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T Cells Can Induce Somatic Mutation in B Cell Receptor-Engaged BL2 Burkitt's Lymphoma Cells Independently of CD40-CD40 Ligand Interactions[1]

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The B cell surface trigger(s) and the molecular mechanism(s) of somatic hypermutation remain unknown, partly because of the lack of amendable in vitro models. Recently, however, we reported that upon B cell receptor cross-linking and coculture with activated T cells, the Burkitt's lymphoma cell line BL2 introduces mutations in its IgV_H gene in vitro. We now confirm the relevance of our culture model by establishing that the entire spectrum of somatic mutations observed in vivo, including insertions and deletions, could be found in the DNA of BL2 cells. Additionally, we show that among four human B cell lines, only two with a centroblast-like phenotype can be induced to mutate. Triggering of somatic mutations in BL2 cells requires intimate T-B cell contacts and is independent of CD40-CD40-ligand (CD40L) interactions as shown by 1) the lack of effect of anti-CD40 and/or anti-CD40L blocking Abs on somatic mutation and 2) the ability of a CD40L-deficient T cell clone (isolated from an X-linked hyper-IgM syndrome patient) to induce somatic mutation in B cell receptor-engaged BL2 cells. Thus, our in vitro model reveals that T-B cell membrane interactions through surface molecules different from CD40-CD40L can trigger somatic hypermutation. ***The Journal of Immunology,*** **2000, 164: 1306–1313.**

Depending on the species, somatic hypermutation, which introduces nucleotides replacement into Ig V(D)J genes, participates in the diversification of the primary Ig repertoire (1–3) and/or to the maturation of the Ab response.[5] In humans and mice, affinity maturation of T cell-dependent Ab responses results from the accumulation of point mutations in the variable region of Ig genes, followed by Ag-driven selection of B lymphocytes expressing high-affinity Abs (3). Accumulation of somatic mutations and B cell selection take place inside germinal centers in secondary lymphoid organs (5–7). In particular, B cells are believed to mutate their IgV genes at the centroblast stage (8, 9) and to face selection at the centrocyte stage (10–12). Selection of high-affinity clones involves follicular dendritic cells which display immune complexes at their surface and deliver cell survival signals (13), whereas low affinity as well as autoreactive B cells are deleted by apoptosis (14, 15).

Even though the phenomenon of somatic hypermutation was observed almost 30 years ago (16), its molecular mechanisms remain unknown. Somatic hypermutation in humans and mouse consists mainly of untemplated single-base changes (17), and to a lesser extent of base insertions and deletions (18), which display the following features: 1) an asymmetrical distribution within a 2-kb region surrounding rearranged IgV genes (19), 2) an intrinsic nucleotide sequence preference (20), 3) a DNA strand polarity (19), 4) an ability to target non-IgV genes inside or even outside an Ig locus (21), 5) a transcription dependence (22), and 6) a requirement for *cis*-acting elements (23). The contribution of DNA repair machinery to the introduction of somatic mutations is currently under scrutiny (24, 25).

Unlike the primary B cell repertoire diversification, which takes place within the bursa of Fabricius in birds or in the ileal Peyer's patches in sheep (26), the maturation of Ab affinity through somatic hypermutation in humans and mouse is Ag driven (27) and requires T cells (28). However, inasmuch as somatic mutation seems to occur exclusively in the specific microenvironment of the germinal center in vivo, the requirements for triggering this unique phenomenon include both the interactions needed to establish the germinal center reaction and those directly involved in the activation of somatic mutation machineries. Regarding the Ag, its binding precedes the recruitment of B cells into the germinal center and underlies the selection of high-affinity Abs (29), but how the parameters of B Cell receptor (BCR)[4] engagement affect the triggering and/or the maintenance of somatic hypermutation has not been addressed yet. T cells, on the other hand, are directly involved in almost every aspect of B cell differentiation, including proliferation, isotype switch recombination, and maturation into plasmocytes or their recruitment into the memory pool. Furthermore, T cells play an indirect role in B cell maturation through their general contribution to the development of secondary lymphoid organs and germinal centers. Several soluble cytokines (including IL-4, IL-6, IL-10, and TNF-α) and membrane-bound molecules [such as CD40 ligand (CD40L), CD27, and OX40L] participate in the different aspects of the help provided by T cells. In particular, CD40

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[5] Abbreviations used in this paper: BCR, B cell receptor; HIGM, hyper-IgM; s, surface; CD40L, CD40 ligand; mut, mutation.

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signaling plays a central role in B cell development (30), but assessing its exact function in somatic mutation is rendered difficult by its absolute requirement for normal B cell development.

Much of what is currently known about somatic mutation has been obtained by studying immune responses either in humans or in normal or transgenic animals (31). We have reported earlier that surface Ig cross-linking followed by coculture with an activated T cell clone induces a fraction of naive tonsilar B cells (32) and BL2 cells (33) to accumulate somatic mutations in their IgV_H genes. After further confirming its relevance, we have now taken advantage of this experimental setting to directly address the role of CD40-CD40L interaction in the triggering of somatic mutation.

Materials and Methods

Cell lines and Abs

Burkitt's lymphomas BL2, BL45, and BL74 (34) and the lymphoblastoid cell line ML1 were obtained from American Type Culture Collection (Manassas, VA). The BL2 cell line was established from a tumor biopsy obtained from a Caucasian child. The BL2 cell line does not carry the EBV genome and has a t(8;22)(q24;q11) reciprocal translocation. Cells were cultured in DMEM/F12 medium supplemented with 10% horse serum and 1% Caryoser (Life Technologies, Cergy-Pontoise, France). Anti-human CD40L monoclonal murine-purified Abs LL2 and LL48 were produced in our laboratory (35) along with anti-human CD40 Ab, mAb89. LL2 and LL48 recognize different epitopes on human CD40L and completely block CD40-CD40L interaction (35). Both anti-CD40 and anti-CD40L Abs are agonists: anti-CD40 Abs can mimic T cell-induced CD40 triggering on B cells and anti-CD40L Abs activate T cells.

T cells

FS35 is a $CD4^+$ T cell clone derived from an allergic patient that recognizes Derp1, the major allergen from *Dermatophagoides pteronyssinus* (36). FS35 is a Th2-like clone which, upon activation, secretes large amounts of IL-4 and IL-10 and only small amounts of IL-2 and IL-5. The $CD4^+$ T cell clone KD20 was derived from PBL of an X-linked hyper-IgM (HIGM) syndrome-affected patient. KD20 cells do not show detectable expression of CD40L and do not secrete IL-4, IL-5, or IL-10 nor IFN-γ upon anti-CD3 activation. KD20 and FS35 cells were expanded in DMEM/F12 medium supplemented with 10% horse serum and 1% Caryoser. T cells were stimulated weekly: they were cultured for 3 days with a feeder cell mixture containing irradiated PBMC, irradiated JY lymphoblastoid B cells, 0.1 mg/ml PHA, and recombinant IL-2 (20 U/ml). T cells were then washed and maintained for 4 days with IL-2 only (20 U/ml). T cells were used 7 days after their last activation by feeder cells.

Cocultures

For the coculture of T and B cells, flat-bottom 96-well culture plates (Falcon 3072; Becton Dickinson, Mountain View, CA) were coated with a 1/1000 dilution of the OKT3 anti-CD3 Ab (American Type Culture Collection) for 2 h at room temperature. FS35 T cells were irradiated with 4000 rad and seeded at 2.5×10^4 cells/well in 100 μl of the same culture medium. BL2 cells were incubated with a soluble anti-human IgM complete Ab (109-005-129; Jackson ImmunoResearch, West Grove, PA) or with the corresponding $F(ab')_2$ fragments (109-006-129; Jackson ImmunoResearch) for 30 min at 4°C. The cells were then washed and seeded at 50 or 500 cells/well in a 100-μl volume of culture medium, alone or over the activated T cells.

When T cell supernatants were tested for their ability to substitute activated T cells for the induction of somatic mutation, anti-IgM-activated BL2 cells were seeded at 50 cells/well over a fresh layer of irradiated FS35 cells in anti-CD3 precoated wells (as an internal control), in medium alone or over a layer of irradiated CD40L-expressing murine fibroblasts (CD40L-Lc), and T cell-derived supernatants were added as indicated. The T cell-derived supernatants utilized were obtained from 1) FS35 cells activated for 48 h through immobilized anti-CD3 Abs (OTK3; American Type Culture Collection) (37) or 2) from peripheral blood-sorted T cells activated 48 h through a combination of two anti-CD2 Abs (CLB-T11 1/1 and CLB-T11 2/1; CLB, Amsterdam, The Netherlands) both used at a concentration of 1/2000 diluted ascites and PMA (Sigma, St. Louis, MO) at a final concentration of 2 ng/ml and anti-CD28 Abs (CLB-CD28/1; CLB) used at a concentration of 1/2000 diluted ascites. The supernatants were used at 10% of the final volume of the coculture.

Coculture in separated chambers

To provide an environment of mutating BL2 cells, anti-BCR-activated BL2 cells were seeded at the bottom level (diameter of 17 mm) of a transwell (Costar, New York, NY) at 500 cells/well over a fresh layer of 2.5×10^5 irradiated FS35 cells in anti-CD3 precoated wells. Separated by a nylon membrane, in the top level of the transwell (diameter of 7 mm), 50 BL2 cells were seeded under three conditions: 1) on a layer of 2.5×10^4 irradiated FS35 cells after anti-CD3 coating of the nylon membrane as an internal control, 2) in medium alone, and 3) over a layer of irradiated CD40L-expressing murine fibroblasts (CD40L-Lc).

Analysis of the Ig V_H transcripts of BL2 cells

Total RNA was extracted using an RNeasy total RNA kit (Qiagen, Hilden, Germany) from all of the cells harvested at the end of the cultures. cDNA was obtained by RT using oligo(dT12-18) (Pharmacia, Uppsala, Sweden) as primer and SuperScript Reverse Transcriptase (Life Technologies, Gaithersburg, MD).

Full-length V_H4-Cμ transcripts were amplified with 5′:LV_{H4} (5′-CTATAACCATGGTTCATGAAACACCTGTGGTTC-3′) and 3′:XbaI-Cμ(5′-TGCATGCATTCTAGAAAGGGTTGGGGCGGATGCACTCC-3′) with cloned PFU polymerase (Stratagene, La Jolla, CA) using the reaction buffer provided by the manufacturers and a DNA thermal cycler (Perkin-Elmer/Cetus, Foster City, CA) with 40 cycles of 1 min denaturation at 94°C, 2 min annealing at 50°C, and 3 min extension at 74°C. The amplification was completed by an additional 10-min extension step at 74°C. The PCR products were gel purified, then extracted using Qiaex beads (Qiagen), digested with *Nco*I and *Xba*I restriction enzymes, gel purified again, and ligated into an *Xba*I-*Nco*I opened plasmid (modified pBluescript; Stratagene). Both DNA strands of plasmids extracted from individual bacterial colonies were sequenced on an automated sequencer (Applied Biosystems, Foster City, CA) using −21M13 and M13RP primers flanking the plasmid cloning sites. The sequences were analyzed using the SeqEdTM program (DNAstar, Madison, WI).

Results

The IgVH DNA of BL2 cells induced to mutate contains base substitutions as well as base insertions and deletions

In our previous study, the IgVH transcripts of BL2 cells were analyzed by RT-PCR, and only single bp substitutions were observed after surface Ig (sIg) cross-linking and coculture with activated T cells (33). However, analysis of mRNA is intrinsically biased by factors that affect the mRNA representation, whereas PCR amplification of BL2 IgV genes allows direct assessment of mutations at the DNA level. Therefore, DNA of BL2 cells was extracted from two culture wells at the end of a 7-day coculture of BCR-engaged BL2 cells with activated FS35 T cells. A total of 128 PCR products including the leader intron as well as the V_H-D-J_H (LVDJ) sequence were obtained and were sequenced, of which 31 (24%) displayed somatic mutations: 40 base substitutions, 3 insertions, and 1 deletion (Fig. 1). This represents a mean of 1.4 mutation per mutated sequence and an overall frequency of 6.5×10^{-4} mutation per total sequenced base pair (mut/bp). To determine the background level of mutations introduced by direct DNA amplification with PFU, LVDJ was amplified from bulk cultured BL2 cells: a single bp substitution was observed in 48 sequences (0.5×10^{-4} mut/bp). Of the 40 base substitutions observed on LVDJ sequences, 31 were independent. Regarding their nature and distribution, these 31 mutations were similar to the 152 previously described in BL2 V_{H4}-D-J_H-Cμ transcripts, and 24 of them had been detected earlier (33). Mutations were distributed over the entire VDJ region and showed a replacement vs silent ratio of 1.7 in the coding region. Unlike mRNA analysis, amplification of BL2 DNA revealed the presence of three single bp insertions and one single bp deletion (Fig. 1*B*). These four mutations introduce premature stop codons into the corresponding transcripts. These events represent 9% (4 of 44) of the mutations affecting LVDJ sequences, which corresponds to the overall frequency of insertions and deletions observed in the DNA of mutating germinal center B lymphocytes (18).

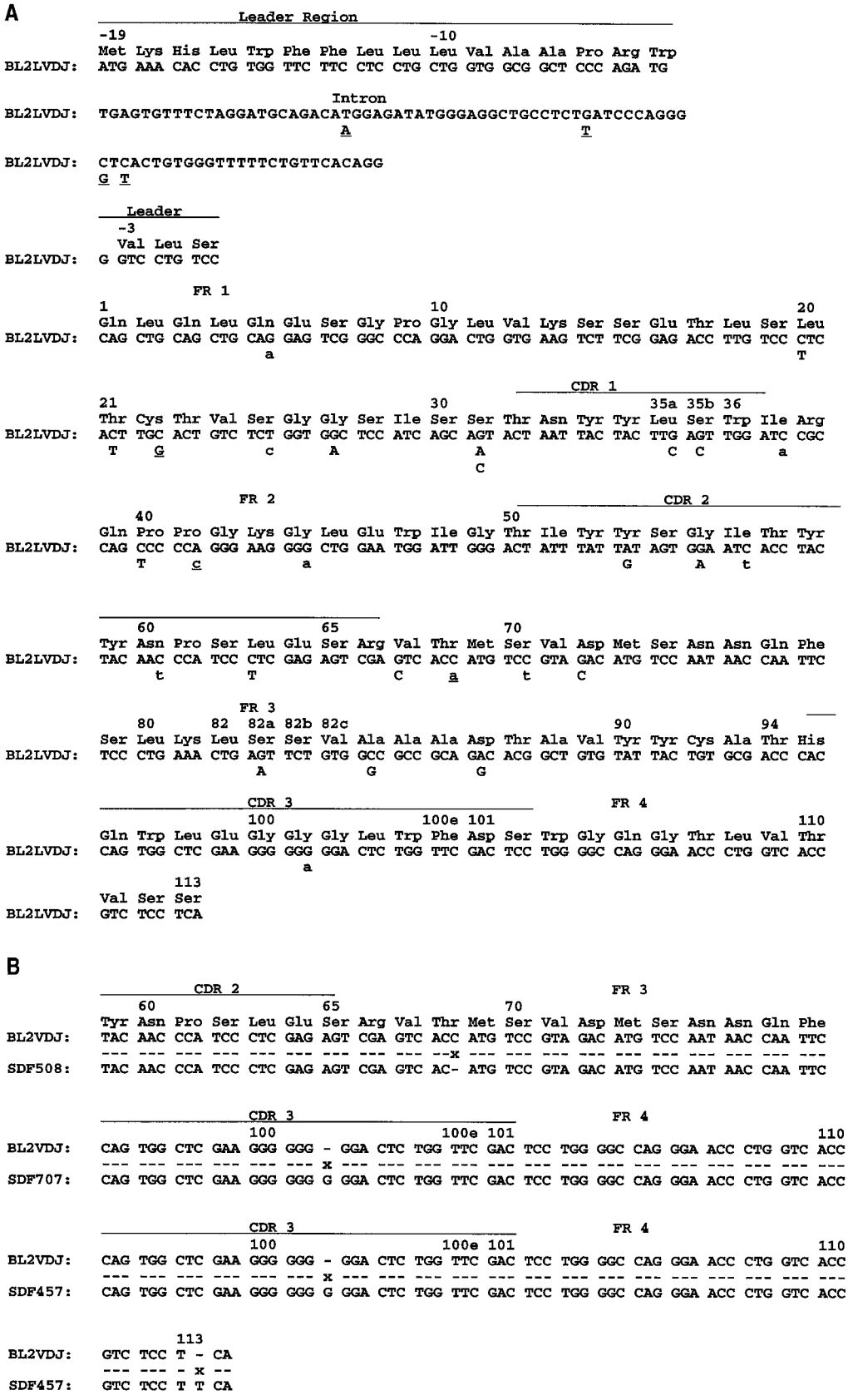

FIGURE 1. After BCR cross-linking and coculture with activated FS35 T cells, BL2 cell DNA contains base substitutions, insertions, and deletion. *A*, The distribution of somatic mutations in activated BL2 cells is similar when analyzed either by direct amplification of the DNA or after RNA RT and amplification. Upper case letters indicate replacement whereas lower case letters indicate silent mutations. Mutations which had not been observed previously on the cDNA (33) are underlined. *B*, Three independent single bp insertions and one single bp deletion have been observed; clones SDF508 and 707 are from the culture well *A*, whereas SDF457 is from culture well *B*.

Table I. *Characteristics of four human B cell lines tested for induction of somatic mutation*

	BL2	BL45	BL74	ML1
Type of B cell line	Type 1 BL	Type 1 BL	Type 3 BL	LCL
Expressed Ig	Mutated V_{H4}-IgM	Mutated V_{H3}-IgM	Mutated V_{H4}-IgA	Germline V_{H6}-IgM
Induction of somatic mutation	yes	yes	no	no
No. of analyzed wells	8	1	6	2
No. of analyzed clones	820	30	148	152
Frequency (mut/bp)	9.6×10^{-4}	10.3×10^{-4}	2.3×10^{-4}	2.1×10^{-4}

Not all human B cell lines can be induced to mutate their IgVH after BCR cross-linking and coculture with activated FS35 cells

In mouse and humans, the somatic mutation machinery is activated only at the centroblast stage (38). We therefore wondered whether induction of somatic mutation was specific to the centroblast-like BL2 Burkitt's lymphoma or not. BL45 is an EBV-negative type I Burkitt's lymphoma cell line with a germinal center phenotype similar to BL2 ($CD10^+CD38^+CD77^+CD44^-$) which does not accumulate spontaneous somatic mutation in vitro (data not shown). After anti-IgM cross-linking and coculture with activated FS35 cells for 7 days, 26% of the 30 V_{H3}-DJ_H transcripts analyzed were mutated with a mean of 1.6 mutation per mutated sequence, leading to an overall frequency of 10.3×10^{-4} mut/bp (Table I). This frequency is not statistically different from the mean (9.6×10^{-4} mut/bp) obtained with BL2 cells after the analysis of 820 clones and 4 independent cultures in the same setting. As observed with BL2 cells, this coculture did not lead the IgM-expressing BL45 cells to switch isotype (data not shown). We also analyzed two EBV-positive B cell lines, BL74 and ML1. BL74 is a type III centroblast-like Burkitt's lymphoma cell line ($CD10^+CD77^+CD23^+$) expressing an IgA encoded by a mutated V_{H4} segment, and results from the transformation of a germinal center B lymphocyte through a c-*myc* translocation into the λ-light chain locus (Table I). BL74 cells were activated with an anti-IgA Ab and cultured over activated FS35 T cells. A total of 148 PCR-amplified V_H-DJ_H transcripts originating from six wells collected after three independent cultures displayed an overall frequency of mutation of 2.3×10^{-4} mut/bp, which is undistinguishable from the background level. ML1 is a fetal liver-derived lymphoblastoid B cell line that expresses unmutated rearranged V_{H6}-DJ_H-C_μ and V_{KA20}-J_{K1}-C_K segments, suggesting that it derives from a naive B cell. Two independent cultures were performed, and a total of 152 transcripts, originating from four culture wells, displayed a total of 12 independent mutations and a mean frequency of mutation of 2.1×10^{-4} mut/bp corresponding to the background level. In conclusion, somatic mutations accumulate in our culture conditions in BL2 and BL45, two B cell lines which closely resemble centroblasts, but not in the centroblast-derived BL74 nor in the naive B cell-derived ML1 (Table I).

Soluble factors cannot substitute for cell contact in the induction of somatic mutation

Having demonstrated that results from the culture system were very similar to data obtained in vivo, we used our culture system to further dissect the mechanisms of induction of somatic mutation. The

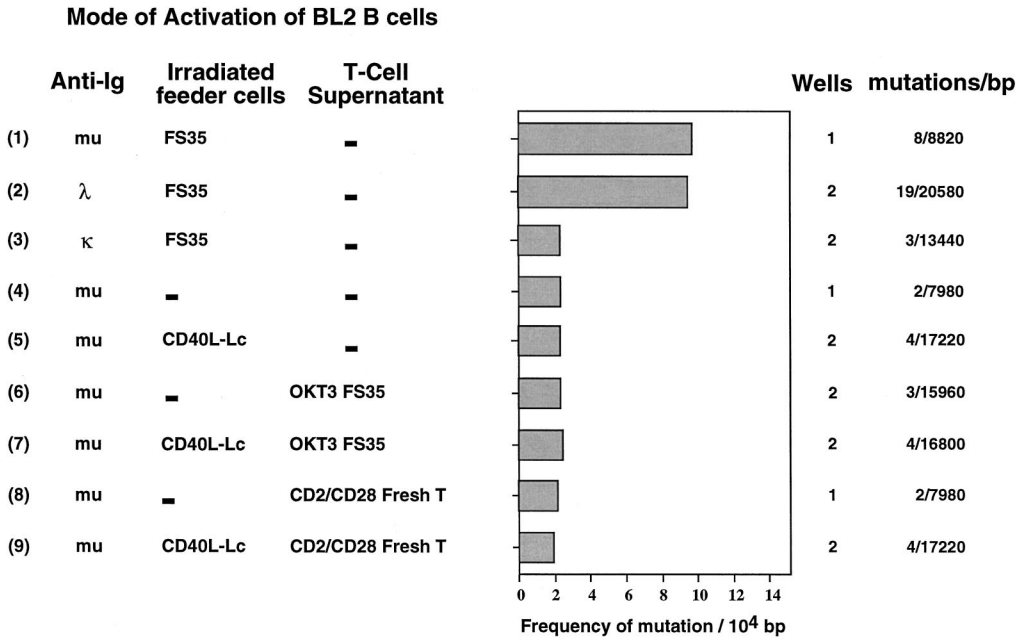

FIGURE 2. T cell supernatants fail to induce somatic mutation in anti-IgM-activated BL2 cells even in the presence of CD40L-expressing murine fibroblasts. BL2 cells (IgM^+, λ^+) were incubated with goat anti-IgM, anti-λ, or anti-κ $F(ab')_2$ fragments at 1 μg/ml for 1 h as indicated. BL2 cells were then seeded at 50 cells/well over a fresh layer of irradiated FS35 cells in anti-CD3 precoated wells (*lines 1–3*), in medium (*line 4*), or over a layer of irradiated CD40L-expressing murine fibroblasts (CD40L-Lc) (5-7-9). T cell-derived supernatants of anti-CD3-activated FS35 cells (OTK3 FS35; *lines 6* and *7*) or anti-CD2- and anti-CD28-activated peripheral blood-sorted T cells (CD2-CD28 fresh T cells; *lines 8* and *9*) were added to the cultures. Cells were collected after 7 days, and total RNA was extracted and reverse transcribed. LV_H4-C_μ PCR amplification products were cloned and sequenced. The numbers of wells analyzed and of mutations/bp sequenced are reported on the *right panel*, and the frequency of mutations is represented as black solid bars.

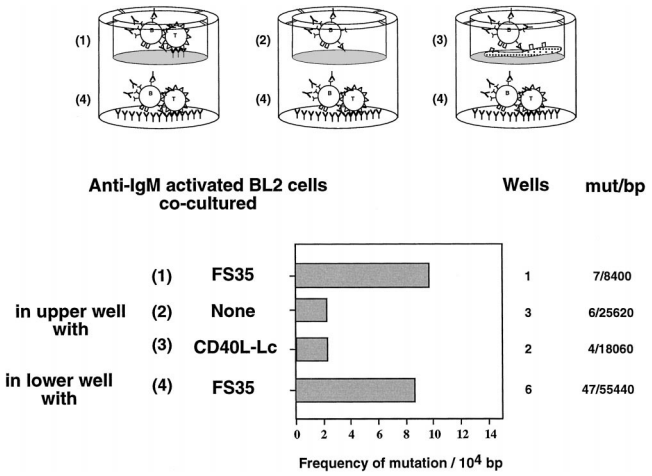

FIGURE 3. Induction of somatic hypermutation requires direct T-B cell surface interactions. BL2 cells were incubated with goat anti-IgM $F(ab')_2$ fragments at 1 μg/ml for 1 h at 37°C, washed, and seeded at the bottom level of a transwell over a fresh layer of irradiated FS35 cells in anti-CD3 precoated wells. In the *top level*, BL2 cells were seeded 1) on a layer of irradiated FS35 cells after anti-CD3 coating of the nylon membrane, 2) in medium alone, and 3) over a layer of irradiated CD40L-expressing murine fibroblasts (CD40L-Lc). Presentation is as in Fig. 2.

combination of CD40 activation signal and T cell-derived cytokines (i.e., IL-4, IL-10, and TGF-β) drives isotype switch recombination (38–40), which represents another B cell-specific DNA alteration. We therefore explored the role of soluble factors and CD40L in the signals provided by the T cells to trigger somatic mutation. First, FS35 cells or freshly sorted peripheral blood T cells were cultured with 1) anti-CD3 Ab or 2) two anti-CD2 Abs plus one anti-CD28 Ab plus PMA, and supernatants were collected after 48 h of culture. The supernatants used in these experiments were biologically active, as they have been found to support human B cell growth and isotype switch recombination (37). However, when added for 7 days in culture of BCR-engaged BL2 cells, those supernatants were unable to induce somatic mutation (Fig. 2, *lines 6* and *8*) in contrast with BL2 cells cocultured with activated FS35 T cells (Fig. 2, *lines 1* and *2*; see Ref. 33). Of note, BCR engagement through either heavy or light chain cross-linking resulted in comparable induction of somatic mutation in BL2 cells (Fig. 2, *lines 1* and *2*). The lack of effect of anti-C_k Abs on the induction of somatic mutation in BL2 cells provides an additional control to these observations (Fig. 2, *line 3*). Furthermore, addition of CD40L-transfected L cells to these cultures did not restore the induction of somatic mutation (Fig. 2, *lines 7* and *9*). The absence of effect of T cell-derived supernatants in the triggering of somatic mutation could have resulted from a transient production and/or high lability of soluble inducer(s), which would have disappeared from cultures of T cells activated for 48 h. Alternatively, the production by T cells of soluble inducers might require close contact with BCR-engaged B cells. To address these possibilities, anti-IgM-activated BL2 cells were seeded in the upper chamber of a transwell while the lower well contained a T/B cell coculture designed to induce somatic hypermutation. BL2 cells separated from a productive T-B cell interaction by a nylon membrane were not induced to mutate their IgVH genes (Fig. 3, *line 2*). Again, addition of CD40L-transfected L cells to the upper chamber of the transwell did not restore the triggering of somatic mutation (Fig. 3, *line 3*). A possible inhibitory effect of the nylon membrane could be eliminated by showing comparable accumulation of somatic mutations in BCR-engaged BL2 cells cocultured with anti-CD3 -activated FS35 cells within the upper or the lower well of the transwell vessel (Fig. 3, *line 1* vs *line 4*). These data show that T cell-derived soluble factors cannot substitute for intimate contact between BCR-engaged BL2 cells and activated T cells to induce somatic mutation.

In BL2 cells, somatic hypermutation is induced independently of CD40-CD40L interaction

CD40-CD40L interactions have been shown to be critical in the generation of germinal centers in vivo (41–44) and in the induction of B cell proliferation, differentiation, and isotype switch recombination in vitro (30). Several strategies were set up to explore the role of CD40-CD40L interactions in somatic mutation triggering in BL2 cells. In our culture system, both blocking anti-CD40 mAb89 (45) and/or anti-CD40L Abs LL2 and LL48 (46, 47) could not block the induction of somatic mutation (Fig. 4). Furthermore, the CD40L-negative T cell clone KD20 isolated from an X-linked HIGM syndrome patient was able to induce BCR-engaged BL2 cells to mutate as efficiently as the CD40L-positive T cell clone FS35 in two independent cultures (Fig. 5). Taken together, these results demonstrate that CD40-CD40L

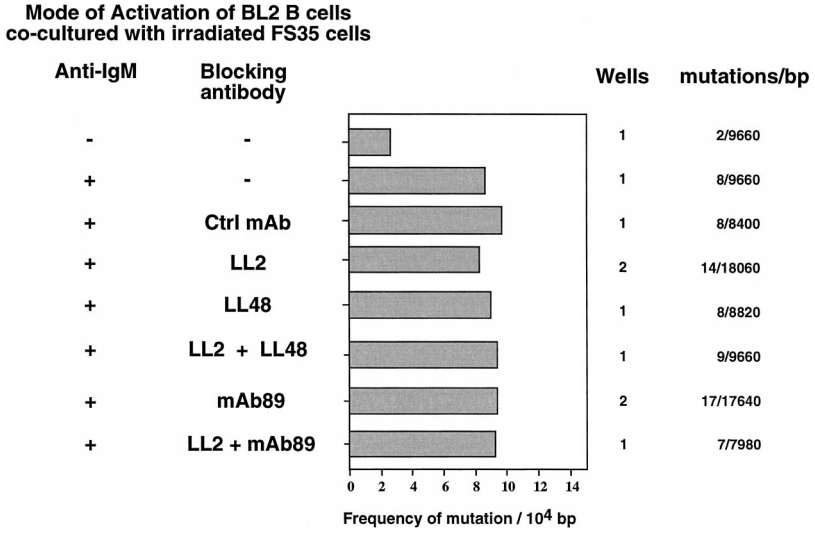

FIGURE 4. Interruption of CD40-CD40L interaction with the use of either anti-CD40L or anti-CD40 Abs does not prevent the induction of somatic mutation in BL2 cells. BL2 cells were incubated with goat anti-IgM $F(ab')_2$ fragments, washed, and seeded over a fresh layer of irradiated FS35 T cells in anti-CD3 precoated wells in the presence or absence of blocking anti-CD40L Ab (LL2 and LL48), or with anti-CD40 Ab (mAb89), or in the presence of a control unrelated mouse mAb (30N) at the concentration of 10 μg/ml. Presentation is as in Fig. 2.

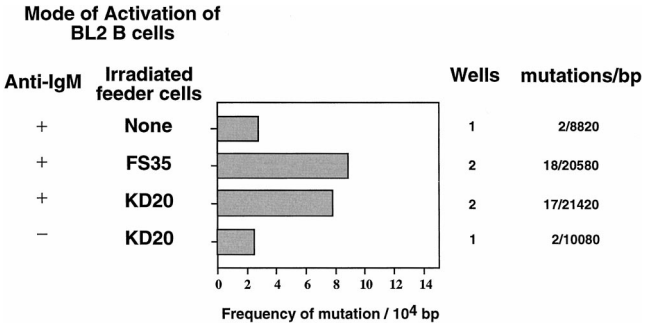

FIGURE 5. Activated CD40L-deficient T cell clone KD20 triggers somatic hypermutation in anti-IgM-activated BL2 cells. BL2 cells were incubated with goat anti-IgM $F(ab')_2$ fragments, washed, and seeded over a fresh layer of irradiated T cells in anti-CD3 precoated wells using either the CD40L-expressing FS35 clone or the X-linked HIGM patient-derived KD20 clone not expressing CD40L. Presentation is as in Fig. 2.

interaction is not required for the triggering of somatic mutation in BL2 cells in vitro.

Discussion

Although our model for inducing somatic mutation in vitro had shown single bp substitutions in V_H transcripts with all of the intrinsic features attributed to the somatic mutation machinery, neither stop codon, nor insertion-deletion had been observed. The analysis of BL2 DNA confirmed the general pattern of mutations (which accumulated with a frequency 4- to 5-fold over background due to PCR errors) and the lack of evidence for Ag-driven selection observed earlier at the RNA level. Furthermore, it revealed several insertions-deletions resulting in the creation of stop codons. Insertions-deletions within viable B cells isolated ex vivo were always found as triplets or multiples thereof, which leave the Ig transcript in frame (18). Stop codons have been observed in V_H genes amplified from mouse centroblasts (48), but, like in our culture system, these sequences might be derived from apoptotic cells. The absence of stop codon in cDNA sequences may have resulted from destabilization of mRNA, leading to their underrepresentation. By extending the similarities with observations made in vivo, the finding of insertions-deletions in DNA of mutated BL2 cells further supports the conclusion that mutations have accumulated in vitro as a result of somatic hypermutation mechanisms.

Active hypermutation appears to be restricted to centroblasts in vivo (6). Our finding that somatic mutation could be triggered in two EBV-negative Burkitt's lymphomas with a centroblast-like phenotype (BL2 and BL45) suggests that they correspond to the window of B cell maturation that is permissive for somatic mutation. However, neither the type III Burkitt's lymphoma BL74 nor the lymphoblastoid B cell line ML1 could be induced to hypermutate in vitro. Therefore, the status of the Ig V(D)J genes (germline vs mutated) at the initiation of the culture does not predict the susceptibility to further mutate, as BL74 had accumulated mutations in its V_H gene in vivo, whereas ML1 had not. Whether EBV infection might prevent induction of somatic hypermutation as it has been shown to interfere with cytokine and sIg signal B cell responsiveness remains unknown. Thus, as observed in vivo, induction of somatic mutation appears to be limited in our in vitro culture conditions.

In addition to BCR-cross-linking, the second signal required to trigger somatic mutation in BL2 cells is provided by activated $CD4^+$ T cells. The present study demonstrates that T cell help requires direct membrane contacts, and that T cell-derived soluble factors, although possibly needed, remain insufficient to induce somatic mutation. CD40-CD40L interaction is not essential for inducing somatic mutation as neither blocking CD40-CD40L interaction by the use of anti-CD40L mAbs nor the replacement of FS35 T cells by CD40L-negative KD20 T cells did prevent the accumulation of somatic mutations. Direct evidence that CD40-CD40L interaction has taken place in the experiments where BL2 cells were cultured with activated T cells are not available. Indeed, no change in division rate, Ig secretion, or BL2 phenotype could be detected when BL2 cells were cocultured with activated T cells. However, there is little doubt that CD40-CD40L interaction has occurred during those experiments, as conditions were identical to those described by several laboratories, including ours, where CD40-CD40L interactions resulted in biological effects. In fact, in the very unlikely hypothesis that no CD40-CD40L had occurred in our culture conditions, this would imply that somatic mutation was triggered independently of CD40-CD40L interaction. Moreover, the lack of inhibition of somatic mutation by anti-CD40 mAb suggests that the activation of T cells which enables them to induce somatic mutation in BL2 cells does not depend on CD40L-mediated signaling either. Evidence has established a critical role for CD40-CD40L interaction during the in vitro proliferation and differentiation of mouse and human B lymphocytes (49–51). However, the direct dependence of somatic mutation on CD40-CD40L interaction in vivo remains a matter of debate. CD40 knockout mice have impaired immune responses and cannot develop germinal centers (41, 42). In humans, the X-linked HIGM syndrome which results from the absence of functional CD40L leads to a defect in germinal center formation, the lack of isotype switch recombination, and the accumulation of IgM with no or very few somatic mutations (46, 52). Indeed, in our previous study, the presence of somatic mutations in V_H6 genes from a peculiar HIGM patient was associated with the transient expression of CD40L at the surface of his activated T cells (32). Interestingly, T cell factors and surface molecules responsible for the induction of somatic mutation in anti-IgM-activated BL2 cells are shared by at least three different T cell clones: Th2-type FS35, Th1-type MT9, and Th0-type KD20. Since the three T cell clones exhibit different cytokine expression patterns, we may conclude that, if T cell cytokines are necessary for the induction of somatic mutation in BL2 cells, these cytokines are either secreted by all three clones or have redundant activities. T cells participate in the selection of high-affinity, non-autoreactive centroblasts mainly through CD40-CD40L interaction, which rescues germinal center B cells from apoptosis (53). However, our results show that undefined molecules are probably involved in triggering somatic mutation. Other members of the TNF-TNF receptor family expressed on T-B cells such as CD27-CD70, 4-1BB-4-1BBL, and OX40-OX40L may represent candidates for triggering of somatic mutation.

Recently, using an in vitro culture model very similar to ours, Zan et al. (54) have reported the triggering of Ag-independent somatic mutation in the CL-01 B cell line upon BCR engagement and coculture with anti-CD3-activated peripheral blood $CD4^+$ T cells. They concluded from blocking experiments with mAbs that both CD80-CD28 and CD40-CD40L interactions are necessary but insufficient for the induction of somatic mutation. The reason for the discrepancy with our results regarding the dispensable role of CD40-CD40L is unclear, but may reflect differences between B cell lines, as indicated by the ability of CL-01 but not of BL2 to switch isotype in vitro. The importance of the cells under consideration is further suggested by the report of Sale and Neuberger (55) who recently observed that BL2 cells could mutate spontaneously in vitro, although at a very low rate. However, we have no evidence for spontaneous mutation from the analysis of more than

8×10^4 bp in our BL2 subclone. The same authors have reported that another EBV-negative Burkitt's lymphoma, Ramos, mutates spontaneously in vitro (55). Once more, the differences between BL2 and Ramos cells, which may explain the accumulation of somatic mutation with or without exogenous triggering, remain speculative. Furthermore, these differences may be quantitative rather than qualitative, since it is not known whether coculture of Ramos cell with activated T cells in the presence of anti-sIg can increase the rate of spontaneous mutation.

In conclusion, we have initiated a systematic analysis of the BCR and T cell-dependent signals that trigger somatic mutation in a culture model, which bypass the requirements for B cell proliferation-survival factors but remains dependent on the nature of the B cell analyzed. We have found that soluble factors cannot replace intimate contact between T and B cell membranes. Furthermore, the direct demonstration that CD40 is not the B cell trigger for somatic mutation in BL2 cells points to potential involvement of other cell surface molecules.

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