Somatic hypermutation and B-cell lymphoma

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During the B-cell response to T-cell-dependent antigens, the B cells undergo a rapid proliferative phase in the germinal centre. This is accompanied by the introduction of mutations into the immunoglobulin (Ig) variable region (V) genes. The B cells are then selected according to the affinity of the encoded immunoglobulin for antigen, resulting in affinity maturation of the response. Analysis of mutations in IgV genes has given insight into the history of individual B cells and their malignancies.

In most cases, analysis of mutations confirms classifications of B-cell lineage designated by studies of cellular morphology and surface antigen expression. However, of particular interest is the subdivision of groups of malignancies by analysis of somatic hypermutation. It is now apparent that there are two subsets of chronic lymphocytic leukaemia (CLL), one with a low load of mutations and poor prognosis, and one with a heavy load of mutations with a much more favourable prognosis. In addition, in Burkitt's lymphoma, sporadic and endemic subtypes are now considered possibly to have a different pathogenesis, reflected in differences in the numbers of mutations.

Hodgkin's disease, which was a mystery for many years, has now been shown to be a B-cell tumour. Although in many cases the Ig genes are crippled by somatic hypermutation, it is thought that failure to express Ig is more likely to be associated with problems of transcription.

It has been proposed that the distribution of mutations in a B-cell lymphoma can be used to determine whether a lymphoma is selected. We have investigated the load and distribution of mutations in one group of lymphomas—marginal zone B-cell lymphomas of mucosa-associated lymphoid tissues (MALT-type lymphoma), which are dependent on *Helicobacter pylori* for disease progression, to investigate the limits of information that can be derived from such studies. Comparison of the load of mutations demonstrates that these tumours have approximately the same load of mutations as normal mucosal marginal zone B cells from the Peyer's patches and mucosal plasma cells. This is consistent with the origin of these cells from mucosal marginal zone B cells with plasma cell differentiation.

To investigate selection in MALT lymphomas we compared a region of the framework region three in ten MALT lymphomas which use the V_{H4} family, with the same codons in groups of V_{H4} genes that are out of frame between V and J. The latter accumulate mutations but are not used and are not selected. A group of V_{H4} genes are in-frame between V and J were also included for comparison. There were no obvious differences in the distribution of mutations between the groups of genes; the same hot spots and cold spots were apparent in each. In the MALT lymphomas, selection was apparent in the framework regions only and the tendency was to conserve. We therefore feel that there is selection to conserve antibody structure and that this does not reflect selection for antigen. We do not believe that antigen selection can be deduced reliably from sequence information alone.

It is possible that somatic hypermutation could be a cause of malignancy since it has been shown that the process may generate DNA strand breaks and is known to be able to generate insertions and deletions. Such events may mediate the translocation of genes—a process that is pivotal in the evolution of many lymphomas.

Keywords: lymphoma; B cells; selection

1. SOMATIC HYPERMUTATION IN NON-HODGKIN'S B-CELL LYMPHOMAS

Normal B cells acquire mutations in their immunoglobulin genes during the germinal centre response, following encounter with T-cell-dependent antigens. This process is followed by a process of selection according to affinity of the encoded Ig for antigen (Jacobs *et al.* 1991).

Thus virgin B cells have germline IgV genes, and germinal centre and postfollicular B cells have mutated IgV genes (Pascual *et al.* 1994). The study of somatic hypermutation in B-cell malignancy has gathered considerable pace over the last decade (Stevenson *et al.* 1998; Klein *et al.* 1998). Initially, data confirmed expectation—tumours of CD5+, IgM+, IgD+ putative virgin B cells, such as mantle cell lymphoma and chronic lymphocytic leukaemia (CLL), had non-mutated IgV genes (Hummel *et al.* 1994; Kipps *et al.* 1989). Tumours of germinal centre

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cells had mutated Ig genes, with evidence of continuing mutation, parodying the events in the normal germinal centre (Bahler & Levy 1992; Kuppers *et al.* 1993). Tumours thought to be of postfollicular B cells carried mutated Ig genes (Vescio *et al.* 1995; Zhu *et al.* 1995; Chapman *et al.* 1995). However, now that the number of cases investigated is increasing, subdivisions in groups of tumours, which could be considered as single entities, are becoming apparent. Some recent advances are discussed below.

CLL was originally found to have germline IgV_H genes, consistent with origin from virgin B cells (Kipps *et al.* 1989). However, it is now apparent that CLL is not a single entity. Groups of patients with CLL can be subdivided into prognostic groups according to the frequency of somatic hypermutation in their IgV_H genes (Hamblin *et al.* 1999; Damle *et al.* 1999); those with mutated IgV_H genes have better survival. It has also been shown that trisomy 12 is associated with poor survival (Juliusson *et al.* 1990) and that trisomy 12 is associated with low frequency of somatic hypermutation (Oscier *et al.* 1997). These data together support the concept of two diseases with different pathogenesis, both of which manifest clinically and histologically as CLL. One is a disease of virgin B cells that involves acquisition of trisomy 12 and has a poor prognosis. The second is a disease of postfollicular B cells that has a more favourable prognosis. Information on the sequence of Ig genes would clearly be of use to haematologists in determining the therapeutic regime required in individual cases of CLL.

Burkitt's lymphoma was originally subclassified as endemic and sporadic largely according to epidemiological criteria, though there are other characteristics that may discriminate between them (Stevenson *et al.* 1998). Detailed knowledge of the frequency of somatic hypermutation in Burkitt's lymphoma has revealed a marked difference in the frequency of somatic hypermutation in these groups; the frequency of somatic hypermutation is higher in endemic than in sporadic Burkitt's lymphoma (Chapman *et al.* 1995, 1996; Klein *et al.* 1998). Although the reason for this is not yet clear, it is likely to reflect significant differences in the origin of the subsets of Burkitt's lymphoma.

Diffuse large B-cell lymphoma represents a group of aggressive tumours with relatively few morphological or phenotypic characteristics that would allow them to be tied to normal counterparts in most cases. Analysis of somatic hypermutation has now been carried out in large groups of sequences, which has confirmed that this is a heterogeneous group (Lossos *et al.* 2000). The load of somatic hypermutation is generally high, though there are exceptions. The genes are functional in most, but not all, cases. In most cases, there is evidence of progressive accumulation of mutations. It is likely that this group of lymphomas emerge from transformation events within a diverse background of B cells.

2. IMMUNOGLOBULIN GENES IN HODGKIN'S DISEASE

The tumour cells in classical Hodgkin's disease, the Reed–Sternberg cells, are morphologically bizarre cells with an unusual phenotype; the only antigens they commonly express are CD30, which is associated with a subset of activated T cells in normal lymphoid tissues and,

in some cases, the granulocytic marker CD15. Tumour cells tend to be isolated cells surrounded by a reactive infiltrate. The use of single-cell microdissection coupled with analysis of the sequence of Ig genes allowed the designation of the majority of cases of Hodgkin's disease as B-cell lymphomas (Kanzler *et al.* 1996). Cases of Hodgkin's disease and follicular lymphoma, in the same patient, which shared the same Ig gene rearrangement have been described (Marafioti *et al.* 1999; Braeuninger *et al.* 1999). In addition, the emergence of unusual Reed–Sternberg-like cells in CLL, which are clonally related to the tumour cells in CLL in some cases, have been reported (Kanzler *et al.* 2000). These observations tie Hodgkin's disease closer still to transformation events in B-cell malignancy, though the precise significance remains obscure. Since the initial description of Hodgkin's disease as a B-cell malignancy, a minority of cases with monoclonal T-cell receptor rearrangements have also been described (Seitz *et al.* 2000; Muschen *et al.* 2000).

The rearranged Ig genes in Reed–Sternberg cells have a high load of mutations, consistent with a postfollicular origin. In many cases this results in disruption of the integrity of the gene. This has been proposed as a reason for the failure of Reed–Sternberg cells to expose their B-cell phenotype by expressing Ig (Kanzler *et al.* 1996). However, the Ig genes are not crippled by somatic hypermutation in all cases and it has been suggested that it may be a failure in transcription rather than the sequence of the Ig genes that results in the failure to express Ig on the surface of Reed–Sternberg cells (Jox *et al.* 1999; Marafioti *et al.* 2000).

Analysis of Ig genes in nodular lymphocyte-predominant Hodgkin's disease, where the tumour cells (lymphocytic and histiocytic; L&H cells) frequently express the B-cell antigen CD20, has demonstrated that these tumours are also of B-cell lineage and that the tumour cells may be related to the large cell lymphomas that often arise in this disease (Greiner *et al.* 1996; Ohno *et al.* 1997; Marafioti *et al.* 1997; Braeuninger *et al.* 1997).

3. TO WHAT EXTENT CAN SOMATIC HYPERMUTATION BE USED TO INVESTIGATE THE BIOLOGY OF B-CELL MALIGNANCIES?

In the following paragraphs, we will briefly review the biology of one group of tumours, extranodal marginal zone B-cell lymphoma (low-grade B-cell lymphomas of mucosa-associated lymphoid tissue type; gastric mucosa-associated lymphoid tissue (MALT) lymphomas), and attempt to define the limits of useful information relating to the biology of these tumours that can be derived from analyses of somatic hypermutation.

(a) *Lineage of gastric MALT lymphomas*

The marginal zone is a zone of B cells surrounding the mantle zone in the spleen and Peyer's patches of the terminal ileum. The majority of marginal zone B cells express IgM and markers of mature B cells such as CD20 but do not express IgD, which is expressed by the majority of virgin B cells (Spencer *et al.* 1985). Marginal zone lineage of MALT lymphomas was based largely on morphological similarities between the malignant cells in MALT lymphomas and normal marginal zone B cells

Figure 1. Comparison of number of mutations in IgV_H genes in IgM plasma cells from human gut lamina propria, salivary gland or spleen (Dunn-Walters *et al.* 2000), microdissected marginal zone from human spleen (Dunn-Walters *et al.* 1995) or Peyer's patches (Dunn-Walters *et al.* 1996) or from high- or low-grade MALT lymphomas (Thiede *et al.* 1998; Hallas *et al.* 1998; Driessen *et al.* 1999; Chapman *et al.* 1996; Qin *et al.* 1995; Bahler *et al.* 1997). Low-grade MALT lymphomas (the majority of which are IgM+ and *ca.* 30% of which show plasma cell differentiation) have a number of mutations, which is not different from that observed in the mucosal IgM plasma cell populations in the gut or salivary gland, or the marginal zone population in the Peyer's patches. However they are more highly mutated than the splenic IgM plasma cells or marginal zone B cells ($p < 0.01$). The same trend is observed in the high-grade MALT lymphomas, though they are also significantly more highly mutated than the Peyer's patch marginal zone B cells ($p < 0.05$). This is consistent with the origin of MALT lymphomas from a lineage of mucosal IgM-only B cells and plasma cells.

(Spencer *et al.* 1985). Both are medium-sized cells with irregular nuclear morphology and a moderate amount of cytoplasm, tend to surround the mantle zone and share a very similar phenotype (CD20+, IgM+, IgD-). Differentiation to IgM-secreting plasma cells occurs in *ca.* 30% of MALT lymphomas (Isaacson *et al.* 1986), a property that may be shared by antigen-stimulated marginal zone B cells (Liu *et al.* 1988).

Normal stomach, however, does not have any organized lymphoid tissue (Wyatt & Rathbone 1988). This may be acquired in response to infection with *Helicobacter pylori*, probably as a consequence of induction of the cytokine BCA-1 that has been detected in *H. pylori* gastritis and MALT lymphomas, together with its receptor on B cells, CXCR5 (Mazzucchelli *et al.* 1999). The acquired lymphoid tissue in the stomach resembles the normal Peyer's patches in the terminal ileum (Wotherspoon *et al.* 1991). In figure 1, we have compared the frequency of somatic hypermutation in IgV_H genes in high and low-grade MALT lymphomas (Thiede *et al.* 1998; Hallas *et al.* 1998; Driessen *et al.* 1999; Chapman *et al.* 1996; Qin *et al.* 1995; Bahler *et al.* 1997) with that observed in postfollicular compartments of normal mucosal and splenic lymphoid tissues, the two sites in man with prominent marginal zone B-cell components (Dunn-Walters *et al.* 1995, 1996, 1997*a,b*). The frequency of mutation in MALT lymphomas is not significantly different to that observed in constitutive gut-associated lymphoid tissue, and

is significantly higher than that observed in either the marginal zone B cells or IgM plasma cells in the spleen, strongly supporting the hypothesis that these malignancies are tumours of mucosal marginal zone B cells which differentiate into IgM plasma cells. Although MALT lymphomas are most commonly IgM+, it has been suggested that this may be due to errors in class switching (Nardini *et al.* 2000). Our analysis would support the hypothesis that MALT lymphomas are malignancies of an IgM-only pathway of B-cell development in normal gut-associated lymphoid tissue (Klein *et al.* 1997; Boursier *et al.* 1999).

(b) Low-grade gastric MALT lymphomas are dependent on antigenic stimulation by *Helicobacter pylori*

Early low-grade B-cell gastric MALT lymphomas (stage E₁) are mostly dependent on an infection with the gastric pathogen *H. pylori* for tumour progression; eradication of *H. pylori* leads to tumour regression in *ca.* 80% of cases (Wotherspoon *et al.* 1993; Morgner *et al.* 2000). The residual clone can often be detected following regression and may continue to diversify by somatic hypermutation, but it does not develop into frank lymphoma (Thiede *et al.* 1998). Because of the responsiveness of low-grade MALT lymphomas to eradication of *H. pylori*, and the generally indolent behaviour of the majority of cases, it has been questioned whether these represent true malignancies. However, transformation from relatively quiescent low-grade to more aggressive high-grade lesions has been observed and confirmed by CDR3 analysis (Peng *et al.* 1997). Some particularly aggressive variants with a t(1;14) chromosomal translocation have been identified (Willis *et al.* 1999). In many cases of MALT lymphoma a t(11;18) translocation has been identified, which results in the juxtaposition of the *API2* gene that encodes an inhibitor of apoptosis and a novel gene *MLT* located at 18q21 (Dierlamm *et al.* 1999). Despite antigen responsiveness, MALT lymphomas are clearly a bona fide group of tumours.

The dependence of MALT lymphomas on *H. pylori* is thought to be due to dependence of the malignant B cells on the T-cell response to *H. pylori* (Hussell *et al.* 1996; D'Elia *et al.* 1999). In contrast, the tumour B cells themselves tend to have specificity for autoantigen (Hussell *et al.* 1993; Greiner *et al.* 1994). There have been no reports of reactivity or cross-reactivity with *H. pylori* antigens. It is not known whether the B cells themselves are selected by antigen or are dependent on antigen (either autoantigen or *H. pylori* antigen) for growth. Since this is a central question in trying to understand the behaviour of these tumours there have been numerous attempts to determine whether MALT lymphomas are antigen selected, by analysis of the imprint of somatic hypermutation on the IgV_H genes. In §3(c), we will consider methods of analysing selection, using MALT lymphomas as the test sample.

(c) Can we conclude that human IgV_H genes are selected by antigen by analysis of the imprint of somatic hypermutation?

The simplest method initially used to investigate selection was to compare the frequency of replacement (R) and silent (S) mutations in the complementary-determining regions (CDRs) and the framework regions (FRs), the

Table 1. *Ratio of replacement to silent mutations*

(Data from a database of 21 in-frame V_{H4-34} genes with 433 mutations and 18 out-of-frame genes with 238 mutations derived from analysis of DNA isolated from postfollicular B-cell and plasma cell populations. A panel of nine lymphomas of mucosa-associated lymphoid tissue were also analysed that contained 160 mutations between the beginning of CDR1 and the end of FR3 (not all of the genes had full FR1 sequence).)

	expected ^a	out-of-frame V_{H4-34} genes	in-frame V_{H4-34} genes	MALT- lymphoma V_{H4} family genes
FR1	2.7	1.6	1.3	—
CDR1	4.5	2.2	2.6	1.3
FR2	2.7	1.5	0.7	0.7
CDR2	4.4	4.8	5.5	3.4
FR3	2.7	2.3	1.4	1.5

^a Expected values were calculated according to Chang & Casali (1994).

Table 2. *Comparisons of distribution of replacement mutations in the CDRs of Ig heavy chain genes*

(*p*-values are given using the binomial distribution model, according to Chang & Casali (1994). A panel of 12 out-of-frame V_{H4-34} genes obtained from studies of microdissected human intestinal plasma cells (Dunn-Walters & Spencer 1998) and in 19 high-grade and 13 low-grade lymphomas, obtained from Thiede *et al.* (1998), Hallas *et al.* (1998), Driessen *et al.* (1999), Chapman *et al.* (1996), Qin *et al.* (1995) and Bahler *et al.* (1997) numbers in bold are significant.)

out-of-frame genes	low-grade MALT lymphomas	high-grade MALT lymphomas
0.14	0.026	0.12
0.028	0.18	0.12
0.14	0.023	0.03
0.19	0.05	0.12
0.16	0.29	0.03
0.15	0.18	0.14
0.21	0.38	0.09
0.19	0.14	0.32
0.027	0.27	0.09
0.036	0.14	0.04
0.23	0.17	0.224
0.11	0.43	0.1
	0.004	0.04
	0.17	
	0.08	
	0.18	
	0.07	
	0.54	
	0.17	
	0.08	
	0.4	
	0.15	
3/12	3/22	4/13

idea being that the FRs would be conserved (a lower R:S ratio than expected), whereas the CDRs would be diversified by selection (a higher R:S ratio than expected). At the time when mutational analysis of the IgV genes in lymphomas was gaining pace, there were no controls available that could indicate the relative tendency for different sites in human IgV genes to become mutated. Since then a number of groups, including ours, has

generated databases of genes that are out of frame between V and J, and which, although subjected to the somatic hypermutation process, are not selected (Dorner *et al.* 1997, 1998; Klein *et al.* 1998; Dunn-Walters & Spencer 1998). This supposition is supported by the presence of stop codons in the database of out-of-frame genes. In table 1 it can be seen that the pattern of R:S mutations along the gene is similar between all three data sets and different to that which would be expected from a purely random process. FR2 consistently has the lowest R:S ratio, and CDR2 consistently has the highest ratio, whereas the expected values are approximately the same between the three FR regions and between the two CDRs. This difference between actual and expected, even in out-of-frame genes, is due to a combination of preferential targeting of mutations by the motif preferences of the hypermutation mechanism and codon structure. Only in Fw3 does the actual R:S ratio of out-of-frame genes approximate to that expected. However, by comparing the in-frame genes and the MALT lymphomas to the out-of-frame genes, conservation of FRs is apparent. Preferential targeting of replacement mutations to the CDRs by the hypermutation mechanism in the out-of-frame genes is very marked and it is not possible to see any consistent superimposed effects of selection in the used genes in terms of accumulation of R mutations. It is interesting to note that conservation of the CDRs as reported previously (Dorner *et al.* 1998) is observed in the MALT lymphomas but not the in-frame genes in this analysis. The possibility that the CDRs may be conserved to some degree, together with the ability of single base changes to modify affinity (Allen *et al.* 1988), suggests that deduction of selection by analysis of CDRs using an R:S ratio, especially in a single or small group of sequences, is not likely to be a viable proposition.

Another method used to investigate selection in lymphomas is the binomial distribution model initially applied to this problem by Chang & Casali (1994). This method takes into account the codon composition of the different regions of the IgV_H genes that affects their inherent mutability. For example, codons which can have any base in third position are less likely to acquire a replacement mutation than codons for which the amino acid encoded depends on the last base. The method also takes into account the sizes of the regions. Replacement and silent mutations in the CDRs and FRs can be analysed

Figure 2. Analysis of mutations from A, G, C or T nucleotides, expressed as percentage of total mutations. (a) All mutations, (b) replacement only and (c) silent only. Genes investigated were 18 out-of-frame genes with 83 mutations in the codons studied (solid bars), 177 in-frame V_{H4-34} genes with 950 mutations in the codons studied (open bars) and ten MALT lymphomas with 73 mutations in the codons studied (shaded bars). MALT lymphomas were from Driessen *et al.* (1999), Thiede *et al.* (1998) and five additional sequences from the authors (C.T. and B.A.). In total these were four V_{4-04} , two V_{4-61} , two V_{4-59} and one each of V_{H4-34} and V_{4-39} . The area investigated was the 22 codons of FR3 sequence common to all the V_{H4} family genes investigated (codons 66–91, but lacking codons 69, 73, 76, 84, 85 and 88). When the numbers were analysed directly using χ^2 -analysis, G nucleotides were significantly more frequently mutated in the in-frame genes, compared with the out-of-frame genes. This was associated with the replacement mutations. (Asterisked bars, $p < 0.001$ for all mutations and $p < 0.003$ for replacement mutations only.)

separately. It has been claimed that this method identifies antigen selection in some cases of MALT lymphoma. However, as shown as table 2, this method identifies 'selection' in a similar proportion of in-frame and out-of-frame genes as well as MALT lymphomas and it is not therefore a reliable method for the analysis of selection.

(d) Analysis of hallmarks of selection in FR3 of IgV_{H4} family genes

The data in table 1 suggest that FR3 is conserved in used genes. Other studies have also highlighted the potential of FRs in analysing the effects of selection (Dorner *et al.* 1998; Klein *et al.* 1998). There is a significant degree of homology between members of the V_{H4} family. This has enabled us to examine the characteristics of mutations

that result in the changed R:S ratio in FR3 in detail, using the same sequence in used genes and out-of-frame controls. We have studied 22 codons of the FR3 sequence that are common to all the V_{H4} family genes investigated (codons 66–91, but lacking codons 69, 73, 76, 84, 85 and 88) (Tomlinson *et al.* 1992). The genes investigated were 18 out-of-frame genes with 83 mutations in the codons studied, 177 in-frame V_{H4-34} genes with 950 mutations in the codons studied and ten MALT lymphomas with 73 mutations in the codons studied. MALT lymphomas were from Driessen *et al.* (1999), Thiede *et al.* (1998) and five additional sequences from authors C.T. and B.A. In total these were four V_{4-04} , two V_{4-61} , two V_{4-59} and one each of V_{H4-34} and V_{4-39} . The data in tables 3 and 4 and in figures 2–4 all relate to this sequence and these genes.

Initial analysis of the genes failed to identify any individual bases or codons within the sequence, which were preferentially mutated or conserved, in any of the data sets. The percentage of total substitutions from each base, in each group investigated, is illustrated in figure 2. In the in-frame genes there is a significant tendency for G nucleotides to acquire proportionately more mutations than the other bases when compared with the out-of-frame genes. This was statistically significant using χ^2 -analysis. The MALT lymphoma sequences followed the same pattern as the in-frame genes but were not statistically significant. There were no substitution preferences other than a tendency to not replace T with A in the in-frame genes (figure 3). However, mutations from T tend to be relatively infrequent and this may not be biologically significant.

Mutations in the last base position of codons are more likely to be silent than mutations that occur in first or second positions. To determine whether the increased number of mutations from G is due to the position of G within the codon structure of the sequence analysed, mutations from each base in each position within the codon were studied (figure 4). In the majority of cases, the in-frame genes and MALT lymphomas follow the same pattern, being either both higher or both lower than the out-of-frame genes in that position. A tendency to conserve the second base and diversify the last base is generally observed. The apparent increase in mutations in C nucleotides in first position is due to the contribution of some silent C to T mutations. To discount the effect of third-base replacement and first-place silent mutations in this analysis, the replacement and silent mutations were analysed separately. The significant increase in mutations from G was in the replacement mutations only, though the same trend was identified in the silent mutations (figures 2 and 3).

Analysis of the R:S ratio for each base individually, as shown in table 3, illustrates that the ratio for each base follows the same pattern in the in-frame genes and the MALT lymphomas. In each base, there is a reduction in the R:S ratio compared to the out-of-frame genes. The lowest change in ratio is in the A and G nucleotides. This occurs in G despite the common occurrence of G nucleotides in the third-base position where they could potentially gather silent mutations, thus providing an opportunity to see large differences in R:S ratio in selected and unselected genes. In contrast, A nucleotides are most common in first- and second-base positions

Figure 3. Analysis of substitutions in the same data set analysed in figure 2. (a) All mutations, (b) replacement only and (c) silent only. Solid bars, out-of-frame genes; open bars, in-frame genes; shaded bars, MALT lymphomas. Apart from a significant tendency to not replace T with A when the in-frame genes are compared with the out-of-frame genes, no significant substitution preferences were observed.

where they may gather replacement mutations (figure 4). This suggests that replacement mutations from G nucleotides may not be so rigorously selected against in this data set. We have analysed the consequences of replacement mutation in terms of the properties of the encoded amino acids (table 4). In comparison to replacements from A and C nucleotides, replacements from G are more likely to be functionally conservative. Replacements from T also have an interesting pattern but are few in number. The data in tables 3 and 4 and figure 4 together suggest that the relatively high proportion of substitutions from G in used genes is likely to be due to the greater tolerance to mutations from G in this sequence, since although they are replacement mutations at the DNA level, they are replacements with amino acids with equivalent properties and may therefore be functionally silent.

(e) *Can somatic hypermutation be used to deduce antigen selection?*

In this paper, we have carried out the first extensive analysis of an area of sequence in FR3 in a group of lymphomas that involves the use of the same sequence in both out-of-frame and in-frame genes as controls. The pattern of mutations in the FRs in MALT lymphomas mirrors that in the in-frame genes and is likely to be selected according to the same criteria, probably conservation of a functional structure. The increase in mutations from G has not been observed as a general phenomenon in selected genes (Dorner *et al.* 1997) and is likely to be a local characteristic of the template studied. Overall, our rigorous analysis of mutations in FR3 has determined that there are no specific signatures of selection that can be tied to individual codons. The bases that are hot spots and

Figure 4. (a) Analysis of composition of sequence analysed, illustrating the number of each nucleotide in first, second and third positions in the codons. (b) Analysis of mutations from each nucleotide, in first, second or third place. Figures are percentage of mutations from each nucleotide, corrected for the relative frequency of that nucleotide in first, second and third codon position. Solid bars, out-of-frame genes; open bars, in-frame genes; shaded bars, MALT lymphomas.

Table 3. Ratio of replacement to silent mutations in FR3 codons that are common to all family members

(From A, C, G and T nucleotides in the FR3 sequence common to all the V_{H4} family genes investigated, i.e. lacking codons 69, 73, 76, 84, 85 and 88. In the FR3 sequence analysed there were 83 mutations in the out-of-frame genes, 950 mutations in the in-frame genes and 73 mutations in the MALT lymphomas. MALT lymphomas were from Driessen *et al.* (1999), Thiede *et al.* (1998) and five additional sequences. In total these were four V_{H4-04} , two V_{H4-61} , two V_{H4-59} and one each of V_{H4-34} and V_{H4-39} .)

	18 out-of-frame V_{H4-34} genes	177 in-frame V_{H4-34} genes	10 V_{H4} family MALT lymphomas
mutations from A	3.0	2.0	2.2
mutations from C	1.2	0.5	0.5
mutations from G	2.4	1.7	1.8
mutations from T	12.0	4.5	1

cold spots in the unused genes retain the same properties in the used genes. The effect of selection is subtle, and unless there are control populations for comparison, as in this analysis, it would be difficult to quantify as a means of determining selection in single genes.

Table 4. Analysis of amino-acid substitutions resulting from replacement mutations from each nucleotide

(Expressed as per cent amino-acid replacements, which are functionally conservative as a consequence of replacement mutation from each base.)

	all	A	C	G	T
out of frame	60	44	38	100	54
in frame	75	54	42	94	81
MALT lymphomas	79	64	80	85	100

4. SOMATIC HYPERMUTATION AS A POSSIBLE CAUSE OF B-CELL MALIGNANCY

The pathogenesis of B-cell malignancies frequently involves chromosomal translocations. In many tumours, translocations directly involve the Ig loci, such as the t(14;18) translocation that is present in the majority of follicular lymphomas (Gaulard *et al.* 1992). This results in the juxtaposition of the anti-apoptosis gene *Bcl-2* and the J region of IgV_H and, as a consequence, resistance of tumour cells to the signals that normally cause the death of germinal centre cells (Tsujimoto & Croce 1986; Hockenbery *et al.* 1990). The generation of translocations is likely to be a consequence of events that normally

generate DNA double-strand breaks, a number of which are involved in the generation of the mature functional Ig repertoire. These processes include gene rearrangement during the development of the primary repertoire when gene segments are deleted—isotype switching—which again involves the deletion of segments of DNA and the most recent addition to this list: somatic hypermutation (Sale & Neuberger 1998). Since somatic hypermutation can occur outside the Ig loci (Shen *et al.* 1998), this process may be involved in distant translocation events. It has been proposed that insertion and deletion events, which can also be generated by somatic hypermutation, may be directly involved in the evolution of light chain disease, where segments of the Ig loci are characteristically deleted (Goossens *et al.* 1998).

Translocations are often not sufficient to generate malignant change. It is known that apparently otherwise normal B cells, which harbour t(14;18) translocations, are detectable in very low levels in the blood of many individuals (Limpens *et al.* 1995). It may be by chance encounter with a stimulating antigen or a supportive T-cell response—or possibly further genetic events or a combination of these—that determines that a B cell should have the advantage over its peers and generate a malignant clone.

5. CONCLUSION

Analysis of somatic hypermutation has provided a valuable means of investigating origin and lineage in B-cell lymphomas. Quantification of mutations is now providing clinically useful information; frequency of IgV mutation in CLL is a new prognostic indicator. However, there is still no reliable method available to deduce whether a mutated gene is derived from an antigen-selected B cell. The large data set over a relatively short sequence studied here enables us to conclude that MALT lymphomas have been through a selective process. However, due to the position and nature of the changes, this is likely to be selection for intact Ig rather than affinity for antigen.

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