AUTOANTIBODIES ENCODED BY THE MOST J_H-PROXIMAL HUMAN IMMUNOGLOBULIN HEAVY CHAIN VARIABLE REGION GENE

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The human Ig heavy chain variable region gene (V_H) locus has been studied in considerable detail at the structural level; it contains ~100–200 Ig heavy chain V_H genes that have been grouped into six families (denoted V_H1 through V_H6) based on amino acid and nucleic acid sequence similarities (1–7). The different families range in size from 1 member (V_H6) to >25 members (V_H3) and contain both functional genes and pseudogenes, interspersed over the entire 2,000-kb locus (2–4, 6).

The contribution of different Ig heavy and light chain variable region gene segments to antibodies of particular specificity has been extensively characterized in murine systems (8–12). In contrast to the murine system, little is known about V_H gene utilization in different human B-lineage cell populations or in antibodies of particular specificity and function (reviewed in reference 13). To begin to characterize the contribution of human V_H gene segments to antibody specificities, we have screened antibodies produced from a large panel of IgM-secreting, EBV-transformed human B cell lines for V_H gene utilization and antigen-binding pattern. The results show that there is no obvious correlation between antigen-binding pattern and expression of members of the families V_H1-V_H5. In contrast, antibodies encoded by the V_H6 gene family displayed binding patterns reminiscent of autoantibodies present in the sera of patients with SLE. Nucleotide sequence analysis revealed that both germline and somatically mutated V_H6 genes are expressed in these antibodies and that the third complementarity-determining region (CDR3)[1] is conserved in length.

Materials and Methods

EBV-transformed Cell Lines. Mononuclear cells from peripheral blood, obtained from three healthy volunteers and from 130-d-old fetal liver and spleen were prepared and depleted of

This work was supported by the Howard Hughes Medical Institute, the Netherlands Organization for the Advancement of Pure Research (ZWO), and by National Institutes of Health grants AI-20047 and CA-40427. T. Logtenberg is a fellow of the Howard Hughes Medical Institute. F. M. Young is a Robert Wood Johnson Fellow.

[1] *Abbreviations used in this paper*: CDR, complementarity determining region; Cyt *c*, cytochrome *c*; PCR, polymerase chain reaction; Pdt, poly(dT).

T cells as described previously (14). EBV-transformed cell lines were generated under limiting dilution conditions as described previously (15).

ELISAs. Culture supernatants of EBV-transformed cell lines were screened for reactivity against a panel of 25 antigens using direct binding ELISA assays. The panel of antigens consisted of BSA, dinitrophenol-BSA, trinitrophenol-BSA, arsonate-BSA, fluorescein-BSA, human IgG, human thyroglobulin, human insulin, ovalbumin, hen egg lysozyme (Hel), tetanus toxoid, rabies virus, cytochrome *c* (Cyt *c*), diptheria toxoid, native DNA (nDNA), single-stranded DNA (ssDNA), poly(dT) (PdT), poly(A:U), bovine heart cardiolipin (Card), RNA, smRNP, pooled pneumococcal polysaccharide (Pneumovax), α1, 6 dextran and *Haemophilus influenzae* type b capsular polysaccharide. Controls, coating, and assay conditions were described elsewhere (14–17; and Logtenberg, T., manuscript submitted for publication). Binding was confirmed in liquid phase inhibition ELISA as described elsewhere (18); immunopurified IgM or crude culture supernatant was preincubated with varying concentrations of competitor and assayed for residual binding to solid-phase bound antigen. The concentration of competitor required for 50% inhibition reflects the relative avidity of the antibody for the competitor.

Northern Blotting. Total RNA from each cell line was separated on a 1% agarose gel and analyzed by Northern blotting for hybridization to probes specific for each of the six known human V_H gene families as described previously (6, 15).

cDNA Cloning and Sequencing. cDNA cloning and dideoxy sequencing were carried out as described (19, 20) with two modifications: (*a*) the starting material for cDNA synthesis was 50 μg of total cellular RNA and (*b*) blunt-ended cDNAs were cloned into Bluescript (Stratagene, La Jolla, CA). In each case both strands of cloned cDNA were sequenced.

Polymerase Chain Reaction (PCR). DNA was amplified by using the Perkin-Elmer Cetus (Norwalk, CT) GeneAmp Kit using Taq 1 polymerase. 2 μg genomic DNA in polymerase buffer was mixed with appropriate primers (final concentration 1 μM) and subjected to 35 cycles of PCR, each consisting of 1 min of denaturation at 94°C, 2 min of cooling to 37°C and 3 min of extension at 72°C. After each cycle the extension time was increased by 5 s. After amplification, one-tenth of the reaction volume was subjected to electrophoresis on a 1% agarose gel, blotted to ZetaProbe membranes and probed with the ^{32}P-labeled V_H6 probe. Primers used were TL20 5′ TCCAAGTGGTATAATG 3′, TL21 5′ TCCAAGTGGAATACTA 3′, TL22 5′ TCTTGCACAGTAATACAC 3′.

Results and Discussion

V_H Gene Utilization in EBV-transformed Cell Lines. We established 187 monoclonal IgM-secreting human B cell lines by EBV transformation of B cells (under limiting dilution conditions) from peripheral blood of three healthy adults (97 cell lines), from a 130-d-old fetal liver (36 cell lines), and from an adult spleen (54 cell lines; reference 15). RNA from these cell lines hybridized to only one of the V_H probes and the frequency of V_H gene utilization in both adult and fetal tissue–derived collections roughly correlated with the complexity of each family (15).

Antibodies from V_H6-expressing Cell Lines Bind to DNA. Antigen binding properties of IgM molecules secreted by the 187 cell lines were assayed by screening culture supernatants in direct binding ELISA for reactivity against a panel of 24 antigens. A number of antibodies that used heavy chains containing V_H segments from families 1–5 bound to particular antigens within the panel (Logtenberg, T., unpublished data); however, there was no obvious correlation between antigen-binding patterns and expression of these V_H gene families (not shown). In contrast, antibodies from each of the four V_H6-expressing cell lines in the collection bound to ssDNA and PdT. Binding to polynucleotides was not simply a matter of interaction with negatively charged molecules because no binding was observed to similarly charged molecules such as RNA and poly(A:U) (Table I, legend). Two of these clonal cell lines

TABLE I

Origin of V_H6-expressing EBV-transformed B Cell Lines and Properties of the Antibodies They Secrete

Clone no.	Origin	Isotype	Antigen					
			ssDNA	nDNA	Card	PdT	Cyt c	Hel
			μg/ml					
A10*	adult PB	IgM,κ	5.0	0.02	0.1	0.02	§	0.02
A431*	adult PB	IgM,λ	5.0	0.3	0.2	5.0	2.0	–
L16*	130 d FL	IgM,λ	5.0	–	0.13	0.001	–	–
ML1‡	130 d FS	IgM,κ	2.5	–	–	10.0	–	–

Liver and spleen cells from a 130-d-old fetus and mononuclear peripheral blood cells from three adults were transformed with EBV under limiting dilution conditions. Supernatants from monoclonal IgM-secreting B cell lines were tested in direct binding ELISA for reactivity against a panel of 24 antigens (see Materials and Methods). Results obtained from the direct binding assay were confirmed in liquid phase inhibition ELISAs with *immunopurified antibodies or ‡crude supernatant. Control competitor antigens included IgG, smRNP, and RNA. Results are expressed as the concentration of competitor required for 50% inhibition of binding to the solid phase-bound antigen. PB, peripheral blood; FL, fetal liver; FS, fetal spleen. § Indicates lack of binding to solid phase-bound antigen.

produced κ light chains with variable regions from different Vκ subgroups (Logtenberg, T., unpublished data) and the other two produced λ light chains (Table I). Therefore, the common binding activities were independent of the light chain used by the antibodies. The V_H6 antibodies from individual clones also displayed various patterns of binding activities with a limited number of antigens in the panel, notably native DNA, cardiolipin, Cyt *c* and lysozyme. Such polyreactivity is a characteristic of some anti-DNA mAbs of both murine and human origin (reviewed in reference 21).

Germline and Somatically Mutated V_H6 Gene Segments in Anti-DNA Autoantibodies. The molecular basis of the observed similarities and differences in antigen binding patterns of antibodies secreted by V_H6-expressing cell lines was investigated by analyzing cDNA clones of the Ig heavy chain mRNA expressed by each cell line. Comparison of the nucleotide sequences of the V_H6 genes expressed by fetal tissue-derived lines L16 and ML1 to that of a germline V_H6 segment (6-1G1; reference 6) revealed 100% homology (Fig. 1). An identical nucleotide sequence has been obtained from four additional germline V_H6 segments from unrelated individuals, demonstrating that the human V_H6 segment is highly conserved in the outbred human population (22, 23; and Logtenberg, T., unpublished). The V_H regions expressed by the adult peripheral blood lymphocyte-derived lines A10 and A431 differed from the prototypic germline sequence by three and six nucleotides, respectively (Fig. 1). Eight of the nine differences were concentrated in the CDR1 and CDR2 (antigen contact) regions of the V_H segment, and in each case, resulted in amino acid replacements (Fig. 2).

The pattern and extent of nucleotide substitutions in the A10 and A431 V_H6 genes suggested somatic mutations (24). To confirm this possibility, we used the PCR to determine whether the "mutated" CDR2 sequence of cell line A431 existed in germline DNA from the same donor. PCR reactions used a common 3′ primer (TL22) that represented sequences conserved in both germline and mutated V_H6 genes and

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          FR1>                                        10
6-1G1     CAG GTA CAG CTG CAG CAG TCA GGT CCA GGA CTG GTG AAG CCC TCG CAG
L16       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
ML1       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
A10       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
A431      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                    20                                                30  CDR1>
6-1G1     ACG CTC TCA CTC ACC TGT GCC ATC TCC GGG GAC AGT GTC TCT AGC AAC
L16       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
ML1       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
A10       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --G
A431      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                         FR2>              40
6-1G1     AGT GCT GCT TGG AAC TGG ATC AGG CAG TCC CCA TCG AGA GGC CTT GAG
L16       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
ML1       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
A10       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
A431      --- --- TT- --- --- --- --- --- --- --- --- --- --- --- --- ---

               CDR2>                                          60
6-1G1     TGG CTG GGA AGG ACA TAC TAC AGG TCC AAG TGG TAT AAT GAT TAT GCA
L16       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
ML1       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
A10       --- --- --- --- --- --- --- --- -A- --- --- --- --- --- --- ---
A431      --- --- --- --- --- --- --- --- --- --- --- --- A-- -C- A-- --- ---

               FR3>              70
6-1G1     GTA TCT GTG AAA AGT CGA ATA ACC ATC AAC CCA GAC ACA TCC AAG AAC
L16       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
ML1       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
A10       -A- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
A431      --- --- --- --- --- --- --- --- -C- --- --- --- --- --- --- ---

          80                                                  90
6-1G1     CAG TTC TCC CTG CAG CTG AAC TCT GTG ACT CCC GAG GAC ACG GCT GTG
L16       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
ML1       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
A10       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
A431      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

6-1G1     TAT TAC TGT GCA AGA GA
L16       --- --- --- --- --- --
ML1       --- --- --- --- --- --
A10       --- --- --- --- --- --
A431      --- --- --- --- ---
```

FIGURE 1. Nucleotide sequence of expressed V_H6 genes. The nucleotide sequence of four V_H6 cDNAs is compared with the sequence of a previously published germline V_H6 sequence (6-1G1). Dashes indicate nucleotide identity.

either a 5′ primer (TL20) specific for CDR3 of the germline V_H6 gene or a 5′ primer (TL21) specific for CDR3 of the "mutated" V_H6 gene (Fig. 3). With the 3′ germline/5′ germline primer combination, amplification from normal granulocyte DNA of the A431 donor generated a band of expected size (135 bp; Fig. 3 *B*, lane *1*) that hybridized to the V_H6 probe (Fig. 3 *C*, lane *1*); but the 3′ germline/5′ mutated primer combination failed to generate a V_H6 hybridizing band from this DNA (Fig. 3, *B* and *C*, lanes *2*). However, from A431 DNA the 3′ germline/5′ mutated combination primed amplification of the expected 135-bp V_H-6 hybridizing band (Fig. 3, *B* and *C*, lanes *3*). Together, these data demonstrate that the mutated sequence exists

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                                                  CDR1
L16       QVQLQQSGPGLVKPSQTLSLTCAISGDSVSSNSAAWNWIRQSOS
ML1       ---------------------------------------------
A10       -------------------------------L-------------
A431      -------------------------------F-------------

                    CDR2
L16       RGLEWLGRTYYRSKWYNDYAVSVKSRITINPDTSKNQFSLQLNS
ML1       ---------------------------------------------
A10       ----------K--------E-------------------------
A431      ----------NTN----------T---------------------

                    CDR3
L16       VTPEDTAVYYCARELGDAFDIWGQGTMVTVSS
ML1       --------------ETGL--Y-----L-----
A10       ----------------G--------------
A431      ------------ GDQG--Y-----L-----
```

FIGURE 2. Amino acid sequence comparison of expressed V_H-6 genes. The single-letter amino acid code is used. Dashes indicate identity with the sequence of clone F19L16.

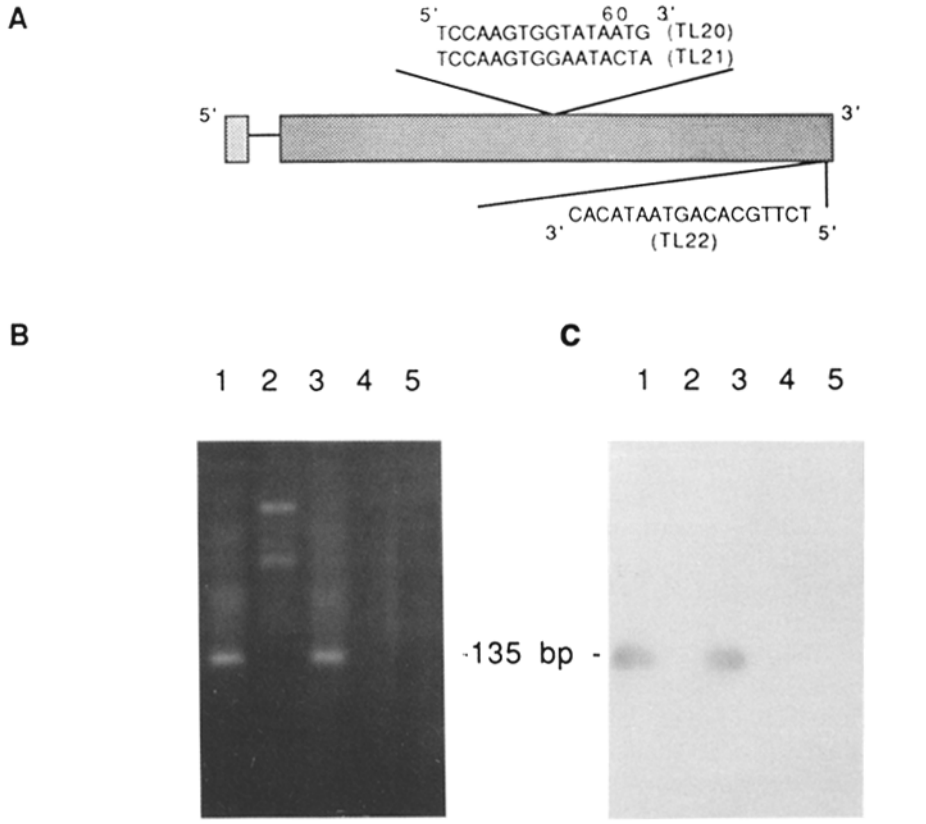

FIGURE 3. PCR Analysis of somatic mutation in expressed V_H6 Genes. (*A*) Diagramatic representation of the prototypic germline V_H6 gene with locations and directions of PCR primers TL20, TL21 (5′ end primers) and TL22 (3′ end primer). (*B*) Ethidium bromide–stained gel showing amplified product. (*C*) Southern blots showing specific amplified products after probing with ^{32}P-labeled V_H6 probe. Genomic DNA was isolated from cell line A431 and from granulocytes of the donor from whom the cell line was established. (*B*) Lane *1*, granulocyte DNA with primers TL 20 and TL22; lane *2*, granulocyte DNA with TL21 and TL22. Lane *3*, A431 cell line DNA with TL21 and TL22; lane *4*, same as lane *1* without Taq I polymerase; lane *5*, same as lane *3* without Taq I polymerase. (*C*) Lanes are numbered as in *B*.

in the DNA of the A431 line but not in normal granulocytes from the same donor. Therefore, the nucleotide sequence substitutions in cell line A431 (and most likely in A10) result from somatic mutations. The pattern and extent of replacements in the A431 and A10 V_H regions suggest that these mutations were selected over multiple generations, characteristic of memory B cells (24, 25). However, unlike memory B cells, A10 and A431 have undergone somatic mutation in the absence of isotype switching. Because T cells play an important role in directing switching, this observation might reflect absence of adequate T cell help for V_H6-expressing B cells in healthy individuals. Also, it is notable that the A10 and A431 antibodies bind with high relative avidity to nDNA, a property not displayed by antibodies that express the germline V_H6 segments; this property may reflect the mutations and/or contributions from the light chains in these antibodies (26).

Conservation of CDR3 Length. All four of the characterized $V_HD_HJ_H$ rearrangements contained short D_H segments, ranging from 9–12 bp in length (Fig. 4). D_H segments from L16 and A10 share extensive sequence homology with the D_HQ52 germline sequence; the D_HQ52 is the most J_H-proximal germline D_H segment (27) and is used frequently in EBV-transformed human fetal liver pre-B cells (28). The D_H segments used in A431 and ML1 rearrangements could not be unequivocally identified but also could have arisen from D_HQ52 rearrangement and N-region insertions. The CDR3 regions in the four V_H6 Ig heavy chains show extensive variation in amino acid sequence but are conserved in length in A10, L16, and ML1. The length of CDR3 of A431 differs by only one amino acid residue. Conservation of CDR3 length is not a property of human anti-DNA autoantibodies per se; extensive variation in CDR3 length has been found in anti-DNA antibodies that use members of different V_H families (29, 30). However, conserved CDR3 length has been noted to be characteristic of certain rheumatoid factors of mice and man (31, 32) and of antibodies that predominate in some well-studied murine immune responses (reviewed in reference 33). Such constraints on heavy chain CDR3-length may reflect regulatory and/or functional (antigen-binding) aspects of antibodies (33, 34).

Several features of the V_H6 gene segment suggest a significant function for antibodies it encodes: (*a*) The germline V_H6 gene segment is extremely well conserved at the nucleotide level in the human population; (*b*) The germline V_H6 segment encodes polyreactive anti-DNA antibodies that also bind additional (auto)antigens; (*c*) Somatically mutated V_H6 genes are contained within autoantibodies that bind with high avidity to native DNA, a property of antibodies diagnostic for SLE (28). (*d*) Based on conserved CDR3 length, V_H6 antibodies may be highly selected. In addition, the V_H6 gene family previously has been found to be unique in several ways: it has only one member (6) and that member is the most J_H proximal of all known human V_H segments (6, 22). In this regard, the organization of Ig heavy chain V_H genes influences their representation in primary antibody repertoires (reviewed in reference 35); for example, differentiating fetal murine pre-B cells preferentially rearrange and, as a result, express J_H-proximal V_H gene segments (36). Similar to proximal murine V_H gene segments, the V_H6 gene appears to be preferentially expressed in fetal liver versus adult peripheral blood lymphocytes (Berman, J., and F. Alt; manuscript in preparation). Significantly, studies of murine antibodies that use J_H-proximal V_H genes demonstrated that a high proportion exhibit binding to self-antigens (37, 38) leading to the proposal that some such antibodies play a role in the early establishment of the murine B cell repertoire (38, 39).

Clone	V_H	D_H	J_H
L16	AGA GAA CTG GGG	GAT GCT TTT GAT ATC	TGG GGC CAA GGG ACA ATG GTC ACC GTC TCT TCA
ML1	AGA GAA GAA ACG GGG	CTC TTT --C TA-	--- --- --G --A --C C-- --- --- --- --- --C ---
A10	AGA GAA CTG GGG	GAT GGT TTT	--- G-- --- --- --- --- --- --- --- --- --- --- ---
A431	AGA	GGG GAT	CAA GGC TTT --C TA- --- --- --G --A --C C-- --- --- --- --- --C ---
D_HQ52		TAA CTG GGGA	

FIGURE 4. Nucleotide sequences of V_H6DJ_H joins. For comparison, the sequence of the germline D_HQ52 segment is shown. Regions of homology with D_HQ52 are underlined. Dashes indicate sequence homology.

The molecular genetic origin of pathogenic autoantibodies in patients with SLE is unknown; they may derive from "natural" autoantibodies encoded by germline V genes present in every healthy individual (40, 41) or they may result from somatic mutations of V genes that encode other specificities (42). The V_H6-expressing B cells isolated in this study were not associated with autoimmune disease; therefore, additional factors must be required for expansion and/or activation of B cells that secrete pathogenic autoantibodies. Because the V_H6-encoded heavy chain appears to be the main contributor to the DNA-binding properties of the antibodies we tested, introduction of a functionally rearranged V_H6 gene segment into transgenic mice may generate a population of B cells that express V_H6-encoded autoantibodies. Such a mouse model may be useful in the study of factors that contribute to the generation of "autoagressive" antibodies and autoimmune disease.

Summary

Little is known about the utilization of human Ig heavy chain variable gene segments (V_H segments) in different B-lineage cell populations or in antibodies of particular specificity and function. We now demonstrate that human antibodies with Ig V_H regions encoded by the most J_H-proximal human V_H segment (V_H6) have specificities resembling those of autoantibodies present in sera of patients with systemic lupus erythematosus (e.g., anti-DNA and anticardiolipin). These specificities appear to be encoded by the germline V_H6 gene because the activity was found in multiple independent V_H6 antibodies in which the light chain varied with respect to isotype and $V\kappa$ subgroup. Features of CDR3 length and somatic mutation patterns in several V_H6 antibodies suggested that they were selected by the immune system.

We thank T. Keith Blackwell and George Yancopoulos, Gary Rathbun, and Jeffrey Berman for critically reading this manuscript.

Received for publication 24 May 1989.

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