase chain reaction; R/S, replacement:silent.

One simple explanation for this paradox is that the antigen-driven developmental transition occurring at the germinal center stage involves a major shift clonal pattern, including the attrition of fetal-type VH3 B cells and positive selection for other favorable VH3 B cells rare in the primary B cell population. During this transition (i.e., the germinal center stage), the net abundance of VH3 B cells would be diminished, since restoration would only occur after the positively selected VH3 cells underwent clonal expansion (which mainly occurs after germinal center emigration). To test this explanation, we have characterized a set of rearranged VH3 genes from normal and CVI adults, with the prediction that these VH3 genes would be novel and fetal type, respectively. However, we were surprised to find that in most respects, a comparable fetal pattern was observed in both groups. Moreover, sequence divergence of these and other VH genes reflected strong selection against coding diversity. These observations raise new issues concerning the nature of the clonal shift during germinal center differentiation, and the mechanism of human versus murine repertoire diversification.

Methods

Subjects and specimen preparation. Peripheral blood mononuclear cells were obtained by venipuncture and Ficoll-Hypaque gradient fractionation from adults (20–65 yr), including healthy laboratory personnel (R.P., G.C., and H.V.) and individuals with common variable immunodeficiency (T.F., D.H., A.W.). One specimen of tonsil mononuclear cells was obtained from the tonsillectomy specimen of a 4-yr-old child (E.P.). Procedures for subject recruitment, consent, and specimen procurement were in accordance with protocols approved by the UCLA Human Subject Protection Committee.

Cloning of rearranged genomic VH3 clones. Genomic DNA was prepared by standard methods, and amplified for rearranged VH3 by polymerase chain reaction (PCR) under quantitative reaction conditions, as previously reported (20–22). 10–100 ng of genomic DNA template was reacted with 50 pmol each of VH3L and JHKOR oligonucleotides (respectively: 5′-dCTGGTGGAGTCTGGGGGAGGC; VH3 coding strand sequence, FR1, codons 6–12; and 5′-dACCTGAGGA-GACGGTGACCAGGGT; JH noncoding strand sequence, codons 108 to 2 nt 3′ of codon 113A) (Dr. Thomas Sutherland, Molecular Biology Institute), 100 µmol dNTP and 0.5 U *Thermus aquaticus* DNA polymerase (Taq) with commercial reaction buffer (Perkins-Elmer/Cetus Corp., Emeryville, CA). The reaction mixture was reacted using a DNA pacer (Bellco Glass, Inc., Vineland, NJ) for 40 cycles, each consisting of 94°C/1 min and 72°C/2 min. PCR products were fractionated using 1%/1% Nu-Sieve/LE agarose (FMC Corp., Rockland, ME) in 1× TAE buffer. Gels were briefly stained with ethidium bromide to visualize the 380-bp VDJ product, and the band was excised and extracted. The material was cloned in some cases (LJ42 and LJ86) by blunting with mung bean exonuclease (New England Biolabs, Beverly, MA) and insertion into the Bluescript SK (Stratagene Inc., San Diego, CA) EcoRV site. In the other cases, extended VH3L and JHKOR oligonucleotides were used for amplification containing terminal HindIII and BamH1 sites, respectively. After complete restriction enzyme digestion, they were cloned in the Bluescript SK HindIII/BamHI site.

In order to limit the possibility of repeat cloning of identical VDJ segments, the PCR reactions were performed under stringent, quantitative reaction conditions (20–22), and a single random clone was chosen for sequencing from each PCR reaction/cloning. Among 14 clones obtained in this manner, only 1 duplicate clone occurred (LJ55). It was identical to another clone (LJ33) obtained and sequenced from a different individual 5 mo earlier, and therefore, was a probable LJ33 contaminant. Overall, this confirms that the strategy maintained a high degree of independence among the set of VDJ clones.

Sequencing and analysis. Inserts were sequenced using flanking vector primers by the dideoxynucleotide chain termination method, both manually and using an automated sequencer (Applied Biosystems Inc., Palo Alto, CA). Sequences were identified with reference VH3 and JH gene segments by comparison to previously reported germline and rearranged sequences (17, 18, 23–27). The CDR3 regions were compared to a large tabulation of DH segments with respect to global homology and local homologies (> 6–7 nucleotides) using the COMPARE, and ALIGN and SEQCOMP programs, respectively (DNA-STAR Inc., Madison, WI) (28). Calculation of replacement:silent (R/S) values was done manually, as described previously (29). Nucleotide sequence data contained in this paper have been submitted to GenBank (M82924–M82936).

Results

The VH regions of the cloned VDJ segments are shown in Figs. 1 and 4. When compared to previously reported sequences, each clone could be readily assigned to 6 of 26 (or more [27]) isolated VH3 germline genes. This limited occurrence of VH3 genes was not attributable to our VH3 amplimer. 19 of these germline genes are perfect matches for the VH3L amplimer sequence, and 6 differ by only 1 nt. Three clones apparently derive from the 30p1/VH26 gene, which differs from the VH3L amplimer by 1 nt. Thus, a minimum of 19, and potentially 24 or more germline VH3 genes are amplifiable by our PCR strategy. In addition, distinct categories of clones were obtained using the same amplification method from normal versus CVI groups (see below). Amplimer selection is thus unlikely to account for the observed restriction in VH3 gene cloning.

For four clones, there was some uncertainty in discerning the source germline genes among the numerous set of highly homologous 56p1-like germline genes represented by humvh3005, 1.9III, and others (27, 30). Using six distinguishing polymorphic sites, we established that three clones (LJ33, LJ34, LJ24) were identical 56p1 (versus 2 and 5 nt mismatches with 3005 and 1.9III), and one clone was identical to 1.9III (versus 4-nt mismatches for 56p1 and 3005). It is particularly notable that while these clones were common in individuals with CVI (4/6 clones and 2/3 individuals), no clones bearing 56p1-like genes were obtained from normal individuals (0/7 clones and 0/4 individuals) (Table I).

The CDR3 (DH and flanking N regions) of each clone is shown in Figs. 2 and 4. As usually observed for human sequences, this region is highly diverse, and in most cases not readily identifiable with known germline DH genes. Correspondence to defined DH genes was therefore assessed by computer matching for partial homologies ("motifs"), and inversions and D–D fusions which can involve DIR genes (see Discussion). In this manner, we detected a striking use of the DXP family among this set of clones: complete or partial germline D genes (in particular 21.9) were used by 8/13 clones overall, and by 8/9 non-56p1/1.9III clones (Table I). It also appeared that the DIR-1 and/or DIR-2 genes were also frequently used; in this small sample, they were limited to clones bearing 30p1/VH26 and 56p1/1.9III VH genes (Table I).

The JH segments are shown in Figs. 3 and 4. With respect to technical issues, the JH segment was inadvertently excised during cloning of LJ14, LJ34, and LJ42 due to a BamH1 site in the CDR3 region. Three other clones (LJ23, LJ54, LJ62) had partial JH deletions due to presumed cloning artifacts. The striking finding was the exclusive use of JH4, even though the
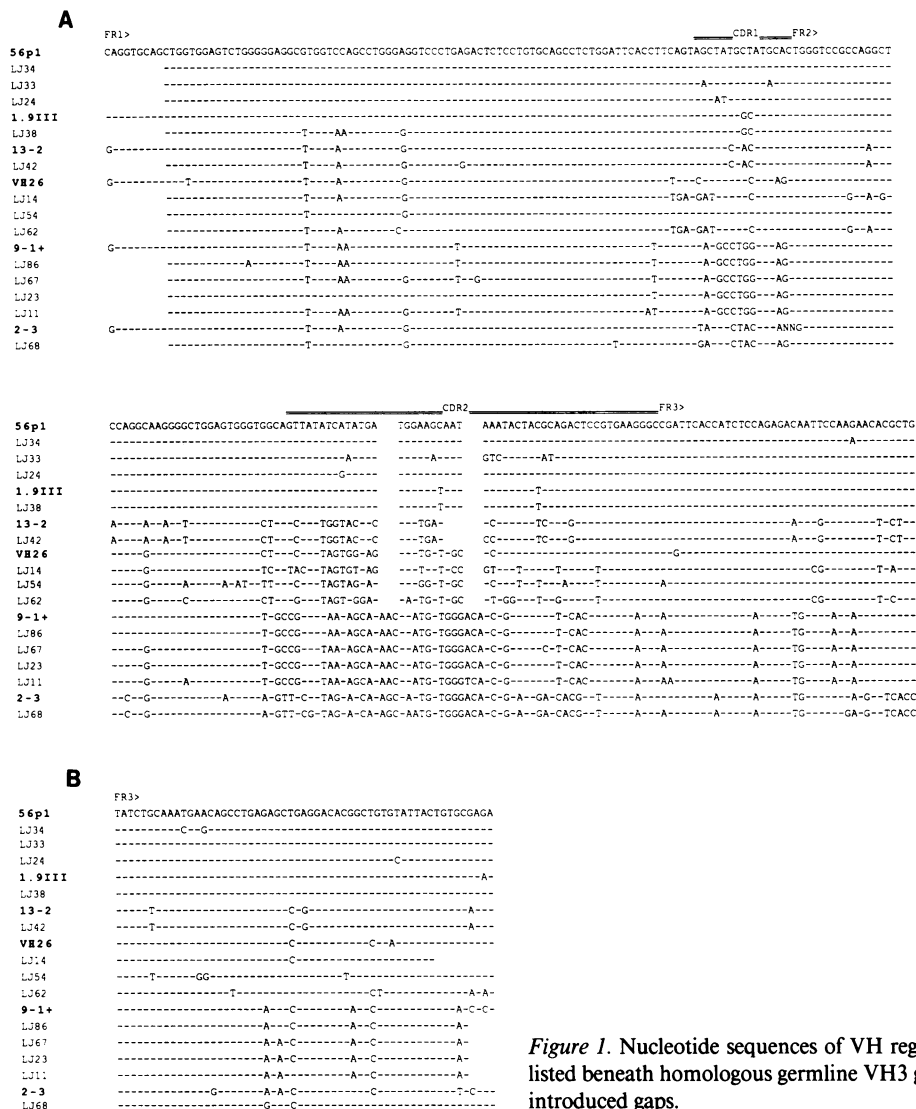
A

FR1>   ___CDR1___FR2>

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56p1    CAGGTGCAGCTGGTGGAGTCTGGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTCAGTAGCTATGCTATGCACTGGGTCCGCCAGGCT
LJ34    ------------------------------------------------------------------------------------------------------------------------
LJ33    ----------------------------------------------------------------------------------A---------A------------------
LJ24    ----------------------------------------------------------------------------------AT------------------------
1.9III  ------------------------------------------------------------------------------------------GC------------------
LJ38    --------------------T----AA--------G-------------------------------------------------GC------------------
13-2    G-----------------------T----A--------G-------------------------------------------C-AC----------------A---
LJ42    --------------------T----A--------G------G-----------------------------------------C-AC----------------A---
VH26    G-----------T--------------T----A--------G------------------------------------T--C-----C---AG--------------
LJ14    --------------------T----A--------G---------------------------------TGA-GAT-----C--------------G--A-G-
LJ54    --------------------T------------G------------------------------------------------------------
LJ62    --------------------T----A--------C------------------------------TGA-GAT-----C--------------G--A---
9-1+    G-----------------------T----AA-------------T-------------------T--------A-GCCTGG---AG----------------
LJ86    -----------A---------T----AA-------------T-------------------T--------A-GCCTGG---AG----------------
LJ67    --------------------T----AA--------G--------T--G------------------T--------A-GCCTGG---AG----------------
LJ23    -------------------------------------------------------------------T--------A-GCCTGG---AG----------------
LJ11    --------------------T----AA--------G--------T----------------AT-------A-GCCTGG---AG----------------
2-3     G-----------------------T----A--------G--------------------------------TA---CTAC---ANNG--------------
LJ68    --------------------T-------------G------------------------T-----------GA---CTAC---AG--------------
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                                                    _____CDR2_____                              FR3>
56p1    CCAGGCAAGGGGCTGGAGTGGGTGGCAGTTATATCATATGA      TGGAAGCAAT    AAATACTACGCAGACTCCGTGAAGGGCCGATTCACCATCTCCAGAGACAATTCCAAGAACACGCTG
LJ34    -----------------------------------------      ----------    --------------------------------------------------A--------
LJ33    -----------------------------------A----      -----A----    GTC------AT---------------------------------------------
LJ24    -----------------------------------G----      ----------    ------------------------------------------------------
1.9III  -----------------------------------------      ------T---    ---------T---------------------------------------------
LJ38    -----------------------------------------      ------T---    ---------T---------------------------------------------
13-2    A----A--A--A--T-----------CT---C---TGGTAC--C   ---TGA-       -C------TC---G---------------------------------A---G---------T-CT--
LJ42    A----A--A--A--T-----------CT---C---TGGTAC--C   ---TGA-       CC------TC---G---------------------------------A---G---------T-CT--
VH26    -----G-------------------CT---C---TAGTGG-AG    ---TG-T-GC    -C----------------------------------G-------------------------
LJ14    -----G-------------------TC--TAC--TAGTGT-AG    ---T--T-CC    GT---T----T----T----------------------------------CG--------T-A---
LJ54    -----G-----A-----A-AT--TT---C---TAGTAG-A-      ---GG-T-GC    -C---T--T--T---A----T---------A----------------------------
LJ62    -----G-----C-------------CT---G---TAGT-GGA-    -A-TG-T-GC    -T-GG---T--G----T----------------------------------T-C---
9-1+    -------------------T-GCCG----AA-AGCA-AAC--ATG-TGGGACA-C-G-------T-CAC-------A---A------------A----TG-----A--A---------
LJ86    -------------------T-GCCG----AA-AGCA-AAC--ATG-TGGGACA-C-G-------T-CAC-------A---A------------A----TG----A--A---------
LJ67    -----G-------------T-GCCG---TAA-AGCA-AAC--ATG-TGGGACA-C-G-------C-T-CAC-------A---A------------A----TG----A--A---------
LJ23    -----G-------------T-GCCG---TAA-AGCA-AAC--ATG-TGGGACA-C-G-------T-CAC-------A---A------------A----TG----A--A---------
LJ11    -----G-----A-------T-GCCG---TAA-AGCA-AAC--ATG-TGGGTCA-C-G-------T-CAC-------AA---------A----TG----A--A---------
2-3     --C--G-------------A-----A-GTT-C--TAG-A-CA-AGC-A-TG-TGGGACA-C-G-A--GA-CACG--T-----A----------A-----A-----TG------A-G--TCACC
LJ68    --C--G-------------A-----A-GTT-CG-TAG-A-CA-AGC-AATG-TGGGACA-C-G-A--GA-CACG--T-----A----A------A-----A-----TG------GA-G--TCACC
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B

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        FR3>
56p1    TATCTGCAAATGAACAGCCTGAGAGCTGAGGACACGGCTGTGTATTACTGTGCGAGA
LJ34    ----------C--G---------------------------------------
LJ33    ------------------------------------------------------
LJ24    -------------------------------------C---------------
1.9III  ---------------------------------------------A-
LJ38    ------------------------------------------------------
13-2    -----T-------------------C-G-----------------------A---
LJ42    -----T-------------------C-G-----------------------A---
VH26    -------------------------C-----------C--A--------------
LJ14    -------------------------C--------------------
LJ54    -----T-------GG---------------------T----------------
LJ62    ---------------T------------------CT-------------A-A-
9-1+    -----------------------A---C--------A--C------------A-C-C-
LJ86    -----------------------A---C--------A--C------------A-
LJ67    -----------------------A-A-C--------A--C------------A-
LJ23    -----------------------A-A-C--------A--C------------A-
LJ11    -----------------------A-A---------A--C------------A-
2-3     ---------------G------A-A-C--------------C------------T-C--
LJ68    -----------------------G---C---------------------------
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Figure 1. Nucleotide sequences of VH regions of rearranged VH3 clones. Clones are listed beneath homologous germline VH3 gene sequences (*boldface*) (17, 23). Spaces are introduced gaps.

JH amplimer was fully homologous to JH1, 4, and 5. Many of the clones showed nucleotide differences, in comparison with the germline JH sequences compiled by Leder and colleagues (31). In some cases, these reflect silent polymorphisms either reported (e.g., CAG in codon 106) (17) or presumed (GTG and TCG in codons 110 and 113), the latter inferred from their recurrence in separate clones from the same individual (LJ33, LJ38). However, the JH segments of two other clones (LJ24 and LJ68) each displayed seven unique replacement-type nucleotide differences unrelated to reported JH genes or pseudogenes. These may represent authentic somatic mutations, and are of additional interest because they represent the two silent genes in this set of clones.

Nucleotide sequence differences were present in the VH segments of all clones when compared with the reference germline VH3 genes (Table II). The frequency of these base changes ranged from 0.3%/1 nt (LJ86) to 11%/30 nt (LJ14), with most clones between 2 and 4%. We note that for each characterized reference gene, there are numerous additional homologous germline genes, most of which remain uncloned (25–27). Therefore, this sequence divergence may be largely due to the unavailability of authentic source genes for optimal sequence comparison.

It is unlikely that these base changes were due to infidelity of PCR amplification. The error rate of Taq polymerase is reported as $\sim 10^{-4}$/bp per duplication (32), hence $< 0.4\%$ for 40 cycles. This is substantially below the observed frequency in this set of clones. In our own tests, several reamplified and sequenced clones did not show new mutations. Moreover, several other groups have used similar strategies to clone and sequence sets of V genes (2, 6, 33, 34), and did not observe a significant frequency of artifactual mutations due to this factor.

It therefore appeared that these divergent sequences were authentic base changes reflecting some combination of somatic mutations, allelic polymorphisms, and newly detected homologous genes. Their localization and effect on amino acid coding are summarized in Table III. As expected, the frequency of base changes in CDRs was approximately threefold greater than in framework regions (FRs). The R/S value is a useful index of coding change due to the nature of the triplet code, this ratio is approximately three–four for a random base change; elevated or decreased ratios typically reflect positive or negative biologic selection for these coding changes, respectively. In the case of FRs, the R/S value was very low (1.0), reflecting the expected selection against coding changes which interfere with critical V region conformation encoded by the framework seg-

Table I. Summary of V_H^3 Clones

Subject	Clone	VH	DH motif	JH
Normal				
RP	LJ54	30p1/VH26	21.9, DIR1/2	JH4, int. del.
GC	LJ11	20p1/9-1	21.9	JH4
	LJ62	30p1/VH26	21.7, DIR1/2.RC	JH4, int. del.
	LJ67	20p1/9-1	21.9, 23.7	JH4
	LJ68*	2-3	21.9, DHQ52	JH4
HV	LJ86	20p1/9-1	21.05/.10, 23.7, 21.9	JH4
EP	LJ42*	38p1/13-2	21.9	
Common variable immunodeficiency				
DH	LJ14	30p1/VH26	DIR1/2, DFL16	
AW	LJ23	20p1/9-1	21.9	JH4, trunc.
	LJ24	56p1/3005	DIR2, DLR1/3.RC, DM2	JH4
TF	LJ33	56p1/3005	DIR2, DIR1/2.RC	JH4
	LJ34	56p1/3005	DFL16	
	LJ38	1.9III	DIR2, DIR1/2.RC	JH4

* Nonexpressed genes due to internal stop codon.

ments. However, we were surprised to also find striking suppression of R/S in the CDRs (0.91).

We wondered whether this unusual R/S suppression of the CDRs was a unique feature of this set of VH3 genes, or a general feature of the VH3 or other human V gene families. Therefore, we recalculated mutation frequencies and R/S values from published sequences of several sets of V genes (Table III).

Figure 2. Nucleotide sequences of DH regions of rearranged VH3 clones. Clones are listed with related germline DH gene (*boldface*) (28). Arrows show introduced deletions; spaces are introduced gaps; underlined sequences are internal BamH1 sites. *Mismatches.

Figure 3. Nucleotide sequences of JH regions of rearranged VH3 clones, compared with germline JH4 (31). Spaces are introduced gaps.

In fact, CDR R/S values were remarkably low among human VH3 and VH4 genes (Table III); similar low values were also observed when we recalculated in intrafamily comparisons for VkIII (35, 36) and Vλ (37) germline genes (data not shown). These findings with human V genes were in striking contrast to mouse germline V genes (both coding and pseudogenes) (38, 29), for which CDR R/S values were elevated substantially above neutral (R/S ~ 7).

Discussion

To our knowledge, this study is the first sequence characterization of an unselected set of rearranged VH genes in the human adult. We had anticipated robust diversity among these clones, since they were drawn from the adult recirculating B cell population, and should be predominantly composed of relatively long-lived postantigenic B cells. However, several features of these clones systematically limited coding diversity in these clones, suggesting a global restriction in the diversity of the adult human antibody repertoire.

Characterization of V, D, and J segments. All except the earliest fetal B cell populations include a major component (> 50%) of cells expressing VH3 genes. Despite the existence of a large number of VH3 genes, we were surprised to find only six VH3 genes in this set of rearranged VH3 clones from the adult. The recurrence of these VH3 genes does not appear to be artifactual: each clone is clearly independent based on sequence features, and by the apparent suitability of the FR1 VH3 amplimer to prime at least 24/26 known germline VH3 sequences. It is striking that five of these genes (when 56p1 and 1.9III are considered together) correspond to the set of VH3 genes comprising the fetal B cell population and the recurrent set of VH3 autoantibodies and neoplastic CLL/WM B cells. The one exception was LJ68, corresponding to the 2–3 pseudogene. However, previous studies have been limited to cDNA libraries or characterization of expressed antibodies, which by design exclude nonproductive VDJ rearrangement. Thus, fetal VH3 genes may actually be the only VH3 genes commonly used in the human antibody repertoire. In the future, it will be important to confirm this unexpected conclusion through additional methodologies in larger series of subjects.

In this regard, the VH4 family (consisting of 12–15 genes and only one pseudogene) is notable, since only three-four members have been found expressed (39). VH4-21, a nonpolymorphic germline VH4 gene, is particularly impressive, since it encodes nearly all cold agglutinins and a large fraction of VH4 autoantibodies (40). Despite the fact that these and most other studies of V gene use have focused on autoreactive or certain antigens, all the evidence thus far points to the recurrent expression of only 20–25 VH genes in humans.

As discussed elsewhere (28), the genetic mechanisms forming human CDR3 regions are complex, including D–D fusions, inversions, DIR genes, gene conversion, and exceptionally long
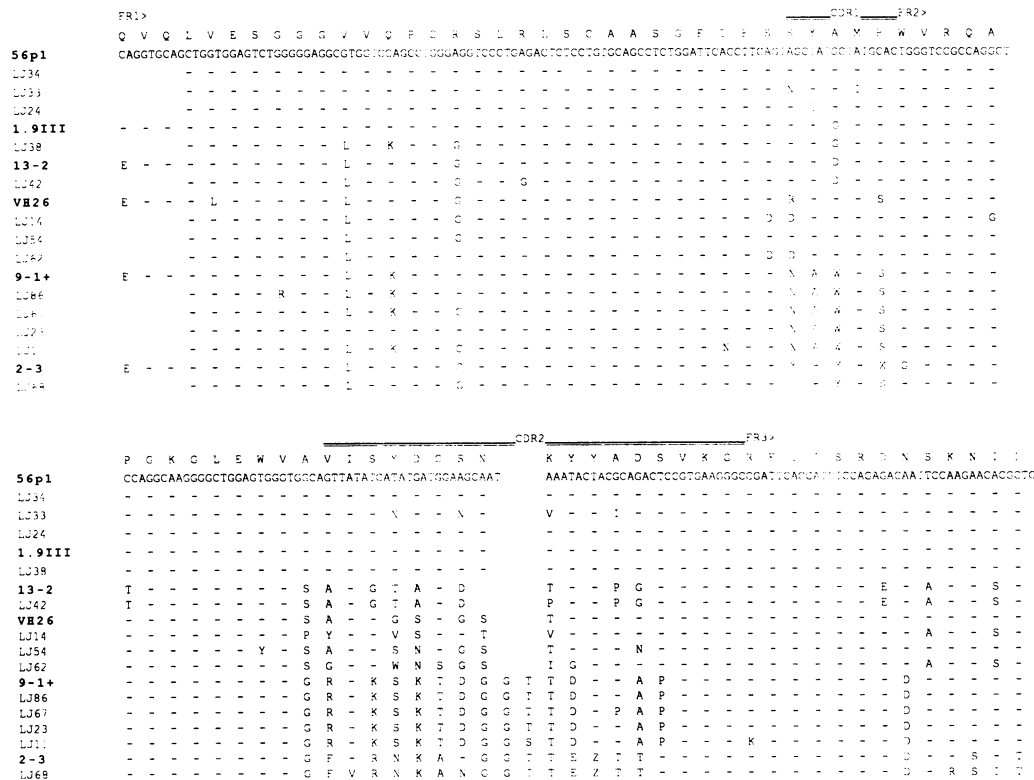
Figure 4. Predicted peptide sequence of rearranged VH3 clones.

N regions. This complexity was manifested by the current set of clones. In addition, certain DH genes recurred among these VH3 clones, including the DXP family (especially the 21.9 gene) and DIR-like genes. The use of JH segments was also remarkably limited to JH4, even though the JH amplimer was completely homologous to JH1, JH4, and JH5, and despite the fact that rearranged VDJ segments with JH5 have been cloned in our lab using the same PCR methodology. JH2, JH3, and JH6 were not evaluable in the present study due to amplimer design (insufficient homology). In other studies, JH1 has been rarely observed, and the relative frequency of JH4 to JH5 appears to increase during ontogeny (2, 17, 18, 33, 34, 41).

Distinct characteristics of VH3 clones from CVI B cells. Clones obtained from subjects with CVI were unusual in their use of VH and DH segments. 56p1-like VH genes were commonly used (4/6 clones), whereas they were absent among the clones from normal individuals (0/7 clones). For DH segments, DIR motifs were common (4/6 clones) and DXP rare (1/6 clones) in CVI, whereas the reciprocal was true for normal subjects (2/7 and 7/7 clones, respectively). Even in this small data set, a paired, two-tailed t test of these comparisons indicates that the difference between normal and CVI clones is statistically significant for 56p1-like genes and DXP ($P = 0.025$ and 0.004, respectively). The predominance of 56p1-like genes in CVI B cells is comparable with the common use of 56p1 in fetal B cells (18), and the reported $\sim$ 10-fold increased abundance of the corresponding B6 idiotope in fetal versus adult B cells (14).

Since the B cell dysfunction in CVI and HIV infection share common features, it is interesting to consider the recent sequence data for VH3 cDNA clones from HIV-positive individuals reported by Marks et al. (2). Of 16 VH3 clones, they observed: 7/16 56p1-like VH genes; 2 DFL16, 2 DXP1, 1 each of DA1, DN1, DN4, and DLR4 (nucleotide sequence was unavail-

able to assess DIR gene usage); 11 JH4, 4 JH6, and 1 JH2 (no JH1, JH3, or JH5). Thus, based on gene segment usage, HIV and CVI B cell populations seem to share the same distinguishing clonal features.

One purpose of this study was to better understand the reason for the depletion of VH3 B cells at the germinal center stage. The present findings of comparable fetal to adult VH3 gene use do not support our previous hypothesis that this stage involves a VH3 clonal shift between progenitor (e.g., fetal) and postantigenic (recirculating blood and mantle zone) B cell populations. The depletion of VH3 B cells in tonsil germinal centers suggests that their entry into the recirculating pool (and apparent somatic mutation; see below) may simply occur independent of germinal centers. Recently, additional clonal members of this germinal center-excluded B cell population have been reported (42, 43). The existence of an independent population of germinal center-derived "memory" B cells has been proposed by Klinman and colleagues (44), and is supported by the rich V gene diversity of B cell clones in non-Hodgkin's lymphoma, thought to derive from the germinal center population (45).

Negative selection restricts cloning diversity. In all clones, base changes (usually 2–4%) were detected when VH regions were compared with their presumed germline VH3 genes. It is unlikely that they reflect allelic polymorphisms, since none have been previously reported, and their frequency exceeds that expected for this class of sequence diversity (< 1%). They also are unlikely to represent PCR-associated mutations, since their frequency is too high (see Results) and they are nonrandom with respect to R/S values and CDR versus FR sequences. It is conceivable that they may in some cases represent previously unknown germline genes very homologous to the presumed reference gene. For example, this is likely to account for the particularly high "mutation" frequency in the 30p1/VH26-

Table II. Sequence Differences in VH3 Clones Compared with Reference Germline VH3 Genes

	FR1		CDR1		FR2		CDR2		FR3		All FRs		All CDRs	
	R	S	R	S	R	S	R	S	R	S	R	S	R	S
LJ34	0	0	0	0	0	0	0	0	2	1	2	1	0	0
LJ33	0	0	2	0	0	0	4	3	0	0	0	0	6	3
LJ24	0	0	2	0	0	0	0	1	1	0	1	0	2	1
LJ38	3	1	0	0	0	0	0	0	1	0	4	1	0	0
LJ42	1	1	0	0	0	0	1	1	0	0	1	1	1	1
LJ14	2	2	2	3	3	2	6	5	2	5	7	9	8	8
LJ54	1	2	2	2	1	4	3	5	2	4	4	10	5	7
LJ62	3	2	2	3	1	2	6	4	4	5	8	9	8	7
LJ86	1	0	0	0	0	0	0	0	0	0	1	0	0	0
LJ67	1	1	0	0	0	1	1	1	1	0	2	2	1	1
LJ23	2	2	0	0	0	1	0	1	1	0	3	3	0	1
LJ11	2	0	0	0	0	1	1	1	2	1	4	2	1	1
LJ68	0	2	1	0	0	1	2	0	7	1	7	4	3	0

Table III. Negative Selection Limits Coding Diversity of Human V Genes

V_H3 clones compared with germline V_H^3 reference genes*

	Replacement	Silent	Total	R/S
FRs	1.6% (44/2822)	1.6% (44/2822)	3.1% (88/2822)	1.0
CDRs	4.0% (35/885)	3.4% (30/885)	8.7% (77/885)	1.2

V_H3 germline genes compared with 1.9III[‡]

	Replacement	Silent	Total	R/S
FRs	4.1% (36/872)	6.0% (53/872)	10.1% (102/872)	0.7
CDRs	16.5% (45/273)	19.8% (54/273)	36% (99/273)	0.8

V_H4 germline genes compared with V_{71-2}[§]

	Replacement	Silent	Total	R/S
FR	2.2% (30/1368)	1.6% (22/1368)	3.8% (52/1368)	1.4
CDRs	4.8% (19/396)	4.3% (17/396)	9.1% (36/396)	1.1

NP-related V_H genes in two strains of mice[‖]

	Replacement	Silent	Total	R/S
FR	1.8% (22/1200)	2.3% (27/1200)	4.1% (49/1200)	0.8
CDRs	9.1% (30/330)	1.3% (4/330)	10.3% (34/330)	7.0

Values are % base change/bp, calculated from: * Table II.
[‡] V_H3 germline genes 13-2, VH26, 9-1+, and 2-3 (23).
[§] V_H4 germline genes V_{71-4}, V_{11}, V_{79}, V_{12G-1}, V_{2-1}, V_{58} (59).
[‖] NP equivalent murine BALB/c V_H genes to C57BL/6 V_H genes (29).

like clones (25, 28, 46, 47). However, it is likely that all the 56p1-like genes have been identified (29), yet our 56p1-like clones nonetheless show substantial sequence divergence. Therefore, most of these base changes (and at least a portion in the other clones) represent authentic somatic mutation. Such mutations apparently occur in LJ68 and LJ24, which would be the first demonstration of this type of mutation in silent alleles in humans.

Despite a substantial mutation frequency, we were surprised to find that the CDR R/S values were strikingly low. Upon reanalysis of published sequences, we found that this feature was shared by all families of human germline V genes (see Results). It is also notable that very low R/S values were found for mutations observed in rearranged V gene segments associated with monospecific rheumatoid factors (48), and clonal siblings of a non-Hodgkin's lymphoma (49). This observation is in distinct contrast to murine V genes, which show a preference of CDR coding diversity at both the germline and somatic level. However, a population-based sequence study has just appeared (50) which reveals much greater restriction in expressed murine V genes than previously appreciated from the existing literature of V family hybridization analysis.

The biologic selection behind this limited coding diversity in human V genes is uncertain. At the germline level, it could reflect the benefit of focusing the antibody repertoire on a set of critical specificities, such as certain environmental pathogens or regulatory idiotypes (18, 29, 51). The notion of Ig clans has also been introduced to account for phylogenetically conserved FR1 segments (52, 53) bearing highly conserved binding sites for unconventional antibody-ligand interactions such as FR1-dependent rheumatoid factor activity and reactivity with Staphylococcus aureus protein A (54–56). In fact, rheumatoid factor B cells have been directly shown to efficiently present immune complexes and elicit fully reciprocal T-B interactions (57). These selection factors presumably contribute to the limited diversity observed at the somatic level, as also suggested in the recent murine study by Gu et al. (50).

A global restriction of human antibody diversity. The expressed antibody repertoire is thought to be limited primarily

by the size of the B cell pool, due to the presumption of a largely random V/D/J recombination, assortment of light chain and heavy chain molecules, and somatic mutation. However, the present paper suggests that the expressed repertoire in the recirculating adult B cell pool is much more restricted: 4/25 VH3 genes, 5/30 DH genes, and 1/3 JH genes. Moreover, coding diversity among germline VH3 genes (by comparing CDR R/S ratios) is 1/6 that of murine VH genes, as is the diversity produced by somatic mutation. Hence, the expressed diversity in the VH3 B cell pool may only be 0.03% of that estimated using the paradigm of maximal diversity. In view of the low CDR R/S values for germline genes of the other VH and VL families, these factors for limited diversity probably hold true for the entire human antibody repertoire.

In this light, how can one account for the satisfactory state of human antibody immunity? First, the total B cell pool in humans is large ($> 10^{11}$), and since the R/S values is greater than zero, the total antibody diversity is substantial. Second, the exceptional CDRIII diversity in humans may compensate for the low VH and JH diversity. Third, highly diversified populations of human B cells (for V/D/J use and high CDR R/S values) have been detected in contexts of apparent antigenic stimulation (58), and in the follicular center cell class of B cell neoplasms (15, 16, 45). However, the relative abundance of such B cells is uncertain, and perhaps small, considering the current study and most other reports of rearranged VH genes. If so, this would be the opposite of adult murine B cell populations in which diversified B cells are predominant. A fundamental issue to resolve will be the relative abundance of these restricted versus diversified B cell populations.

Acknowledgments

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