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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 \( V_H \) joining-constant region (\( J_H \)) transcripts, and paired \( V_H J_H -C_H \) 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:CD82 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 CA. These mutations were more abundant in secondary \( V_H \) \( J_H -\gamma \) than primary \( V_H J_H -\mu \) transcripts and in V(D)J-C than \( V_H J_H -A \) transcripts. These mutations were associated with coding DNA strand polarity and showed an overall rate of \( 2.42 \times 10^{-4} \) base changes/cell division in \( V_H J_H -C_H \) 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 > 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⁺ IgD⁺ 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-transfected human embryonic kidney 293 cells, CD40L-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 differentiate 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). 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 IgG1, IgA1, and IgE, but also to effectively mutate the VsDJH and VgJh gene segments, while sparing the C4 and C6 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, biasing targeted 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 sIgM+ sIgD+ and monoclonal, as shown by blotting and Southern hybridization with labeled Jβ3 probes and by the expression of unique VβDJβ4-Cα and VβDJβ5-Cα transcripts (27). These cells display on both chromosomes the switch (S)μ, Sδ, and Sγ3, Sγ1, Sγ2, Sγ4, Sε, and Sο2 regions in germline configuration (15). They also express the phenotype of GC founder centroblasts, including CD38 and CD77. Upon engagement of CD40 by CD40L and exposure to appropriate cytokines, CL-01 cells undergo a coordinated program of GC differentiation involving characteristic phenotypic changes and switching to all downstream isotypes, i.e., IgG3, IgG1, IgA1, IgG2, IgG4, IgA2, and IgE, eventually giving rise to plasma-cytoid elements and memory-like B cells (15, 16, 25). sIgM+ sIgD+ CL-01 cells were cultured in RPMI (160 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated FCS. 2 mM MgCl₂, 100 μM penicillin, and 100 μg/ml streptomycin (FCS-RPMI) at 10⁶/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 FCS-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 μM of human rIL-2 (Genzyme, Cambridge, MA) for 2 weeks. A T:B cell coculture experiment, 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 FITC (Sigma) and 500 ng/ml of Iono- mycin (Calbiochem-Novabiochem, San Diego, CA) before culture with B cells. The experiment described above. The PCR cDNA products were purified with the PCR purification kit (Qiagen) and then diluted 1:15 in 10 mM EDTA, 0.1% SDS. The PCR cDNA products were amplified with the VH 3 leader-specific sense primer LVA1(I) (5′-ATGGACCTGATTGGGAAAAAGTGGG-3′) and the consensus JH antisense primer (5′-GGTTCAGGCTCAACGACCTG-3′) (Cy, 209–223), or Cy1-I′-ε (5′-CGGAGGTCGCCAGAACTGCTATTTTAAAAGGTGTCCAGTGT-3′) (Taq, 201–223), using Cloned Pfu DNA polymerase (Strategene, La Jolla, 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-BRAB, 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 T:B 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: IL-4 (Genzyme) and IL-10 (Scherling-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 B cells with saturating amounts (30 μg/ml) of mouse IgG1 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, Carpenteria, CA), or mouse B1/B7-1 mAb to human CD28 (PharMingen). PCR amplification of V(D)J transcripts

RNA was extracted from 2 × 10⁶ cells using the RNeasy total RNA kit (Qiagen). mRNA was reverse transcribed using the SuperScript premiunction system for first strand cDNA synthesis (Life Technologies). Vβ3DJβ4-Cα transcript cDNAs were amplified with a Vβ3 leader-specific sense primer LVA3(I) (5′-ATGGACCTGATTGGGAAAAAGTGGG-3′) and the antisense-specific primers Cy1-I′-ε (5′-GGTTCAGGCTCAACGACCTG-3′) (Cy, 209–223), or Cy1-I′-ε (5′-CGGAGGTCGCCAGAACTGCTATTTTAAAAGGTGTCCAGTGT-3′) (Taq, 201–223), using Cloned Pfu DNA polymerase (Strategene, 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 Vα1-Jα-Cα transcripts, the Vα1 leader-specific sense primer LVA1(I) (5′-ATGGACCTGATTGGGAAAAAGTGGG-3′) and the CA-specific antisense primer LVA1(I′-ε) (5′-CGGAGGTCGCCAGAACTGCTATTTTAAAAGGTGTCCAGTGT-3′) (Taq, 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 (Strategene). The (positive) white bacterial colonies were screened by PCR (28) using the Vβ3 leader sense primer LVA1(I) (5′-GGTTCAGGCTCAACGACCTG-3′) (CL-01 heavy chain leader, 21–57) and the consensus JH antisense primer (5′-CGGAGGTCGCCAGAACTGCTATTTTAAAAGGTGTCCAGTGT-3′) for Vβ3DJβ4-Cα clones and the internal Vα1 leader sense primer LVA1(I′-ε) (5′-ACCCTCCTACACTCTGCTG-3′) (CL-01 α-chain leader, 23–45) together with the internal CA-antisense primer CA(I′) (5′-TTGGCTGTTAGGCTTACGAGGA-3′) (CA, 42–64) for VA-Jα-CA transcripts. 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 Vβ3DJβ4-Cα or VA-Jα-CA transcripts were selected for single-stranded conformational polymorphism (SSCP) analysis.

Detection of mutated Vβ3DJβ4-Cα and VA-Jα-CA transcripts by SSCP

Mutated Vβ3DJβ4-Cα and VA-Jα-CA 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 cloned cDNA 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 μM primer [α-32P]dCTP (NEN Life Sciences, Boston, MA) (3000 Ci/mmol). The internal Vα3 leader sense primer LV3(I) and CA(I′-ε) antisense primer CA(I′-ε) (5′-AGACAGGGGAAAAAGTGGG-3′) (Cμ, 18–37), Cε (5′-TGGAGAACCTACACCGCACGGCTGTG-3′), Cg (5′-GGAAGCTACACCGCACGGCTGTG-3′), Cγ (5′-GGAAGCTACACCGCACGGCTGTG-3′) (Cy, 24–33), or Ce (5′-CCAAGGTCGCCAGAACTGCTATTTTAAAAGGTGTCCAGTGT-3′) (Cy, 54–77) were separately used for Vβ3DJβ4-Cα, Vβ3DJβ4-Ce, Vβ3DJβ4-Cg, or Vβ3DJβ4-Cγ analysis. To analyze the λ-chain, the Vα1 leader sense primer LVA1(I′-ε) and the CA antisense primer CA(I′-ε) were used. Optimal sensitivity is achieved when SSCP is performed using 100–300 bp DNA fragments. Therefore, our amplified PCR products were digested with KpnI and then diluted 1:15 in 10 mM EDTA, 0.1% SDS. The labeled, cleaved, and diluted DNAs were mixed with an equal volume of sequencing stop solution containing 95% formamide, 20 mM NaOH, 20 mM EDTA, 0.05% bromophenol 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/bis) 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. Downloaded from www.jimmunol.org by guest on October 29, 2017
for 18 h at 6 W. Gels were autoradiographed on Kodak X-Omat AR film (Eastman Kodak, Rochester, NY).

**Sequencing Ig V(D)J-C transcripts**

The clones displaying an altered electrophoretic mobility in SSCP gel were analyzed by sequenced to confirm and characterize the nature of the mutations. Plasmids were extracted using QIAprep Spin Miniprep kit (Qiagen) and sequenced on both strands using Taq dideoxy terminator cycle sequencing kit and a 373 automatic sequencer (Applied Biosystems, Foster City, CA). Sequences were compared with the unmutated CL-01 V_HDJ_H, C_H1 and VAJA-CA sequence from CL-01 cells cultured in medium only using the MacVector v.5.0 software (International Biotechnologies, New Haven, CT). Sequencing of a total of 20 heavy chain and 10 λ-chain transcripts defined as negative by SSCP analysis revealed a lack of somatic mutations in all of them (not shown). Sequencing of a total of 15 heavy chain and 15 λ-chain transcripts defined as positive by SSCP analysis yielded 100% concordance with the SSCP analysis results and demonstrated that a single nucleotide change in the 375 bp of the V_HDJ_H or the 336 bp of the VAJA cDNA sequence was sufficient to alter DNA mobility in the SSCP gel (not shown).

**Mutational analysis**

The census of the somatic point-mutations was performed by counting identical mutations in more than one transcript (of any isotype) only. 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 V_HDJ_H 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 (χ² 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.

**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 λ-chain. The V_HDJ_H segment consists of a V_H26–3.7-like gene (90% identity) (31), rearranged to DN4 and D5 genes, and an unmutated J_H6c gene. The VAJA segment consists of a 1c.10.2/DPL2 V_A1-like gene (94% identity) (32), rearranged to a J_A2 gene with a single change, probably a point-mutation. Multiple sequences at different times of V_HDJ_H-C_H, V_HDJ_H-C_δ, 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 cDNAs were amplified using the CL-01 V_H or Vλ gene leader sense primer in combination with an antisense C_H, C_δ, C_α, C_ε, 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), V_HDJ_H-C_γ, V_HDJ_H-C_γ-C_α, and

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**Table I. In vitro somatic point-mutations in Ig V_HDJ_H and VAJA gene sequences expressed by induced CL-01 cells**

<table>
<thead>
<tr>
<th>V_HDJ_H</th>
<th>Mutated/Total Transcripts</th>
<th>Mutated Transcripts Sequenced</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 V_HDJ_H</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.
* Identical mutations in different transcripts of the same and different isotypes were assumed not to be independent and were counted only once.

**Table II. Somatic point-mutations induced in CL-01 cells do not accumulate preferentially in V(D)J CDRs**

<table>
<thead>
<tr>
<th>CDRs</th>
<th>R</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>V_HDJ_H</td>
<td>21</td>
<td>8</td>
</tr>
<tr>
<td>[22]</td>
<td>[6]</td>
<td>[50]</td>
</tr>
<tr>
<td>VAJA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>[2]</td>
<td>[1]</td>
<td>[7]</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>9</td>
</tr>
<tr>
<td>[24]</td>
<td>[7]</td>
<td>[57]</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).


V\textsubscript{H} DJ\textsubscript{H}-C\gamma-C\epsilon c\DNAs were readily amplified, in addition to V\textsubscript{H}-DJ\textsubscript{H}-C\delta and V\textsubscript{H} DJ\textsubscript{H}-C\theta c\DNAs, from CL-01 cultured with T cells after BCR engagement. By SSCP analysis, 16 of 112 (14.3%) V\textsubscript{H}-DJ\textsubscript{H}-C\delta 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\textsubscript{H} DJ\textsubscript{H}-C\delta and 50 identical V\textsubscript{L} J\textsubscript{L}-C\lambda transcripts (Table I and Fig. 1B). Also mutated were 20 of 110 (18.2%) V\textsubscript{H} DJ\textsubscript{H}-C\epsilon, 17 of 120 (14.2%) V\textsubscript{H} DJ\textsubscript{H}-C\theta, 9 of 86 (10.5%) V\textsubscript{H} DJ\textsubscript{H}-C\gamma transcripts, and 18 of 179 (10.1%) V\textsubscript{L} J\textsubscript{L}-C\lambda 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 V\textsubscript{H} DJ\textsubscript{H}-C\delta, 50 V\textsubscript{H} DJ\textsubscript{H}-C\epsilon, and 50 V\textsubscript{H} DJ\textsubscript{H}-C\gamma transcripts analyzed).

Table III. Nature of the base substitutions in the Ig V\textsubscript{H} DJ\textsubscript{H} 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>
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<tr>
<td>Transitions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>19\textsuperscript{*}</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>[32]</td>
<td>[9.7]\textsuperscript{b}</td>
<td>[7.6]</td>
<td>[7.8]</td>
</tr>
</tbody>
</table>

Transversions | G → C | A → C | C → A | T → A |
<table>
<thead>
<tr>
<th></th>
<th></th>
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<td>23</td>
<td>9</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>[32]</td>
<td>[9.7]</td>
<td>[7.6]</td>
<td>[7.8]</td>
</tr>
<tr>
<td></td>
<td>G → T</td>
<td>A → T</td>
<td>C → G</td>
<td>T → G</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>17</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>[32]</td>
<td>[9.7]</td>
<td>[7.6]</td>
<td>[6.8]</td>
</tr>
</tbody>
</table>

Total mutations | G → N | A → N | C → N | T → N |
<table>
<thead>
<tr>
<th></th>
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<th></th>
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</thead>
<tbody>
<tr>
<td>96</td>
<td>45\textsuperscript{*}</td>
<td>7\textsuperscript{**}</td>
<td>31</td>
<td>13\textsuperscript{**}</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>

\textsuperscript{a} Identical mutations in different transcripts of the same and different isotypes were assumed not to be independent and were counted only once.

\textsuperscript{b} 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\textsubscript{H} DJ\textsubscript{H} 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\textsubscript{H} DJ\textsubscript{H} sequence) by 96, divided by 3 (as G → A, G → C, and G → T mutations have all the same theoretical probability to occur) = 9.7.

\textsuperscript{*}, p < 0.05; \textsuperscript{**}, p < 0.001.

Table IV. Somatic point-mutations induced in the V\textsubscript{H} DJ\textsubscript{H} gene segment of CL-01 cells preferentially target AG and CT dinucleotides

<table>
<thead>
<tr>
<th>Dinucleotides in Unmutated Sequence</th>
<th>Number of occurrences\textsuperscript{a}</th>
<th>Percent of the total dinucleotides</th>
<th>Observed number of mutations\textsuperscript{b}</th>
<th>Expected number of mutations\textsuperscript{c}</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\textsuperscript{*}</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\textsuperscript{*}</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>
<td>0.72</td>
</tr>
<tr>
<td>AA</td>
<td>17</td>
<td>4.6</td>
<td>4</td>
<td>8.6</td>
<td>0.47\textsuperscript{*}</td>
</tr>
<tr>
<td>AT</td>
<td>16</td>
<td>4.3</td>
<td>2</td>
<td>8.3</td>
<td>0.24\textsuperscript{*}</td>
</tr>
<tr>
<td>CT</td>
<td>26</td>
<td>7.0</td>
<td>25</td>
<td>13.4</td>
<td>1.87\textsuperscript{*}</td>
</tr>
<tr>
<td>GT</td>
<td>26</td>
<td>7.0</td>
<td>17</td>
<td>13.4</td>
<td>1.27</td>
</tr>
<tr>
<td>CA</td>
<td>30</td>
<td>8.0</td>
<td>5</td>
<td>15.4</td>
<td>0.32\textsuperscript{*}</td>
</tr>
<tr>
<td>TG</td>
<td>27</td>
<td>7.2</td>
<td>12</td>
<td>13.8</td>
<td>0.87</td>
</tr>
<tr>
<td>CC</td>
<td>22</td>
<td>5.9</td>
<td>15</td>
<td>11.3</td>
<td>1.33</td>
</tr>
<tr>
<td>GG</td>
<td>44</td>
<td>11.8</td>
<td>22</td>
<td>22.7</td>
<td>0.97</td>
</tr>
<tr>
<td>TT</td>
<td>12</td>
<td>3.2</td>
<td>0</td>
<td>6.2</td>
<td>0.00\textsuperscript{*}</td>
</tr>
<tr>
<td>TC</td>
<td>25</td>
<td>6.7</td>
<td>12</td>
<td>12.9</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Total 374 100 192 192 192 1.00

\textsuperscript{a} The V\textsubscript{H} DJ\textsubscript{H} 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).

\textsuperscript{b} Identical mutations in different transcripts of the same and different isotypes were assumed not to be independent and were counted only once.

\textsuperscript{c} 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.

\textsuperscript{d} The expected number of mutations for any given dinucleotide takes into account the base composition of the unmutated V\textsubscript{H} DJ\textsubscript{H} 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\textsubscript{H} DJ\textsubscript{H} region.

\textsuperscript{*}, p < 0.001.
Table V.  Somatic point-mutations induced in the V_HDJ_H gene segment of CL-01 cells preferentially target GAG trinucleotidesa

<table>
<thead>
<tr>
<th>Number of occurrences</th>
<th>7</th>
<th>11</th>
<th>9</th>
<th>6</th>
<th>8</th>
<th>18</th>
<th>12</th>
<th>8</th>
<th>7</th>
<th>8</th>
<th>10</th>
<th>7</th>
<th>5</th>
<th>4</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trinucleotide sequence</td>
<td>AGA</td>
<td>GAG</td>
<td>AGG</td>
<td>GGC</td>
<td>CTC</td>
<td>GGG</td>
<td>CCT</td>
<td>GGA</td>
<td>ACC</td>
<td>CCT</td>
<td>AGC</td>
<td>GAC</td>
<td>ACC</td>
<td>CTT</td>
<td>AGG</td>
</tr>
<tr>
<td>Nucleotide changes</td>
<td>GA</td>
<td>A</td>
<td>G</td>
<td>AAG</td>
<td>GCT</td>
<td>TTT</td>
<td>TAT</td>
<td>A</td>
<td>G</td>
<td>A</td>
<td>ATC</td>
<td>GGC</td>
<td>GCT</td>
<td>G</td>
<td>AGA</td>
</tr>
<tr>
<td>GA</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>G</td>
<td>T</td>
<td>A</td>
<td>G</td>
<td>ATC</td>
<td>GA</td>
<td>T</td>
<td>TC</td>
<td>T</td>
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<td>A</td>
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<td>A</td>
<td>C</td>
<td>T</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Number of mutations targeting the trinucleotideab,cd

| Number of mutations targeting the underlined dinucleotide in the context of the trinucleotideab,cd | 100 | 21* | 10* | 11* | 11 | 16 | 11* | 10* | 11 | 10 | 2 | 8* | 5 |
|------------------------------------------------------------------------------------------------|-----|------|------|------|----|-----|------|------|----|----|----|----|----|----|----|
|                                                                                                  | [5.5] | [8.4] | [6.9] | [4.6] | [6.0] | [13.8] | [10.7] | [6.0] | [6.0] | [6.0] | [7.8] | [5.5] | [3.7] | [3.2] | [4.6] |
| Number of mutations targeting the underlined dinucleotide out of the context of the trinucleotideab,cd | 21 | 22 | 32 | 5* | 16 | 13 | 13 | 16 | 40 | 26 | 18 |
|                                                                                                  | [22] | [17] | [22] | [9] | [17] | [13.5] | [17] | [20] | [23] | [21] |

The trinucleotides shown in this table are those targeted by 10 or more point-mutations and those that have been suggested to be hotspots (36).

1 The [expected] number of mutations was normalized for the base composition of the unmutated V_HDJ_H sequence. The [expected] number of mutations targeting any given trinucleotide was calculated by multiplying the frequency of occurrence of the dinucleotide in or out of the context of the trinucleotide, respectively, by the total number of point-mutations.

2 The [expected] number of mutations targeting the trinucleotide in or out of the context of the trinucleotide, respectively, was calculated by multiplying the frequency of occurrence of the dinucleotide in or out of the context of the trinucleotide, respectively, by the total number of point-mutations that occurred in that given dinucleotide.

a, p < 0.01.

without BCR engagement (50 V_HDJ_H-Cuc, 50 V_HDJ_H-Csh, and 50 V_HDJ_H-Cy transcripts analyzed). Thus, after BCR engagement and exposure to activated T cells, CL-01 cells effectively Ig class switch and mutate both primary (V_HDJ_H-Cuc and V_HDJ_H-Csh) and secondary (V_HDJ_H-Cy, V_HDJ_H-Csh, and V_HDJ_H-Cy) heavy chain transcripts, as well as VAJA-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_HDJ_H-Cuc, 50 V_HDJ_H-Csh, and 50 V_HDJ_H-Cy 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_HDJ_H-Cuc and 40 VAJA-CA transcripts analyzed from anti-CD30 Ab cultures and 50 V_HDJ_H-Ccy and 52 VAJA-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_HDJ_H-Cuc, 50 V_HDJ_H-Csh, and 50 V_HDJ_H-Cy 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_HDJ_H-Cuc, 50 V_HDJ_H-Csh, and 50 V_HDJ_H-Cy 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_HDJ_H-Cuc, -Csh, -Ccy, -Cca, -Cce, and VAJA-CA sequences were compared with those of the V_HDJ_H-Cuc and VAJA-CA transcripts expressed by the CL-01 cultured alone (unmutated templates) (Fig. 2). A total of 123 mutations were found in the 38 V_HDJ_H-Cuc, V_HDJ_H-Csh, V_HDJ_H-Ccy, V_HDJ_H-Cca, and V_HDJ_H-Cce transcripts (575 bp each). A total of 121 were point-mutations confined to the V_HDJ_H sequence (375 bp), one was a deletion of three nucleotides in the framework region (FR) 3 of a V_HDJ_H-Ccy transcript (γφ13), and one was a C→G S mutation at residue 87 of the C_h1 sequence of a V_HDJ_H-Cca transcript (µφ15) (Fig. 2A). The 121 V_HDJ_H point-mutations corresponded to an overall frequency of 1.22 × 10⁻³ 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) \((p < 0.001)\). The single nucleotide change in the C1 region of VDJCHC1 transcript m015 represented a frequency of \(1.35 \times 10^{-4}\) changes/base, which was threefold the PCR error rate. The 121 VDJCHC1 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 VlJl-CL transcripts sequenced (539 bp each). Sixteen were point-mutations confined to the VlJl sequence (336 bp), and one was a 3-nucleotide deletion in the FR2 of transcript l013. No mutations were found in the CL1

FIGURE 1. Induction of somatic hypermutation and Ig class switching in human IgM+ IgD+ CL-01 cells. A, Strategy used to analyze somatic mutations in induced CL-01 cell Ig VDJ genes. B, BCR engagement and CD40:CD40L and CD80:CD28 coengagements are crucial for the induction of hypermutation. Bars indicate percent of VDJ gene transcripts mutated as determined by SSCP in CL-01 cells cultured for 14 days with medium only; CD40L-293 cells, TCM, IL-4 (100 U/ml), and IL-10 (100 ng/ml) after BCR engagement; activated T cells alone; activated T cells after BCR engagement; activated T cells and anti-CD28 mAb after BCR engagement; activated T cells; anti-CD40L mAb after BCR engagement; and activated T cells and anti-CD30 mAb after BCR engagement. The number of transcripts analyzed from CL-01 cells cultured activated T cells upon BCR engagement are shown in the Table I. The numbers of transcripts analyzed from all other culture conditions are given in Results.
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_{1\mu}DJ_{\mu}-C_{\mu} and the V\text{AJA}-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_{1\mu}DJ_{\mu}-C_{\mu} and the 16 point-mutations in the 9 V\text{AJA}-CA transcripts from CL-01 cells cultured with activated T cells after BCR engagement as compared with the sequences of the unmutated V_{1\mu}DJ_{\mu} and V\text{AJA} 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_{1\mu}DJ_{\mu} and 2 rows for V\text{AJA}, 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 (p < 0.01) (Table III), and the 14 unique V\text{AJA} point-mutations consisted of almost twice as many transitions (n = 9) as transversions (n = 5) (p < 0.01). In the V_{1\mu}DJ_{\mu} 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_{1\mu}DJ_{\mu} 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
frequency of mutations in C (Table III). A preference for transitions over transversions and G nucleotide targeting could be discerned in the V_{\text{J}} sequence, 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 V_{\text{H}} D_{\text{H}} sequence and made a census of all the point-mutations targeting these dinucleotides (the V_{\text{J}} 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 the 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 V_{\text{H}} D_{\text{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 ($p < 0.001$) (Table V). In the unmutated V_{\text{H}} D_{\text{H}} 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.

FIGURE 3. Stepwise accumulation of somatic point-mutations correlates with class switching from IgM to IgG, IgA, or IgE in CL-01 cells. Depicted is the genealogical tree constructed using related V_{\text{H}} D_{\text{H}} -C sequences of V_{\text{H}} D_{\text{H}} -C, V_{\text{H}} D_{\text{H}} -C, V_{\text{H}} D_{\text{H}} -C, V_{\text{H}} D_{\text{H}} -C, and V_{\text{H}} D_{\text{H}} -C transcripts from a single culture of CL-01 cells incubated, after BCR engagement, with activated T cells for 14 days. Thin frames depict putative intermediate sequences, and point-mutations are indicated by their codon number and the nature of the base change. Vertical bars depict S mutations, and lollipops depict R mutations.
Somatic point-mutations accumulate stepwise concomitant with Ig class switching

The ability of sIgM+ sIgD+ 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 two second-generation shared mutations plus a third mutation (336 G → A). The second 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 two second-generation shared mutations plus a third mutation (336 G → A). 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 Ig VDJ 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 sIgM+ sIgD+ switch to IgG, IgA, and IgE and eventually give rise to plasma-cell differentiation, whereby CL-01 cells that are sIgM+ sIgD+ 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_{J}A-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.

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 indispensible 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 mAbs 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 isotype 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_{H} and -C_{L} transcripts bore a load of somatic mutations approximately twofold greater than that of V_{H}DJ_{H}-C_{H} transcripts or their primary V_{H}DJ_{H}-C_{L} and V_{H}DJ_{H}-C_{L} counterparts (Table I). The higher frequency of mutations in CL-01 cell V_{H}DJ_{H}-C_{H} transcripts in vitro extends findings in vivo showing, in centroblasts, a heavier load of mutations in V_{H}DJ_{H}-C_{H} than in V_{H}DJ_{H}-C_{L} 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 a V_{H}DJ_{H}-C_{H} construct than in its V_{H}DJ_{H}-C_{L} 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_{H}, V_{H}DJ_{H}-C_{L}, and V_{H}DJ_{H}-C_{L}, but not V_{H}DJ_{H}-C_{L}, transcripts was about two- to fourfold higher than in V_{J}A-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} 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 transitions, which are especially copious in all transcripts, is consistent with either preferential G targeting by the mutational machinery in the transcription strand or selectivity for G in the AG dinucleotide, whether isolated or in the context of AGA, GAG, or AGG trinucleotides. Other structural features, such as palindromes, may also be important (62). Likewise, a scarcity of mutations was found in the different iterations of the RGYW motif in the 5′-CL-01 cell V VH 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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