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Differential Regulation of Immunoglobulin Class Switching and Somatic Hypermutation During B-Cell Proliferation In Vitro

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Research Article

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Abstract

Antibody V genes in antigen-stimulated B cells pick up somatic mutations, and these changes create the clonal diversity from which memory B cells are later selected during responses to T-cell-dependent antigens. Such mutations don't seem to arise when B cells are stimulated with mitogens or respond to T-cell-independent antigens. T-cell-dependent humoral immunity rests on T cells recognizing processed antigen presented by class II major histocompatibility glycoproteins (Ia) on the B-cell surface. We therefore asked whether engaging Ia on B cells is enough to trigger somatic mutation. We stimulated B cells in vitro under conditions where their growth depended on antibody ligation of surface Ia. Hybridoma V genes derived from these cells carried no somatic mutations, even after prolonged stimulation, and even though the cells were driven to secrete immunoglobulin and switch to the isotypes typical of T-cell-dependent responses. This suggests that Ia-mediated signalling and isotype switching do not cause somatic mutation. The differentiation route that produces somatic mutation in memory B cells therefore appears distinct from the one that drives proliferation, immunoglobulin secretion and class switching.

INTRODUCTION

High-affinity serum antibodies result from strong clonal selection, in which B cells whose receptors bind the antigen most tightly are the ones that expand [1]. A V region's affinity depends on three things: which VH, D, JH and JL gene segments were combined to build it [2], the exact sequences at the joins between those segments [3], and the somatic mutations that arise within its coding regions [4]. Of the three, only somatic mutation is acquired later, during the antigen-driven phases of B-cell differentiation.

In vivo, mutations build up quickly in and around expressed V regions, at an estimated rate of about 10^{-3} per base pair per generation [5]. Lineage studies of large B-cell clones from individual mice show that mutations accumulate across many rounds of division and can continue after isotype switching [6]. Monoclonal IgM antibodies sometimes carry mutations too, but it is unclear whether these arise independently of switch signals. A switch can occur on only one of the two sister chromatids, and these separate at mitosis [7].

Mutations appear to arise through a point-mutation process whose mechanism remains largely unknown. Because of the broad interest in how this process works and what triggers it, we have tried to build polyclonal in vitro systems in which somatic mutation is active. Proliferation driven by a mitogen (lipopolysaccharide) is not enough to induce mutation [8]. A separate report found no somatic mutations in V genes used in responses to T-cell-independent antigens. Together, these findings point to T-cell control of the process, either through secreted lymphokines or through T-cell engagement of B-cell surface molecules [9].

The most plausible B-cell surface candidate for relaying T-cell signals is Ia, since T-helper cells are thought to recognize processed antigen presented by Ia on the B cell. In murine B cells primed with anti-IgM and interleukin-4 (IL-4), cross-linking surface Ia triggered tyrosine kinase activation, calcium

mobilization and entry into the cell cycle [10]. Class II molecules have a comparable role in human B cells, that anti-Ia drives CH12 cells to secrete IgM [11]. We therefore tested whether signalling through Ia could induce mutational differentiation. We primed B cells polyclonally in vitro with a non-mitogenic monoclonal IgM antibody and IL-4, then stimulated proliferation with immobilized anti-Ia. To characterize the antibodies these cells produced, we generated hybridomas, determined their isotypes and sequenced the defined Vκ genes expressed by selected clones.

MATERIALS AND METHODS

Mice

(CAL-20 × A/J)F1 mice (I-A□/I-A□) were bred at the department of microbiology, University of North Bengal and were used when they were 7 to 12 weeks old.

B-cell isolation and stimulation in vitro

B cells were isolated and stimulated in vitro as follows. To rule out preactivated B cells that might already have acquired somatic mutations in vivo, we purified resting B cells on discontinuous Percoll gradients after removing erythrocytes and T cells [12]. We started with 3×10^8 nucleated spleen cells from four adult female mice and recovered quiescent B cells at a density above 1.085. Flow cytometry of cells stained with anti-Ig or acridine orange showed that over 98% were surface immunoglobulin (mIg)-positive and over 97% were in G0. The cells were cultured at 10^6/ml in complete Iscove's modified Dulbecco's medium with 10% foetal calf serum and 5×10^{-5} M 2-mercaptoethanol [13].

Stimulation followed the published protocol (ref. 14). For the first 16 hr, the cells were primed with IL-4 (40 U/ml) and 20 μg/ml of the monoclonal rat anti-IgM antibody Bet-2. At 16 hr they were transferred, still in Bet-2 and IL-4, to wells precoated with 10 μg/ml of the monoclonal anti-Ia□,□ antibody D3.137.5.7 [15]. On day 3 the cultures were washed and moved into medium containing IL-4 (40 U/ml), IL-5 (40 U/ml) and 10% mixed lymphocyte culture (MLR) supernatant [16]. On day 7, half of the cells were fused with SP2/O-Ag14 to make hybridomas by a standard method. The rest were kept in 5 ml of fresh medium with 10% MLR supernatant, IL-4 and IL-5, and another 5 ml of the same medium was added on day 9. On day 15, half of the remaining cells went into a second fusion, and the final cells were cultured the same way until a third and last fusion on day 19. Hybridomas were maintained in RPMI-1640 with 5×10^{-5} M 2-mercaptoethanol and 15% iron-supplemented calf serum [17].

Hybridoma screening

Hybridoma screening was done by replica plating the clones and testing them for mRNA that hybridized to a $VkId^{CR}$ DNA probe, using a lysate hybridization protocol (ref. 18). The probe covered the full VK coding sequence, including 6 bp of 5' sequence and 5 bp of 3' sequence from JK1, plus 450 bp of

flanking M13 KS+ vector DNA. Hybridizations and washes were run under low-stringency conditions, in 750 mM NaCl, 75 mM sodium citrate (5× SSX) and 50% formamide at 42–45°C. Under these conditions, any of the three members of the VKO family, which includes $VkId^{CR}$, gives a signal (ref. 19). Clones that gave a signal were subcloned twice by limiting dilution and screened again the same way. The subclones were then checked for immunoglobulin isotype and, by Southern blotting, for rearrangement of the $VkId^{CR}$ gene.

To find hybridomas that expressed the $VkId^{CR}$ member specifically, we extracted hybridoma DNA, digested it with HindIII and ran Southern blots with the $VkId^{CR}$ probe as previously described. Bands at 4.4, 4.0, 3.4 and 3.1 kilobases (kb) mark rearranged $VkId^{CR}$ genes joined to JK1, JK2, JK4 and JK5, respectively (ref. 20). Hybridomas without these rearrangements must express a different VKO gene or a closely related VK9 family gene.

Isotype analysis

Hybridomas selected for producing $VkId^{CR}$ or related VK transcripts were tested for the isotype of the immunoglobulin (Ig) they secreted, using an enzyme-linked immunosorbent assay (ELISA). Microtitre wells were first coated (50 µl, 2 µg/ml) with isotype-specific rabbit antibodies. For the IgE well, an affinity-purified monoclonal rat anti-mouse IgE was supplied by Dr Anju Das (Department of Microbiology and Immunology, North Bengal Medical College & Hospital, Darjeeling). Hybridoma culture supernatants (50 µl) were then added to the wells. The controls were TEPC 183 (IgM), TEPC 15 (IgA), MOPC 21 (IgG1), RPC 5 (IgG2a), MOPC 195 (IgG2b) and FLOPC 21 (IgG3), each at 2 µg/ml. Bound antibody was detected with a polyspecific goat anti-rabbit reagent (anti-IgG, IgA and IgM) conjugated to horseradish peroxidase, added at 25 ng in 50 µl, and the signal was developed with 50 µg of peroxidase substrate, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) [21]. Except for the coating step, all dilutions were made in phosphate-buffered saline (PBS) containing 0.05% Tween-20, 3 mg/ml bovine serum albumin and 2 mg/ml gelatin. Wells were washed thoroughly with PBS between steps.

V-gene sequencing analyses

Rearranged $VkId^{CR}$ genes were amplified by polymerase chain reaction (ref. 22) and sequenced directly, without cloning. In brief, hybridoma DNA was amplified for 35 cycles with primers matching sequences in the $VkId^{CR}$ leader exon and in the intron 3' of the JK5 exon. The products were run on a 1.5% agarose gel, stained with ethidium bromide, and visualized by ultraviolet transillumination [23]. Bands of amplified DNA were cut out as plugs with a Pasteur pipette, melted at 95°C, and 1-µl samples were reamplified for 25 cycles with nested primers, which sit inside the first primer pair relative to the $VkId^{CR}$-JK5 product. The 3' primer in this second round carried a phosphate group at its 5' end. The phosphorylated strand of the product was then digested with exonuclease (ref. 24) to leave single-stranded DNA for sequencing. The amplified $VkId^{CR}$ genes were sequenced by the dideoxy chain-termination method with Sequenase,

the nested sequencing primers were end-labelled with ^{32}P, and the termination reactions were carried out at 45°C to reduce compression artifacts.

RESULTS

Polyclonal stimulation and hybridoma production

An in vitro polyclonal model of B-cell stimulation that depends on engagement of surface Ia on the B cell (ref. 25). It was built to mimic the cognate interactions that are thought to take place in vivo among B cells, T cells and antigen. Resting B cells are first primed for 12–16 hr with IL-4 and soluble, non-mitogenic anti-Ig antibodies, which stand in for antigen. We then proliferate when placed on plastic coated with anti-Ia antibodies, which stand in for the T-cell receptor. Adding lymphokines (IL-4, IL-5 and MLR supernatant) afterwards drives these cells to differentiate and secrete IgG and keeps them proliferating over the long term. Here, quiescent splenic B cells were stimulated with anti-IgM, IL-4 and anti-Ia as described in Materials and Methods, then kept in culture for 19 days in medium conditioned with IL-4, IL-5 and MLR supernatant. We made B-cell hybridomas by fusing the cells with SP2/0 on days 7, 15 and 19. As a first screen, we hybridized a VKO probe to RNA from whole-cell lysates of the hybridomas. This yielded 27 hybridomas expressing VKO-related genes, and each was subcloned twice by limiting dilution before further analysis.

Isotype analysis

We determined the isotypes of the immunoglobulins secreted by the 27 cloned hybridomas by ELISA, and the results are listed in Table 1. The data suggest that the stimulated B cells differentiate in culture by switching isotype. Most hybridomas made on day 7 secreted IgM, whereas most of those made on days 15 and 19 produced IgG1 and IgE, respectively.

Several lines of evidence point to switching, rather than selection of pre-existing subsets, as the cause of this shift. First, anti-IgM must be present during priming for the cells to proliferate when later placed on immobilized anti-Ia. The starting B cells must therefore express IgM when the culture begins. Second, a switch to IgG1 and IgE fits with IL-4 being present in the medium, and differs from the isotype profile of hybridomas made from B-cell blasts stimulated with the mitogen lipopolysaccharide (refs. 26). Third, the sequence of IgM, then IgG1, then IgE hybridomas across successive fusions matches the order of the corresponding genes at the Ig locus, which suggests stepwise switching from IgM to IgG1 to IgE in culture. This stepwise pattern is backed by studies of switched Ig genes and their circular excision products, and by work showing that IgE-secreting cells arise from IgG1 precursors in vitro (refs. 27).

Table 1
Isotype expression and Vk10 gene usage by hybridomas

Hybridoma	Day of cell fusion	Isotype	$Vkld^{CR}$ expression
KL2.2	7	IgM	No
KL2.6	7	IgM	No
KL2.8	7	IgM	No
KL2.9	7	IgM	No
KL2.10	7	IgM	No
KL2.13	7	IgM	No
KL2.14	7	IgM	No
KL2.16	7	IgM	No
KL2.18	7	IgM	Yes
KL2.19	7	IgM	No
KL2.21	7	IgM	Yes
KL2.22	7	IgGI	No
KL2.25	7	IgM	No
KL2.27	7	IgM	No
KL2.28	7	IgM	Yes
KL2.29	7	IgM	Yes
KL2.30	7	IgM	No
KL2.32	7	IgM	No
KL2.33	7	IgGI	Yes
KL3.5	15	IgGI	No
KL3.8	15	IgGI	Yes
KL3.15	15	IgM	No
KL3.26	15	IgGI	No
KL4AI	19	IgE	Yes
KL4B10	19	IgE	Yes
KL4C8	19	IgE	Yes

Hybridoma	Day of cell fusion	Isotype	$Vkld^{CR}$ expression
KL4CII	19	IgE	Yes

Sequencing analysis

To test whether the differentiated B cells had mutated their antibody V genes, we first identified which hybridomas expressed a particular VKO gene, $Vkld^{CR}$, whose germline sequence is known (refs. 28). Screening for VKO-related expression has a practical advantage: the family has only three members, and one of them has already been cloned and sequenced. On average, about one in three randomly obtained hybridomas expressing a VKO gene should therefore be using $Vkld^{CR}$.

Figure 1. Sequences of the $Vkld^{CR}$-JK genes. The top line shows the germline $Vkld^{CR}$-JK gene as expressed by hybridoma 36–65 (ref. 29). Dashes mark positions identical to this germline sequence, and dots are inserted to align sequences whose junctional regions differ in length. All differences between sequences are explained by junctional diversity and by diversity among the germline JK segments (ref. 30).

Rearranged $Vkld^{CR}$ genes can be identified specifically by Southern blotting. HindIII fragments of 4.4, 4.0, 3.4 and 3.1 kb indicate rearrangement of $Vkld^{CR}$ to JK1, JK2, JK4 and JK5, respectively (JK3 is a pseudogene). Ten of the 27 hybridomas expressing VKO-related genes carried $Vkld^{CR}$ rearrangements. Five of these came from the day 7 fusion, one from day 15 and four from day 19. $Vkld^{CR}$ was joined to JK1 in six cases, to JK2 in three and to JK5 in one. We amplified the rearranged $Vkld^{CR}$-JK genes from hybridoma DNA by PCR and sequenced them directly. Every $Vkld^{CR}$ gene was rearranged in a functional reading frame. Across the 10 complete $Vkld^{CR}$ -JK sequences, totalling 3209 nucleotides, we found no difference at all from the germline sequence (Fig. 1). The sequences came from $Vkld^{CR}$ genes expressed with IgM (four sequences), IgG1 (two) and IgE (four). Differences in the VK-JK junctional sequences (Fig. 1) and in non-productive heavy-chain rearrangements show that the 10 hybridomas arose from at least nine different precursor cells.

DISCUSSION

The most direct reading of these results is that signalling through Ia is not enough to trigger somatic mutation. We also show that mutational differentiation can be separated from isotype-switch differentiation, even when the cells undergo several switch events (IgM to IgG1 to IgE).

A strength of this experiment is that the VK genes we analysed come from a germline precursor that takes part in a well-characterized immune response to p-azophenylarsonate (Ars) in vivo. The absence of mutations in the $Vkld^{CR}$ genes sequenced here contrasts sharply with how common they are in $Vkld^{CR}$ genes recovered from fusions of splenic B cells responding to Ars in vivo. 26 $Vkld^{CR}$ -JK genes from

hybridomas made from spleen cells 16 days after immunization carried 90 somatic mutations in total, which works out to about 0.22 mutations per VkId^{CR} -JK gene per day (ref. 31). Had mutation proceeded at a similar pace in our cultures, we would have expected roughly 28 mutations across the 10 VkId^{CR} -JK genes from our hybridomas [32]. Finding none strongly suggests that the mutation machinery was inactive.

One caveat is that mutation might have been induced but left the VkId^{CR} genes nonfunctional, for instance through premature stop codons. Because heavy-chain expression without light chain seems to be toxic to hybridoma cells (ref. 33), such mutated genes might never survive in viable hybridomas. Even so, if mutation were occurring, we would expect some hybridomas to carry mutated VK genes that still encode a kappa chain able to pair with a heavy chain, as happens in vivo. We saw none, which argues that mutation did not occur at all. The alternative, that some cells mutated extensively enough to destroy their V genes while others did not mutate at all, is very unlikely, since every cell received the same signals (anti-Ig, anti-Ia and interleukins) in a polyclonal stimulation. Our sequencing and blotting data show that the 10 VKIdCR genes were expressed by B cells from at least nine independent precursors, which supports the polyclonal nature of the stimulation. We therefore favour the simplest conclusion: somatic mutation was not occurring in these cultures at any measurable frequency.

T-cell recognition of Ia probably has a central role in delivering help specifically to antigen-stimulated B cells, yet the signals generated by Ia ligation apparently cannot activate the somatic mutator on their own. Additional signals seem to be needed, either through direct contact between B-cell membrane molecules and those of other cells in the immune response, or through other interleukins. Our findings further suggest that what the somatic mutator needs to be switched on differs from what sustains long-term proliferation or drives related events such as isotype switching and Ig secretion.

Limitations

A number of limitations of the study should be noted. Firstly, our analysis was entirely dependent on the successful isolation of live hybridomas and so it's entirely possible that we have missed clones which have accumulated a great deal of mutation (some of which may have resulted in structural defects or stop codons that would have rendered the heavy and light chains non-functional when paired). Secondly, our sequencing was restricted to the rearranged VκId^{CR} genes. That gives us a very solid and reliable benchmark, but looking at a wider range of heavy and light chains across the whole repertoire would have given us a much broader view. Thirdly, our in vitro system is very good at isolating the Ia mediated signal and the cytokine exposure. It is still, however, an in vitro system and so it can't replicate the full 3D environment, the cellular architecture or the direct membrane to membrane contacts (e.g. CD40-CD40L) that are present in the live cells within the germinal centres in vivo. Even with all of these caveats, the complete absence of mutations in the sequences is very strong evidence that Ia ligation and class switching occur independently of somatic hypermutation in this situation.

DECLARATIONS

Ethics approval and consent to participate: This study was approved by the Institutional Animal Ethics Committee (IAEC) of University of North Bengal (Protocol No.: IAEC/NBU/2025/04).

Ethical Permissions

Not Applicable

Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author contributions

AKS: Data curation, Project administration, Conceptualization, Visualization, Writing – original draft, Supervision, Validation, Writing – review & editing, Investigation, Software, Formal analysis, Methodology, Resources.

AD: Data curation, Project administration, Conceptualization, Visualization, Writing – review & editing.

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Figures

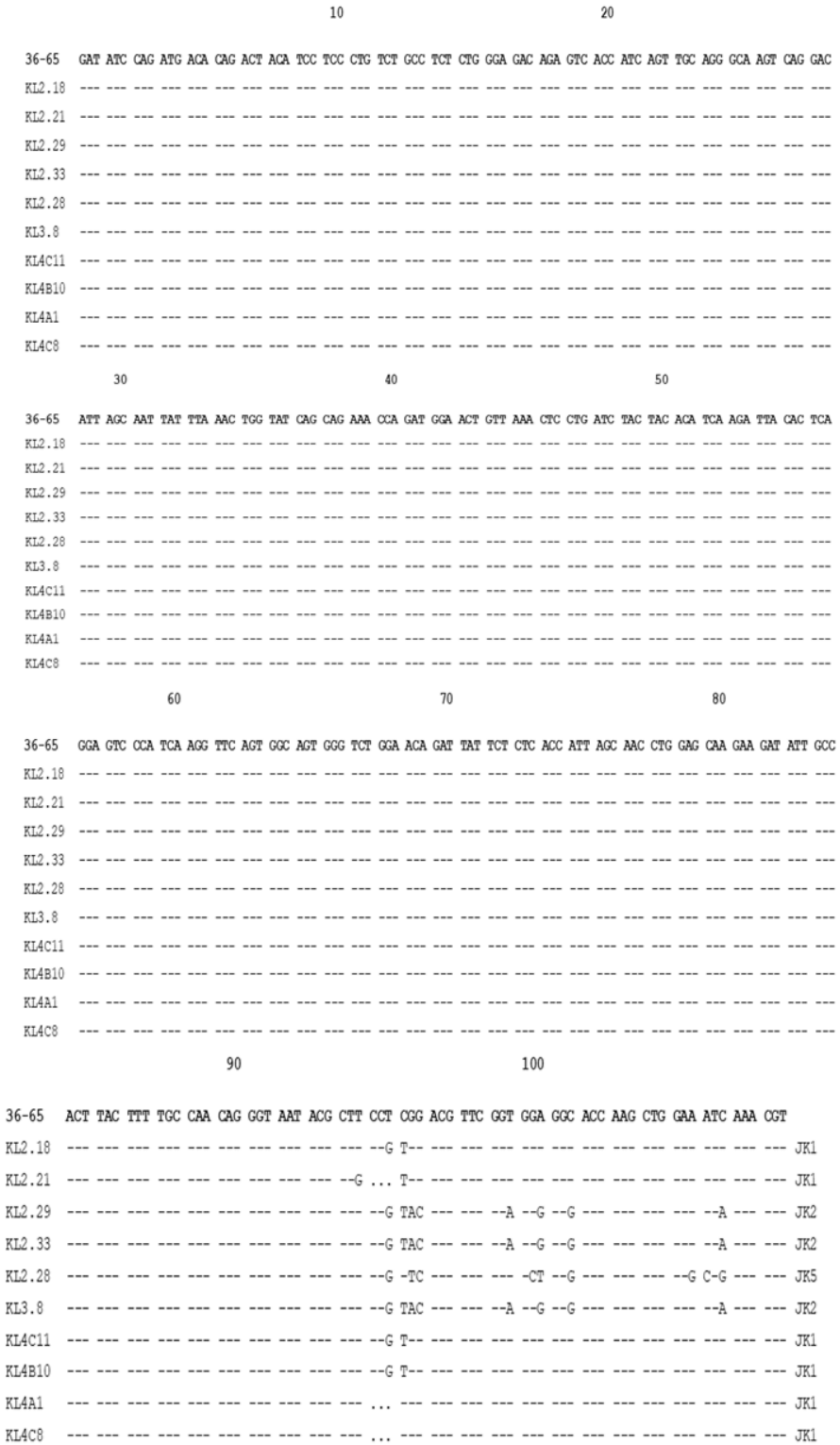


Figure 1

Sequences of the VkId^{CR}-JK genes. The top line shows the germline VkId^{CR}-JK gene as expressed by hybridoma 36-65 (ref. 29). Dashes mark positions identical to this germline sequence, and dots are inserted to align sequences whose junctional regions differ in length. All differences between sequences are explained by junctional diversity and by diversity among the germline JK segments (ref. 30).