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Signals sustaining human immunoglobulin V gene hypermutation in isolated germinal centre B cells

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SUMMARY

Affinity maturation of antibody responses depends on somatic hypermutation of the immunoglobulin V genes. Hypermutation is initiated specifically in proliferating B cells in lymphoid germinal centres but the signals driving this process remain unknown. This study identifies signals that promote V gene mutation in human germinal centre (GC) B cells *in vitro*. Single GC B cells were cultured by limiting dilution to allow detection of mutations arising during proliferation *in vitro*. Cells were first cultured in the presence of CD32L cell transfectants and CD40 antibody (the 'CD40 system') supplemented with combinations of cytokines capable of supporting similar levels of CD40-dependent GC B-cell growth [interleukin (IL)-10 + IL-1β + IL-2 and IL-10 + IL-7 + IL-4]. Components of the 'EL4 system' were then added to drive differentiation, providing sufficient immunoglobulin mRNA for analysis. Analysis of VH3 genes from cultured cells by reverse transcription–polymerase chain reaction (RT–PCR)-based single-strand conformation polymorphism indicated that the combination IL-10 + IL-1β + IL-2 promoted active V gene mutation whereas IL-10 + IL-7 + IL-4 was ineffective. This was confirmed by sequencing which also revealed that the *de novo* generated mutations were located in framework and complementarity-determining regions and shared characteristics with those arising *in vivo*. Somatic mutation in the target GC B-cell population may therefore be actively cytokine driven and not simply a consequence of continued proliferation. The experimental approach we describe should facilitate further studies of the mechanisms underlying V gene hypermutation.

INTRODUCTION

One of the hallmarks of the T-dependent immune response is the increased affinity of antibodies produced upon repeated antigenic challenge.[1,2] This affinity maturation is attributable to a combination of somatic hypermutation in the immunoglobulin V genes and selection of those B cells which consequently produce immunoglobulin with an improved affinity for antigen.[2–4] While understanding of the mechanisms of the B-cell selection process are now well advanced (reviewed in 5), the mechanisms underlying V gene-directed somatic hypermutation are still largely unknown.

Molecular aspects of the somatic hypermutation process have been studied in immunized mice by using polymerase chain reaction (PCR) amplification of immunoglobulin germline families whose use is antigen-restricted.[1–3,6,7] Such studies have revealed that the process produces mainly single point mutations and has a number of characteristic features including a preference for transitions over transversions, strand polarity, and targeting of mutations to hotspots (reviewed in 4). Study of hypermutation during immune responses in man, however, is more difficult. Clonal analysis of some human responses has been achieved by making hybridomas from Epstein–Barr virus (EBV)-transformed B cells from immunized donors.[8] Transgenic mice bearing human immunoglobulin miniloci have been used as an alternative to immunization to study somatic mutation during antibody responses in humans.[9,10] This approach however, has the disadvantages that only a limited repertoire of human V regions can be

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Abbreviations: CDR, complementarity determining region; EBV, Epstein–Barr virus; FR, framework region; GC, germinal centre; M-MLV, murine Moloney leukaemia virus reverse transcriptase; SSCP, single strand conformation polymorphism.

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introduced resulting in bias of the germline usage and that murine, not human, hypermutation machinery is used to induce the mutations.

Sequence analysis of B-cell subsets and single B cells picked from histological sections of peripheral lymphoid tissues indicates that somatic hypermutation is initiated in the rapidly proliferating centroblasts in the germinal centres (GC) of B-cell follicles.[11–13] The molecular characteristics of somatic hypermutation have been studied in human follicular and Burkitt's lymphoma cell lines because some show appreciable rates of spontaneous mutation in culture.[14,15] Attempts to study the mutation process in normal centroblasts however, have been hampered by the fact that GC B cells are programmed for apoptosis and die rapidly upon culture.[16]

Previously we have shown that single human GC B cells can be maintained *in vitro* by culture with mouse EL4 thymoma cells (in the 'EL4 system',[17,18]). Although these culture conditions initially sustain proliferation and maintain the phenotypic features of the freshly isolated GC B cells, they later promote progressive differentiation to plasma cells and, importantly, the cells do not accumulate somatic mutations. Cycling human GC B cells can be maintained for longer periods without terminal differentiation in bulk cultures supported by CD32 transfected mouse fibroblasts and CD40 antibody (the 'CD40 system',[19]) supplemented with appropriate combinations of cytokines.[20]

Here we have combined these two *in vitro* culture systems in order to study the signals regulating somatic hypermutation in human GC B cells. We used reverse transcription (RT)–PCR-based single-strand conformation polymorphism (SSCP) analysis to screen for mutational activity and confirmed mutations in positive clones by sequencing. We show that of two cytokine combinations that can maintain GC B-cell proliferation in CD40 stimulated cultures only one (IL-10 + IL-1β + IL-2) sustains active V gene-directed mutation. The characteristics of the mutations observed are consistent with those reported to be found *in vivo*.

MATERIALS AND METHODS

Monoclonal antibodies

AC2 (CD39) and G28-5 (CD40) were produced from hybridomas in the Department of Immunology, University of Birmingham (Birmingham, UK). G28-5 hybridoma was obtained from American Type Culture Collection (ATCC; Rockville, MD). The monoclonal antibodies were purified by ion exchange chromatography on DE52 cellulose (Whatman Ltd, Madison, UK). Non-conjugated anti-human immunoglobulin D (IgD) and R-phycoerythrin (RPE)-conjugated CD38 and CD3 antibodies were purchased from Becton-Dickinson (San Jose, CA). Fluoroscein isothiocyanate (FITC)-conjugated CD19 antibody and anti-IgD were purchased from Dako A/S (Glostrup, Denmark).

Cells

B cells from human tonsils were purified as previously described.[18,21] The tonsils were obtained from patients undergoing routine tonsillectomy at Lund University Hospital (Lund, Sweden) or at Malmö Academic Hospital (Malmö, Sweden). Briefly, tonsils were minced and mononuclear cells isolated using density gradient centrifugation on Ficoll-Isopaque (Pharmacia, Uppsala, Sweden). T cells were removed by two rounds of rosetting with neuraminidase-treated sheep red blood cells.[22] B cells were then fractionated by centrifugation on isotonic Percoll (Pharmacia Biotech) 60% (v/v) in RPMI. The buoyant cell fraction which was enriched for GC B cells was depleted of contaminating resting B cells and follicular mantle cells by negative selection using anti-IgD and CD39 antibody coated onto magnetic beads (Dynal, Norway). The resultant GC B fraction, defined as $CD38^+$ IgD^- $CD19^+$ was $<3\%$ $CD38^-$ IgD^+, $>96\%$ $CD19^+$ and $<0{\cdot}3\%$. $CD3^+$ T cells. The expression of CD19, CD38 and IgD on each population was estimated using flow cytometry as previously reported.[18]

Cell cultures

Culture medium was RPMI-1640 (Gibco, Paisley, UK) supplemented with 10% calf serum, 2 mM L-glutamine, 1% non-essential amino acids, 50 μg/ml gentamicin (Biological Industries, Hamek, Israel). GC B cells were seeded at a limiting dilution to give one cell/well in starting cultures for efficient evaluation by SSCP. GC B cells were first cultured for five days on γ-irradiated (5000 rad) human CD32 transfected murine L cells (DNAX Research Institute of Cellular and Molecular Biology; Palo Alto, CA) (2×10^4/well) with CD40 antibody (G28-5; 0·5 μg/ml) in 100 μl complete medium. The cultures were set up in flat-bottomed 96-well plates (Costar, Cambridge, MA). Cultures were supplemented with two different combinations of recombinant human cytokines: (i) IL-10, IL-1β and IL-2 or (ii) IL-4, IL-7 and IL-10 (R & D Systems Ltd, Oxford, UK), respectively, previously shown to promote optimal growth conditions for the cells preserving the GC B cell phenotype.[20] Cytokines were used at concentrations previously published to give optimal synergistic effect on GC B cells in the CD40 system.[20] After five days of culture, the wells were supplemented with γ-irradiated (5000 rad) murine EL4 thymoma cells (50×10^3/well), 100 U/ml IL-2 (Genzyme, Cambridge, MA), 3 ng/ml 4-phorbol 12-myristate 13-acetate (PMA; Sigma, Poole, UK) and 50 μM 2-mercaptoethanol (Merck, Darmstadt, Germany) together with 5% T-cell replacing factor [pokeweed mitogen (PWM)-TRF[23]]. After an additional five days of culture with the EL4 cells, the expanded B-cell clones were washed once in ice-cold phosphate buffered saline and lysed in Tris–HCl buffered 4 M GuSCN, pH 7·5 for RNA extraction.

Table 1. Oligonucleotide primers used for RT-PCR analysis of V genes

SGS13	5′-CTG AGG AGA CGG TGA CCA-3′	J_H universal 3′
SGS14	5′-A(AG)G (AT)AG (TC)CC T(TG)(TG) (GA)C(GC) AGG CAG-3′	Consensus,G,A,M H chain, aa141–147 3′
SGS15	5′-GAG GTG CAG CTG GTG GAG (AT)CT-3′	VH3 5′ FR1, aa1–7
SGS16	5′-TCC CTG AGA CTC TCC TGT-3′	VH3 FR1 reamplification, aa17–22

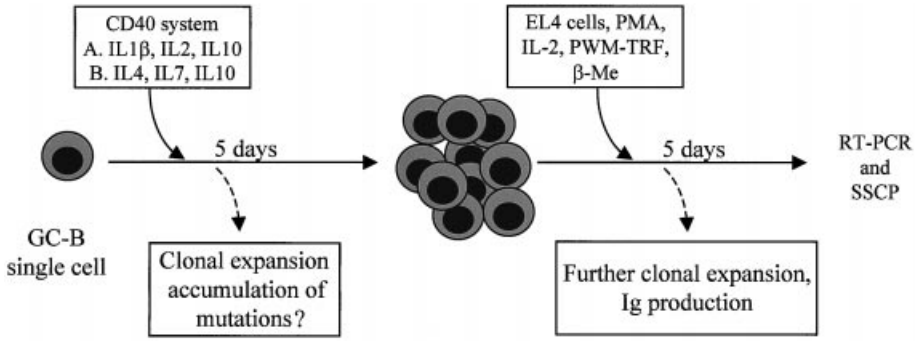

Figure 1. Schematic illustration of culture system employed. Germinal centre B cells, at limiting dilution, were cultured for five days with CD32 transfectants and CD40 antibody supplemented with (a) IL-1β, IL-2 and IL-10 or (b) IL-10, IL-7 and IL-4. After 5 days EL4 cells, PMA, IL-2, PWM-TRF and 2-mercaptoethanol were added. After a further 5 days of culture, cells were lysed for RNA extraction and RT–PCR-based SSCP.

RT-PCR analysis of V genes

mRNA was extracted according to a previously described protocol[23] using magnetic capture on oligo-d$(T)_{25}$-beads. Following magnetic capture (MPC-9600; Dynal) the oligo-d$(T)_{25}$ bound poly-A RNA was reverse transcribed using murine Moloney leukaemia virus (M-MLV) for 1 hr at 37° in a 20-µl reaction. Five µl of the cDNA reaction was amplified in a nested PCR using VH3-specific primers (Table 1). Primers were designed to bind 5′ in framework region 1 (FR1) (SGS15) and 3′ using a consensus for IgM, A and G (SGS14). Each cycle (total of 25) consisted of denaturation (96°, 30 s), primer annealing (55°, 30 s) and DNA synthesis (72°, 30 s (120 s for final cycle)). One µl of this PCR product was reamplified (25 cycles) using a 5′ primer downstream in FR1 (SGS16) and a universal 3′ primer in the J segment (SGS13). The products were then analysed on a preparative 4·5% Nusive GTG low melting point agarose gel and ethidium bromide was used for visualization. Lanes containing positive bands were cut out and purified from the gel (Wizard, Promega, Madison, WI). All PCR reactions were performed in a Perkin Elmer Gene Amp PCR system 2400.

Single-strand conformation polymorphism (SSCP) analysis

SSCP[24] was used as a rapid screen for mutations in the PCR-amplified samples. The analysis was performed using 1 µl of PCR-amplified and gel-purified product double-stranded DNA. Briefly, heat-denatured and ice-cooled PCR product was separated on a 12·5% native polyacrylamide PHAST gel using the PhastSystem™ (Pharmacia LKB Biotechnology, Uppsala, Sweden) with native buffer strips (native polyacrylamide gel electrophoresis; PAGE). The separated strands were detected with silver staining. The separation was run according to Application Note no. 383 (PhastSystem™) and the silver staining according to Application Note no. 210 (PhastSystem™).

In order to confirm the sensitivity of SSCP for mutational analysis cDNA from two closely related human hybridomas with known Igκ gene sequences was analysed. These hybridomas (anti-TF[25]; TF=Thomsen Friedenreich) which differed by one triplet in their IgV sequences were mixed at different cell ratios, mRNA prepared from the mixtures and cDNA obtained by VH3 specific nested PCR.

Cloning and sequence analysis

PCR-products for sequencing were cloned into a pGEM®-T vector using T/A cloning approach (Promega). To avoid further analysis of cloned Taq-errors, the originality of cloned PCR products were re-analysed after cloning using SSCP to identify the original banding-pattern of the cloned product. All sequencing was performed with BigDye™ (Perkin Elmer) reaction using M13 forward and reverse primers. The samples were collected and analysed on a ABI Prism 377 DNA Sequencer (Perkin Elmer).

Statistical analysis

Cell densities were calculated from the number of positive PCR products, reflecting outgrowth of VH3 positive cell clones, in relation to total number of wells screened.[26] SSCP data were analysed using the Poisson approximation to the binomial distribution and the significance assessed by the χ^2 test.

RESULTS

Single cell culture of human GC B cells

We seeded cultures with single GC B cells by limiting dilution[26] to allow detection of mutations arising in clonally related cells during proliferation *in vitro*. For the first 5 days single GC B cells were cultured in the 'CD40 system' supplemented with two different combinations of cytokines, each capable of supporting similar levels of CD40-dependent GC B-cell growth.[20] In order to obtain sufficient mRNA from the clones for RT–PCR-based SSCP analysis components of the 'EL4 system'[17] were then added to cultures to promote terminal differentiation.[18]

The details of the culture system employed to maintain GC B cells are outlined in Fig. 1. EL4 system components were added after 5 days culture in the CD40 system and mRNA was extracted after a further 5 days. These culture intervals were chosen in the light of previously reported kinetics of DNA synthesis and growth in the two systems when used independently for GC B-cell culture.[18,20] RNA was amplified for VH3 immunoglobulin by nested RT–PCR and double-stranded PCR products were analysed for mutations by SSCP. The appearance of more than two bands in SSCP analysis of PCR products as performed in this study is indicative of either mutation of the clone or the presence of more than one

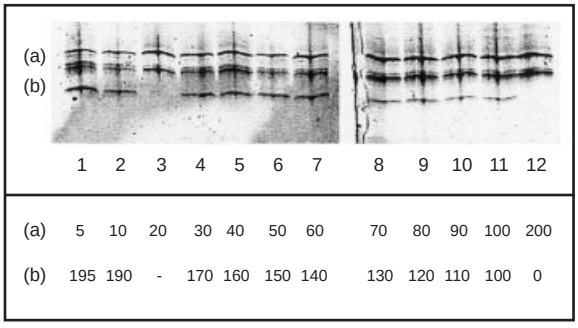

Figure 2. Sensitivity of SSCP in detecting mutations. SSCP analysis of cDNA obtained from mixtures of anti-TF hybridomas A and B that differed in one triplet in their IgV genes was performed as described in Materials and Methods. The relative numbers of each hybridoma in the mixtures are shown below each lane. No PCR product was obtained from hybridoma B in lane 3. As few as five cells of hybridoma A could be detected in the presence of 195 cells of hybridoma B (lane 1).

precursor (the likelihood of which follows the Poisson distribution).

We were able to detect as few as 2·5% hybridoma cells differing in one triplet in their V regions[25] by this approach (Fig. 2) suggesting that the sensitivity of the assay to detect mutations was as previously reported for SSCP.[27,28]

Detection of V gene mutation in cultured germinal centre B cells

SSCP analysis revealed clear differences between GC B cells that had been cultured with different cytokine combinations (Table 2, Fig. 3a,b). PCR products from cultures supplemented with IL-10 + IL-1β + IL-2 gave a greatly increased proportion of lanes containing more than two bands. After analysis of the proportion of cultures that failed to yield PCR products we concluded that the presence of more than two bands in those containing IL-10 + IL-1 + IL-2 was a result of mutations arising during growth *in vitro* and not seeding more than one precursor ($P < 0{\cdot}001$) (Table 2). Cells cultured with IL-10 + IL-1β + IL-2 gave 32/210 (15%) lanes with multiple bands compared with 1/90 (1·1%) for those cultured with IL-10 + IL-7 + IL-4. The frequency of single cell cultures containing IL-10 + IL-7 + IL-4 that gave more than two bands in SSCP was not significantly different from that which would be expected by chance (from the Poisson approximation to the binomial distribution, $P > 0{\cdot}3$), suggesting no mutations had occurred, whereas for cultures containing IL-10 + IL-1β + IL-2 there was a highly significant difference ($P < 0{\cdot}001$). Culture of GC B in the EL4 system alone failed to sustain mutational activity (Table 2).

Confirmation of immunoglobulin V gene mutations arising *in vitro*

In order to determine whether multiple bands in SSCP analysis of the PCR amplified samples were due to the presence of cells with different clonal origins growing in a single culture well or to changes within one clone that occurred during culture as a result of mutation, the DNA was cloned and sequenced. Before sequencing, the origin of the clone picked for sequencing was re-confirmed by SSCP to exclude the possibility of cloned Taq-errors.

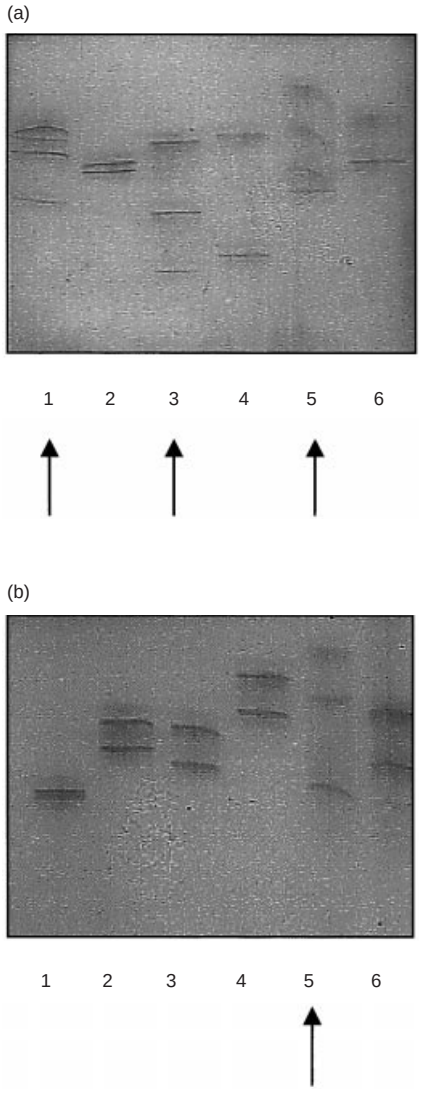

Figure 3. Representative gels of SSCP-analysis of cultured GC B cells. Culture conditions were as described in Fig. 1. After amplification of VH3 in a nested PCR, double-stranded PCR products were denatured and separated on a native polyacrylamide gel. Lanes containing more than two bands are indicated by arrows. Multiple bands (more than two) were found more frequently in cultures containing IL-10 + IL-1β + IL-2 (a: lanes 1, 3 and 5) compared with IL-10 + IL-7 + IL-4 (b: lane 5). Cloning and sequencing showed that the additional 2 bands in (b) lane 5 were due to different VH3 germline genes. Additional bands in (a) were due to *in vitro* mutations.

Bands from the representative SSCP-gels in Fig. 3(a) (lane 1, 3 and 5) and Fig. 3(b) (lane 5) (samples from experiment I, Table 2) were sequenced, as well as some of the lanes containing multiple bands from cells cultured with IL-10 + IL-1β + IL-2 to confirm differences at the DNA level between the clones. Table 3 shows that in one experiment two clones out of three represented material with one and two mutational differences, respectively. In a second experiment (Exp. II, Table 3) four of nine clones analysed represented mutated clones and one to three mutations were found in each

Table 2. SSCP analysis of cultured GC B cells

Cytokines	Expt.	No. of PCR products (no. of wells screened)	Cell density*	No. of wells giving >2 bands/lane in SSCP
IL-10 + IL-1β + IL-2	I	8 (30)	0·31	3
	II	21 (60)	0·43	9
	III	13 (60)	0·24	7
	IV	25 (60)	0·53	13
IL-10 + IL-7 + IL-4	I	13 (60)	0·24	1
	II	10 (30)	0·41	0

GC B cells were cultured for 5 days in the CD40 system supplemented with the cytokines indicated and then for further 5 days with the EL4 system. RNA was amplified for VH3 immunoglobulin by nested RT–PCR and double stranded PCR products were analysed for mutations by SSCP. For cultures containing IL-10 + IL-7 + IL-4 the frequency of samples giving >2 bands in SSCP was no more than would be expected by chance for seeding cultures with >1 precursor ($P > 0{\cdot}3$), whereas for those from cultures containing IL-10 + IL-1β + IL-2 it was significantly higher ($P < 0{\cdot}001$). When GC B cells were cultured in the EL4 system alone 2/27 PCR positive wells from 180 screened gave lanes with >2 bands in SSCP ($P > 0{\cdot}45$).

*Cell density (the estimated number of cells per well at start of cultures) was calculated according to the Poisson distribution, using the proportion of VH3 positive wells.

of these clones sequenced. Also, five of the wells contained sequences belonging to cells of different clonal origin, i.e. they were of different germlines and VDJ-junctions (Table 3). The one well that gave more than two bands in SSCP analysis of cells cultured with IL-10 + IL-7 + IL-4 (lane 5, Fig. 3b) contained sequences belonging to cells with a different clonal origin. The number of expected clones with different germlines was found to be consistent with calculations given for a 95% confidence interval at the cell density analysed (Table 3).

Onset of somatic mutation *in vitro*

When DNA samples which did not differ in VH3 germline configuration gave more than two bands in SSCP analysis the additional bands were always of similar strength to the originals (e.g. Fig. 3) This suggests that somatic mutations were introduced during the first cycle(s) of division in culture.

Figure 4(a) outlines the distribution of confirmed mutations in clones that were analysed. Two clones from well 2E demonstrated three single base pair mutations and showed some important features when compared to the nearest related germline sequence: two of the mutations belonged to one clone and the third to the other. This suggests a scenario illustrated in Fig. 4(b) in which a cell division occurred before somatic mutation of the V gene for that particular clone. This contrasted with other clones in which sequence comparisons suggested that mutations were likely to have occurred before cell division (Fig. 4c).

Similarity between V gene mutations generated *in vitro* and *in vivo*

Extended sequence analysis of the mutated clones was performed and the position of mutations was compared to sequence motifs previously described to be targets for somatic hypermutation.[29–31] The number of silent to replacement mutations, and their positions, was also analysed. The overall distribution of mutations is shown in Fig. 4(a) and Table 4. All mutations found within the complementarity-determining regions (CDR) represented replacement mutations, whereas most of those in the FRs were silent (Table 4). These features are reported to be characteristic of somatic hypermutation of immunoglobulin V genes.[4] Also, some of the mutations we observed occurred in motifs which have been reported to be targeted by the hypermutation process. For example, Table 4 shows that clone 5C which contains two G→A mutations corresponds to a motif suggested by Dunn-Walters *et al.*[29] in which G is the target nucleotide for non-selected, out-of-frame human immunoglobulin genes. The surrounding sequence in clone 5C (mutated nucleotide in bold) is A**G**AG and T**G**AG, respectively, while the suggested motif in this case would be D**G**HD (D = A/G/T; H = A/C/T). Also, there was partial agreement (three nucleotides out of four) with the suggested RGYW motif[30] for antigen-selected genes (clone 2E and 5B, Table 4).

DISCUSSION

Activation of somatic hypermutation is tightly regulated *in vivo*. The process is initiated in proliferating B cells in GCs but

Table 3. Sequence analysis of SSCP-positive clones to confirm mutations

Cytokines	Experiment	>2 bands/lane in SSCP	Clones with confirmed mutations (no. per clone)	Different germlines (95% CI)
IL-10 + IL-1β + IL-2	I	3	2 (1,2)	1 (0·7–1·7)
	II	9	4 (1,1,1,3)	5 (2·7–5·8)
IL-10 + IL-7 + IL-4	I	1	0	1 (0·9–2·2)

SSCP-positive clones were cloned, and re-confirmed with SSCP before sequencing.

The overall distribution of mutations is given in Fig. 4 and in Table 4.

The number of clones expected to contain different germlines according to Poisson distribution are given for a 95% confidence interval (CI) at the calculated cell density.

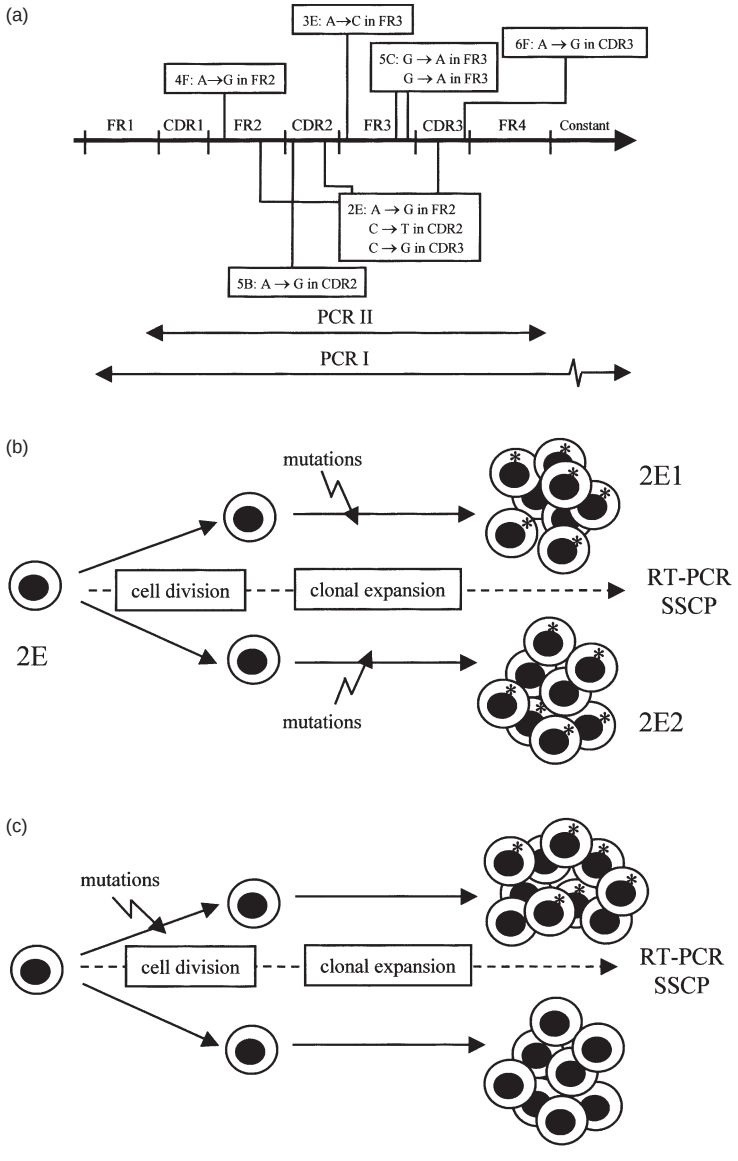

Figure 4. Distribution and origin of immunoglobulin V gene mutations arising *in vitro*. (a) The single nucleotide substitutions found within the V region of the PCR-amplified immunoglobulin genes. Three point mutations were found in the two clones in well 2E. Comparison with the nearest related germline sequence revealed that two of these mutations belonged to one of the two clones (2E1 in b) and the third mutation to the other clone (2E2). This indicates a scenario outlined in (b), in which cell division occurred before further accumulation of somatic mutations. In contrast, mutations in other clones are likely to have occurred before cell division (c). The design of the nested PCR employed is also shown in (a): PCR I denotes the first, and PCR II the second amplifications. *indicates a cell containing mutated IgVH3 genes.

not those in extrafollicular sites.[13] Study of ongoing mutation in the GC B cells has hitherto been prevented by an inability to maintain these cells in culture. We recently reported conditions for the *in vitro* maintenance of proliferating human GC B cells that retain the phenotypic characteristics of centroblasts[18,20] and in the present study we used these culture conditions to investigate whether signals that maintain proliferation and phenotype also sustain active mutation.

The two cytokine combinations investigated were able to support similar levels of CD40-dependent GC B-cell growth[20] yet only one (IL-10 + IL-1β + IL-2) was able to sustain active mutation. Our finding argues against a simple requirement for proliferating GC B cells (centroblasts) to remain in cycle for mutation to continue. Taken together, our results suggest that the process may be actively cytokine driven. As IL-10 was common to both cytokine combinations this implicates IL-1β and/or IL-2 as possible key regulatory cytokines in this regard. IL-2 and IL-10 have each been identified at the mRNA level in GC T cells and IL-1β in follicular dendritic cells at both the mRNA and protein level.[32] Tingible body macrophages that are found in the GC dark zone are another possible source of IL-1β. Soluble factors released by the CD32 fibroblast transfectants used in the CD40 system contribute to the cytokine-dependent maintenance of GC B cell proliferation[20] and we cannot exclude the possibility that they may also play a part in the observed mutational activity. However, as such

Table 4. Sequence analysis of mutated clones

Clone	Mutation	Position (amino acid)	Codon	Amino acid shift (S or R*)
2E	A→G	FR2 (41)	CCA→CCG	pro→pro (S)
	C→T	CDR2 (70)	TCC→TTC	ser→phe (R)
	C→G	CDR3 (88)	GCC→GGC	ala→gly (R)
5B	A→G	CDR2 (64)	AAG→AGG	lys→arg (R)
5C	G→A	FR3 (82c)	CTG→CTA†	leu→leu (S)
	G→A	FR3 (71)	AGA→AAA	arg→lys (R)
3E	A→C	FR3 (71)	AGA→CGA	arg→arg (S)
4F	A→G	FR2 (41)	CCA→CCG	pro→pro (S)
6F	A→G	CDR3 (87)	AGA→GGA	arg→gly (R)

*S, silent; R, replacement.

†The surrounding sequences in clone 5C (mutated nucleotide in bold) are **AG**AD and T**G**AG, while the motif for hypermutation suggested in 29 would be D**G**HD (D = A/G/T; H = A/C/T).

factors are common to both the mutating and nonmutating conditions identified for GC B cells, their role – if any – must be subsidiary only.

The ability of the cytokine combination IL-10 + IL-1β + IL-2 to sustain mutational activity was, *de facto*, CD40-dependent: GC B cells could not be maintained in culture in the absence of CD40 engagement.[20] The dependence of mutational activity on CD40 signals is consistent with current models of B-cell development. First, to enter a GC reaction – the exclusive site for somatic mutation *in vivo* – antigen-primed B cells must engage cognate T cells in a CD40L-dependent manner.[33] It is of interest to note that in the reports to date describing the *in vitro* induction of somatic mutation, either in B-cell lines or in primary B-cell populations, all demonstrated a requirement for activated T cells.[34–36] Second, it has been suggested that having entered a follicular reaction, differentiating centroblasts forming the dark zone might sustain their proliferation by means of autocrine, B-cell associated CD40L expressed, or released, in the locality.[37–39] Clearly, when established at the limiting cell numbers needed for our analysis, any CD40 signalling that would normally be provided by juxtacrine engagement *in vivo* would have to be provided exogenously *in vitro*. Finally, centrocytes, the non-dividing progeny of centroblasts, upon antigen-rescue, receive cognate signals from CD40L-containing $CD4^+$ T cells in the GC outer zone. The delivery of a CD40 signal here is thought to direct a proportion of the T-cell selected centrocytes to return to the dark zone (DZ) as centroblasts and thereby undergo further rounds of somatic mutation.[33] Each of these can be considered, since the cells we cultured were an approximately equal mix of centroblasts and centrocytes.

In addition to a requirement for T cells, the reports describing the induction of mutational activity in B cells *de novo* have highlighted a strict dependence on B-cell receptor (BCR) engagement.[34–36] Our finding that, for GC B cells, mutations can be sustained in the absence of BCR-derived signals is likely to reflect the distinctive origin and biology of these cells: centroblasts express, at best, low levels of surface immunoglobulin and opportunities for productive engagement of BCR on centroblasts by antigen in the follicular dendritic cell (FDC)-poor dark zone *in vivo* would not only be scarce but also inappropriate. Our findings indicate that a distinction exists between signals that can *initiate* mutation and those that *maintain* it. We cannot, however, exclude the possibility that engaging the BCR on cultured GC cells may alter the mutation rates observed, a possibility that – along with other considerations – can now be examined.

As the SSCP analysis indicated that the mutation of IgV genes *in vitro* was likely to have been confined to the first cycle(s) of culture it is possible that the number of cell divisions that occurred set a limit on the extent of mutations we observed. The culture conditions we employed may not have been optimal to maintain growth of single centroblasts nor promote re-entry of centrocytes into cell cycle.[40]

Importantly, as RT–PCR-based SSCP was employed to screen for mutations generated *in vitro* we verified that cultures giving positive results had contained single cells by cloning and sequence analysis of amplified DNA. We also excluded the possibility of mutations arising from Taq polymerase errors: cloned PCR products were always re-confirmed by SSCP analysis before sequencing.

The characteristics of the mutations we observed in cultured GC B cells (Table 4) were consistent with those reported *in situ*, for example the preponderance of replacement mutations within the CDRs and silent mutations in the FRs. However, we have not yet analysed sufficient sequences to draw firm conclusions regarding similarities with reported target motifs. We are currently performing an extended study of mutated clones from cultured GC B cells to analyse for motifs in mutated immunoglobulin V genes.

The approach described here should enable further study not only of the mechanisms of somatic hypermutation in the cells in which the process occurs physiologically but also of other molecular aspects of GC B-cell biology.

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