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Induction of Ig Somatic Hypermutation and Class Switching in a Human Monoclonal IgM⁺ IgD⁺ B Cell Line In Vitro: Definition of the Requirements and Modalities of Hypermutation

Hong Zan,* Andrea Cerutti,* Patricia Dramitinos,* András Schaffer,*† Zongdong Li,* and Paolo Casali*††

Partly because of the lack of a suitable in vitro model, the trigger(s) and the mechanism(s) of somatic hypermutation in Ig genes are largely unknown. We have analyzed the hypermutation potential of human CL-01 lymphocytes, our monoclonal model of germinal center B cell differentiation. These cells are surface IgM⁺ IgD⁺ and, in the absence of T cells, switch to IgG, IgA, and IgE in response to CD40:CD40 ligand engagement and exposure to appropriate cytokines. We show here that CL-01 cells can be induced to effectively mutate the expressed V\_H DJ\_H-C\_H, V\_H DJ\_H-C\_G, V\_H DJ\_H-C\_D, V\_H DJ\_H-C\_e, and VA\_J\_A-CA transcripts before and after Ig class switching in a stepwise fashion. In these cells, induction of somatic mutations required cross-linking of the surface receptor for Ag and T cell contact through CD40:CD40 ligand and CD80:CD28 coengagement. The induced mutations showed intrinsic features of Ig V(D)J hypermutation in that they comprised 110 base substitutions (97 in the heavy chain and 13 in the \(\lambda\)-chain) and only 2 deletions and targeted V(D)J, virtually sparing C\_H and C\_A. These mutations were more abundant in secondary V\_H DJ\_H-C\_\(\gamma\) than primary V\_H DJ\_H-C\_\(\mu\) transcripts and in V(D)\_J-C than VA\_J\_A-CA transcripts. These mutations were also associated with coding DNA strand polarity and showed an overall rate of \(2.42 \times 10^{-4}\) base changes/cell division in V\_H DJ\_H-C\_H transcripts. Transitions were favored over transversions, and G nucleotides were preferentially targeted, mainly in the context of AG dinucleotides. Thus, in CL-01 cells, Ig somatic hypermutation is readily inducible by stimuli different from those required for class switching and displays discrete base substitution modalities. The Journal of Immunology, 1999, 162: 3437–3447.

The process of V(D)J gene somatic hypermutation diversifies Abs, thereby providing the structural basis for selection by Ag of higher-affinity mutants and the maturation of the immune response. This process occurs within the germinal center (GC), where it requires T cell help and engagement of the surface B cell receptor for Ag (BCR) and remains one of the most intriguing features of the T cell-dependent immune response (1, 2). Somatic Ig V(D)J gene hypermutation is thought to be operative at the centroblastic stage (3). At the centrocytic stage, B clones with a BCR with high affinity for the inducing Ag would undergo Ag-driven positive selection, while autoreactive B cells or low-affinity clones undergo negative selection through apoptosis (4). In vivo and in vitro studies have suggested that Ig hypermutation displays: 1) a prevalence of point-mutations together with occasional insertions and deletions (5, 6); 2) an intrinsic preference for certain “hotspots” (7); 3) a dependence on initiation of transcription, A \(\times\) T bias, and DNA strand polarity (2, 5, 8, 9); 4) a dependence on cis-acting elements, including the intronic and 3′ enhancers in the \(\kappa\) locus (10, 11); and, finally, 5) a preference for secondary Ig isotypes (12). However, the lack of a well-defined in vitro model of GC differentiation has limited our understanding of the requirements for the induction, the modalities, and the mechanisms of hypermutation.

CD40:CD40 ligand (CD40L) engagement in association or not with BCR cross-linking in the presence of various cytokines has led to the induction of proliferation and isotype switching, but not somatic hypermutation (13–16). Consistent with the primary role of T cells in GC formation in vivo (1, 17, 18), Ig somatic mutations have been induced in vitro in mouse and human B cells in the presence of T cell help and upon BCR engagement (19–21). This, together with the finding that certain monoclonal B cell lines, such as the murine 18.81 cells (22) and a human follicular lymphoma line (23), mutate spontaneously in vitro in the absence of specific triggers, provided impetus for the identification of the Burkitt’s lymphoma cell line BL2, which was found to accumulate somatic mutations in the expressed IgM upon BCR cross-linking and coculture with activated T cells (24). However, these cells appear to be frozen at the surface (s)IgM⁺ sIgD⁻ phenotype and are incapable of switching to downstream Ig isotypes and undergoing concomitant phenotypic differentiation. A cell line that enables analysis of the requirements and the modalities of somatic

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‡ Abbreviations used in this paper: GC, germinal center; BCR, B cell surface receptor for Ag; CD40L, CD40 ligand; CD40L-293 cells, CD40L-transfected human embryonic kidney 293 cells; CDR, complementarity determining region; FR, framework region; R, replacement (mutation); S, silent (mutation); SSCP, single-strand conformational polymorphism; TCM, T cell conditioned medium; s, surface.
hypermutation as it relates to Ig class switching and other GC differentiative processes would constitute a more appropriate and useful model of physiological Ig hypermutation.

We have analyzed the Ig somatic hypermutation potential of our recently identified monoclonal model of GC B lymphocyte differentiation, human CL-01 cells. These B cells express a founder centroblast-like phenotype, including sIgM, sIgD, CD38, and CD77 (15, 16, 25, 26). Following engagement of CD40 by CD40L and exposure to the appropriate cytokines, they undergo a coordinated maturation program that includes Ig class switching to all seven downstream isotypes, progression through phenotypic GC stages, and differentiation to memory-like B cells and plasma cells. We show here that CL-01 cells can be induced to not only switch to IgG, IgA, and IgE, but also to effectively mutate the V_{H}DJ_{H} and V_{L}J_{L} gene segments, while sparing the C_{H} and C_{L} regions. CD40 and CD80 coengagement by T cell CD40L and CD28 is necessary, in addition to BCR engagement, for the induction of Ig hypermutation. Mutations accumulated in a stepwise fashion before and after class switching and were distributed throughout the entire V(D)J gene segment, indicating a lack of selection by Ag. These mutations showed preference for transitions over transversions, biased targeting of G within the AG dinucleotide, and evidence of strand polarity.

Materials and Methods

CL-01 cells

The human B cell line CL-01 has been described (15). CL-01 cells are grown in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (FCS-RPMI) at <10^4/ml.

T cells

CD4^+ T cells were positively selected from normal PBMCs, prepared by fractionation through Histopaque 1077 (Sigma, St. Louis, MO) using anti-CD4 mAb-conjugated magnetic beads (Miltenyi Biotech, Auburn, CA). They were cultured in PCS-RPMI and expanded by weekly stimulation with a feeder cell mixture containing irradiated (1200 rad) PBMCs, 100 μg/ml of PHA (Life Technologies), and 100 U/ml of human rIL-2 (Genzyme, Cambridge, MA) for 10 days. For C: B cell coculture experiments, CD4^+ T cells were used at least 2 wk after their last activation with feeder cells and were incubated for 6 h with 20 ng/ml of PMA (Sigma) and 500 ng/ml of ionomycin (Calbiochem-Novabiochem, San Diego, CA) before use with B cells.

T:B cell cultures

CL-01 cells were cultured at 0.5 × 10^5 cells/well in the presence or absence of 2.5 × 10^5 irradiated (4000 rad) CD4^+ T cells or 1 × 10^5 irradiated (4000 rad) human CD40L-transfected 293 cells (CD40L-293 cells) in a flat-bottom 6-well plate (5.0 ml FCS-RPMI volume). For B:T cell cocultures, plates were previously coated with 1,800 OKT3 mAb (Ortho Diagnostics Systems, Raritan, NJ) for 2 h at room temperature. To cross-link the BCR, CL-01 cells were reacted for 2 h at 4°C with Sepharose-conjugated rabbit Ab to human Ig light chain (2 μg/ml; Irvine Scientific, Santa Ana, CA) and then washed with cold PBS. After 7 days of culture, CL-01 cells were collected, freed of dead cells and debris by fractionation through Histopaque 1077 (Sigma), reacted again with anti-BCR Ab, washed, and reseeded over a new layer of irradiated T cells or CD40L-293 cells (in plates coated or not coated with anti-OKT3 mAb) in the presence or absence of cytokines. At day 14 of culture, CL-01 cells were harvested for total RNA extraction. In selected B:T cell cocultures, T cell conditioned medium (TCM) obtained from the culture fluids of T cells activated for 1 wk with 1,800 OKT3 mAb and IL-2 (100 U/ml) was used at a concentration of 1.5:1 IL-4 (Genzyme) and IL-10 (Schering-Plough, Kenilworth, NJ) were used at 100 U/ml and 100 ng/ml, respectively. The CD40-CD40L, CD80:CD80L, and CD30:CD30L interactions were blocked by preincubating the B cells with saturating amounts (30 μg/ml) of mouse anti-human CD80 mAb to human CD40L (Ancell, Bayport, MN), mouse CD28.2 mAb to human CD28 (PharMingen, San Diego, CA), mouse Ber-H2 mAb to human CD30 (Dako, Carpinteria, CA), or mouse B1/B7-1 mAb to human CD80 (PharMingen).

PCR amplification of V(D)J transcripts

RNA was extracted from 2 × 10^6 cells using the RNeasy total RNA kit (Qiagen, Valencia, CA). mRNA was reverse transcribed using the SuperScript preamplification system for first strand cDNA synthesis (Life Technologies). V_{H}DJ_{H}-C_{H} transcripts were amplified with a V_{H}3 leader-specific sense primer LV_{H}3(5’)-ATGGAGCATTTGGGCCTGGAC(GC)TTGGCTCACATGGCCTGCA(5’)(Cathy 3) and the antisense-specific primers C_{H}1-μ- (5’)-GGTCCGTTGCGCTGGTCGAG3 ’ (spanning C_{H} mRNAs 268–288), C_{H}3-TCCAGAGGCTGCAAGGCGAG3’ (C_{H}, 200–225), universal primer C_{H}-1y (5’)-CAGGCTGGTCCGGACGGCTGAC3’ (Cy, 206–226), C_{H}1-α (5’)-CGTGGACTGCTGGTTGCGGCGACGGCTGAC3’ (Cy, 209–223), or C_{H}1-ε (5’)-CGGAGGCTGACAAAGCAGTCATGGAC3’ (Cy, 201–223), using Cloned Phi DNA polymerase (Stratagene, La Jolla, CA), and the reaction buffer provided by the manufacturers with 30 cycles, each consisting of a 1-min denaturation at 94°C, a 1-min annealing at 58°C, and a 1-min extension at 72°C. The amplification was completed by an additional 10-min extension at 72°C. To amplify V_{A}1-JA-CA transcripts, the V_{A}1 leader-specific sense primer LVA1(II)-ATGGAGCATTTGGGCCTGGAC(GC)TTGGCTCACATGGCCTGCA (Cathy 3) and the CA-specific antisense primer CA(II)-CGTGGACTGCTGGTTGCGGCGACGGCTGAC3’ (Cy, 202–223) were used with the PCR conditions described above. The PCR cDNA products were purified with the PCR purification kit (Qiagen) and ligated into pCR-Script SK vector (pCR-Script Cloning Kit, Stratagene, La Jolla, CA), which was used to transfect in XLI-Blue MRF supercompetent cells (Stratagene). The (positive) white bacterial colonies were screened by PCR (28) using the V_{H}3 leader sense primer LV_{H}3(II) (5’)-GTTCTATTTTAAAAGGTGTCCAGTGT-3’ (CL-01 heavy chain leader, 21–57) and the consensus J_{H}1 antisense primer (5’)-CGTGCACCTGCTCCTCTCA-3’ for V_{H}DJ_{H}C_{H} clones and the internal V_{A}1 leader sense primer LVA1(II)-ACCCCTCTCCTACTCAGTGGACG3’ (Cy, 23–45) together with the internal CA antisense primer CA(II)-3’-TGTCAGCTGGACAGTCCTGCAGAGGA-3’ (CA, 42–64) for V_{A}CA clones. The individual colonies that had been directly used as PCR templates were seeded onto fresh Luria-Bertani medium plates and expanded overnight. The clones containing the V_{H}DJ_{H}C_{H} or V_{A}CA-SC transcripts were selected for single-stranded conformational polymorphism (SSCP) analysis.

Detection of mutated V_{H}DJ_{H}C_{H} and V_{A}CA-SC transcripts by SSCP

Mutated V_{H}DJ_{H}C_{H} and V_{A}CA-SC transcripts were identified by SSCP analysis (29). cDNAs for SSCP analysis were amplified by PCR (30 cycles of a 1-min denaturation at 94°C, a 1-min annealing at 58°C, and a 1-min extension at 72°C) using the cDNA product inserted into pCR-Script SK vector as template in a 10-μl reaction volume with Taq DNA polymerase (Life Technologies) in the presence of 1 μCi [α-32P]dCTP (NEF Linen Sciences, Boston, MA) (300 Ci/mmol). The internal V_{H}3 leader sense primer LV_{H}3(II) and C_{H}1 antisense primer C_{H}(5’)-AGAGCAGGGGGGGAAGGGGTG3’ (C_{H}, 18–37), C_{H}(5’)-TTGAGAAACACTTCCCGGCTCGAG3’ (C_{H}, 8–30), universal primer C_{H}(5’)-GGAAGTGACGATCCCTTGTTGGAG3’ (C_{H}, 9–27), C_{H}(5’)-GACCTTGGGGTGGCTGCGG3’ (C_{H}, 23–34), or C_{H}(5’)-CCGGACGGTGATCCTGGAG3’ (C_{H}, 4–27) were separately used for V_{H}DJ_{H}C_{H} or V_{H}DJ_{H}C_{V}, V_{H}DJ_{H}C_{H} or V_{H}DJ_{H}C_{V}, or V_{H}DJ_{H}C_{H} or V_{H}DJ_{H}C_{V} analysis. To analyze the λ chain, the V_{A}1 leader sense primer LVA1(II) and Ca antisense primer CA(I) were used. Optimal sensitivity is achieved when SSCP is performed using 100- to 300-ng DNA fragments. Therefore, our amplified ~500-bp fragments were digested with KpnI and then diluted 1:15 in 10 mM EDTA, 0.1% SDS. The labeled, cleaved, and diluted DNA samples were mixed with an equal volume of sequencing stop solution containing 95% formamide, 20 mM NaOH, 20 mM EDTA, 0.05% bromophenyl blue, and 0.05% Xylene cyanol. Samples were denatured for 10 min at 98°C, chilled on ice, and immediately loaded in 3-μl aliquots onto a 6% acrylamide gel (20:1 acrylamide:fd) with 1× Tris/boric acid/EDTA buffer (100 mM Tris, 90 mM boric acid, and 1 mM EDTA) containing 10% glycerol. Electrophoresis was at room temperature.
in the SSCP gel (not shown). 336 bp of the Vl frequency of mutation was calculated with Inh. Sus. Calc. program v 1.0 (30).

Tated V(D)J sequence (unstimulated CL-01 cells). The Ig V(D)J gene inherent fre-

The census of the somatic point-mutations was performed by counting identical mutations in more than one transcript (of any isotype) only once. It was assumed that identical base changes in different transcripts were shared mutations, although some of these mutations might have arisen as hotspots and therefore could actually be independent mutations. The obtained values were used for the compilation of Tables I (last column), II, III, IV, and V. For the compilation of the fourth column of Table I, all point-mutations found in all sequenced transcripts were counted. For the fifth column of Table I, the somatic point-mutations found in different transcripts of the same isotype were counted only once.

Comparison of the observed with the expected frequency of replacement (R) and silent (S) somatic point-mutations was performed using the inherent mutation rate of the CL-01 VμDJH and VAJA sequences, calculated by the Inh. Sus. Calc. program version 1.0 for the Macintosh (30) and a binomial distribution model, as reported by Chang and Casali (30). The comparisons of the observed with the expected number of mutations for each individual nucleotide residue to each of the three other nucleotides were performed using a contingency table ($\chi^2$ test). The expected frequency of mutations was calculated by taking into account the base composition of the unmutated CL-01 V(D)J sequence; that is, it was corrected by the frequency of occurrence of the individual nucleotides, or dR-, tR-, or tetranucleotides considered within the CL-01 V(D)J sequence assuming randomness.

<table>
<thead>
<tr>
<th>VμDJH</th>
<th>Mutated/Total Transcripts</th>
<th>Mutated Transcripts</th>
<th>Mutations in All Sequenced Transcripts</th>
<th>Unique Mutations in Transcripts of Different Isotypes$^*$</th>
<th>Unique Mutations in Transcripts of All Isotypes$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>μ</td>
<td>16/112</td>
<td>7</td>
<td>15</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>δ</td>
<td>20/110</td>
<td>14</td>
<td>30</td>
<td>29</td>
<td>28</td>
</tr>
<tr>
<td>γ</td>
<td>17/120</td>
<td>12</td>
<td>59</td>
<td>49</td>
<td>43</td>
</tr>
<tr>
<td>α</td>
<td>9/86</td>
<td>4</td>
<td>10</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>e</td>
<td>3/24</td>
<td>1</td>
<td>7</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Total VμDJH</td>
<td>65/452</td>
<td>38</td>
<td>121</td>
<td>108</td>
<td>96</td>
</tr>
<tr>
<td>VAJA</td>
<td>18/179</td>
<td>9</td>
<td>16</td>
<td>13</td>
<td>13</td>
</tr>
</tbody>
</table>

$^*$ Identical mutations in different transcripts of the same isotype were assumed not to be independent and were counted only once.

<table>
<thead>
<tr>
<th>CDRs</th>
<th>FRs</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>VμDJH b</td>
<td>21</td>
</tr>
<tr>
<td>[22] r</td>
<td>[6]</td>
</tr>
<tr>
<td>VAJA a</td>
<td>1</td>
</tr>
<tr>
<td>[2]</td>
<td>[1]</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
</tr>
<tr>
<td>[24]</td>
<td>[7]</td>
</tr>
</tbody>
</table>

$^*$ In addition to the R and S mutations, an amber stop codon was found in the heavy chain FR3 region.

$^*$ Identical mutations in different transcripts of the same and different isotypes were assumed not to be independent and were counted only once.

The [expected] number of mutations was calculated by multiplying the number of observed total (R and S) mutations by the R mutation frequency inherent to the V(D)J gene CDR or FR sequence and the relative size of those regions in the unmutated V(D)J sequence (unstimulated CL-01 cells). The Ig V(D)J gene inherent frequency of mutation was calculated with Inh. Sus. Calc. program v 1.0 (30).

**Results**

CL-01 cells switch to IgG, IgA, and IgE, and hypermutate the V(D)J gene in both primary and secondary isotypes upon BCR engagement and exposure to T cells

Human CL-01 cells are monoclonal and express sIgM and sIgD with a chain. The VμDJH segment consists of a Vμ26–3.7-like gene (90% identity) (31), rearranged to D4N and D5 genes, and an unmutated Jμ6c gene. The VAJA segment consists of a 1e.10.2/ DPL2 VA1-like gene (94% identity) (32), rearranged to a λ2 gene with a single change, probably a point-mutation. Multiple sequences at different times of VμDJH-Cμ, VμDJH-Cδ, and VAJA-CA cDNAs from cultured CL-01 cells revealed no somatic mutations (not shown). To determine whether CL-01 cells could be induced to hypermutate the expressed Ig V(D)J genes, these cells were reacted with an anti-light chain Ab to cross-link the BCR and then cultured with activated human T cells. After 14 days, mRNA was extracted from the cultured cells and reverse transcribed. Ig V(D)J-C cDNAs were amplified using the CL-01 Vμ or VA gene leader sense primer in combination with an antisense Cμ, Cδ, Cα, Ce, or CA primer and were cloned into appropriate vectors for further “nested” PCR amplification (in the presence of [32P]dCTP) of V(D)J-C cDNAs. Amplified [32P]-labeled V(D)J-C cDNAs were digested with KpnI for SSCP analysis. Those V(D)J-C cDNAs defined as mutated by SSCP were then sequenced (Fig. 1A).

As expected from the detection of abundant IgG, IgA, and IgE in the culture fluids (not shown), VμDJH-Cγ, VμDJH-Cγ-Cα, and
cDNAs were readily amplified, in addition to V<sub>H</sub> DJ<sub>H</sub>-C<sub>m</sub> and V<sub>H</sub> DJ<sub>H</sub>-C<sub>d</sub>cDNAs, from CL-01 cultured with T cells after BCR engagement. By SSCP analysis, 16 of 112 (14.3%) V<sub>H</sub> DJ<sub>H</sub>-C<sub>m</sub> transcripts were mutated, i.e., they displayed a gel mobility different from that of corresponding transcripts from CL-01 cultured in medium alone, based on the analysis of 50 identical V<sub>H</sub> DJ<sub>H</sub>-C<sub>m</sub> and 50 identical V<sub>L</sub> J<sub>L</sub>-C<sub>L</sub> transcripts (Table I and Fig. 1B). Also mutated were 20 of 110 (18.2%) V<sub>H</sub> DJ<sub>H</sub>-C<sub>d</sub>, 17 of 120 (14.2%) V<sub>H</sub> DJ<sub>H</sub>-C<sub>g</sub>, 9 of 86 (10.5%) V<sub>H</sub> DJ<sub>H</sub>-C<sub>a</sub>, 3 of 24 (12.5%) V<sub>H</sub> DJ<sub>H</sub>-C<sub>e</sub> transcripts, and 18 of 179 (10.1%) V<sub>L</sub> J<sub>L</sub>-C<sub>L</sub> transcripts (Table I and Fig. 1B). In contrast, no mutated transcripts were found in CL-01 cells cultured with anti-BCR Ab in the absence of activated T cells (50 VH DJ<sub>H</sub>-C<sub>m</sub>, 50 VH DJ<sub>H</sub>-C<sub>d</sub>, and 50 VH DJ<sub>H</sub>-C<sub>g</sub> transcripts analyzed).

### Table III. Nature of the base substitutions in the Ig V<sub>H</sub>DJ<sub>H</sub> gene segment of induced CL-01 cells

<table>
<thead>
<tr>
<th>Transitions</th>
<th>G → A</th>
<th>A → G</th>
<th>C → T</th>
<th>T → C</th>
</tr>
</thead>
<tbody>
<tr>
<td>44</td>
<td>19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>[32]</td>
<td>[9.7]&lt;sup&gt;b&lt;/sup&gt;</td>
<td>[7.6]</td>
<td>[7.8]</td>
<td>[6.8]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Transversions</th>
<th>G → C</th>
<th>A → C</th>
<th>C → A</th>
<th>T → A</th>
</tr>
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<tbody>
<tr>
<td>23</td>
<td>9</td>
<td>1</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>[32]</td>
<td>[9.7]</td>
<td>[7.6]</td>
<td>[7.8]</td>
<td>[6.8]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total mutations</th>
<th>G → N</th>
<th>A → N</th>
<th>C → N</th>
<th>T → N</th>
</tr>
</thead>
<tbody>
<tr>
<td>96</td>
<td>45&lt;sup&gt;*&lt;/sup&gt;</td>
<td>7&lt;sup&gt;**&lt;/sup&gt;</td>
<td>31</td>
<td>13&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>[96]</td>
<td>[29.2]</td>
<td>[22.8]</td>
<td>[23.5]</td>
<td>[20.4]</td>
</tr>
</tbody>
</table>

<sup>a</sup> Identical mutations in different transcripts of the same and different isotypes were assumed not to be independent and were counted only once.

<sup>b</sup> The [expected] number of mutations (from a given nucleotide residue to another given nucleotide residue) was normalized for the base composition of the unmutated V<sub>H</sub> DJ<sub>H</sub> sequence. It was calculated by multiplying the frequency of occurrence of the nucleotide target of mutation in the unmutated sequence (unstimulated CL-01 cells) by the total number of observed mutations, and dividing this product by three. For instance, the expected number of G → A mutations was calculated by multiplying 0.30 (G frequency of occurrence in the unmutated V<sub>H</sub> DJ<sub>H</sub> sequence) by 96<sup>5</sup>29.2, divided by 3 (as G → A, G → C, and G → T mutations have all the same theoretical probability to occur) = 9.7.

<sup>*</sup>, p < 0.05; **, p < 0.001.

### Table IV. Somatic point-mutations induced in the V<sub>H</sub>DJ<sub>H</sub> gene segment of CL-01 cells preferentially target AG and CT dinucleotides

<table>
<thead>
<tr>
<th>Dinucleotides</th>
<th>Number of occurrences&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent of the total dinucleotides</th>
<th>Observed number of mutations&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Expected number of mutations&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Observed to expected mutation ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG</td>
<td>28</td>
<td>7.5</td>
<td>32</td>
<td>14.4</td>
<td>2.22&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>GA</td>
<td>26</td>
<td>7.0</td>
<td>14</td>
<td>13.4</td>
<td>1.04</td>
</tr>
<tr>
<td>TA</td>
<td>16</td>
<td>4.3</td>
<td>2</td>
<td>7.8</td>
<td>0.26&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>GC</td>
<td>18</td>
<td>4.8</td>
<td>13</td>
<td>9.2</td>
<td>1.41</td>
</tr>
<tr>
<td>CG</td>
<td>14</td>
<td>3.7</td>
<td>7</td>
<td>7.1</td>
<td>0.99</td>
</tr>
<tr>
<td>AC</td>
<td>27</td>
<td>7.2</td>
<td>10</td>
<td>13.8</td>
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Total: 374 100 192 192.0 1.00

<sup>a</sup> The V<sub>H</sub>DJ<sub>H</sub> segment of CL-01 cells comprises 375 nucleotides. Therefore, the number of occurrences is 375–1, as dinucleotides are identified in two different frames (i.e., beginning with residue 1 and residue 2).

<sup>b</sup> Identical mutations in different transcripts of the same and different isotypes were assumed not to be independent and were counted only once.

<sup>c</sup> The number of mutations in any dinucleotide cohort is twice the number of actual somatic point-mutations because each mutation is counted twice as being shared by two partially overlapping dinucleotides.

<sup>d</sup> The expected number of mutations for any given dinucleotide takes into account the base composition of the unmutated V<sub>H</sub>DJ<sub>H</sub> sequence (i.e., it was corrected for the sequence base composition). It was calculated by multiplying the frequency of occurrence of each of the dinucleotides considered in the unmutated sequence by the total number of observed point-mutations in the V<sub>H</sub>DJ<sub>H</sub> region.

<sup>*</sup>, p < 0.001.
without BCR engagement (50 V<sub>H</sub>D<sub>I<sub>H</sub></sub>-C<sub>M</sub>, 50 V<sub>H</sub>D<sub>I<sub>H</sub></sub>-C<sub>S</sub>, and 50 V<sub>H</sub>D<sub>I<sub>H</sub>-C<sub>Y</sub> transcripts analyzed). Thus, after BCR engagement and exposure to activated T cells, CL-01 cells effectively Ig class switch and mutate both primary (V<sub>H</sub>D<sub>I<sub>H</sub></sub>-C<sub>M</sub> and V<sub>H</sub>D<sub>I<sub>H</sub></sub>-C<sub>S</sub>) and secondary (V<sub>H</sub>D<sub>I<sub>H</sub>-C<sub>Y</sub>, V<sub>I<sub>H</sub></sub>D<sub>I<sub>H</sub>-C<sub>Y</sub>, and V<sub>I<sub>H</sub></sub>D<sub>I<sub>H</sub>-C<sub>Y</sub>-Ce) heavy chain transcripts, as well as V<sub>J<sub>A<sub>-CA transcripts.

**CD40:CD40L and CD80:CD28 coengagement is required for the induction of Ig somatic hypermutation**

As T cell-derived soluble factors have been implicated in sustaining Ig V gene somatic hypermutation (21), we assessed the ability of these factors to substitute T cells in the induction of Ig mutation in CL-01 cells. No mutated primary or secondary Ig transcripts were detected in CL-01 cells cultured after BCR engagement with TCM further enriched with IL-4 and IL-10 (not shown), even upon CD40 engagement by CD40L-293 cells (50 V<sub>H</sub>D<sub>I<sub>H</sub></sub>-C<sub>M</sub>, 50 V<sub>H</sub>D<sub>I<sub>H</sub>-C<sub>S</sub>, and 50 V<sub>I<sub>H</sub></sub>D<sub>I<sub>H</sub>-C<sub>Y</sub> transcripts analyzed) (Fig. 1B). To further explore the role of CD40 in the induction of hypermutation, CD40L on the activated T cells was blocked using a mouse mAb to human CD40L. The failure of the CL-01 cells cultured with this anti-CD40L mAb, but not the putatively irrelevant anti-CD30 mAb, to hypermutate the expressed Ig even upon BCR engagement (60 V<sub>H</sub>D<sub>I<sub>H</sub></sub>-C<sub>M</sub> and 40 V<sub>I<sub>H</sub></sub>D<sub>I<sub>H</sub>-C<sub>Y</sub> transcripts analyzed from anti-CD30 Ab cultures and 50 V<sub>H</sub>D<sub>I<sub>H</sub>-C<sub>Y</sub> and 52 V<sub>J<sub>A<sub>-CA transcripts from anti-CD30 Ab cultures) indicates that CD40 engagement by CD40L, not mere T:B cell contact, is necessary to induce the mutational machinery in CL-01 cells (Fig. 1B).

Because the CD28 activation pathway has been reported to be essential for the Ab response to T cell-dependent Ags in the mouse (33), we addressed the role of this costimulatory molecule in the induction of somatic hypermutation. Blocking of CD28 on the surface of activated T cells using an anti-CD28 Ab made these T cells ineffective inducers of hypermutation (50 V<sub>H</sub>D<sub>I<sub>H</sub></sub>-C<sub>M</sub>, 50 V<sub>H</sub>D<sub>I<sub>H</sub>-C<sub>S</sub>, and 50 V<sub>I<sub>H</sub></sub>D<sub>I<sub>H</sub>-C<sub>Y</sub> transcripts analyzed) when added to CL-01 cells after BCR engagement (Fig. 1B). That CD28 was required to engage CD80 (the CD28 complement on the B cell surface) to induce Ig hypermutation was further indicated by the lack of mutations (50 V<sub>H</sub>D<sub>I<sub>H</sub>-C<sub>M</sub>, 50 V<sub>I<sub>H</sub></sub>D<sub>I<sub>H</sub>-C<sub>S</sub>, and 50 V<sub>I<sub>H</sub></sub>D<sub>I<sub>H</sub>-C<sub>Y</sub> transcripts analyzed) in CL-01 cells cultured with activated T cells in the presence of a blocking mouse anti-CD80 mAb (Fig. 1B). Thus, in addition to BCR engagement, engagement of the CD40:CD40L and CD80:CD28 costimulatory pairs is necessary for the induction of somatic Ig hypermutation in human B cells.

**The mutations induced in CL-01 cells show intrinsic features of Ig V(D)J somatic hypermutation, but not evidence of Ag-driven selection**

The 38 heavy chain and 9 λ-chain transcripts that appeared mutated by SSCP analysis were sequenced, and all contained somatic point-mutations. These V<sub>H</sub>D<sub>I<sub>H</sub></sub>-C<sub>M</sub>, -C<sub>S</sub>, -C<sub>Y</sub>, -Ca, -Ce, and V<sub>J<sub>A<sub>-CA transcripts expressed by the CL-01 cultured alone (unmutated template) (Fig. 2). A total of 123 mutations were found in the 38 V<sub>H</sub>D<sub>I<sub>H</sub></sub>-C<sub>M</sub>, V<sub>I<sub>H</sub></sub>D<sub>I<sub>H</sub>-C<sub>Y</sub>, V<sub>I<sub>H</sub></sub>D<sub>I<sub>H</sub>-C<sub>Y</sub>-Ce, and V<sub>I<sub>H</sub></sub>D<sub>I<sub>H</sub>-Ce transcripts (575 bp each). A total of 121 were point-mutations confined to the V<sub>H</sub>D<sub>I<sub>H</sub></sub>-C<sub>M</sub> sequence (375 bp), one was a deletion of three nucleotides in the framework region (FR) 3 of a V<sub>H</sub>D<sub>I<sub>H</sub></sub>-C<sub>M</sub> transcript (γ913), and one was a C→G S mutation at residue 87 of the C<sub>H</sub>1 sequence of a V<sub>H</sub>D<sub>I<sub>H</sub>-M<sub>1</sub> transcript (μ105) (Fig. 2A). The 121 V<sub>H</sub>D<sub>I<sub>H</sub></sub>-C<sub>M</sub> point-mutations corresponded to an overall frequency of 1.22 × 10<sup>−3</sup> changes/
base (Table I), >30-fold the PCR amplification error rate with high-fidelity Pfu DNA polymerase (~$10^{-6}$ bases/cycle, i.e., $4.0 \times 10^{-5}$ changes/base in 30 cycles) ($\rho < 0.001$). The single nucleotide change in the C_H1 region of V_HDJ_H-C_H1 transcript $\mu_{015}$ represented a frequency of $1.35 \times 10^{-4}$ changes/base, which was threefold the PCR error rate. The 121 V_HDJ_H nucleotide changes comprised 96 independent mutations, 17 of which were observed in multiple transcripts.

The 96 independent point-mutations consisted of 68 R, 27 S, and one stop codon mutations and yielded an overall mutation rate of $2.42 \times 10^{-4}$ base changes/cell division.

Seventeen mutations were found in the 9 V_lJ_l-C_l transcripts sequenced (539 bp each). Sixteen were point-mutations confined to the V_lJ_l sequence (336 bp), and one was a 3-nucleotide deletion in the FR2 of transcript $\lambda_{013}$. No mutations were found in the CA1
sequence (203 bp). The 16 V\alpha\lambda point-mutations corresponded to an overall frequency of 5.4 × 10^{-4} changes/base (Table I), a rate >10-fold the PCR error rate for 30 cycles (p < 0.001). These mutations comprised 13 independent mutations, 3 of which were observed in multiple transcripts. The 13 independent mutations consisted of 7 R and 6 S mutations and yielded an overall mutation rate of 1.08 × 10^{-2} base changes/cell division, a value lower than that in V\gamma DJ\gamma (p < 0.05).

The highest load of mutations was found in the single V\gamma DJ\gamma -C\gamma transcript sequenced (Table I). Overall, the V\gamma DJ\gamma -C\gamma transcripts bore a mutational load two- to threefold greater than that of the V\gamma DJ\gamma -C\mu, -C\delta, -C\alpha, or V\alpha\lambda -CA transcripts (Table I). In the V\gamma DJ\gamma -C\gamma transcripts, the 96 independent point-mutations targeted 83 nucleotide residues scattered throughout the V\gamma DJ\gamma sequence, with no preferential segregation to complementarity-determining regions (CDRs) or FRs (Fig. 2 and Table II). Likewise, in the V\alpha\lambda -CA transcripts, the 13 independent mutations targeted 13 nucleotide residues scattered throughout the V\alpha\lambda sequence, with no preferential segregation to CDRs or FRs (Fig. 2). In both the V\gamma DJ\gamma and V\alpha\lambda segments, the number of R mutations in CDRs was lower than that theoretically expected by chance alone, and, therefore, inconsistent with a positive selection of R mutations by Ag (Fig. 2 and Table II).

The somatic mutations induced in vitro in CL-01 cells are scattered through the entire Ig V(D)J gene sequence and preferentially target selected nucleotides. A, Schematic depiction of the V\gamma DJ\gamma -C\gamma and the V\alpha\lambda -CA sequences of mutated transcripts (as defined by SSCP analysis) from CL-01 cells cultured with activated T cells after BCR engagement. Bars depict S mutations, lollipops depict R mutations, solid boxes (■) depict deletions, and the cross (×) depicts a stop codon. B, Sequences containing the 121 point-mutations identified in the 38 V\gamma DJ\gamma -C\gamma and the 16 point-mutations in the 9 V\alpha\lambda -CA transcripts from CL-01 cells cultured with activated T cells after BCR engagement as compared with the sequences of the unmutated V\gamma DJ\gamma and V\alpha\lambda templates (unstimulated CL-01 cells). Different nucleotide changes found in different transcripts are in many cases listed in the same row, resulting in a total of only 6 rows for V\gamma DJ\gamma and 2 rows for V\alpha\lambda, instead of a total of 38 and 9, respectively, actual mutated transcripts sequenced.

The mutations induced in CL-01 cells show a bias for transitions over transversions and preferentially target G nucleotides and AG and CT dinucleotides

Randomly occurring point-mutations are expected to be one-third transitions and two-thirds transversions, but the 96 unique V\gamma DJ\gamma point-mutations were equally divided between transitions and transversions (p < 0.01) (Table III), and the 14 unique V\alpha\lambda point-mutations consisted of almost twice as many transitions (n = 9) as transversions (n = 5) (p < 0.01). In the V\gamma DJ\gamma transcripts, G nucleotides were mutated at a frequency (46.9% of total mutations) about 50% higher than that expected by chance alone (33.3%) after correcting for base composition, i.e., normalizing for the relative occurrence of G in the unmutated V\gamma DJ\gamma sequence template (p < 0.05), with G → A transitions accounting for 42.2% of the total G mutations and 43.2% of the total transitions (Table III). Preferential mutation of G was associated with scarcity of mutations in A (p < 0.001) and T (p < 0.001) and stochastic
A preference for transitions over transversions and G nucleotide targeting could be discerned in the V_{\text{VJ}} transcripts, but the small sample size did not allow a meaningful statistical conclusion.

Certain sequence motifs have been suggested to be preferentially targeted by the hypermutation machinery (2, 6, 8, 34–36). We considered the 16 possible dinucleotides identifiable in the VH DJ H sequence and made a census of all the point-mutations targeting these dinucleotides (the V_{\text{VJ}} sequence was not considered because of the small number of point-mutations) (Table IV). All 16 dinucleotides were targeted by mutations except for TT. The AG dinucleotide and its inverse repeat CT were found to be mutated at a frequency significantly higher than that expected by chance alone (p < 0.001) (Table IV). This was not due to a mere G and C preference (Table IV), as G and C were mutated at a significantly lower frequency when occurring outside than within AG and CT (38 and 45% vs 62 and 55% of total G and C mutations, respectively). In addition, GA and TC, which contain the same nucleotides in inverse order, contained 2.3- and 3.4-fold fewer G mutations than AG and CT (Table IV).

GAG and AGG trinucleotides are preferential targets of point-mutations as a result of the preferential targeting of G within the AG dinucleotide

We examined all occurrences of AGN, NAG, CTN, and NCT trinucleotides to verify whether the observed concentration of somatic point-mutations in the AG and CT dinucleotides could in fact reflect a preference for the trinucleotides that harbor these dinucleotides (Table V). In addition, GA and TC, which contain the same nucleotides in inverse order, contained 2.3- and 3.4-fold fewer G mutations than AG and CT (Table IV).

The RGYW tetranucleotide motif is virtually spared by somatic point-mutations

The consensus RGYW motif, where R is a purine (A or G), Y is a pyrimidine (C or T), and W is A or T, has been identified as a mutational hotspot (35). Census of the CL-01 Ig VH DJ H sequence for the presence of the RGYW tetranucleotide revealed that this motif occurred 11 times in 6 different iterations (AGCA twice, AGCT once, AGTA twice, AGTT none, GGCA once, GGCT three times, GGTA twice, and GGTT none) and was mutated more frequently than expected only in the GGCT iteration (p < 0.001). In the unmutated V_{\text{HiDJH}} sequence, G occurred 69 times outside AG, AGA, GAG, or AGG and 36 times as part of these di- or trinucleotides, but was the target of mutation 16 and 29 times, respectively, in different transcripts (p < 0.001). AG was targeted by mutations at the expected frequency whether occurring in or outside AGA, GAG, and AGG trinucleotides. Thus, the high frequency of mutations in AGA, GAG, and AGG trinucleotides reflected the preferential targeting of G within the AG dinucleotide.
Somatic point-mutations accumulate stepwise concomitant with Ig class switching

The ability of slgM⁺ slgD⁺ CL-01 cells to switch to IgG, IgA, and IgE allowed us to address the relationship between somatic hypermutation and Ig class switching. A genealogical tree was constructed using the V_{H}DJ_{H} sequences derived from CL-01 cells cocultured with T cells in a single 14-day culture after BCR engagement. Identical mutations in different transcripts were considered to be shared mutations, although some of these might have arisen as hotspots and therefore could actually be independent mutations. There are three branches to the genealogical tree we generated, spanning four, three, and two generations (Fig. 3). The first branch stemmed from a first-generation putative intermediate bearing a 56 G → A transition, which could be tracked down to two fourth-generation IgG-switched elements bearing 5 and 10 point-mutations (γ013 and γ002), through a putative second-generation intermediate bearing the 56 G → A and a 267 G → A transition and a third-generation putative intermediate bearing four shared mutations, flanked by an IgD intermediate bearing the second-generation shared mutations plus a third mutation (336 G → A). The second branch stemmed form a first-generation putative intermediate bearing a 20 G → A transition, which gave rise to two third-generation elements, one still expressing IgD (δ0075), the other switched to IgE (ε001), both with seven point-mutations, of which two shared the 115 C → A transition and the original 20 C → A transition. An unswitched IgD element bearing five point-mutations and a putative IgD/IgM second-generation element with two mutations that were conserved in the third-generation elements were also part of this branch. Finally, the third branch consisted of a first-generation element characterized by a 355 C → A transversion, which gave rise to IgM (μ015) and IgA (α006) second-generation elements bearing three and five point-mutations, of which only the original 355 C → A transversion was shared. Thus, the mutational machinery was active throughout class switching in CL-01 cells, and point-mutations accumulated in a stepwise fashion in IgG, IgA, and IgE.

Discussion

The present findings show that human CL-01 cells can be induced to hypermutate the expressed V_{H}DJ_{H} genes in vitro with modalities that are congruent with those inferred from studies in vivo. Together with our other findings (15, 16, 25, 26), they also show that in this monoclonal cell line somatic hypermutation is induced in the context of a coordinated program that recapitulates GC B cell differentiation, whereby CL-01 cells that are slgM⁺ slgD⁺ switch to IgG, IgA, and IgE and eventually give rise to plasma-cytes and memory-like B cells. In CL-01 cells, somatic hypermutation is induced in all primary and secondary V_{H}DJ_{H}-C_{H} as well as V_{\Lambda_\Lambda}-CA transcripts, when the appropriate stimuli are applied, i.e., BCR engagement and T cell contact, allowing for engagement of at least the two costimulatory molecule pairs CD40:CD40L and CD80:CD28, whereby CL-01 cells that are sIgM⁺ differentiate, spanning four, three, and two generations (Fig. 3). The first branch stemmed from a first-generation putative intermediate bearing a 56 G → A transition, which could be tracked down to two fourth-generation IgG-switched elements bearing 5 and 10 point-mutations (γ013 and γ002), through a putative second-generation intermediate bearing the 56 G → A and a 267 G → A transition and a third-generation putative intermediate bearing four shared mutations, flanked by an IgD intermediate bearing the second-generation shared mutations plus a third mutation (336 G → A), the other switched to IgE (ε001), both with seven point-mutations, of which two shared the 115 C → A transition and the original 20 C → A transition. An unswitched IgD element bearing five point-mutations and a putative IgD/IgM second-generation element with two mutations that were conserved in the third-generation elements were also part of this branch. Finally, the third branch consisted of a first-generation element characterized by a 355 C → A transversion, which gave rise to IgM (μ015) and IgA (α006) second-generation elements bearing three and five point-mutations, of which only the original 355 C → A transversion was shared. Thus, the mutational machinery was active throughout class switching in CL-01 cells, and point-mutations accumulated in a stepwise fashion in IgG, IgA, and IgE.

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The ability of CL-01 cells to undergo Ig somatic hypermutation and class switching as part of an integrated differentiation program has allowed us to determine that the requirements for the induction of these two central GC maturational processes are different. As we have shown (15, 16, 25), CD40 engagement by soluble trimeric CD40L or CD40L-expressing 293 cells in the absence of T cells effectively induces CL-01 cells, as well as freshly isolated normal human IgM⁺ IgD⁺ B cells, to switch to IgG, and IgA, and, in the presence of IL-4, to IgE. As we show here, CD40:CD40L engagement is necessary but not sufficient for the induction of Ig somatic hypermutation. CD40L-expressing 293 cells cannot substitute for T cells in inducing somatic hypermutation, even in the presence of TCM, further enriched with IL-4 and IL-10, and after BCR engagement. The failure of these switched B cells to accumulate mutations further points to somatic hypermutation and class switching as two independent and discrete processes and indicates that engagement of additional B:T cell costimulatory molecule pair(s) is dispensable to trigger the mutational machinery.

Our present findings strengthen those showing that both BCR engagement and T cell help are required to trigger Ig hypermutation (21, 24) and extend them by providing evidence that B:T cell contact allowing for CD40:CD40L and CD80:CD28 coengagement is necessary, in addition to BCR engagement, to induce this process. Furthermore, by showing that anti-CD30 mAb fails to interfere with the induction of the mutational machinery, they provide an explanation for the putatively normal somatic hypermutation process observed in CD30-deficient mouse mutants (37) and indicate that the anti-CD40L, anti-CD80, and anti-CD28 mAbs ablated Ig hypermutation not by merely reducing or abrogating B:T cell contact, but rather by specifically interfering with CD40: CD40L or CD80:CD28 coengagement and related signaling. Whether, after BCR engagement, CD40:CD40L and CD80:CD28 coengagement is sufficient to induce Ig hypermutation or the co-engagement of these costimulatory pairs mediates the induction of crucial T cell surface molecules and/or factors remains to be determined.

Ig somatic hypermutation and class switching are intimately linked in the GC, where point-mutations account for a vast majority of the changes, which include only scarce insertions and deletions (38). Ig hypermutation is thought to begin in centroblasts before class switching (15, 39). It is not known whether high-affinity GC centrocytes that have undergone isotype switching undergo further hypermutation, although it has been suggested that isotype switching does not terminate hypermutation (40). Our present findings at the clonal level suggest that the onset of somatic hypermutation is not related to Ig class switching to IgG, IgA, or IgE and further emphasize that these are two independent processes. They also show that the modalities of in vivo GC hypermutation are reflected in CL-01 cells, in which, vis-à-vis of 137 somatic point-mutations, only two codon deletions were found, both involving triplets and both leaving the transcripts in frame. The conservation of shared mutations among primary and secondary isotypes and the observation of nonmutated Ig secondary iso-type transcripts suggest that B cells accumulate somatic mutations along cell divisions before and after isotype switching occurs.

In induced CL-01 cells, V_{H}DJ_{H}-C_{\Lambda_\Lambda} and -C_{\mu} transcripts bore a load of somatic mutations approximately twofold greater than that of V_{H}DJ_{H}-C_{\mu} transcripts or their primary V_{H}DJ_{H}-C_{\mu} and V_{H}DJ_{H}-C_{\delta} counterparts (Table I). The higher frequency of mutations in CL-01 cell V_{H}DJ_{H}-C_{\Lambda_\Lambda} transcripts in vitro extends findings in vivo showing, in centroblasts, a heavier load of mutations in V_{H}DJ_{H}-C_{\Lambda_\Lambda} than in V_{H}DJ_{H}-C_{\mu} transcripts (3, 41) and indicates that this differential mutational load may reflect an inherent feature of the integrated hypermutation and class-switching processes. This contention would be further supported by the 1000-fold higher mutation rate in V_{H}DJ_{H}-C_{\Lambda_\Lambda} construct than in its V_{H}DJ_{H}-C_{\mu} counterpart after stable integration into an established B cell line (42, 43). In CL-01 cells, the frequency of mutations in V_{H}DJ_{H}-C_{\Lambda_\Lambda}, V_{H}DJ_{H}-C_{\mu}, and V_{H}DJ_{H}-C_{\delta}, but not V_{H}DJ_{H}-C_{\mu} transcripts was about two- to fourfold higher than in V_{\Lambda_\Lambda}-CA transcripts. A similar higher load of mutations in the heavy chain than the light chain of Ag-selected Abs has been interpreted to reflect the dominant role of the V_{H}DJ_{H} over the V_{\Lambda_\Lambda}L segment in providing the structural correlate for Ag binding (44, 45). Our in vitro findings in a system putatively devoid of nominal antigenic pressure suggest
that more efficient targeting of mutations to the heavy chain is an intrinsic property of the hypermutation machinery.

In vivo studies have shown that Ig V(D)J somatic point-mutations are not generated randomly in terms of base substitution, preference, and distribution (hotspots) (6, 36, 46). In induced CL-01 cells, G bases were preferentially targeted with G → A transitions, which are especially copious in all transcripts of different heavy chain isotypes. The preferential G targeting in CL-01 cells differs from the lack of such a bias in productive and non-productive human V_{H}DJ_{H} rearrangements ex vivo (27, 47–53) and contrasts with the preferential targeting of A in the mouse (8, 36). A preferential targeting of G nucleotides has been observed in vitro in human BL2 cells (24), Chinese hamster cells (54), and mouse B cells (55) and in vivo in exothemers that do not have GCs (56), suggesting the mechanism(s) driving such a mutational preference is phylogenetically conserved and is operational in the absence of the GC microenvironment. A preferential G targeting has also been found in MSH2 mismatch repair protein-deficient mice (57, 58) and may be related to the activity of this protein, which corrects mismatches mainly at G and C (58).

The identification of sequence-specific preferences by the somatic hypermutation machinery is important as it may provide clues in determining the molecular mechanism of this process. In addition to base targeting bias, mutational hotspots have been identified throughout Ig V(D)J gene sequences (6, 36, 46). While some of these hotspots may reflect the application of selection force(s) on the gene product, others may be inherent to the nucleotide sequence targeted by mutations, as suggested by the analysis of “passenger” or nonselectable genes (59, 60). The putative lack of the application of a nominal positive or negative selective pressure to CL-01 cells under the culture conditions used here should allow for an insight into the base preference(s) and hotspot(s) that are inherent to the mutational machinery. In our experiments, the striking bias for G mutations (more than half of all VH DJ H point-mutations) would reflect a marked mutational preference for the AG dinucleotide, whether isolated or in the context of AGA, GAG, and AGG trinucleotides.

For convention, all the present mutations in CL-01 cells were recorded from the coding strand, although it is not known on which strand mutations occur. The high G:C mutation ratio (1.6) in CL-01 cells resembles the marked bias toward mutation of purines in the coding strand, especially G → A transitions as in sheep V_{A} regions (61). As emphasized by Storb, one cannot detect a strand bias if there is not also a G/C or A/T bias (8). In CL-01 cells, the higher than expected frequency of G mutations recorded from the coding strand is consistent with either preferential G targeting by the mutational machinery in the nontranscribed (“top”) strand or preferential C targeting by the mutational machinery in the transcribed (“bottom”) strand. In CL-01 cells, this strand polarity is further supported by the finding that T accumulated almost twice as many mutations as A and is in agreement with the proposed strand polarity of somatic mutations in the experimental mouse (2, 5, 8), although, in the mouse, strand polarity has been associated with an A > T bias.

The Ser encoded by AGC or AGT, particularly at position 31 of Ig V(D)J sequences, has been reported to constitute a hotspot both in vivo (2, 36) and in vitro (24). AGC and AGT codons occur five times in the CL-01 Ig V_{H}DJ_{H} sequence, and only two of them were mutated, neither at codon 31 (AGC). A comparable sparing of AGC and AGT codons by mutations has been found in several human Ig V(D)J sequences in vivo (38) and in vitro (20). In general, many AGC/T sequences are not mutational hotspots, and the local targeting of mutations is not simply dependent on the two or three bases surrounding the hotspot (2). Other structural features, such as palindromes, may well be important (62). Likewise, a scarcity of mutations was found in the different iterations of the RGYW motif in the of CL-01 cell V_{H}DJ_{H} gene sequence, despite the originally proposed hotspot nature of this sequence (35). This suggests that the hypermutation process has been subjected to evolutionary pressure to yield substitutions over the whole V(D)J region and that aspects of local secondary structure are also likely to contribute to the formation of mutational hotspots. These and other mechanistic issues, including those related to the activation of the mutational machinery, can be optimally addressed by further use of CL-01 cells.

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References

action of the immunoglobulin mutator system. Genome 31:118.
lymphoma B cell line hypermutates its functional immunoglobulin genes in vitro.
24. Deenopoux, S., D. Razanajaona, D. Blanchard, G. Meffe, J. D. Capra, J. Banchereau,
cell line in vitro. Immunity 6:35.
25. Cerutti, A., A. Schaffer, S. Shah, H. Zan, H-C. Liou, R. G. Goodwin, and
P. Casali. 1998. CD30 is a CD40L-inducible molecule that negatively regulates
CD40-mediated immunoglobulin class switching in non-antigen-selected human
sequence upstream of the human Ig H chain S3y region is a functional promoter:
synergistic activation by CD40L and IL-4 via cooperative NF-kB and STAT-6
1997. V, DJ, V genes sequences and antigen reactivity of monoclonal antibodies
produced by human B-1 cells: evidence for somatic selection. J. Immunol. 158:
2477.
and colonies by the polymerase chain reaction. Nucleic Acids Res. 17:4000.
detection of point mutations and DNA polymorphisms using the polymerase
chain reaction. Genomics 5:874.
30. Chang, B., and P. Casali. 1994. The CDRI sequences of a major proportion of
human germline Ig V genes are inherently susceptible to amino acid replace-
USA 94:5284.
35. Rogozin, I. B., and N. Kolchanov. 1992. Somatic hypermutagenesis in immun-
oglobulin genes. II. Influence of neighbouring base sequences on mutagen-
37. Sasseville, G., H. Jacobs, M. Meiering, R. Kuhn, J. Roes, W. Muller, S. Gilti
can, H. Fujitaka, H. Kikutani, N. Yoshida, R. Amakawa, C. Benoist, D. Mathis,
T. Kishimoto, T. W. Mak, and K. Rajewsky. 1996. Somatic hypermutation occurs
in B cells of terminal deoxynucleotidyl transferase-, CD23-, interleukin-4-, IgD-
39. Danon, D., K. Johnson, and P. Casali. 1996. The CDRI sequences of a major proportion of
human germline Ig V genes are inherently susceptible to amino acid replace-
40. Phung, Q. H., D. B. Winter, A. Cranston, R. E. Tarone, V. A. Bohr, R. Fishel, and
P. J. Gearhart. 1998. Increased hypermutation at G and C nucleotides in immu-
noglobulin genes. II. Influence of neighbouring base sequences on mutagen-
41. Sohn, J., A. Berger, and M. Apel. 1991. Maturation of the immune response in
42. Harndranath, N., I. S. Goldfarb, H. Ikematsu, S. E. Burastero, R. L. Wilder,
A. L. Notkins, and P. Casali. 1991. Complete sequence of the genes encoding the
V and V regions of low- and high-affinity monoclonal IgM and IgA rheu-
matoi factors produced by CDS B cells from a rheumatoid arthritis patient. Int.
Immunol. 3:865.
43. Kasaian, M. T., H. Ikematsu, J. E. Ballow, and P. Casali. 1994. Structure of the
V and V segments of monoreactive and polyreactive IgA autoantibodies to DNA
44. Kasaian, M. T., H. Ikematsu, J. E. Ballow, and P. Casali. 1994. Structure of the
V and V segments of monoreactive and polyreactive IgA autoantibodies to DNA
46. Phung, Q. H., D. B. Winter, A. Cranston, R. E. Tarone, V. A. Bohr, R. Fishel, and
P. J. Gearhart. 1998. Increased hypermutation at G and C nucleotides in immu-
noglobulin genes. II. Influence of neighbouring base sequences on mutagen-
47. Harndranath, N., I. S. Goldfarb, H. Ikematsu, S. E. Burastero, R. L. Wilder,
A. L. Notkins, and P. Casali. 1991. Complete sequence of the genes encoding the
V and V regions of low- and high-affinity monoclonal IgM and IgA rheu-
matoi factors produced by CDS B cells from a rheumatoid arthritis patient. Int.
Immunol. 3:865.
V and V segments of monoreactive and polyreactive IgA autoantibodies to DNA
50. Phung, Q. H., D. B. Winter, A. Cranston, R. E. Tarone, V. A. Bohr, R. Fishel, and
P. J. Gearhart. 1998. Increased hypermutation at G and C nucleotides in immu-
noglobulin genes. II. Influence of neighbouring base sequences on mutagen-
51. Harndranath, N., I. S. Goldfarb, H. Ikematsu, S. E. Burastero, R. L. Wilder,
A. L. Notkins, and P. Casali. 1991. Complete sequence of the genes encoding the
V and V regions of low- and high-affinity monoclonal IgM and IgA rheu-
matoi factors produced by CDS B cells from a rheumatoid arthritis patient. Int.
Immunol. 3:865.
52. Dorner, T., H.-P. Brezinschek, R. I. Brezinschek, S. J. Foster, R. Domiati-Saad,
and P. E. Lipsky. 1997. Analysis of the frequency and pattern of somatic muta-
tions within nonproductively rearranged human variable heavy chain genes. J.
sequence of the genes encoding the V and V regions of low- and high-affinity
monoclonal IgM and IgA rheumatoid factors produced by CDS B cells from a rheumatoid arthritis patient. Int.
Immunol. 3:865.