Article No. jmbi.1998.2396 available online at http://www.idealibrary.com on IDEAL J. Mol. Biol. (1999) 285, 895–901

JMB

COMMUNICATION

Analysis of Heavy and Light Chain Pairings Indicates that Receptor Editing Shapes the Human Antibody Repertoire

Ruud M. T. de Wildt[1]*, René M. A. Hoet[1]*, Walther J. van Venrooij[1] Ian M. Tomlinson[2] and Greg Winter[2]

[1]*Department of Biochemistry University of Nijmegen, PO Box 9101, 6500 HB, Nijmegen The Netherlands*

[2]*MRC Centre for Protein Engineering and MRC Laboratory for Molecular Biology, Hills Road, Cambridge CB2 2QH, UK*

**Corresponding authors*

In the bone marrow, diversity in the primary antibody repertoire is created by the combinatorial rearrangement of different gene segments and by the association of different heavy and light chains. During the secondary response in the germinal centres, antibodies are diversified by somatic mutation and possibly by further rearrangements, or ''receptor editing''. Here, we have analysed the pairings of heavy and light chain variable domains (V_H and V_L) in 365 human IgG^+ B cells from peripheral blood, and established that these pairings are largely random. The repertoire is dominated by a limited number of pairings of segments and folds. Among these pairings we identified two identical mutated heavy chains in combination with two different mutated light chains (one kappa and one lambda). This shows that receptor editing occurs in the human periphery and that the same antibody lineage can be subjected to both receptor editing and somatic hypermutation. This suggests that receptor editing may be used together with somatic mutation for the affinity maturation of antibodies. We also propose that receptor editing has shaped variable gene segment use and the evolution of V gene families.

Keywords: heavy and light chain pairings; receptor editing; secondary light chain rearrangement; somatic hypermutation

Diversity in the primary B cell repertoire is created by the combinatorial rearrangement of different variable (V), diversity (D) and joining (J) gene segments (Tonegawa, 1983), by junctional diversity and by the association of different heavy and light chains. Additional diversity can be created by somatic hypermutation (Berek & Milstein, 1988). The sequencing of all the human V gene segments has made it possible to dissect the contributions of germline diversity and somatic mutation to the diversity of human antibody heavy or light chains (Tomlinson *et al.*, 1996; Ignatovich *et al.*, 1997). However, the contributions of heavy and light chain association and of secondary rearrangements, or receptor editing (Tiegs *et al.*, 1993; Gay *et al.*, 1993), are unknown. To investigate this, we analysed V_H and V_L pairings in human peripheral IgG^+ B cells. Blood was taken from three donors, single IgG^+ B cells were sorted and cultured for 10-11 days using mouse EL4 B5 T-cells (see the legend to Figure 1). Using PCR amplification of cDNA, we were able to identify V_H and V_L pairs in 365/442 (83%) independent IgG^+ clones: V_κ and V_λ were never found together in the same clone.

Sequencing of the 365 V_H and V_L pairs indicated that the use of κ (61%) and λ (39%) chains and of gene segments and families was consistent with previous analyses in which a few segments dominate the repertoire (Ignatovich *et al.*, 1997; Brezinschek *et al.*, 1997; Huang *et al.*, 1996; Cox *et al.*, 1994; Corbett *et al.*, 1997). The pairings are

Present addresses: R. M. T. de Wildt, MRC Centre for Protein Engineering, Hills Road Cambridge CB2 2QH, UK; R. M. A. Hoet, CESAME, Department of Pathology, University Hospital Maastricht, PO Box 5800, 6202 AZ, The Netherlands.

Abbreviations used: V, variable gene segment; D, diversity gene segment; J, joining gene segment; CDR3, third complementarity determining region; RAG, recombinase activating genes; PBMC, peripheral blood mononuclear cells.

E-mail addresses of the corresponding authors: rdw@mrc-lmb.cam.ac.uk; rhoet@lpat.azm.nl

0022-2836/99/030895–07 $30.00/0

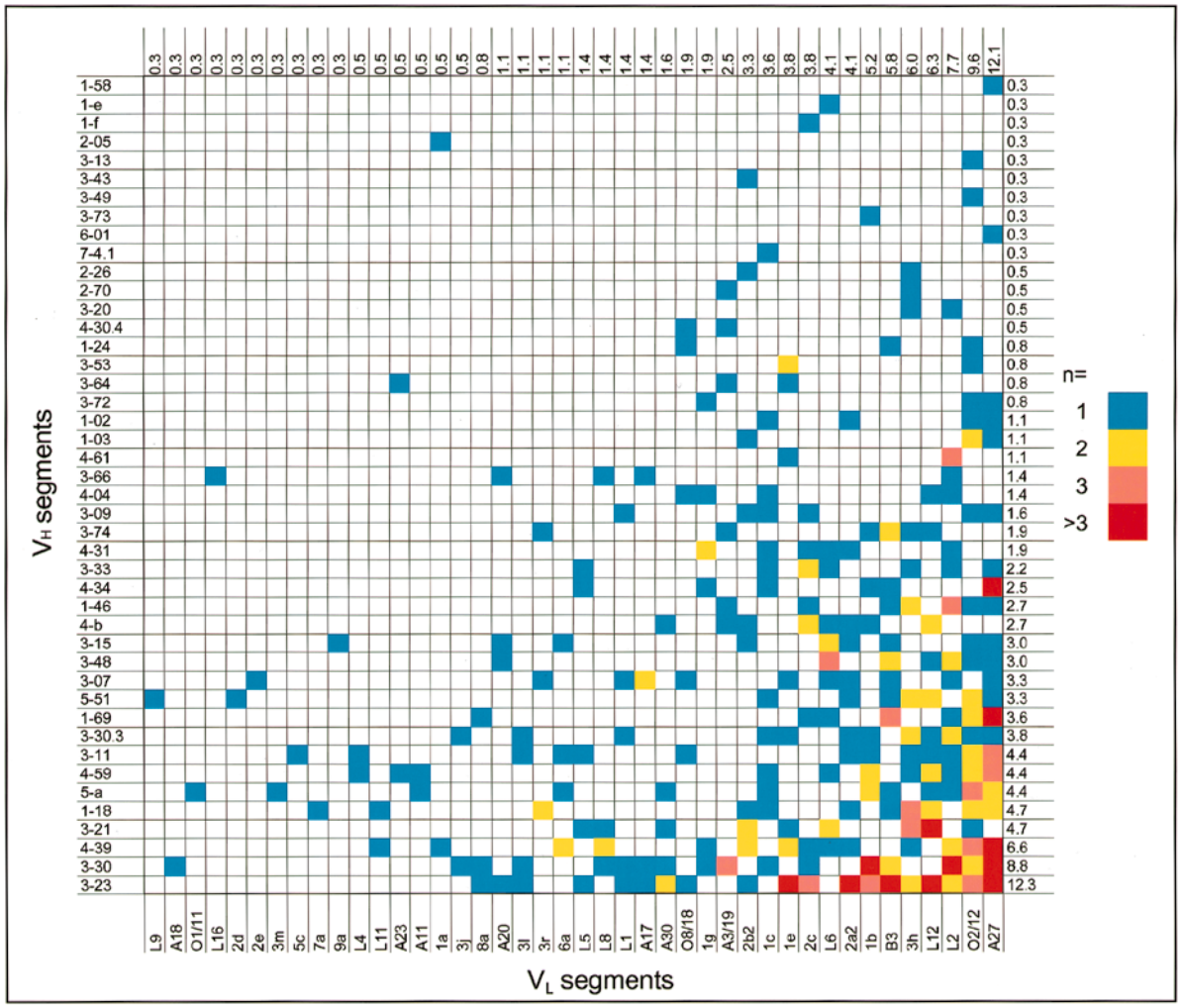

Figure 1 *(legend opposite)*

summarised in the matrix of Figure 1 in which the V_H and V_L gene segments have been ordered by frequency. The picture is broadly consistent with random pairing of heavy and light chains, and as expected, frequently used V gene segments tend to be paired. We also analysed the pairings for loop (canonical) structures (Figure 2). As expected for random combination of the V gene segments, the most popular structures are represented by the combination of V_H 1-3 with V_κ 2-1 (21%) and with V_λ 14-7(A) (10%). The repertoire is dominated by a few structures with half of the pairings corresponding to only five combinations. As noted for V_H and V_κ pairings in human IgM^+ B cells (Brezinschek *et al.*, 1998), there was no evidence for preferential pairings of specific families. Furthermore, there appears to be no association between the heavy and light chain third complementarity determining region (CDR3) lengths and sequences nor between V and D and J gene segments.

In contrast, the mutations in V_H and V_L for each IgG^+ B cell are correlated (Figure 3). The V_H genes are on average more mutated than the V_L genes, with 10.9% (7.6% for V_L) amino acid substitutions or 6.1% (3.9% for V_L) nucleotide substitutions. Indeed, 82% of the B cells have more mutations in the V_H gene than in the V_L gene. There are several possible explanations for the excess of heavy chain mutations (Storb, 1996), including secondary light chain rearrangement (see below).

During our analysis, we identified two B cell clones from the same healthy individual with identical (and highly mutated) heavy chains but different light chain partners, one κ and the other λ. This was confirmed by a second RT-PCR from the same mRNA. The heavy chain V_H segment (3-09) has 19 nucleotide (nt) substitutions and the light chains V_L segments (V_κ L1 and V_λ 1c) five and four nucleotide substitutions, respectively (Figure 4). As the occurrence of identical V (D) J rearrangements and the chances of the same multiple mutations arising in two independent B cell clones are very small, they must belong to the same lineage. This indicates that secondary light chain rearrangement has occurred in one of the two B cells. The presence of mutations in the heavy and light chains indicates that the secondary rearrangement must have occurred in the periph-

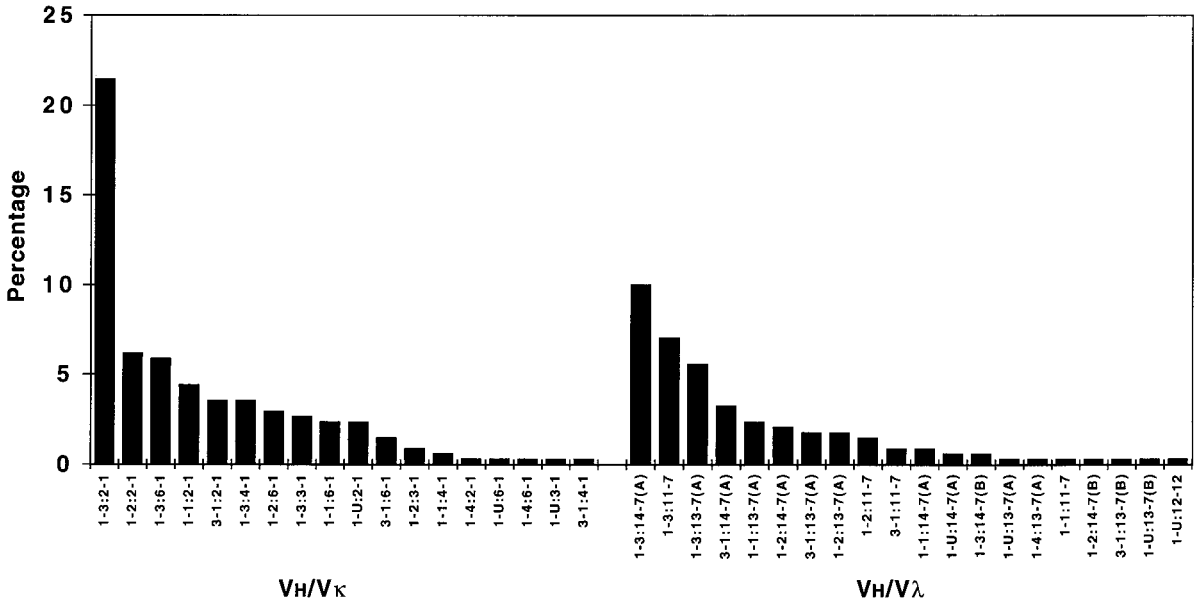

Figure 2. Antibody structures of the human repertoire. The frequencies of pairings were analysed according to the (canonical) loop structures encoded by V_H (Chothia *et al.*, 1992), V_κ (Tomlinson *et al.*, 1995) and V_λ (Williams *et al.*, 1996) segments. Numbers refer to the canonical structures (for V_H and V_κ) or the CDR lengths (for V_λ) of the H1, H2, L1 and L2 loops, respectively. U indicates a canonical structure that is currently unknown.

ery. As the heavy chain must have been mutated before the secondary rearrangement, and the rearranged light chain must have been mutated after the secondary rearrangement, the processes of somatic mutation and receptor editing are interwoven. Four additional pairs of highly related clones were isolated with several shared somatic mutations indicating a common lineage: however,

Figure 1. V_H and V_L segment pairings. Pairings present in 365 rearranged antibodies from the peripheral IgG^+ B cells of three individuals, with V gene segments ordered according to their detected frequencies, as indicated on the *x* and *y*-axes. The probability of observing this table in 100,000 randomly generated tables having the same V_H and V_L segment frequencies was calculated under the hypothesis that pairings are not associated. No evidence was found to reject this hypothesis and to conclude the presence of an association, because the *p*-value exceeded the 5% level of statistical significance ($p = 0.087$, Fisher's exact test statistic). Because the table is sparsely populated and the *p*-value is close to the 5% level, more data are necessary to conclude, beyond an acceptable level of chance error, whether pairings are favoured or disfavoured. Culturing of the B cells and amplification and sequencing of V_H/V_L regions from B cell clones was performed as follows: blood was obtained from three donors (male, 41 years old; female, 34 years old; and female, 29 years old). Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) and washed with PBS. Cells were labelled with FITC conjugated anti-human IgG (Kallestadt) and PE conjugated anti-CD19 (Dako, Denmark), and single IgG^+, $CD\text{-}19^+$ lymphocytes were sorted into 96-well plates using a Coulter Epics Elite flow cytometer (Coulter, Hialeah, Florida, USA) equipped with an automatic cell deposit unit. Culturing of the B cells on the EL4 B5 T cells (Zubler *et al.*, 1985) was performed as described (De Wildt *et al.*, 1997). In theory, a bias introduced by the culture system cannot be ruled out, but as these single B cell cultures appeared to grow out very efficiently (>60% Ig^+ cultures), this is not very likely. The culture supernatants were screened by ELISA, and RNA from IgG^+ wells was used for cDNA synthesis as described (De Wildt *et al.*, 1997). Aliquots of the cDNA were used in separate PCRs to amplify V_H, V_κ and V_λ genes using family specific 5′ primers and 3′ constant region primers. Primers were identical with those described (Marks *et al.*, 1991) except for the following: VH1back, CAG (GA)T(GTC) CAG CTG GTG CAG TCT GG; VH2back, CAG (AG)TC ACC TTG AAG GAG TCT GG; VH4back, CAG GTG CAG CTG CAG GAG T(GC)(GC) GG; VH5back, GAG GTG CAG CTG GTG CAG TCT GG; Vκ1back, G(AC)C ATC C(AG)G ATG ACC CAG TCT CC; Vκ3back, GAA ATT GTG (AT)TG AC(GA) CAG TCT CC; Vλ1back, CAG TCT GTG (CT)TG AC(TG) CAG CC; Vλ3aback, TCC TAT GAG CTG AC(TA) CAG CC; Vλ4back, CAG C(CT)T GTG CTG ACT CAA TC; Vλ5back, CAG (CG)CT GTG CTG ACT CAG CC; Vλ7/8back, CAG (AG)CT GTG GTG AC(TC) CAG GAG; Vλ9/10back, CAG (CG)C(TA) G(TG)G CTG ACT CAG CCA. As a negative control for the RT-PCR, mRNA isolated from wells in which no B cells were present was used. PCR products were purified using PCR purification columns (Qiagen, CA, USA). Sequencing reactions were performed using CH1.lib.seq primer (Griffiths *et al.*, 1994) for the heavy chains, Cκlib.seq (Griffiths *et al.*, 1994) for the κ light chains and CL.SEQ (Ignatovich *et al.*, 1997) for the λ light chains, respectively, and run on an automated sequencer (Applied Biosystems 373A, Perkin Elmer, CA, USA). Nucleotide sequences were aligned to their germline counterparts using the V-BASE Sequence Directory (Tomlinson *et al.*, 1998; http://www.mrc-cpe.cam.ac.uk/imt-doc/index.html). Since the results obtained from the three donors were very similar, all data were treated as one group in this analysis. GenBank accession numbers, AF103026-103725.

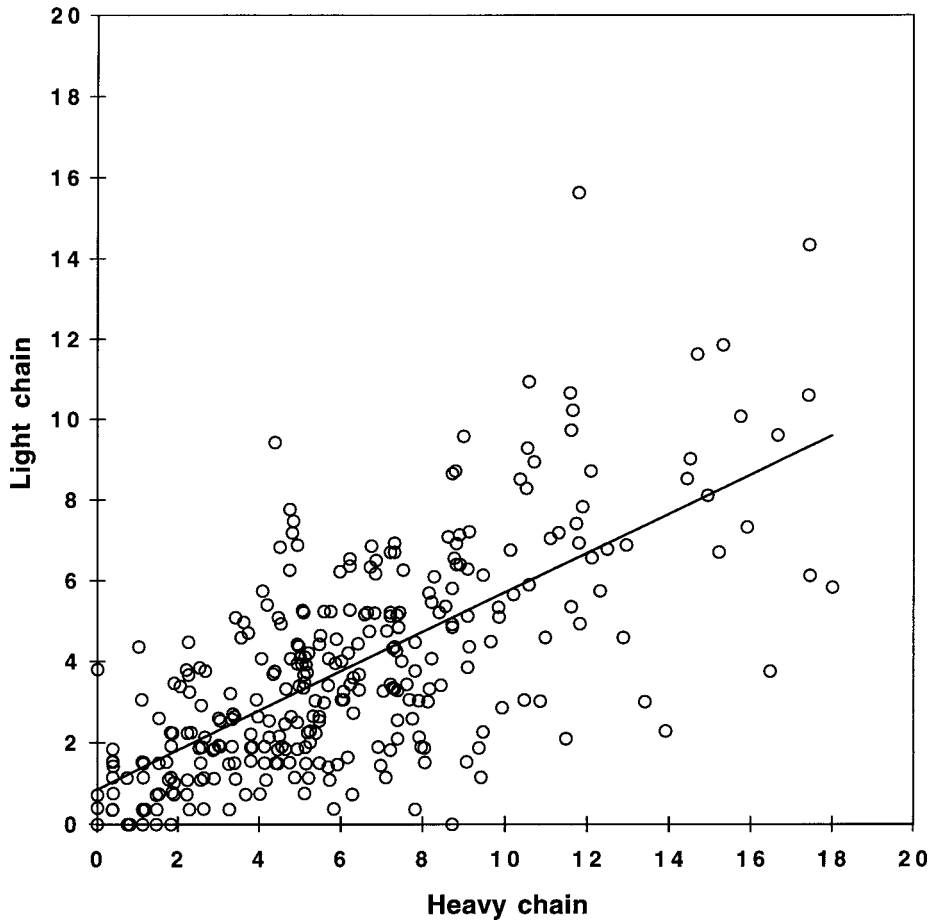

Figure 3. Scatter plot of the somatic nucleotide mutations in the V_H and V_L segments of heavy and light chain pairs. The V_H and V_L mutations are correlated with a Pearson correlation coefficient of 0.7 for the whole repertoire. For the statistical analysis, the first eight codons of the framework 1 region (spanning the region corresponding to the primers) were excluded.

in these cases the heavy as well as the light chains were derived from the same segments. As a secondary rearrangement was observed in 20% of the pairs identified (1/5), this may be a frequent event and would help to explain the lower overall mutation rate of the light chain compared to the heavy chain.

Until recently, receptor editing was thought to play a role in central tolerance (Tiegs *et al.*, 1993; Gay *et al.*, 1993; Pelanda *et al.*, 1997; Chen *et al.*, 1997). There is now evidence for the expression of recombinase activating genes (RAG) and receptor editing in germinal centre B cells (Han *et al.*, 1997; Papavasiliou *et al.*, 1997; Hikada & Ohmori, 1998; Meffre *et al.*, 1998), and that in mice this is associated with affinity maturation (Hertz *et al.*, 1998). Our results show that receptor editing also occurs in the human periphery and may be interwoven with somatic mutation, suggesting an involvement in the affinity maturation of antibodies. Heavy chains have been shown to pair with a range of light chain partners leading to the changes of affinity (Radic *et al.*, 1991) and indeed, the improvements in affinity (Clackson *et al.*, 1991). In principle, the new light chains could be derived from the same germline V gene segment (at the other allele or from an identical duplicated segment at the same allele), from germline segments of the same family, from germline segments of a different family (at either allele), or from a different sub-class.

Replacement by the same germline segment provides a means of segregating heavy chains from a disadvantageous set of mutations on the light chain. It also provides a fresh start for light chain mutations. Replacement by segments from the same family (highly related sequences and generally with loops of the same fold) provides both a means of segregation and of targeting multiple simultaneous mutations to the loops. Replacement by segments from different families provides a means of generating antibodies that bind to the same epitope (as directed by the heavy chain), but with a very different set of light chain contacts (Figini *et al.*, 1994). Similar arguments could also apply to the secondary rearrangement of heavy chains *via* a heptamer-like sequence adjacent to the third hypervariable loop (Radic & Zouali, 1996;

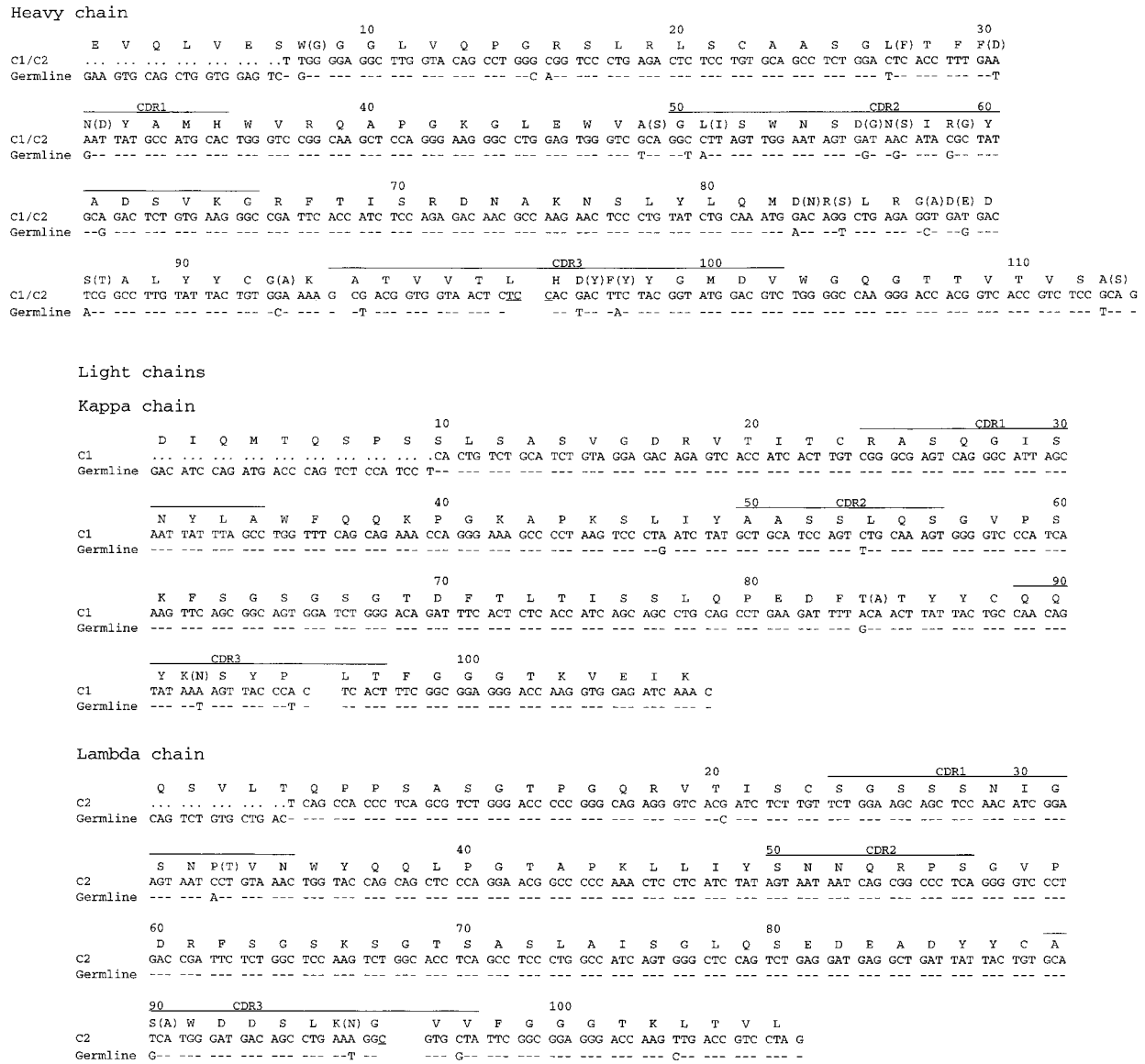

Figure 4. V_H and V_L sequences of the clonal pair (C1 and C2) with a secondary rearranged light chain. The three lines represent the deduced amino acid sequence, the nucleotide sequence and its corresponding germline gene, respectively. The original (germline) residues are given in parentheses. Dashes indicate sequence identity. Germline sequences are: V_H 3-09 (containing 19 nt replacements), D3-24 (1 nt replacement) and J_H6 (3 nt replacements) for the heavy chain (GenBank accession numbers: C1, AF103107 and C2, AF103276); V_κ L1 (5 nt replacements) and $J_\kappa4$ (no replacements) for the κ light chain (C1, AF103429) and V_λ 1c (4 nt replacements) and $J_\lambda2/3$ (2 nt replacements) for the λ light chain (C2, AF103687). N-nucleotides are underlined and CDRs are indicated. Segment designations and amino acid numbering are according to the V BASE Sequence Directory (Tomlinson *et al.*, 1998).

Stamatopoulos *et al.*, 1996). We therefore envisage that receptor editing operates in conjunction with somatic mutation to improve antibody affinities both by removing undesirable mutations and by creating further diversity.

The intrinsic frequency of rearrangement is known to be associated with, for example, location on the chromosome (Cox *et al.*, 1994) or the sequence of the recombination signal (Akamatsu *et al.*, 1994). A segment favoured in the primary rearrangement should also be favoured in the secondary rearrangement and will, therefore, have a greater chance of segregating undesirable mutations. In turn, this should favour repertoires dominated by a limited number of segments, as is observed (Figure 1). Likewise, the representation in the antibody repertoire of a limited number of folds with highly related sequences (families; Figure 3) is also consistent with receptor editing: the chances of secondary rearrangement to generate highly related antibodies are greater in repertoires comprising segments organised as families. We therefore suggest that receptor editing has helped shape the repertoire and the evolution of V gene segments.

Acknowledgements

We thank A. H. M. Pennings and G. Vierwinden for flow cytometry analyses, F. H. J. van den Hoogen for patient materials, P. G. A. Steenbakkers for help with the B cell cultures, A. T. Prevost for help with the statistical analysis and M. Neuberger and C. Milstein for discussions. This work was supported in part by the Netherlands Foundation for Chemical Research (SON) and the Technology Foundation (STW) with financial aid from the Netherlands Organisation for Scientific Research.

References

Akamatsu, Y., Tsurushita, N., Nagawa, F., Matsuoka, M., Okazaki, K., Imai, M. & Sakano, H. (1994). Essential residues in V(D)J recombination signals. *J. Immunol.* **153**, 4520-4529.

Berek, C. & Milstein, C. (1988). The dynamic nature of the antibody repertoire. *Immunol. Rev.* **105**, 5-26.

Brezinschek, H. P., Foster, S. J., Brezinschek, R. I., Dorner, T., Domiati-Saad, R. & Lipsky, P. E. (1997). Analysis of the human VH gene repertoire. Differential effects of selection and somatic hypermutation on human peripheral CD5(+)/IgM+ and CD5(−)/IgM+ B cells. *J. Clin. Invest.* **99**, 2488-2501.

Brezinschek, H. P., Foster, S. J., Dorner, T., Brezinschek, R. I. & Lipsky, P. E. (1998). Pairing of variable heavy and variable κ chains in individual naive and memory B cells. *J. Immunol.* **160**, 4762-4767.

Chen, C., Prak, E. L. & Weigert, M. (1997). Editing disease-associated autoantibodies. *Immunity,* **6**, 97-105.

Chothia, C., Lesk, A. M., Gherardi, E., Tomlinson, I. M., Walter, G., Marks, J. D., Llewelyn, M. B. & Winter, G. (1992). Structural repertoire of the human VH segments. *J. Mol. Biol.* **227**, 799-817.

Clackson, T., Hoogenboom, H. R., Griffiths, A. D. & Winter, G. (1991). Making antibody fragments using phage display libraries. *Nature,* **352**, 624-628.

Corbett, S. J., Tomlinson, I. M., Sonnhammer, E. L. L., Buck, D. & Winter, G. (1997). Sequence of the human immunoglobulin diversity (D) segment locus: a systemic analysis provides no evidence for the use of DIR segments, inverted D segments, ''minor'' D segments or D-D recombination. *J. Mol. Biol.* **270**, 587-597.

Cox, J. P. L., Tomlinson, I. M. & Winter, G. (1994). A directory of human germ-line Vκ segments reveals a strong bias in their usage. *Eur. J. Immunol.* **24**, 827-836.

De Wildt, R. M. T., Steenbakkers, P. G., Pennings, A. H. M., van den Hoogen, F. H. J., Van Venrooij, W. J. & Hoet, R. M. A. (1997). A new method for the analysis and production of monoclonal antibody fragments originating from single human B cells. *J. Immunol. Methods,* **207**, 61-67.

Figini, M., Marks, J. D., Winter, G. & Griffiths, A. D. (1994). *In vitro* assembly of repertoires of antibody chains on the surface of phage by renaturation. *J. Mol. Biol.* **239**, 68-78.

Gay, D., Saunders, T., Camper, S. & Weigert, M. (1993). Receptor editing: an approach by autoreactive B cells to escape tolerance. *J. Exp. Med.* **177**, 999-1008.

Griffiths, A. D., Williams, S. C., Hartley, O., Tomlinson, I. M., Waterhouse, P., Crosby, W. L., Kontermann, R. E., Jones, P. T., Low, N. M., Allison, T. J., Prospero, T. D., Hoogenboom, H. R., Nissim, A., Cox, J. P. L. & Harrison, J. L., *et al*. (1994). Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO J.* **13**, 3245-3260.

Han, S. H., Dillon, S. R., Zheng, B., Shimoda, M., Schlissel, M. S. & Kelsoe, G. (1997). V(D)J recombinase activity in a subset of germinal center B lymphocytes. *Science,* **278**, 301-305.

Hertz, M., Kouskoff, V., Nakamura, T. & Nemazee, D. (1998). V(D)J recombinase induction in splenic B lymphocytes is inhibited by B cell receptor signalling. *Nature,* **394**, 292-295.

Hikada, M. & Ohmori, H. (1998). Rearrangment of λ light chain genes in mature B cells in vitro and in vivo. Function of reexpressed recombination-activating gene (RAG) products. *J. Exp. Med.* **187**, 795-799.

Huang, S. C., Jiang, R., Glas, A. M. & Milner, E. C. (1996). Non-stochastic utilization of Ig V region genes in unselected human peripheral B cells. *Mol. Immunol.* **33**, 553-560.

Ignatovich, O., Tomlinson, I. M., Jones, P. T. & Winter, G. (1997). The creation of diversity in the human immunoglobulin Vλ repertoire. *J. Mol. Biol.* **268**, 69-77.

Marks, J. D., Hoogenboom, H. R., Bonnert, T. P., McCafferty, J., Griffiths, A. D. & Winter, G. (1991). By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J. Mol. Biol.* **222**, 581-597.

Meffre, E., Papavasiliou, F., Cohen, P., de Bouteiller, O., Bell, D., Karasuyama, H., Schiff, C., Banchereau, J., Liu, Y. J. & Nussenzweig, M. C. (1998). Antigen receptor engagement turns off the V(D)J recombination machinery in human tonsil B cells. *J. Exp. Med.* **188**, 765-772.

Papavasiliou, F., Casellas, R., Suh, H. Y., Qin, X. F., Besmer, E., Pelanda, R., Nemazee, D., Rajewsky, K. & Nussenzweig, M. C. (1997). V(D)J recombination in mature B cells: a mechanism for altering antibody responses. *Science,* **278**, 298-301.

Pelanda, R., Schwers, S., Sonoda, E., Torres, R. M., Nemazee, D. & Rajewsky, K. (1997). Receptor editing in a transgenic mouse model: site, efficiency, and role in B cell tolerance and antibody diversification. *Immunity,* **7**, 765-775.

Radic, M. Z. & Zouali, M. (1996). Receptor editing, immune diversification, and self tolerance. *Immunity,* **5**, 505-511.

Radic, M. Z., Mascelli, M. A., Erikson, J., Shan, H. & Weigert, M. (1991). Ig H and L chain contributions to autoimmune specificities. *J. Immunol.* **146**, 176-182.

Stamatopoulos, K., Kosmas, C., Stavroyianni, N. & Loukopoulos, D. (1996). Evidence for immunoglobulin heavy chain variable region gene replacement in a patient with B cell chronic lymphocytic leukemia. *Leukemia,* **10**, 1551-1556.

Storb, U. (1996). The molecular basis of somatic hypermutation of immunoglobulin genes. *Curr. Opin. Immunol.* **8**, 206-214.

Tiegs, S. L., Russell, D. M. & Nemazee, D. (1993). Receptor editing in self-reactive bone marrow B cells. *J. Exp. Med.* **177**, 1009-1020.

Tomlinson, I. M., Cox, J. P., Gherardi, E., Lesk, A. M. & Chothia, C. (1995). The structural repertoire of the human Vκ domain. *EMBO J.* **14**, 4628-4638.

Tomlinson, I. M., Walter, G., Jones, P. T., Dear, P. H., Sonnhammer, E. L. L. & Winter, G. (1996). The imprint of somatic hypermutation on the repertoire

of human germline V genes. *J. Mol. Biol.* **256**, 813-817.

Tomlinson, I. M., Williams, S. C., Ignatovich, O., Corbett, S. J. & Winter, G. (1998). *V BASE Sequence Directory*, MRC Centre for Protein Engineering, Cambridge, UK.

Tonegawa, S. (1983). Somatic generation of antibody diversity. *Nature,* **302**, 575-581.

Williams, S. C., Frippiat, J. P., Tomlinson, I. M., Ignatovich, O., Lefranc, M. P. & Winter, G. (1996). Sequence and evolution of the human germline Vλ repertoire. *J. Mol. Biol.* **264**, 220-232.

Zubler, R. H., Erard, F., Lees, R. K., Van Laer, M., Mingari, C., Moretta, L. & MacDonald, H. R. (1985). Mutant EL-4 thymoma cells polyclonally activate murine and human B cells *via* direct cell interaction. *J. Immunol.* **134**, 3662-3668.

Edited by J. Karn

(Received 24 August 1998; accepted 16 October 1998)