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Evidence That Amyloidogenic Light Chains Undergo Antigen-Driven Selection

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AL amyloidosis is characterized by fibrillar tissue deposits (amyloid) composed of monoclonal light chains secreted by small numbers of indolent bone marrow plasma cells whose ontogenesis is unknown. To address this issue and to provide insights into the processes that accompanied pathogenic light chain formation, we isolated the complete variable (V) regions of 14 light (VL) and 3 heavy (VH) chains secreted by amyloid clones at diagnosis (10 Bence Jones and 4 with complete Igs, 9 λ and 5 κ) by using an inverse polymerase chain reaction-based approach free of primer-induced biases. Amyloid V regions were found to be highly mutated compared with the closest germline genes in the databases or those isolated from the patients' DNA, and mutations were not associated with intraclonal diversification. Apparently high usage of the λIII family germline gene V λIII.1 was observed (4 of 9 λ light chains). Analysis of the nature and distribution of somatic mutations in amyloid V regions showed that there was statistical evidence of antigen selection in 8 of 14 clones (7 in VL and 1 in VH). These results indicate that a substantial proportion of the amyloid clones developed from B cells selected for improved antigen binding properties and that pathogenic light chains show evidence of this selection.

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IN AL AMYLOIDOSIS, monoclonal light chains accumulate in fibrillar tissue deposits (amyloid), leading to progressive dysfunction of target organs.[1] Light chains, most frequently of the λ class, are secreted by small numbers of indolent bone marrow plasma cells (PC).[2] The natural history of the amyloidogenic clone is at present unknown. Information might be obtained from an analysis of the nature (silent [S] or amino acid replacing [R]) and distribution of somatic mutations in Ig variable (V) regions.[3] For example, evidence of clustering of R mutations in the antigen-binding loops (CDR), together with their scarcity in the framework (FR) regions (conserved areas with structural importance), is consistent with a role for antigen[4] in selecting and expanding the B cell that will eventually give rise to the amyloidogenic PC.

To provide insights into the processes that accompanied the formation of amyloidogenic light chains and PC, we isolated the complete nucleotide sequences of the Ig V regions of 14 light (VL) and 3 heavy chains (VH) secreted by amyloid clones using an inverse polymerase chain reaction (PCR)-based strategy that uses only primers specific for constant regions, so as to avoid biases for certain sequences.[5] We focused on light chains and principally on Bence Jones (BJ) proteins for the following reasons: (1) the primary structure of VL is implicated in amyloid deposition[6]; (2) secretion of free light chains only is a classic feature of AL amyloidosis (up to 40% of the cases)[7]; (3) gene usage at the germline level is unknown in AL amyloidosis; and (4) a study on multiple myeloma recently stressed the importance of VL in the characterization of the role of antigenic selection on the B cell.[8]

MATERIALS AND METHODS

Patients and bone marrow studies. Fourteen patients with biopsy-proven light chain amyloidosis were studied at diagnosis. Bone marrow aspirates were obtained after informed oral consent was given. Analysis of bone marrow PC light chain κ/λ ratios[7] and labeling indexes[9] were performed with immunofluorescence procedures. Monoclonal components were detected by immunofixation of serum and urine using anti–isotype-specific rabbit antisera (Dako, Glostrup, Denmark).

Inverse-PCR amplification; cloning and sequencing of bone marrow monoclonal light and heavy chains. The inverse PCR-based procedure used to isolate the Ig V regions of AL amyloidosis patients has recently been described in detail.[5] Briefly, double-stranded cDNA from Ficoll-separated bone marrow mononuclear cells was blunt-ended, ligated upon itself to form a circle with T4 DNA ligase (GIBCO-BRL, Life Technologies, Grand Island, NY), and PCR-amplified using oligonucleotides specific for the 5′ and 3′ of the heavy and light chain constant region isotypes. Amplimers are oriented toward the V region (A primers) or toward the 3′ end of constant regions (B primers); consequently, the amplification product consists of (5′→3′): namely, the 3′ end of the constant region and the untranslated region, poly-A tail, 5′ untranslated region, leader, V region, and the 5′ end of the constant region. The PCR products obtained from 3 to 4 independent amplification rounds were pooled, gel-purified, and cloned into plasmid. Several plasmid inserts were then sequenced from both sides using an automated DNA sequencer[5] and compared with each other. The presence of the same V region sequence in several clones indicates its monoclonal origin, because no primer-induced bias can be introduced during amplification and the fraction of plasmid clones with a given V region sequence is proportional to the amount of its mRNA in the bone marrow.[5] The accuracy of this method was tested by amplifying, cloning, and sequencing a plasmid containing a V region fragment of known sequence; only one mismatch of a total of more than 2,000 bases was noted.[5]

Data analysis and identification of mutations in the monoclonal V regions of AL amyloidosis. To identify the presumed germline gene of monoclonal V regions, alignment was made with the current releases of EMBL-GenBank and V-BASE (V BASE Sequence Directory; Tomlinson et al, MRC Centre for Protein Engineering, Cambridge, UK) sequence directories using the BLAST[10] and DNAPLOT (H.-H. Althaus, University of Cologne, Cologne, Germany) search tools, respectively. A binomial distribution model[4] was used to determine the

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likelihood that the observed R mutations in a gene segment occurred by chance. The formula predicts the expected number of R mutations and is based on the total observed mutations (R+S), the R mutations found in the CDR or FR, the relative lengths of the CDR or FR, and the expected proportion of R mutations (Rf). Nucleotides in the CDR show greater susceptibility to generating R mutations, ie, substitutions in these regions are more likely to produce amino acid replacements.[11] For this reason, specific Rf values were calculated for each germline segment according to Chang and Casali.[11]

Isolation of VL germline genes. After identification of the presumed VL germline gene through database searching, the patients' own germlines were isolated by means of an adaption of established procedures.[12,13] DNA from the peripheral blood neutrophils of 5 patients was PCR-amplified using 5′ primers complementary to both the leader sequences of the monoclonal V regions and germline genes VλIII.1 (5′-ttcctcggcgtccttgctta-3′: patients SEM, PAP, DIB, and DEP) and B3 (primer VκIV LEA[13]: patient QUA) and 3′ primers that anneal to regions of the 3′ recombination signals that are highly conserved within gene families.[12,13] The generality of the 3′ primers allows coamplification of related germline genes.[12,13] After cloning, six plasmid inserts were sequenced as described above.

Amino acid sequencing of light chains from amyloid fibrils and BJ proteins. Amyloid fibrils from patients CAR (IgGκ) and DEP (IgAλ) were extracted from biopsy specimens. Light chains were digested with trypsin and peptides were purified by reverse-phase chromatography following reported procedures.[14] PAP and SEM λ BJ proteins were isolated from urine. Amino acid sequencing was performed by adsorptive biphasic column technology using an HPG-1000 A protein sequenator (Hewlett Packard, Palo Alto, CA) as previously described.[15]

RESULTS

Isolation of VL and VH regions secreted by amyloid clones. Table 1 reports the characteristics of the 14 patients at diagnosis of AL amyloidosis. Bone marrow PC κ/λ ratios showed expansions of PC with the same isotype as the monoclonal component. PC in the S-phase of the cell cycle (PC labeling indexes) were extremely rare, a typical finding in AL amyloidosis.[16] There was no evidence of associated multiple myeloma. In 7 cases, it was possible to observe patients for more than 2 years; the disease remained stable with only slight modifications in the bone marrow PC numbers.

V regions from bone marrow cDNA were inverse-PCR amplified and cloned into plasmid, and multiple inserts were sequenced. The number of identical/sequenced plasmid inserts for each patient is included in Tables 2 (VL) and 3 (VH). In each case it was possible to identify a single, identical, repeated V region. The other sequences were different from one another. A minimum of 3 (Table 2; SET IgGλ) and an average of 6.6 repeated plasmid clones were sequenced for each patient. Even when 9 repeated clones were sequenced from the same patient (Table 2; QUA BJκ, CAR IgGκ, DEP IgAλ), no significative nucleotide substitution was observed. We detected only 11 mismatches in more than 42,000 sequenced basepairs (112 plasmid clones), a result that is fully compatible with the estimated error of the cloning procedure.[5] No clones presented more than one mismatch with the predominant sequence. Nucleotide changes were present mainly in the FR (9/11) and none in the CDR3; the latter, subject to the greatest in vivo variation, were isolated to single plasmid inserts, ie, they were not found to occur more than once and were distributed in different patients. Patient SET had only 3 of 10 identical plasmid inserts (Table 2); this result is compatible with a small λ amyloid PC clone, as documented by the slight deviation in the bone marrow PC κ/λ ratio (Table 1).

Table 1. Clinical Findings and Laboratory Values of the 14 Patients With AL Amyloidosis Studied

Patients	Age (yr)	Main Organ Involved*	Monoclonal Component	BMPC %	BMPC k/λ Ratio†	BMPC LI%
QUA	59	Heart	BJκ	7	49.0	0.2
TAU	49	Kidney, liver	BJκ	10	5.41	ND
IAC	55	Liver	BJκ	4	61.5	0.2
ORR	65	Heart	BJκ	10	7.3	0.0
DIB	70	Kidney	BJλ	7	0.28	0.1
PAP	53	GI	BJλ	10	0.2	ND
SEM	46	Muscle, GI	BJλ	5	0.02	0.2
FER	51	Muscle, GI	BJλ	3	0.11	0.0
GHE	78	Heart	BJλ	10	0.02	0.1
FIL	57	Heart	BJλ	4	0.07	0.0
CAR	62	Skin, nerves	IgGκ + BJκ	10	40.67	0.0
SET	56	Lung	IgGλ	3	0.67	0.4
MAR	73	Heart	IgAλ	7	0.13	ND
DEP	48	Skin, heart	IgAλ + BJλ	10	0.23	0.2

Abbreviations: GI, gastrointestinal tract; BMPC, bone marrow PC; LI%, labeling index; ND, not determined.

*Main organ involved at diagnosis.

†Normal values are ≥1.1, ≤2.6.[7]

Cloned VL regions correspond to the light chains isolated from fibrils and urine. In 4 cases, we compared the derived amino acid sequences of cloned VL regions with partial protein sequences of light chains extracted from amyloid deposits (residues: CAR κ = 1 to 30; DEP λ = 2 to 40 and 62 to 87) and of BJ proteins (residues: PAP λ = 5 to 21; SEM λ = 2 to 38). Protein-derived sequences confirmed identity in both germline coded and somatically mutated residues (see ''Light and heavy chains from amyloid clones are highly mutated. Analysis of somatic mutations''), thus demonstrating that the correct monoclonal VL regions had been identified.

Gene usage by amyloid clones. For the most part, λ and κ amyloid light chains used genes that belong to the most numerous gene families: VλIII (5 of 9 light chains) and VκI (3 of 5; Table 2). The germline gene VλIII.1,[17] also known as DPL23[12] and 3r,[18] was rearranged in 4 of 9 cases. By contrast, κ light chains used various germline genes. There was almost constant usage of Jλ2/3 (8 of 9 λ light chains; Fig 1A), the most commonly rearranged Jλ segment in general,[17,19] whereas 2 of 5 κ light chains used Jκ3 (Fig 1B), which is not frequently employed in peripheral blood κ-positive lymphocytes (<10% of functional rearrangements).[20]

VH region analysis is reported in Table 3. Two heavy chains used members of VHIII gene family and one used a member of VHI. The JH4b segment was employed in all cases (Fig 2).

CDR3 formation mechanism. Figure 1 illustrates the most likely mechanism of amyloid light chain CDR3 formation. Trimming of VL and/or JL segments occurred in 9 of 14 cases, and in 5 instances (~36%), nongermline nucleotides were found at junctions. Both templated (P; Fig 1, nucleotides in parentheses)[21] and nontemplated (N) nucleotides were apparently observed. Figure 2 shows the CDR3 formation of VH. In this case, too, trimming of JH and N nucleotides were found.

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Table 2. The VL and JL Germline Segments Used by Amyloidogenic Light Chains and the Number of Identical/Sequenced Plasmid Inserts

Amyloid Clones	VL Germline (family)	Nucleotide Identity* (%)	CDR			FR			Identical/ Sequenced Inserts
			R	S	*P* (CDR)†	R	S	*P* (FR)	
QUA BJκ	B3 (κIV)	94.1	9 (4.4)‡	3	.012	3 (9.4)	3	.002	9/9
TAU BJκ	O2/O12 (κI)	91.6	9 (5.0)	3	.029	6 (13.4)	6	.002	8/9
IAC BJκ	L2 (κIII)	91.9	4 (4.7)	0	.202	13 (12.8)	6	.165	8/8
ORR BJκ	L1 (κI)	94.4	4 (3.3)	1	.205	7 (8.9)	4	.125	7/7
DIB BJλ	λIII.1 (λIII)	94.0	9 (3.6)	2	.003	5 (9.2)	1	.025	7/7
PAP BJλ	λIII.1 (λII)	89.8	15 (6.1)	2	.0002	8 (15.7)	4	.002	5/6
SEM BJλ	λIII.1 (λIII)	94.7	6 (3.2)	2	.053	5 (8.1)	2	.057	6/6
FER BJλ	1b (λI)	95.9	7 (2.8)	1	.008	2 (6.1)	2	.014	5/5
GHE BJλ	2b2 (λIII)	93.3	6 (4.5)	2	.144	8 (10.4)	4	.1	6/6
FIL BJλ	4b (λIV)	96.0	7 (2.8)	1	.008	2 (6.3)	2	.01	5/6
CAR IgGκ	O8/O18 (κI)	95.8	5 (2.5)	1	.063	4 (6.7)	2	.070	9/10
SET IgGλ	λ3.1 (λIII)	95.4	7 (2.7)	1	.008	3 (7.0)	2	.019	3/10
MAR IgAλ	2b2 (λII)	92.6	7 (5.0)	0	.111	10 (11.4)	5	.140	7/11
DEP IgAλ	λIII.1 (λIII)	95.4	5 (2.8)	2	.082	4 (7.0)	2	.055	9/9

*When compared with the closest germline gene. The leader is excluded from identity calculations to allow comparison with other studies.
†Probability that the observed R mutations occurred by chance (calculations according to Chang and Casali[11]).
‡Number in parentheses indicates number of R mutations expected by chance.

Light and heavy chains from amyloid clones are highly mutated. Analysis of somatic mutations. To test amyloid V regions for the presence of somatic mutations, their nucleotide sequences were compared with the closest germline genes in the databases. In 4 λ cases (DEP, PAP, SEM, and DIB) and 1 κ case (QUA), the corresponding germline segment was also looked for in the patients' neutrophil DNA and a gene identical to the sequences published was found (data not shown; sequences available from GenBank [accession nos. AF026934-38]).

The deduced amino acid sequences of the amyloid VL regions are depicted in Fig 3; their nucleotide differences as compared with the corresponding germline gene are summarized in Table 2. VL sequences deviated substantially from the closest germline genes, with a median percentage of mutation of 5.7% and a wide range (4.0% to 10.2%). The mutation rate was apparently similar in λ and κ light chains, BJ proteins, and light chains that are part of a complete Ig. By contrast, the portion coding for the leader peptide (which is deleted from the mature V region) showed no, or rare, nucleotide differences from the germline gene sequence (the median mutation rate decreases from 5.7% to 5.0% [range, 3.4% to 8.8%] when the leader is included in the comparison), thus substantiating correct identification of the corresponding germline gene.

The derived amino acid sequences of the VH regions of the amyloid clones are shown in Fig 4, and the results of detailed analysis of somatic mutations and gene usage are recapitulated in Table 3. These findings were similar to those reported for light chains, with a wide range in somatic mutation (from 3.7% to 10.5% of nucleotide substitutions).

Somatic mutations were also present in JL and JH segments (Figs 1 and 2).

Nucleotide sequences of the V regions were submitted to the EMBL-GenBank databases (accession nos. Z66542, AF026918-33).

Replacement mutations and antigen selection in amyloid Igs. Clonal expansion takes place in the germinal center cells that acquired mutations that improve antigen binding while preserving the correct folding of the Ig V region. Ig subjected to several rounds of antigenic selection are expected to exhibit clustering of R mutations in the antigen-binding loops (positive selective pressure for amino acid changes), whereas, on the contrary, R mutations would be less frequent in the FR (negative selection for amino acid substitutions).[4] A binomial model tests whether the observed distribution of R mutations follows this pattern.[4] The results of this statistical analysis are included in Tables 2 (VL) and 3 (VH). Consistent with selection by Ag, 13 of 14 VL and 2 of 3 VH segments displayed higher numbers of R mutations in the CDR than those theoretically expected (Tables 2 and 3). In accordance with the preservation of amino acids in the FR regions, all but one of the VL and VH segments showed lower numbers of R mutations than those expected for a randomly mutating gene segment (Tables 2 and 3). This difference was statistically significant in 7 of 14 VL and 1 of 3 sequenced VH segments ($P \leq .01$ in 5 VL; $P \leq .05$ in 2 VL and 1 VH; Tables 2 and 3). That 7 of 14 amyloidogenic light chains

Table 3. The VH and JH Germline Segments Used by Three Amyloidogenic Clones and the Number of Identical/Sequenced Plasmid Inserts

Amyloid Clones	VH Germline (family)	Nucleotide Identity* (%)	CDR			FR			Identical/ Sequenced Inserts
			R	S	*P* (CDR)†	R	S	*P* (FR)	
CAR IgGκ	3-48 (VHIII)	92.2	3 (4.2)‡	1	.193	12 (13.2)	7	.144	6/7
MAR IgAλ	3-30 (VHIII)	89.5	8 (5.4)	3	.082	12 (17.9)	8	.015	6/8
DEP IgAλ	1-18 (VHI)	96.3	5 (1.9)	0	.025	5 (6.4)	1	.165	6/6

*When compared with the closest germline gene. The leader is excluded from identity calculations to allow comparison with other studies.
†Probability that the observed R mutations occurred by chance (calculations according to Chang and Casali[11]).
‡Number in parentheses indicates number of R mutations expected by chance.

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A

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                <----------------------CDR3---------------------->
                Vλ                                                Jλ
          89  90  91  92  93  94  95   95A  95B     95C  96  97  98  99  100 101 102 103 104 105 106 107 108

1b        gga aca tgg gat agc agc ctg  agt  gct       t  gtg gta ttc ggc gga ggg acc aag ctg acc gtc cta ggt Jλ2/3
FER       --- --- --- --- --- G-- ---  -C-  --c       *  --- --c --- --- --- --- --- --- --- --- --- --- ---

2b2       tgc tca tat gca ggt agt agc  act  ttc
MAR       --- --- --- --- --- C-- GC-  ---  -** G     *  **- --g --- --- --- --- --- --- t-- --- --- --- ---
GHE       --- --- --- --- --- --- -At  GG-  ***       *  --- --t --- --- --- --- --- --- --- --- --- --g ---

III.1     cag gcg tgg gac agc agc act  gca
DIB       --- -T- --- -C- --- --t -G-  -A*            -  --a A-- --- --- --- --- --- --- --- --- --- --- ---
PAP       --- --- --- --- GTt -A- C--  -A-   GTC(CA)  -  --- --- --t --- --- --- --- --- --- --- --- --- ---
SEM       --- --- --- --- --- --- G--  --g     TTT    *  *** *** --- --- --- --- --- --- --- --- --g ---
DEP       --- --- --- --- -C- -C- ---  --*            *  **- --- --- --- --- --- --- --- --- --- --t ---

3.1       aac tcc cgg gac agc agt ggt  aac cat         t  tat gtc ttc gga act ggg acc aag gtc acc gtc cta ggt Jλ1
SET       --- --- --- --- -C- --- ---  --- ---         *  --- --- --- --- --- --- --- --- --- --- --- --g ---

4b        cag acc tgg ggc act ggc att  ca
FIL       --- --- --- AA- --- -A- --c  A-              *  **- --g --- --- --- --- --- --- t-- --- --- --- --a Jλ2/3
```

B

```
                <----------------------CDR3---------------------->
                Vκ                                                Jκ
          89  90  91  92  93  94  95   95A     95B   96  97  98  99  100 101 102 103 104 105 106 107 108

O12/O2    caa cag agt tac agt acc cct  cc        g   tgg acg ttc ggc caa ggg acc aag gtg gaa atc aaa cgt   Jk1
TAU       --- --a --- -C- -C- TA- --c  -*        *   *-- --- --- --- --- --- --- --- --- --- --G --- --a

O18/O8    caa cag tat gat aat ctc cct  cc        g   ctc act ttc ggc gga ggg acc aag gtg gag atc aaa cgt   Jk4
CAR       --- --a --- A-- G-- --- ---  --  (G)   *   *** --- --- --- --- --- --- --- --- --- --- -C- ---

L1        caa cag tat aat agt tac cct  cc        a   ttc act ttc ggc cct ggg acc aaa gtg gat atc aaa cgt   Jk3
ORR       --- --- --- --- -T- --- --*  **        -   --- --- --- --- --- --- --- -C- --- --- --- --- ---

L2        cag cag tat aat aac tgg cct  cc        g   tgg acg ttc ggc caa ggg acc aag gtg gaa atc aaa cgt   Jk1
IAC       --- --- --- --- --- --- ---  --        -   --- --- --- --- --- --- --- --- --- --- --- --- ---

B3        cag caa tat tat agt act cct  cc        a   ttc act ttc ggc cct ggg acc aaa gtg gat atc aaa cgt   Jk3
QUA       --- --g --- --- --- --- ---  --  (GG)GG   *   --- --- --- --- --- --- --- --- --- --G --- --- --a
```

Fig 1. Formation of amyloidogenic light chain CDR3. The most likely mechanism is reported. Sequences are compared with the closest VL and JL segments. (A) λ light chains; (B) κ light chains. R mutations, upper case letters; S mutations, lower case letters. Nontemplate coded nucleotides (N) are shown in bold; P nucleotides are in parentheses. *Nucleotides apparently removed at recombination.

originated from selected somatic mutations accumulated during an antigenic response is further substantiated by the significant rarity of FR-R mutations ($P \leq .01$ in 4 and $P \leq .05$ in 3 VL; Table 2).

In the remaining 7 amyloid VL, the observed R mutations in the CDR and FR almost reached significativeness in 3 (Table 2; SEM BJλ, CAR IgGκ, and DEP IgAλ), whereas the others demonstrated CDR-R and FR-R mutations close to the numbers predicted by chance.

In the case of DEP IgAλ, significant clustering in the CDR was present in VH rather than in VL (Table 3). Therefore, our analysis showed that 8 of 14 (57%) amyloidogenic PC clones manifested statistical evidence of antigenic selection.

Overall, the frequency of CDR-R mutations (8.8×10^{-2} CDR-R/base) found in the 14 amyloidogenic light chains was about twice that expected (4.7×10^{-2} CDR-Rexp/base; $P < 1 \times 10^{-4}$, χ^2 test). In accordance with structural preservation of FR regions, the rate of R mutations in these areas (2.8×10^{-2} FR-R/base) was 1.6 times lower than expected (4.5×10^{-2} FR-Rexp/base; $P < 3 \times 10^{-4}$).

DISCUSSION

Our results indicate that many amyloid clones develop from B cells whose Ig were subjected to antigenic selection and that pathogenic light chains manifest evidence of this process.

V regions varied substantially from their germline counterparts and the differences were attributed to somatic mutations. Isolation of the corresponding VL germline gene from the DNA

```
                          N - D - N                          JH segments
                |----------------CDR3---------------------||----------------FR4-------------|
                F  I  T  V  V  R  G  V  V  Y  R  S  F  D  F  W  G  Q  G  T  L  V  A  V  S  S
CARγ      tttataactgtggttcggggagttgtttatcgttcttttgacttctggggccagggaaccctggtcgccgtctcctca
DXP'1       ---t---A--------------A--          -------A----------------------A----------- JH4b

MARα      S  S  R  R  T  L  D  L                          W  G  Q  G  T  L  V  S  V  S  S
          agttcacggcgcactcttgacctg                         tggggccagggaacgctggtcagcgtctcctca
DHQ52                      -A---                         --------------c-------C---------- JH4b

DEPα      V  Q  Y  S  D  T  S  G  Y  Y  N  P               W  G  Q  G  T  L  V  T  V  S  S
          gtccaatactctgatactagtggttattacaatccg              tggggccagggaaccctggtcaccgtctcctca
D21-9        -T----A-----G-------------T-                 -------------------------------- JH4b
```

Fig 2. Formation of heavy chain CDR3 from amyloid clones. The most likely mechanism is shown. Sequences are compared with the closest DH and JH segments. R mutations, upper case letters; S mutations, lower case letters.

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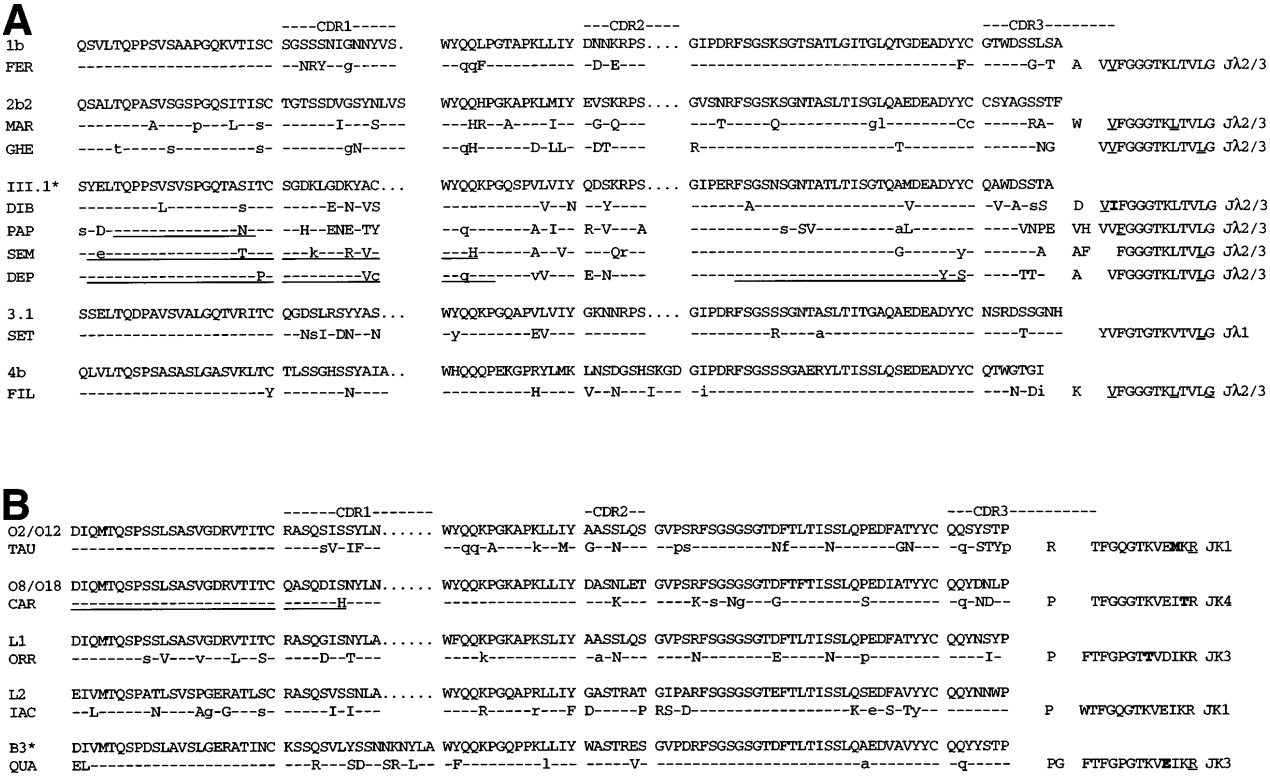

Fig 3. Deduced amino acid sequences of the VL regions of amyloidogenic light chains. Comparisons are made with the most homologous germline gene. (A) λ light chains; (B) κ light chains. R mutations, upper case letters; S mutations, lower case letters; R mutations in JL are shown in bold, S mutations are underlined. *Germline segment isolated from patient DNA. A continuous line indicates the amino acid sequence that was also found in the monoclonal proteins isolated from amyloid fibrils (DEP and CAR) and urine (PAP and SEM). Protein sequencing of the N-terminal portion of λ light chains showed the absence of the first amino acid in DEP and SEM and of 4 residues in PAP. The absence of the first residue occurs in many λIII light chain protein sequences,[22] whereas multiple amino acids were missing in an amyloid λ light chain recently reported.[23]

of patients' neutrophils ruled out possible genetic factors such as allelic variants or better-matching new germline genes in 5 cases. Furthermore, a major contribution of allelic polymorphism or undiscovered germline genes to nucleotide changes has been reported to be highly unlikely in Vκ[24,25] and most probably in Vλ as well.[18] In addition, the discrepancy between the monoclonal V sequences and their germline counterparts was too great (median of 5.7% in VL and from 3.7% to 10.5% in VH) to be attributed exclusively to polymorphism. That somatic hypermutations occurred on amyloid V regions is further substantiated by the presence of deviations from the germline sequence in JL and JH segments.

Sequencing of multiple plasmid clones containing PCR-amplified VH and VL regions showed the identity of repeated sequences. Somatic mutations were therefore homogeneous; there was no significant in vivo intraclonal variation in the V regions expressed by these amyloid clones at diagnosis. This is consistent with a clonal cell that is no longer under the influence of the hypermutation process in the germinal center.[26] This result was expected in clones secreting free light chains only (the lack of heavy chains prevents antigen receptor formation and, consequently, Ig hypermutation), but homogeneity of somatic mutations was also observed in the few cases in which complete Ig were available. Recently, a study documented a modest degree of intraclonal diversification in a minority of patients with monoclonal gammopathy of undetermined significance.[27] Although we cannot exclude the possibility that this phenomenon may also occur in some amyloid clones, our results suggest that this finding, if present, must be rare in amyloidosis, in which BJ clones can constitute up to approximately 40% of all cases.[7]

Our data show frequent usage of the most numerous germline

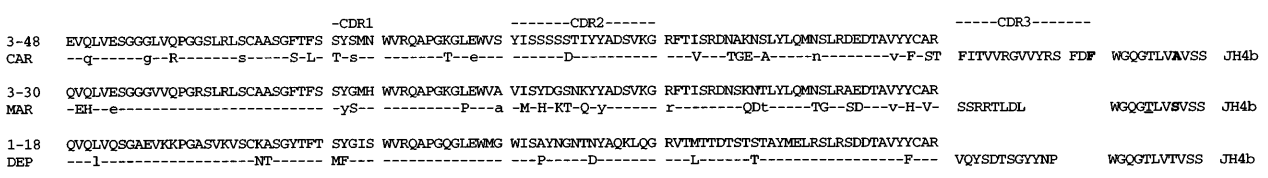

Fig 4. Deduced amino acid sequences of the VH regions secreted by amyloid clones. Comparisons are made with the most homologous germline gene. R mutations, upper case letters; S mutations, lower case letters; R mutations in JH are shown in bold; S mutations are underlined.

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gene families, namely VκI and VλIII. At the level of germline genes, we found that the VλIII.1 alone (of a total of 30 functional Vλ genes)[18] accounted for 4 of 9 amyloid λ light chains and for 4 of 5 amyloid light chains of the λIII family. By contrast, such a bias was not observed in amyloid κ light chains (Table 2) or in myeloma λ light chains.[8] Sequencing of the coding portions of VλIII.1 germline gene from these four unrelated amyloid patients showed their identity to nonamyloid subjects.[12,17] Usage restriction of germline genes appears to be a feature of the normal humoral response.[3,19] This has been shown for VH[28] and Vκ genes,[13,29] whereas Vλ are only now being investigated. Overrepresentation of VλIII.1 in AL amyloidosis might therefore be apparent, due to its preferential expression in λ light chains in general or to limited patient sampling. VλIII.1 is the closest gene to the Jλ-Cλ cluster, being only 14 kb away,[17] and this proximity may predispose it to rearrangement. However, only 1 of 6 myeloma λ light chains used this V gene segment,[8] and analysis of a compilation of antibodies to various complex antigens from hybridomas/B-cell clones showed involvement of VλIII.1 in just 2 of 26 λ light chain rearrangements.[19] Taken together, this evidence suggests that Vλ gene usage in AL amyloidosis might be unique and that the high frequency of VλIII.1 expression may reflect some intrinsic amyloidogenic properties of this gene. More nucleotide sequencing of amyloid and nonamyloid λ light chains is needed to test the association between VλIII.1 and amyloidosis.

Light chain CDR3 showed trimming of V and J segments and insertion of P and N nucleotides, thus showing, besides mutations, intense exonucleasic and transferasic activity during both κ and λ amyloidogenic light chain rearrangements (Fig 1). Therefore, junctional variation (due to truncations or insertions) appears to be a general phenomenon of antibody diversification that involves κ[29,30] as well as λ light chains.

According to the type and distribution of somatic mutations and the binomial distribution model of probabilities, there was statistical evidence of antigenic selection in 8 of 14 clones (~57%). VL alone was sufficient to show selection in 7. Analogously to light chains that are part of a complete Ig, BJ proteins were highly mutated and showed nucleotide changes compatible with antigen-driven selection; because this process can only operate on B lymphocytes with surface Ig receptors, which are composed of both light and heavy chains, selection of the BJ clone most likely occurred before the heavy chain was lost.

Sahota et al[8] analyzed myeloma VH and VL regions and found that 10 of 15 cases (~67%) showed evidence of clonal selection (VL contributed to 4 cases and VH to the other 6), a result that is quite similar to what was found in our study (8 of 14 amyloid clones with significant concentration of R mutations in the CDR). The mutation rate of amyloid light chains is also comparable to that observed in myeloma, with a median of 5.7% for amyloidogenic VL and 5.8%[8] and 8.2%[31] for myeloma VL and VH, respectively. A similar degree of somatic mutations was also found in the VH regions of patients with monoclonal gammopathy of undetermined significance, but evidence of clone selection is still not clear in this condition.[27] These results suggest that myeloma[8,27,31-33] and AL amyloidosis progenitors similarly undergo somatic hypermutation and antigenic selection and, together with the apparent absence of significant intraclonal diversification, indicate that the transformation generating the expanded amyloidogenic marrow PC population probably occurs very late, after completion of B-cell maturation and selection.

Despite numbers of R mutations in the CDR generally higher than those predicted by chance (Tables 2 and 3), clustering of CDR-R mutations was not significant in about 40% of amyloid clones. However, it should be kept in mind that this type of analysis is limited to the study of the V segment, which comprises only part of the antigen binding loops in the mature V region: CDR1 and CDR2 and, in light chains, the 5′ portion of CDR3. Therefore, the contribution of CDR3, which is often essential for optimal antigen recognition,[34] could not be addressed fully in this study.

Light chain deposition disease, another condition characterized by monoclonal light chain tissue deposition, though most frequently of the κ type and lacking the characteristic birefringence of amyloid deposits, has been studied using biochemical and genetic approaches.[35] Similarly to our findings in AL amyloidosis, sequencing data[36] in this latter condition also suggest possible overrepresentation of a germline gene, B3, the only member of the VκIV subgroup. R substitutions, likely caused by somatic hypermutation, were also found here preferentially in the CDR of two VκIV light chains.[37,38]

Dimers of free light chains may function as primitive antibodies, because they can structurally mimic the combining site,[34] and it has been proposed that the initial event leading to amyloid formation might be an antigen-antibody interaction involving amyloid light chains and tissue components and that this phenomenon may account for the heterogeneity of organ involvement typically observed in AL amyloidosis.[39] According to mutation analysis, a substantial proportion of amyloidogenic light chains have genetic features that are not incompatible with this hypothesis; evidence is shown that many amyloid forming light chains were synthesized by clones selected during antibody response to a T-cell–dependent antigen and may therefore possess the capacity to interact with specific ligands, albeit to a lesser extent than intact Ig.

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