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Tracking of the V_{4-34} (V_H4–21) gene in human tonsil reveals clonal isotype switch events and a highly variable degree of somatic hypermutation

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SUMMARY

The V_{4-34} (V_H4–21) gene has been found to encode certain IgM autoantibodies, and is mandatory for pathological IgM anti-erythrocyte antibodies of I/i specificity. The gene is also commonly used by normal IgM-positive B lymphocytes, but its involvement in B cells which have undergone class switching to IgG or IgA is less clear. In order to track V_{4-34} gene usage and class switching events during a normal immune response, we have probed RNA in a limited area of human tonsil. Results indicate that the V_{4-34} gene undergoes class switching to IgG or IgA, with the progeny either remaining unmutated or containing large numbers of somatic mutations. Mutational patterns indicate possible 'hot spots', and some mutations appear deleterious. At the level of individual B cells, we have tracked a clonal isotype switch event from IgM to IgA, with each retaining close to germ-line configuration. In addition, we have followed a clonal switch from a mutated IgM to IgG, with no further accumulation of somatic mutations. These data indicate that the V_{4-34} gene is involved in a maturing immune response, and that the routes to production of IgG or IgA antibodies are various.

Keywords V_H genes B cells somatic mutation class switching

INTRODUCTION

The V_{4-34} (V_H4–21) gene segment, a member of the V_H4 family, has been widely studied. One of the reasons for this has been the availablility of our MoAb, 9G4, which has been found to be highly specific for immunoglobulins encoded by V_{4-34} [1–3]. Using this reagent, we were able to show that the gene is active early in development, and that a significant percentage of B cells in normal lymphoid tissue expresses immunoglobulins encoded by V_{4-34} [1]. A further interesting aspect, confirmed by sequencing the involved V_H genes, is that the gene is mandatory for the pathological anti-erythrocyte antibodies of I/i specificity [4,5]. The V_{4-34} gene is also able to encode anti-DNA antibodies, and certain other antibodies [6,7]. One of the structural features of the gene product is that recognition of erythrocyte antigen appears to be via a framework sequence [8]. V_{4-34}-encoded antibodies therefore can bind autoantigen via unconventional sites in a manner similar to binding of Staphylococcal protein A by V_H3-encoded antibodies [9]. In contrast, recognition of DNA by V_{4-34}-encoded immunoglobulin appears to be via conventional sites in CDR3 [6], indicating that this V_H gene, and possibly others, can recognize autoantigens through different regions of the V_H sequence.

Involvement of V_{4-34} in immune responses to exogenous antigens has not been as thoroughly investigated. However, the gene is used in response to immunization with blood group Rh antigens, mainly by those of IgM class [7]. It has also been found to encode antibodies against bacterial lipid A, again of IgM class [10,11]. The fact that V_{4-34} is used early in development, and continues to be expressed by $\approx$ 6% of normal adult B cells [12,13], suggests that the encoded immunoglobulin represents an important component of the repertoire. However, levels of V_{4-34}-encoded immunoglobulin are surprisingly low in normal serum IgM or IgG [1], suggesting that the circulating B cells may be anergic. If that is the case, anergy can be easily broken by infection with Epstein–Barr virus (EBV) or *Mycoplasma pneumoniae*, where serum levels can increase dramatically [2].

The ability of B cells using the V_{4-34} gene to undergo immunoglobulin class switching to IgG in blood lymphocytes has been analysed at the genetic level [14,15]. In normal blood, V_{4-34}-Cγ transcripts were found, although in small numbers, and with evidence of expanded clonally related B cells [15]. Following infection with EBV, V_{4-34}-Cγ transcripts were again identified, and cells secreting V_{4-34}-encoded IgG were immortalized in culture [14]. However, in both studies, only limited numbers of distinct V_{4-34} sequences which have switched to IgG were identified. One of the reasons for this may be that there are few IgG-positive B cells in blood. Interestingly, V_{4-34} has so far not been found to encode an IgG or IgA myeloma protein ([16], and our own unpublished study of over 200 myeloma proteins), raising the question of the frequency with which these B cells undergo isotype switching. In fact, one study of V_H4 gene expression in the tonsil of

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a 4-year-old child revealed a predominence of IgM sequences and only a single IgG sequence, which showed a relatively low degree of somatic hypermutation [17]. In order to extend these observations in a site where immunoglobulin class switching is known to occur, and to follow accumulation of somatic mutations in a wider profile of immunoglobulin classes, we analysed V_{4-34}-Cμ, V_{4-34}-Cα and V_{4-34}-Cγ transcripts in tonsil.

MATERIALS AND METHODS

Cell preparation

Tonsils were removed from a 6-year-old boy undergoing a routine tonsillectomy. Approximately 5–10 mg of solid tissue were dissected and lysed in RNAzol B (Cinna Biotecx, Houston, TX). The RNA was isolated and reverse transcribed using an oligo dT primer according to the manufacturer's protocol (Promega, Madison, WI).

Amplification and sequencing of the V_{4-34} genes

A nested polymerase chain reaction (PCR) approach was used to allow amplification all the V_{4-34} sequences present within the tissue section. One tenth of the cDNA was initially amplified with primers specific for the V_H4 leader [2] together with either a Cμ [2], Cα (CαII) or Cγ (Cγ100) primer [14]. One twentieth of this initial PCR product was then further amplified with a V_{4-34}-specific primer [14] and internal primers for Cμ [18], Cα [14] and Cγ [19]. PCR conditions in both cases consisted of an initial denaturation step of 95°C for 5 min followed by 25 cycles of 94°C, 1 min; 56°C, 1 min; and 72°C, 1 min, followed by a final extension step of 72°C for 5 min. Amplified products were gel-purified, cloned by ligation into pGEM-T vector and transfected into JM109 competent bacteria (Promega). Nucleotide sequence analysis was by the dideoxy chain termination method using the -20 and reverse M13 primers. Alignment was made to V-BASE (Tomlinson *et al.*, MRC Centre for Protein Engineering, Cambridge, UK) using MacVector 4.0 sequence analysis software (IBI, New Haven, CT).

Mutational analysis of the V genes

The number of expected replacement (R) mutations in the complementarity determining regions (CDR) was calculated according to the methods of Chang & Casali [20]. The binomial distribution model was used to calculate whether the excess or scarcity of R mutations in CDRs and framework regions (FWRs) resulted by chance [20].

RESULTS

Forty-four V_{4-34} IgM-, IgG- and IgA-derived individual sequences were obtained from the tonsil section, of which 31 unique sequences were observed, and are shown in Figs 1–3. In each case a number of nucleotides from the constant region gene were sequenced to confirm the immunoglobulin class used. Nucleotide sequences have been deposited in the EMBL database.

V_{4-34}-Cμ-derived sequences

The deduced amino acid sequences of 12 independent IgM-expressing clones are shown in Fig. 1. Eleven appear to be potentially functional, whilst one, ME3, is highly mutated and contains three stop codons (Fig. 1). Three copies of one of these transcripts (MD1) were identified, possibly indicative of clonal expansion.

Eight of the sequences utilize the V_{4-34} gene in germ-line configuration. The C-terminal amino acid of the V_H gene segment in two of these cases appears mutated, but the changes are probably due to recombination events. Analysis of the other four clones shows that the number of somatic mutations varies from one silent mutation in MF4 (which also has a change in the C-terminal amino acid) to at least 15 replacement and two silent mutations in the non-functional ME3 transcript. This gives an overall mean mutation frequency of 1·0% for the V_{4-34}-Cμ-derived sequences, with the mean mutation frequency being 0·4% when the ME3 transcript is removed from the calculation. No concentration of replacement mutations in the CDRs was observed in these clones (Table 1).

A variety of D segment genes was used by these transcripts (Fig. 5), giving a variable range of length of the CDR3 from 6 to 20 amino acids. A predominance of J_H4b and J_H5b genes was also used, with the J_H genes all being in germ-line configuration (Fig. 1). A similar predominance of J_H4b gene usage has been noted in normal B cells [21].

V_{4-34}-Cγ-derived sequences

The deduced amino acid sequences of 14 independent IgG-expressing clones are shown in Fig. 2. All of these appear to be potentially functional. Eleven copies of one of these transcripts, GA4, were identified, indicative of a proliferating B cell clone. This over-representation is unlikely to be due to PCR bias, since a similar analysis of V_{4-34}-Cγ sequences in splenic tissue found no duplicated sequence among 20 clones (unpublished data).

Two of the V_{4-34}-Cγ sequences were in germ-line configuration (Table 1), indicating that isotype switching can occur with no accumulation of somatic mutations. Mutational analysis of the transcripts revealed very variable numbers of somatic mutations with a mean mutation frequency of 3·1%, but which ranges from 0% (in GB3 and GD6) to 8·9% (in GA3). The pattern of mutations revealed a significant concentration of R amino acids in the CDRs of the IgG-expressing clones GA4, GE1 and GG5, suggestive of a role for antigen selection (Table 1).

One of the V_{4-34}-Cγ sequences (GD3) was found to be clonally related to a V_{4-34}-Cμ sequence (MC7) (Fig. 4). Each used the same D-segment gene derived from D2, with identical N-additions. The two sequences also shared several mutations in V_H and both used J_H5. However, there were six nucleotide differences between them, with a pattern indicative of independent mutational events in IgM-expressing B cells, not found in the isotype-switched population.

As with the IgM clones a variety of D region genes were used (Fig. 5) and a variety of J_H genes were also utilized, both in germ-line, and mutated forms.

V_{4-34}-Cα-derived sequences

The deduced amino acid sequences of six independent IgA-expressing clones are shown in Fig. 3. All of these appear to be potentially functional. One of these sequences is in germ-line configuration, indicating that isotype switching to IgA can also occur in the absence of somatic hypermutation. However, 5/6 sequences showed a considerable degree of somatic hypermutation (mean 10·3%), with the overall level of deviation from germ-line sequence in IgA (8·6%) being higher than in IgG (3·1%). In fact, the percentage mutation ranges from 0% (in AF3) to 16·7% (in AB4).

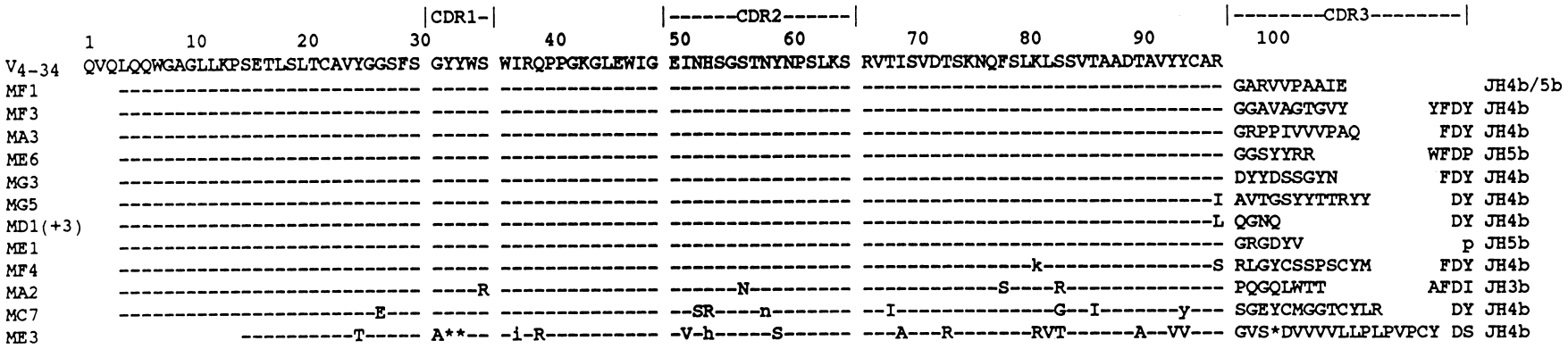


Fig. 1. Deduced amino acid sequences of the V_{4-34} gene utilized by IgM-positive tonsillar B lymphocytes. Upper case, replacement mutations; lower case, silent mutations; *Stop codon. (+3) denotes that four copies of this sequence were identified. Comparison is made with the germ-line gene and J_H usage is indicated.

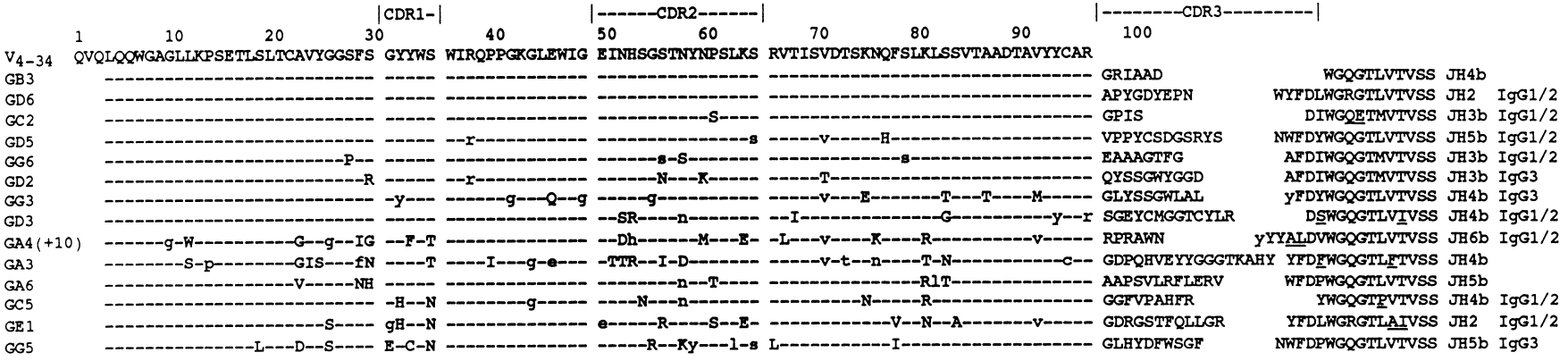


Fig. 2. Deduced amino acid sequences of the V_{4-34} gene utilized by IgG-positive tonsillar B lymphocytes. Upper case/underlined, replacement mutations; lower case, silent mutations. (+10) denotes that 11 copies of this sequence were identified. Comparison is made with the germ-line gene and J_H usage and IgG subclass are indicated where known.

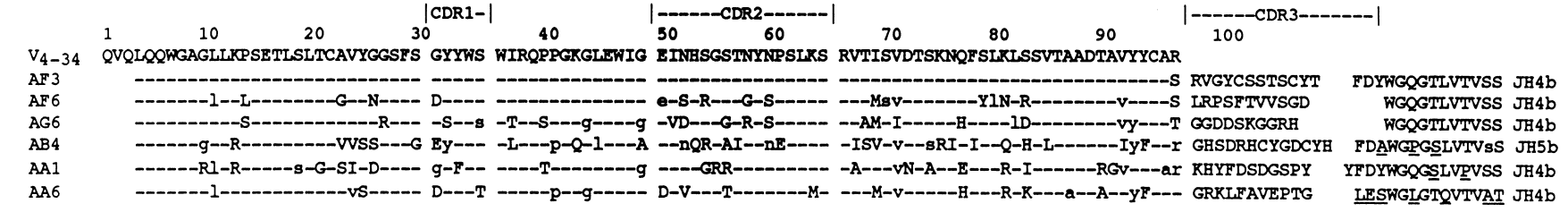


Fig. 3. Deduced amino acid sequences of the V_{4-34} gene utilized by IgA-positive tonsillar B lymphocytes. Upper case/underlined, replacement mutations; lower case, silent mutations. Comparison is made with the germ-line gene and J_H usage is indicated.

Table 1. Analysis of distribution of mutations in $V_{4–34}$ sequences

$V_{4–34}$-Cμ	Percent mutation from gl	R:S_{CDR} obs* R:S_{FWR} obs	R_{CDR} exp†	$P_{(CDR)}$‡
MF4	0·4	0 : 0 0 : 0	0·2	
MC7	3·2	2 : 1 4 : 2	1·6	0·288
MA2	1·4	2 : 0 2 : 0	0·7	0·254
$V_{4–34}$-Cα	Percent mutation from gl	R:S_{CDR} obs* R:S_{FWR} obs	R_{CDR} exp†	$P_{(CDR)}$‡
AF6	7·1	6 : 1 8 : 5	3·6	0·078
AG6	8·9	7 : 1 11 : 6	4·5	0·078
AB4	16·7	9 : 3 23 : 12	8·4	0·141
AA1	10·3	4 : 1 17 : 7	5·2	0·179
AA6	8·5	7 : 1 8 : 8	4·3	0·031
$V_{4–34}$-Cγ	Percent mutation from gl	R:S_{CDR} obs* R:S_{FWR} obs	R_{CDR} exp†	$P_{(CDR)}$‡
GD5	1·4	0 : 1 1 : 2	0·7	0·458
GG6	1·4	1 : 1 1 : 1	0·7	0·394
GD2	1·4	2 : 0 2 : 0	0·7	0·174
GG3	3·5	0 : 2 5 : 3	1·8	
GD3	2·5	2 : 1 3 : 1	1·3	0·279
GA4	6·0	6 : 1 7 : 3	3·0	0·044
GA3	8·9	6 : 1 9 : 9	4·5	0·135
GA6	3·2	3 : 1 4 : 1	1·6	0·145
GC5	2·5	3 : 1 2 : 1	1·2	0·089
GE1	4·3	5 : 2 4 : 1	2·1	0·035
GG5	5·0	6 : 3 4 : 1	2·5	0·019

* R:$S_{CDR/FWR}$ obs is the observed number of replacement (R) and silent (S) mutations seen in the CDRs and FWRs of the potentially functional, somatically mutated IgM, IgG and IgA sequences.

† R_{CDR} exp is the total expected number of replacement mutations in CDRs.

‡ $P_{(CDR)}$ is the probability that an excess of R mutations, in the CDRs, resulted by chance. Significant P values are underlined.

Analysis of the pattern of mutations in the IgA-derived clones (Table 1) revealed that all the sequences had a high R:S ratio in the CDRs. However, in only one case (AA6), did the concentration of R mutations in CDRs reach the significance required to indicate a role for antigen selection [20].

The $V_{4–34}$-Cα sequence which was unmutated in the V_H region, AF3, was found to be clonally related to a $V_{4–34}$-Cμ sequence, MF4 (Fig. 4). Each used the same D-segment gene derived from D4, with similar N-additions. However, the IgA-derived sequence used the D-segment gene in germ-line configuration, whereas the IgM-derived sequence had a single nucleotide change, converting threonine to proline (Fig. 4). There were three further nucleotide differences between them, with two in the N-regions. This pattern would again be consistent with independent mutational events occurring in IgM-positive cells, which may not be found in the isotype-switched clonal relatives.

A variety of D region genes (Fig. 5) and J_H genes were also utilized, both in germ-line, and mutated forms.

Mutational 'hot spots'

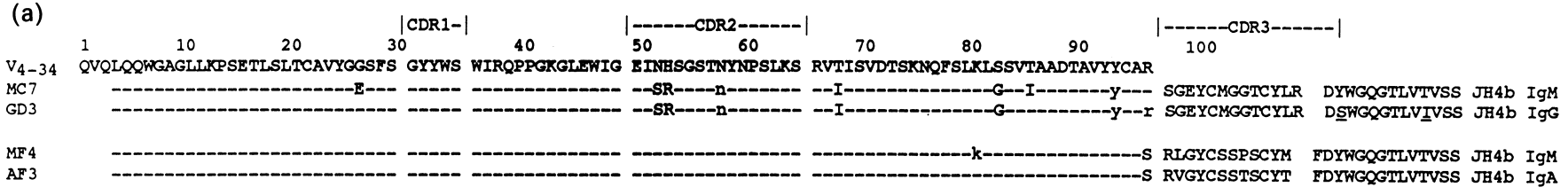
Inspection of Figs 1–3 indicates that a number of codons in $V_{4–34}$ are particularly prone to mutational change, often resulting in the same amino acid substitution. Examples include Lys81, which undergoes the same nucleotide change (AAG to AGG) in a total of six sequences among all isotypes, with one of these sequences, AA6, also having an additional silent mutation. A further site of high mutational activity is Ser35 in CDR1, in which the nucleotide substution, AGC to ACC, occurs in three of the sequences, and AGC to AAC in three others. Hot spots of silent mutations are also seen, with the codon at Val71 undergoing 8S and 5R changes, and that at Val92 undergoing 5S and 3R.

DISCUSSION

Events occurring in V-genes of B cells as they traverse the germinal centre of the lymph node are being unravelled. Investigations in the mouse have revealed that somatic hypermutation takes place mainly in the germinal centre, providing an array of sequences which can be selected by local antigen for generation of plasma cells or memory cells [21]. In the human, similar events occur, and analysis of single cells has shown that germinal centres may be dominated by a few large B cell clones which are undergoing somatic hypermutation [23]. In contrast, B cells in the follicular mantle tend to be clonally diverse and less mutated [23]. Such studies of human material are invaluable, particularly as there may be differences between mouse and man in the detailed routes to generation of high-affinity antibodies. It is now clear that the human V_H gene repertoire is much smaller than that of mouse [24,25], and recombination events and somatic hypermutation may therefore be more important in creating a diverse expressed repertoire in man.

In a recent study of V_H gene patterns in B cell subpopulations in human tonsil, it was found that the somatic hypermutation machinery was activated only after B cells reach the germinal centre at the centroblast stage [26]. At this point, the IgM-positive cells had accumulated a mean of 5·7 point mutations per V_H gene segment, demonstrating that the mutator was activated prior to isotype switch [26]. Similar conclusions had been reached by tracking the V_H6 gene in splenic lymphocytes [27]. The tonsil study went on to show that IgG-positive cells

(a)



(b)

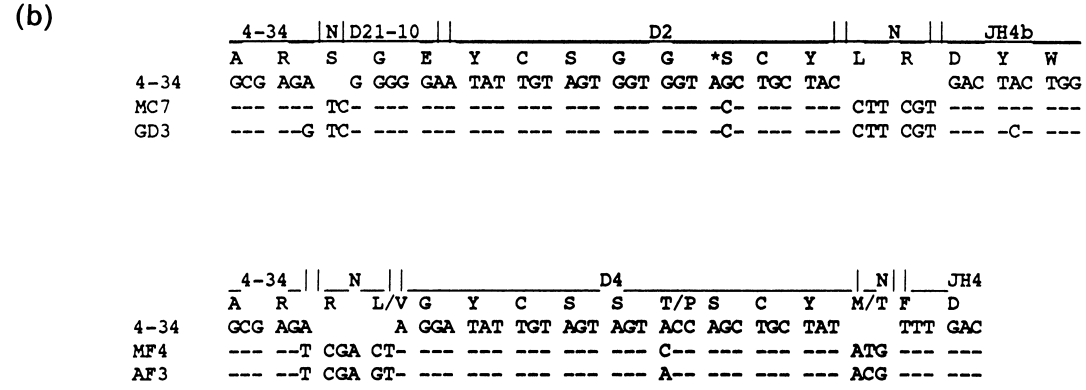


Fig. 4. (a) Deduced amino acid sequences of the V_{4-34} gene utilized by the clonally related tonsillar B lymphocytes. Upper case, replacement mutations; lower case, silent mutations. Comparison is made with the germ-line gene and J_H usage and immunoglobulin class are indicated. (b) Nucleotide sequences of the $V_HD_HJ_H$ recombination site showing the clonal relatedness.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IgM
MF1             ggC GCC CGA GTG GTA CCA GCT GCC ATT GAG
D4                        . ..A ... ... ... ..T ..

MF3             ggC GGA GCA GTG GCT GGT ACG GGG GTC TAC
DN1                   . ... .CA ... ... ..
DXP'1                           . ... T.. ...

MA3             ggC CGC CCT CCG ATT GTA GTA GTA CCA GCT CAA
D4                              ... ... ... ... ... ...
D23-7rc               . A.. ..

ME6             ggT GGG AGC TAC TAC AGG AGA
D2              ... ..T ... .G. ...
DA4                      .. ... ..T .

MG3             ggG TAC TAT GAT AGT AGT GGT TAT A
D21-9               ... ... ... ... ... ... ...

MG5             gCT GTT AGT GGG AGC TAC TAC ACT ACA AGA TAC TAT
D21-7             . ... C.- ... ..T ..T ..T .
D2                     .. ..T ... .G. ..
D2rc                                .. ... ... .T. .C. .

MD1             TTG CAA GGT AAC CAG
DXP1rc          ..A T.. --. ... ...
D21-10rc             .. C.. ..T ..

ME1             ggG AGA GGT GAC TAC GTG
DXP4/D23-7       .. ..T ...
DA1                    . ... ...

MF4             T CGA CTA GGA TAT TGT AGT AGT CCC AGC TGC TAT ATG
DXP'1/D2        . T.. ..- ..
D4                     . ... ... ... ... ... A.. ... ... ...

MA2             CCC CAG GGA CAG CTA TGG ACA ACG
DK4                    . ... ... ...
DHQ52 rc        ... ...


MC7             TCG GGG GAA TAT TGT AGT GGT GGT ACC TGC TAC CTT CGT
D21/7           ..- ... ..
D2                     . ... ... ... ... ... .G. ... ...


IgA
AF3             T CGA GTA GGA TAT TGT AGT AGT ACC AGC TGC TAT ATG
D4                     . ... ... ... ... ... ... ... ... ...

AF6             TCC CTT CGT CCC TTC GGT ACA GTC GTG AGT GGG G
DN4                   . ... ..
D2                              .A. .TT ..A ... -... ..
DLR1rc                    ..A ..- A.. ... A.

AG6               T GGA GAT GAC TCC AAG GGG GGC CGG C
D21-10                            . ... .
D23-7rc/DXP4rc              .. ... ..

AB4             ggC CAC TCC GAC CGC CAT TGT TAT GGT GAC TGT TAT CAC
D23-7rc/DXP4rc    . ... ... A.
D3                              .. ... GG. ... ..T ..C ..
D1rc                       .. .A. ..G .

AA1             AAA CAT TAT TTT GAC AGT GAT GGC TCC CCG TAC
DXP1              . .GA ... ... ...
D21-7rc/DXP'1rc                          .. AA. ... ... A..

AA6             ggC CGG AAG TTG TTT GCA GTT GAA CCA ACT GGT
DM2               . ... ..
DM5-a/D                 . ... -.. C.. ...
D4                            .. .T. ..A .T. ... G.. .C.
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(see next page for caption.)

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IgG
GB3             ggC CGT ATA GCA GCA
DN1/DN4         .. ... ... ...

GD6             gCG CCC TAC GGT GAC TAC GAA CCC AAC
DA1/GA4               . ... A.. A.. ...
DXP'1rc                           . ... ..

GC2             ggC CCG ATT TCC
DXP4                 .. ... .

 GD5             gTG CCC CCT TAT TGT AGT GAT GGT AGC CGC TAC TCC AAC
 D21-10rc             ... ..
 D2                           ... ... ... .G. ... ... T.. ... ..

 GG6             GAA GCA GCA GCT GGT ACA TTC GGG
 DN1               . ... ... ... ... ... ..
 D21-7                                  ... ...

 GD2             CAA TAT AGC AGT GGC TGG TAC GGG GGT GAT
 DN1                  ... ... ..C A.. ... ...
 DLR3                                      ..T ... ...

 GG3             ggC CTT TAT AGC AGT GGC TGG CTG GCG TTA TAT
 DN1                       ... ... ..C A.. ...
 D21-10                                 ... -G. .A. ... .

 GD3             TCG GGG GAA TAT TGT AGT GGT GGT ACC TGC TAC CTT CGT
 D21-7           ..- ... ..
 D2                        .G. ... ... ... ... ... .G. ... .

 GA4             CGC CCC CGG GCA TGG AAC
 D21-7rc              ... ..A A.. .
 D5rc                   . ... .A. ..

 GA3             ggG GAC CCT CAG CAC GTC GAG TAT TAT GGT GGA GGT ACA AAG GCC CAC T
 DN4                      ... .T. ...
 D2                                     ..- ... .G. A.. ..T ... .
 D21-9rc                                                          ..T AA. ... .

 GA6             gCA CCC CCT TCT GTA TTA CGA TTT TTG GAG AGA GTC
 D21-10rc         .T ... ..
 DXP4                           ... ... ... ... ... ... T.

 GC5             ggC GGC TTC GTA CCA GCA CAC TTT CGA
 D4                       .A ... ... ..
 D1rc                       . ... .T. ..A .A. .

 GE1             ggA GAT CGG GGG TCC ACC TTC CAA TTA CTG GGG CGG
 DXP'1/D21-7          .T. ... .
 DM1rc                                  ... ..G ...
 DHQ52                                        .A. ... ..

 GG5             ggC CTT CAT TAC GAT TTT TGG AGT GGT TTC AAC
 DXP4                      .. ... ... ... ... ... ... .
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Fig. 5. Nucleotide sequence comparison between the D_H segments expressed in the IgM, IgG and IgA clones and their closest germ-line D segment genes. –, Missing nucleotide; rc, reverse complement; lower case, nucleotides contributed from V_{4-34} germ-line gene.

had accumulated a mean of 9·5 somatic mutations, in line with the concept that somatic mutation accompanies isotype switching [26].

Our study has focused on a more limited segment of tonsillar lymphoid tissue, and has allowed us to follow events occurring within single clones. We have concentrated on the non-polymorphic V_{4-34} gene segment because it represents a V_H gene which is prominent in the early immune repertoire [12] and which is involved in encoding autoantibodies [4,6,13]. Studies of blood lymphocytes have indicated that the ability of the B cells, which use this gene, to undergo isotype switching is limited [15], and V_{4-34} usage has not been detected in the isotype-switched cells of myeloma. However, V_{4-34}-positive cells are able to enter the lymph nodes, and there is also evidence that the gene is

over-represented in IgM-positive follicular lymphoma [28] and diffuse large cell lymphoma [29].

The results presented confirm and extend initial observations in tonsillar B cells [17] that cells utilizing the V_{4-34} gene are capable of undergoing isotype switch events to both IgG and IgA. Since the source material was RNA, the identified sequences have been successfully transcribed, and all but one of the switched sequences appeared potentially functional. In a separate study, we obtained IgG-secreting hybridomas from tonsil tissue, which supports the likelihood that the encoded IgG is functional (unpublished data). The general increase in mutational level is typical of isotype-switched cells using other V_H genes [22]. IgA is probably of major importance in immunity in the tonsil, and the mean level of mutations in IgA was high.

Numerous common mutations among the V_{4-34} transcripts were observed, suggestive of hot spots seen in other genes such as V_H5 [30–32]. Many of these mutations are common to different clones both within and between isotypes, particularly Ser35 in CDR1, and Lys81 in FWR3. In fact, identical hot spots of mutation can be observed in V_{4-34}-expressing transcripts, isolated both from patients with cold agglutinin disease ([4] and unpublished data) and patients with EBV infection [14].

The finding of clonally related IgM and IgA sequences which carry very few mutations indicates that isotype switching can occur in the absence of somatic mutations, presumably because the unmutated sequence is able efficiently to bind to antigen. A different situation exists in the clonally related IgM and IgG sequences which share a similar pattern of mutation, indicating that considerable somatic mutation can occur before switching, and need not increase. Clearly, we have obtained a 'snap-shot' of events during an immune response. The V_{4-34} gene is not locked into its role as an IgM autoantibody, but it appears to be as capable as other V_H genes of following a wide variety of routes to effective antibody.

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