Heavy-Chain Class Switch Does Not Terminate Somatic Mutation

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Summary

We studied the H-chain class switch rearrangement in four groups of clonally related B cell hybridomas, to test the hypothesis that class switch terminates somatic mutation in a B cell. Using switch region-specific probes in Southern blot analysis individual μ-γ switch rearrangement events can be distinguished. We show that clonally related IgG-producing hybridomas that differ by mutations often share a common switch rearrangement. This indicates that class switch in these cells did not terminate somatic mutation.

Materials and Methods

Hybridomas. All hybridomas were derived from fusions of spleen cells from autoimmune MRL/lpr mice (obtained from The Jackson Laboratory, Bar Harbor, ME) with myeloma cell lines. The generation and initial characterization of clone 1, 3, and 4 hybridomas were described previously (5, 14, 15). The hybridomas in clone 2 were prepared according to the procedure of Marshak-Rothstein et al. (16). Hybridomas in clone 2 secrete IgG2a-specific rheumatoid factors. The characterization of clone 2 hybridomas will be described elsewhere (Shan, H., M. Shlomchik, manuscript in preparation).

Probes. pJ14c (17) was a gift from Dr. B. Birshstein, (Albert Einstein College of Medicine, Bronx, NY). pγ7,BgH2.5 (18) and pγ7,EH10.0 (19) were kindly provided by Dr. W. Dunnick (University of Michigan Medical School, Ann Arbor, MI).

Southern Blot Hybridization. Cell line and MRL/lpr liver DNA were prepared as described (20). Southern blots were performed essentially as described (21). Briefly, 10 μg of DNA was digested with the appropriate restriction enzyme(s) according to the manufacturer’s instructions, and electrophoresed through a 0.8% agarose gel. To compare the size of the hybridizing bands on pJ14c blot and pγ7,BgH2.5 (pγ7,EH10.0) blot, digested DNA samples were loaded on the same agarose gel twice in the same order. After the DNA was transferred to Hybond membranes (Amersham Corp., Arlington Heights, IL), the membranes were cut in half, and each half was hybridized to pJ14c probe and either pγ7,BgH2.5 or pγ7,EH10.0 probe. After overnight hybridization, the membrane received a final wash in 0.2x SSC, 0.1% SDS at 68°C and was exposed to x-ray film at ~70°C, using an intensifying screen.

To detect γ1 switch rearrangements, hybridoma DNA was digested with EcoRI and BamHI and probed with a 5’ Sμ probe (pJ14c) and Sγ1 probe (pγ7,BgH2.5). γ1 switch rearrangements were detected in Southern blots of DNA digested with EcoRI and probed with pJ14c and a γ1 probe (pγ7,EH10.0) (the map of the switch regions and the probes used are shown in the figures).

Sequencing Analysis of the V Region of mAbs. Total cellular RNA was purified from tissue culture cells as described by Chirgwin et al. (22). Antibody V regions were sequenced from poly(A) + RNA as described (20). The V region nucleotide sequences and the derivation of genealogical trees from these sequences will be described in detail (Shan, H., et al., manuscript in preparation).

Results

To test the hypothesis that somatic mutation does not occur after H-chain class switch, we studied clonally related B cell hybridomas that differ from each other by somatic mutations.
Figure 1. Mechanism of H-chain class switch recombination. (VDJ) Rearranged immunoglobulin V region; (Sp and Cu) switch region and constant region of p; (Sy and Cy) switch region and constant region of y. During H chain class switch, part of an upstream switch region of one isotype (Sp switch region) is joined to the downstream switch region of another isotype (Sy), with deletion of the intervening DNA (6, 7, 28). The switch rearrangement is contained on a novel, uniquely sized restriction fragment (28, 29).

In sets of clonally related hybridomas that share H-chain isotype, we can test whether clone members of the same isotype have switched independently from each other or are the result of a single switch event. As described below, Southern blot analysis of the context of switch recombination sites allows us to distinguish these two possibilities. If the clonally related hybridomas express the same isotype because of a single switch event, then the somatic mutations that distinguish them must have occurred after class switch.

Clonally Related Hybridomas. Clones were defined by the usage of the same V, D, and J gene segments and identity of VDJ (H chain) and VJ (L chain) junctional sequences, as determined by sequencing the antibody V regions. In addition, cell lines from clones in which nonproductive rearrangements could be detected, share these rearrangements on either or both H chain and L chain genes, as shown by Southern blot analysis using J, (pJ11) (23) and J, (pECK) (24) probes (data not shown). Each one of these characteristics is highly idiosyncratic, and the chance of independent hybridomas meeting any one of these criteria is extremely small (25).

H Chain Class Switch Recombinations. Antibody H-chain class switch occurs by the recombination of switch sequences: tandemly repeated DNA sequences which reside 5' of H-chain constant region genes (26, 27). As a result of recombination, part of an upstream switch region of one isotype (μ switch region) is joined to the downstream switch region of another isotype, and the intervening DNA is deleted (see Fig. 1 for diagram) (4, 5, 28). Different switch events can be distinguished because rearrangement breakpoints are distributed over the entire switch regions (29). Indeed, independentswitch events rarely result in comigrating bands (29–32). Since switch regions are large (1–10 kb) (28), the difference in size between independent switch recombination fragments is readily de-
detectable on Southern blots (28, 29). Thus, we assume that co-migrating switch rearrangement bands from clonally related hybridomas are the products of a shared single switch recombination, not the result of chance comigration of bands generated by independent events.

Southern blot analysis using $\gamma_{\mu}$ and $\gamma_{\gamma}$ probes was performed on DNA from cell lines of three $\gamma_{3}$-expressing clones and one $\gamma_{1}$-expressing clone. As expected, we found the size of the switch rearrangement bands were all different between clones (data not shown), confirming that the sizes of independent switch rearrangements are distributed over a wide range. The results of the analysis of each of the individual clones are summarized below.

**Clone 1 (Fig. 2).** The band labeled S1 represents the $\gamma_{3}$ switch rearrangement shared by all the clone members sequenced (34-8.1 is from an independent clone). The use of the 5' $\gamma_{\mu}$ probe (pJ14c) in conjunction with $\gamma_{\gamma}$ switch probes allows distinction of bona fide switch rearrangements from deletions within a switch region (intraswitch deletions) (33-35). In bona fide switch rearrangements the 5' $\gamma_{\mu}$ and $\gamma_{\gamma}$ sequences will be linked on the same novel restriction fragment (see Fig. 1). Intraswitch deletions will also change the size of the restriction fragment hybridizing to the relevant switch probe, but in contrast to bona fide switch rearrangements, the novel restriction fragments detected by $\gamma_{\gamma}$ and $\gamma_{\mu}$ probes would be of different size. Note that the S1 band is the same size on both pJ14c and pY3 blots, demonstrating that it is a bona fide switch rearrangement. The second allele in this clone remains in germline configuration at the $\gamma_{\gamma}$3 (G1) and $\gamma_{\mu}$4 (G2) sites. Thus S1 represents the expressed allele. All the clone members are products of that single switch event (S1); thus, the mutations that distinguish these clone members from each other (depicted in the genealogical tree, Fig. 2) must have occurred after the class switch event.

**Clone 2 (Fig. 3).** Most IgG3 clone members in this clone share one $\gamma_{3} + pJ14c+$ band (S1). Therefore, as in clone 1, the mutations that distinguish these clone members must have occurred after that class switch event (S1). The second allele in all clone members remains germline at $\gamma_{\gamma}$3 (G1). The S3 band on the blot probed with pJ14c most likely represents the result of an intraswitch ($\gamma_{\mu}$) deletion; this interpretation is supported by the fact that the C$\mu$ ($\mu$ constant region) gene is not deleted (Southern blot data not shown).

Two hybridomas do not share S1: 5-7 is the product of an independent $\gamma_{3}$ switch event (S2). The mutations on 5-7, which are shared with 10-2 (a IgM clone member), must have occurred before class switch. Therefore, this clone provides evidence that somatic hypermutation occurs both before and after class switch.

The $\gamma_{3} + pJ14c+$ band in 1-6 migrated slightly faster than S1. Given the position of 1-6 in the lineage, the faster migration of the $\gamma_{3} + pJ14c+$ band in 1-6 is most likely due to a secondary intraswitch deletional event.

**Clone 3 (Fig. 4).** In this clone, S2($\gamma_{3} + pJ14c+$) represents the single bona fide $\gamma_{3}$ switch event shared by all three clone members shown in the tree, providing another example of somatic hypermutation continuing after class switch. The bands S1 and S3 presumably represent unlinked intraswitch deletional rearrangements. The possibility that these could represent bona fide isotype switch events is ruled out because the C$\mu$ gene is retained (data not shown).

**Clone 4.** Fig. 5 shows the mutations and analysis of class switch in an IgG1-expressing clone. AM4 and AM5 share one $\gamma_{1} + pJ14c+$ band (S1), showing that they originate from a precursor cell that had already undergone this isotype switch event. The seven mutations by which these two cells differ occurred after that event, showing that somatic hypermutation can be an active process not only in B cells that

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**Figure 3.** Clone 2. The figure is organized as in Fig. 2. Digestions were with EcoRI and BamHI. SP2/0 and Ag8 are hybridoma fusion partners. When cell fusion was performed, part of the spleen was fused with SP2/0 cell line as fusion partner, while the other part of the spleen was fused with Ag8 cell line. S1 is the bona fide $\gamma_{3}$ switch rearrangement band that is shared by most of the clone members. S2 is the $\gamma_{1}$ switch rearrangement band of 5-7. S3 probably represents an intraswitch ($\gamma_{\mu}$) deletion (see Results). The slightly smaller (than S1) $\mu$-$\gamma_{1}$ switch rearrangement band of 1-6 is most likely the result of a secondary deletional event.
have switched from IgM to IgG3, but also in IgG1-producing B cells. AM2 and AM3 have unique, smaller switch rearrangement bands that either represent independent γ1 switches or secondary, intraswitch rearrangements of a common switch shared with AM4 and AM5.

Discussion

Somatic mutation and class switch have been considered to be milestones in B cell development (8, 12, 13). It is generally accepted that somatic mutation occurs in the IgM-expressing B cells which are the dividing precursors of memory B cells (12). These cells, which are responding to antigen for the first time, are said to be in the “memory cell generation” pathway. Rajewsky and colleagues have proposed that the end of this pathway and the end of somatic hypermutation occur when such B cells switch isotype (12, 13). Thus, in their model, B cells responding to secondary antigenic challenge are already class-switched and have completed the somatic mutation process.

As our data unlink the processes of isotype switching and mutation stopping, this model must either be modified or abandoned to account for the fact that mutation also occurs after class switch. In a modified model, either somatic mutation or class switch, but not both processes, could be restricted to a particular differentiation stage (i.e., the memory cell gener-
ation pathway or the transition from memory generation to memory B cell). However, this model may be fundamentally incorrect. An alternative way to account for all the data is that neither mutation stopping nor class switch are deterministic markers of the memory or secondary response, but are events that are either independently regulated or occur stochastically. In a stochastic model, mutation stopping and class switch are more likely to have occurred as time goes on in clonal expansion.

In a cell transfer experiment, Siekevitz et al. (3) failed to observe mutation after class switch. Instead they observed that members of clones had stopped mutating and had identical switch rearrangements (3). We have analogous observations in clones from an autoimmune mouse (5, and data not shown). Perhaps these failures to observe mutation after class switch are because the cells immortalized were relatively old and likely to have undergone both mutation stopping and class switching. Additionally, the cell transfer system could have selected for cells that had stopped mutating; cells that do not mutate will not sustain detrimental somatic mutations and thus will have a higher growth rate than mutating cells (5).

Our data also argue against a role for deletion of the Cα locus causing mutation to stop, as was proposed by Rajewsky et al. (12). In clones 1-3, which express IgG3, there is only one productive switch rearrangement that juxtaposes Sα and Sγ3. On this productive allele, the remaining DNA between Sα and Sγ3 is presumably deleted. As this rearrangement is shared by many members of the clones, deletion of the Cα locus preceded the mutations which distinguishes clone members. Thus, the presence of the Cα locus on the productive allele is not required for maintenance of somatic mutation. Since somatic mutation only occurs in mature B cells after antigen stimulation (8, 36-39), there must be signals that trigger the initiation of somatic mutation. Thus, while this result shows that the Cα gene is not necessary for the continuation of somatic mutation, it cannot rule out that the Cα gene (or membrane-bound IgM) may be needed for the induction of somatic mutation in a B cell, in the sense that the heavy chain enhancer may be required for the induction but not maintenance of high levels of IgH mRNA expression (40).

Although the antibodies analyzed here are autoantibodies from autoimmune MRL/lpr mice, we believe that our findings reflect general features of antibody responses since rheumatoid factor and anti-DNA responses in MRL/lpr mice are similar to secondary immune responses: they are oligoclonal, consist mostly of antibodies of switched isotype, and have many somatic mutations in antibody V regions with the pattern of mutation strongly indicative of the effect of antigen positive selection (5, 15). It seems unlikely then, that mutation occurring after class switch would be a feature only of B cells participating in autoimmune responses and not of B cells in secondary immune responses.

Thus, mutation not only occurs before and after class switch, but mutation stops both before (41) and after (5) class switch. Neither the initiation nor termination of mutations seems to be determined by class switch. These features favor a stochastic model to explain the relationship between mutation and class switch.

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