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Immunology

Somatic diversification of immunoglobulins

(IgM/mutation/amino acid sequence)

STUART RUDIKOFF, MICHAEL PAWLITA, JANET PUMPHREY, AND MARY HELLER

Laboratory of Genetics, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205

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ABSTRACT **A series of three IgM, κ monoclonal antibodies arising from a fusion of BALB/c spleen cells from mice immunized with β-(1,6)-galactan-containing antigens have been analyzed. These three lines were found (*i*) to have homologous protein sequences in the heavy chain D region and at the sites of recombination between the heavy chain variable and D segment (V_H–D) and the D and joining segment (D–J_H), although amino acid substitutions were observed in both the heavy and light chain variable regions; (*ii*) to use identical heavy and light chain joining segments; and (*iii*) to demonstrate two identical (productive and nonproductive) κ-chain rearrangements. A likely explanation for these observations is that the three lines are clonally related (arise from a common precursor) and that the observed heavy and light chain variable segment substitutions represent somatic point mutations. Because these antibodies are all of the IgM class, the results indicate that a somatic mutational mechanism is activated early in B-cell ontogeny and operates at both the heavy and light chain loci. Furthermore, the somatic mutation process appears to continue during the development of a given cell line, but is independent of class switching.**

Immunoglobulins are encoded by large multigene families that potentially express an almost unlimited degree of diversity at the level of serum antibody. It is now clear that a number of processes contribute to this phenomenon. First, there are an apparently large number of light (L)- and heavy (H)-chain germ-line variable (*V*)-region genes (1–4). Second, both L and H chains are encoded by multiple genetic elements: V_K and J_K in the L chain (5, 6) and V_H, D, and J_H (7, 8) in the heavy chain. The number of protein structures that can be generated by various combinations of these segments constitutes a major portion of the total diversity. Third, the joining of the various gene segments is imprecise, creating sequence variation at the points of recombination (5, 6, 9–12). Fourth, interaction (i.e., gene conversion) may occur between related members of immunoglobulin families (13–15). Fifth, somatic point mutation provides an additional means by which structural alterations can be generated (16–23).

The last of the above mentioned processes, somatic mutation, has been a subject of interest and controversy in immunology for a number of years. Early studies in mouse λ chains (16, 17) provided the first evidence for somatic mutation and revealed a concentration of such mutations in complementarity-determining regions (CDR). Subsequent experiments in other systems have further documented the occurrence of somatic mutation in immunoglobulin genes, but it is presently unclear whether this process is random or directed to specific regions. While little is actually known about the precise mechanism and time of occurrence of somatic mutation in lymphocyte ontogeny, studies in two systems (19–21) have suggested that somatic mutation is linked, in some manner, to class switching in that immunoglobulins that have switched from IgM to other classes (i.e., IgG, IgA) frequently, but not always, express somatic mutations, whereas no mutations have been observed in IgM molecules. We have approached this question by examining the structure of a series of IgM hybridomas produced from a single fusion of a pool of spleen cells from two BALB/c mice immunized with β-(1,6)-galactan-containing antigens. The results of these experiments indicate that somatic mutation is likely to be a continuous process occurring throughout the ontogeny of a B-cell line committed to antibody production and, furthermore, is probably not associated with class switching.

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MATERIALS AND METHODS

Proteins. The production of hybridoma lines and purification of monoclonal antibodies were done as described (24). All hybridoma proteins in the present study were derived from a single fusion of pooled spleens from two BALB/c mice given one injection of galactan–bovine serum albumin followed by a second injection of gum ghatti [gum containing a high content of β-(1,6)-galactan].

Sequence Determination. HyGal 8 H and L chains were isolated, cleaved with cyanogen bromide, and amino acid sequences were determined as described (24–26).

RESULTS AND DISCUSSION

Antibodies to β-(1,6)-galactan-containing antigens have been used in this laboratory as models for antibody–antigen interactions (27, 28), idiotypy (26, 29), diversity (10, 24, 26, 30), and three-dimensional structure (31, 32). The current investigation involves an analysis of three hybridoma proteins derived from a fusion of 2 BALB/c spleens with the nonsecreting Sp2/0 cell line (24). The structures from two of these proteins, HyGal 6 and 10, have been reported (24, 26). The determination of the third sequence, HyGal 8, establishes a pattern of variation that links these three hybridoma lines in ontogeny and provides new insights into the occurrence of somatic mutation.

V-Region Relationships. The developmental relationship between hybridoma proteins HyGal 6, 8, and 10 is inferred from a comparison of their V region sequences. An examination of the H chain V region sequences (Fig. 1) reveals that HyGal 8 is identical in the V_H segment (amino acids 1–94) to the translated sequence of a V_H gene (V_H 441) described by Ollo *et al.* (33). HyGal 6 differs from this sequence at three amino acid positions (45, 46, and 91), and HyGal 10 differs at three positions (60, 88, and 91). The phenylalanine-91 substitution is shared between HyGal 6 and 10. These three pro-

Abbreviations: L, immunoglobulin light chain; H, immunoglobulin heavy chain; V, variable region of immunoglobulin L or H chain; V_K, amino acids 1–95 of the L chain V region; V_K, gene encoding the V_K protein segment; J_K, amino acids 96–108 of the L chain V region; J_K, gene encoding the J_K protein segment; V_H, amino acids 1–94 of the H chain V region; V_H, gene encoding the V_H protein segment; J_H, amino acids 100a–113 of the H chain V region; J_H, gene encoding the J_H protein segment; D, portion of the third complementarity-determining region; *D*, gene encoding the D protein segment; CDR, complementarity-determining region; kb, kilobase(s).

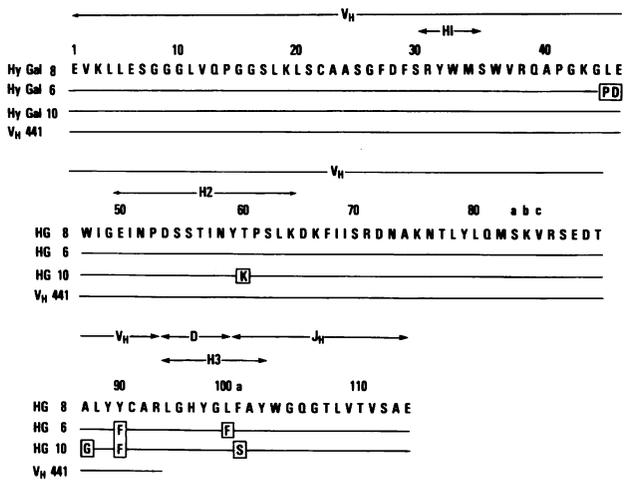

FIG. 1. H-chain V-region sequences from hybridoma proteins HyGal 6, 8, and 10 and the translated sequence of a BALB/c germline V_H gene (33). Numbering and assignment of CDR regions in all figures are according to Kabat *et al.* (34).

teins are also homologous in the H-chain CDR-3 region (amino acids 95–102), which normally exhibits the greatest amount of sequence variation among related H chains, as this portion of the molecule originates from the *D* gene segment and the two recombination events, V_H–*D* and *D*–J_H. In fact, the CDR-3 sequence associated with these three molecules is not found in H chains from six other hybridoma and four myeloma proteins that bind β-(1,6)-galactan or in any other murine H chains (34). A comparison of the CDR-3 regions from HyGal 6, 8, and 10 with BALB/c *D*-region sequences (35) indicates that only tyrosine-98 and glycine-99 are encoded in known BALB/c *D* genes. Since the V_H 441 germ-line gene ends at position 94, it is therefore likely that one or more of the amino acids leucine-95, glycine-96, and histidine-97 are generated during V_H–*D* recombination and that leucine-100 (HyGal 8) is generated during *D*–J_H recombination. Phenylalanine-100 (HyGal 6) and serine-100a (HyGal 10) are suggested to result from somatic point mutations. Leucine-100 and phenylalanine-100a are believed to be the two amino acids encoded at these positions in the original clone, because these amino acids are both present in two of the three sequences. An alternative explanation, which would obviate the clonality interpretation developed below, is that the two substitutions are generated during *D*–J_H recombination. The observation that these three proteins share sequences generated by the two recombination events and the *D* gene infers that they may be clonally related (i.e., progeny of a single precursor cell). Furthermore, these proteins all use the J_H3 (J_H, amino acids 100a–113 of the H chain V region) joining segment, again supporting the argument of clonality, since both J_H1 and J_H2 are found in other galactan-binding antibodies (26, 30), and J_H2 is used as frequently as J_H3, while J_H1 has occurred in a single instance. For these proteins to not be clonally related would require that they have independently produced essentially similar sequences at the two recombination sites and/or use a *D* gene product that has not been previously identified in any murine H chain. A similar analysis of BALB/c phosphocholine binding antibodies (19) reveals that of 17 complete H-chain sequences, 10 show junctional diversity at either or both the V_H–D or D–J_H sites. In no instance are two of these proteins identical at both junction sites. One example is found in which two myeloma proteins share the same single amino acid replacement at the V_H–D junction, but these proteins appear to use different D segments and differ at the site of D–J_H recombination. We therefore feel it is quite unlikely that these three closely related anti-galactan proteins have arisen independently.

The L-chain sequences from HyGal 6, 8, and 10 (Fig. 2) similarly display only minimal variation. The HyGal 10 V_K region (amino acids 1–96) is identical in sequence to 11 other V_K regions from galactan-binding antibodies (24) and presumably represents a germ-line sequence. HyGal 6 differs by a single substitution at position 12, and HyGal 8 differs by two substitutions at positions 51 and 92. Based on the argument of clonality, we suggest that these three L-chain replacements represent somatic mutations. All three proteins express the same J_K sequence (amino acids 97–108), again supporting the contention of clonality as all four functional J_K segments have been found to be potentially used in other anti-galactan L chains.

To further define the relationship among these three lines, endonuclease restricted DNA was hybridized with a probe (5) containing all four functional κ-chain *J* genes. This probe would be expected to hybridize with all germ-line and rearranged fragments containing *J* genes. The hybridization patterns of HyGal 6, 8, and 10 were identical (Fig. 3*a*) indicating that, in addition to the bands contributed by the BALB/c and SP2/0 fusion partners, these lines have two rearranged fragments of ≈9.6 and 5.7 kilobases (kb) in common. The 9.6-kb band is most likely to contain the productively rearranged V_K gene as it is similar in size to X44, which uses the same J_K segment (Fig. 3*b*). This same pattern was not found in three other myeloma and five hybridoma lines secreting galactan-binding antibodies. The sharing of one rearranged band would be expected among lines expressing the same V_K and J_K genes, but a second (nonproductive) rearrangement should be random. Therefore, the finding of two shared rearrangements in HyGal 6, 8, and 10 again indicates a common origin.

In the above discussion we have used three criteria—common H chain CDR-3 sequences, common J segments, and common restriction fragment rearrangement patterns—to identify a putative clonal relationship among hybridoma lines. The crux of this analysis rests on whether these criteria are adequate for such conclusions. It is thus informative to apply these criteria to other anti-galactan lines and observe the resulting patterns. We have previously reported (26) H-chain sequences from a number of galactan-binding antibodies, which reveal that two myelomas (T601 and X24) and one hybridoma (HyGal 3) express nearly identical CDR-3 sequences, although this sequence is quite different from the HyGal 6, 8, and 10 group. T601 uses the J_H1 joining segment, whereas J_H2 is found in X24 and HyGal 3. However,

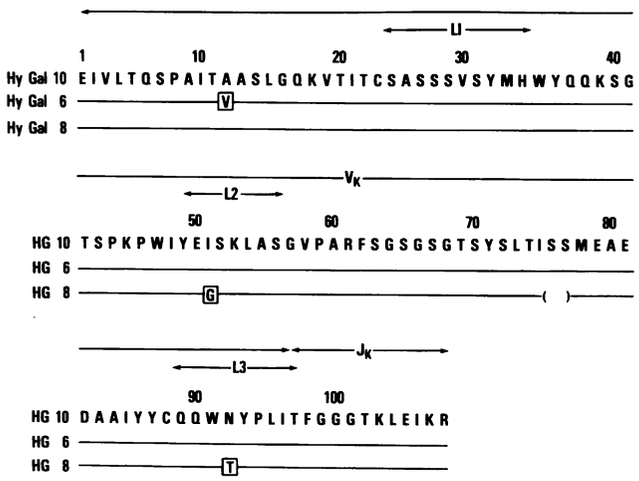

FIG. 2. L-chain V-region sequences from hybridomas HyGal 6, 8, and 10. Parentheses indicate unidentified amino acids.

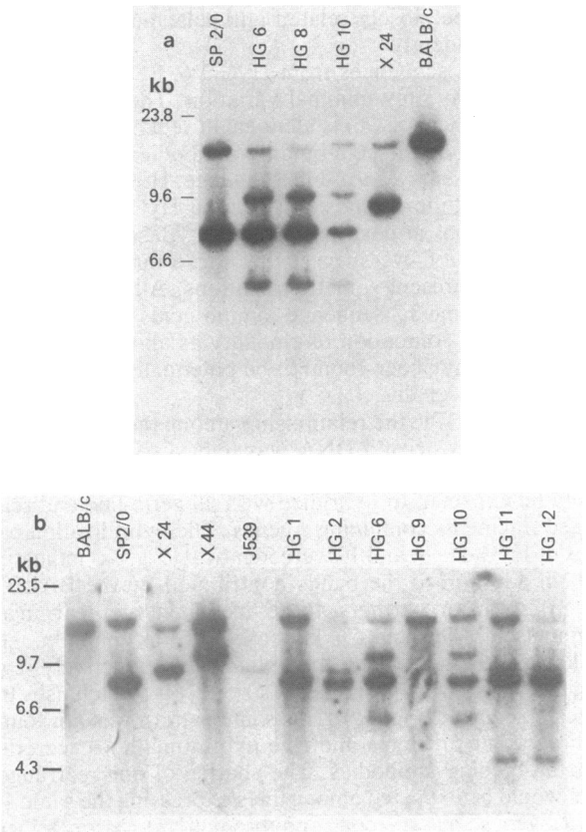

FIG. 3. (*a*) Southern blot analysis of hybridoma lines HyGal (HG) 6, 8, and 10 and myeloma X24. DNA was digested with *Bam*HI and hybridized with a 2.7-kb ^{32}P-labeled *Hin*dIII/*Hin*dIII fragment (5) containing all four J_K genes. (*b*) Southern blot analysis as in *a* including galactan-binding myelomas X24, X44, and J539 and hybridomas HyGal 1, 2, 6, 9, 10, 11, and 12.

X24 and HyGal 3 use different J_K segments (24), so that no two of these proteins meet the three criteria used in the present study. Similarly, myeloma proteins X44 and J539 differ by only one amino acid in CDR-3, yet use different J_K segments and have different rearrangement patterns (Fig. 3*b*). Only hybridoma proteins HyGal 11 and 12 appear identical by all three criteria in addition to sharing one V_H and one J_H amino acid substitution. These two lines arose from the same fusion and we have also proposed (26) that they are likely to be clonally related. Thus, only two sets of proteins (HyGal 6, 8, and 10 and HyGal 11 and 12), which in each case arose from a single fusion, meet the three requirements used to assess clonality. Other lines derived from the same fusion also fail to fulfill all criteria further distinguishing these two sets.

Ontogeny of Galactan-Binding Hybridomas. While none of the above arguments alone presents a compelling case for clonality, the various data taken together strongly suggest that this is indeed the case. Otherwise, by chance, a number of unrelated events would have had to occur coincidentally in three independent cell lines from the same fusion: (*i*) these lines would have had to use a *D* gene never before seen in a murine H chain, and two of these H chains would have incurred the same point mutation at position 91; (*ii*) these lines would have had to use the same J_K and J_H genes although there appears to be no preferential use of J segments in this system; (*iii*) these three lines would have had to generate the same nonproductive κ-chain rearrangement. Based on the relationships described above, which indicate a common origin, it is possible to construct a genealogical tree representing development of the line including hybridomas HyGal 6, 8, and 10 (Fig. 4). The original clone in this line is presumed

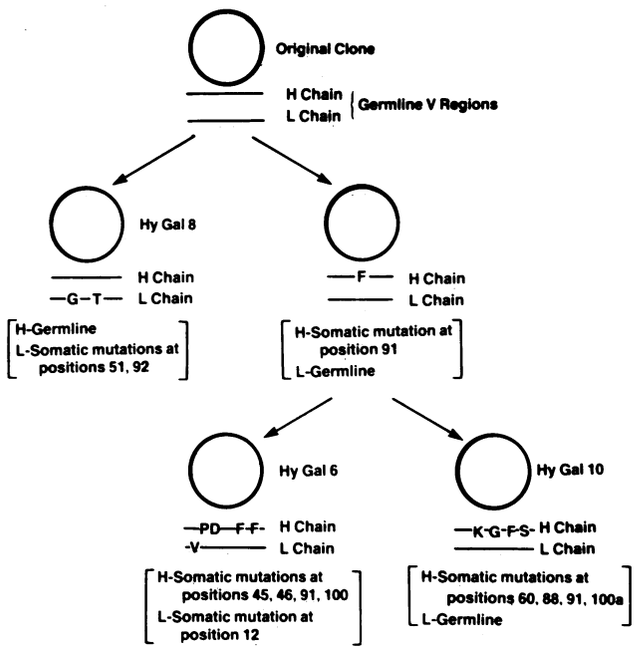

FIG. 4. Hypothetical genealogy describing the generation of hybridomas HyGal 6, 8, and 10.

to have expressed both germ-line L- and H-chain sequences. The HyGal 8 representative would have derived from this cell (or similar daughter cells) in that it displays two substitutions in the V_K segment and the H chain is germline. The threonine interchange at position 92 has also been found in the myeloma protein J539, although J539 has at least two additional substitutions and uses a different J_K segment (10). It thus appears as though this mutation has occurred independently two times in the anti-galactan proteins. An alternative explanation, which cannot be ruled out at present, is that these antibodies use at least two very similar V_K genes, which differ at a minimum by the amino acid encoded at position 92 (asparagine in one gene and threonine in the second). This possibility seems unlikely since the L-chain rearrangements in HyGal 8 are identical to those of HyGal 6 and 10 (Fig. 3). Because neither HyGal 6 nor 10 expresses the two V_K substitutions observed in HyGal 8, these proteins must originate from a subline of daughter cells derived from the original clone and expressing both germ-line L and H chains. This subline presumably acquired a mutation at position 91 in the H chain, representing the shared phenylalanine-91 found in HyGal 6 and 10. The remaining sequence in this cell would be germ line in both L and H chains. Two of the progeny from this cell then accumulated the additional mutations indicated, making HyGal 6 and 10 unique. Alternatively, if the phenylalanine-91 substitution was the result of a random event that had occurred twice independently, HyGal 6 and 10 may have been derived from the original clone or from separate daughter lines. All of the above proposed somatic mutations, with the exception of the L-chain substitution at position 51 in HyGal 8, can be generated by single-base nucleotide changes.

The lineage model presented in Fig. 4 makes no attempt to define the number of cell divisions occurring between any two progeny. For example, the unique mutations arising in HyGal 6 and 10 may have been generated at a single point in time or may have accumulated over a number of generations. It is tempting to suggest that substitutions such as proline-45 and aspartic acid-46 in the HyGal 6 H chain may have arisen at the same time due to their proximity. If this were the case, the presumption would be that once a mutational event of this nature has occurred, the probability is in-

creased that additional such events will occur proximally in the same time frame. However, the glycine-88 and phenylalanine-91 substitutions in HyGal 10 are also proximal and, according to the scheme presented, would have occurred in two different cells. The clustering of somatic mutations has been suggested in both L (36) and H (37) chains from phosphocholine-binding antibodies although additional data are required to substantiate this interpretation. Alternatively, the positions of the putative mutations in the anti-galactan proteins may result entirely from chance distribution.

Somatic Mutation. Analysis of a series of hybridomas originating from a single clone has potentially significant advantages over other types of studies addressing somatic mutation in immunoglobulins. In many instances somatic mutations have been defined by a comparison of hybridoma and myeloma sequences to germ-line genes from the same strain of mouse.

A point, which may not be trivial, is that in such cases these structures have been derived from animals representing different mouse colonies and possibly different sublines of the same strain. Thus, the potential does exist for genetic polymorphism to be interpreted as somatic mutation. This subject has been raised, and similarly addressed, by Weigert and colleagues (38) in studies of hybridomas reacting with determinants on the influenza hemagglutinin. Considering this potential problem of polymorphism, the most rigorous demonstration of somatic mutation has been provided by Scharff and co-workers (22, 23) who have identified single amino acid substitutions arising in cloned lines *in vitro*.

Based on the analysis of the three galactan-binding hybridoma proteins in the present study, a number of points can be made concerning the nature of somatic mutations. These conclusions are based on the observed amino acid substitutions and do not include potential silent mutations not detected at the protein level. First, these mutations occur in IgM cells, and thus the mutational process is activated early in the development of the B-cell lineage. We have previously postulated that the difficulty in detecting such mutations may be related to the fact that IgM-producing cells are thought not to be derived from a memory population and are thus turned over so rapidly that there is little probability of "fixing" by the hybridoma process (24) an IgM cell that has incurred somatic mutations. If somatic mutations accumulate with time, as reflected by cell division or cell longevity, the probability of detecting such events is much higher in cells expressing other immunoglobulin classes, because these cells are derived from a memory population and have probably undergone considerably more cell divisions as well as having "lived" longer. However, the actual mutation and repair process need not, *per se*, require cell division—i.e., scheduled DNA synthesis. It is noteworthy that the three hybridoma lines in our series, HyGal 6, 8, and 10, were generated after two immunizations with galactan-containing antigens. The second immunization may have served to "push" IgM cells, which would normally turn over, into a new series of divisions increasing both the probability of detecting somatic mutations and of fixing such cells by the hybridoma process. In mice receiving only a single immunization, we have characterized four hybridoma proteins expressing germ-line sequences in both V_H and V_K segments and one protein that displays a germ-line V_K segment and possibly a single mutation in the V_H region (26).

Second, the somatic mutation process is continuous over many cell generations, as indicated in Fig. 4. Data from the influenza system (38) indicate that a similar progression of mutations is found in hybridomas that have successively switched classes, indicating that once the mechanism is activated it may persist throughout the ontogeny of a given line. However, these results do not imply that the mechanism is activated in all B-cell lines.

Third, the same mutational mechanism appears to operate at both the H- and L-chain loci as evidenced by the accumulation of substitutions in both chains, as has been seen in other studies (19). Interestingly, it has been suggested that in both H (37) and L (36) chains somatic mutations appear to radiate from the points of V–J joining—i.e., the number of mutations decreases as the distance increases from the site of recombination. Based on the current analysis, the occurrence of point mutations is suggested to be continuous and the positions are likely to be random. Therefore, the basis for such a gradient is not obvious. However, immunoglobulins are the only genes analyzed to date that undergo patterned rearrangements to produce functional transcripts. Although completely speculative, it would be intriguing if these rearrangements created a DNA structure that subsequently focused a somatic mutational mechanism, thus assuring that a high number of mutations would occur in immunoglobulin V regions concomitantly expanding the diversity of the system. An alternative explanation is that there exist nucleotide sequences near the immunoglobulin constant region loci that either promote or focus somatic mutational mechanisms (38).

Fourth, since all three of the galactan-binding hybridomas are of the IgM class, somatic mutation in this system is not associated with class switching as has been previously suggested (19, 20). The present data provide structural evidence for the occurrence of somatic mutation in the absence of class switching, as has been inferred from serological analysis (39). In addition, a single substitution has been reported in an IgM anti-phosphocholine hybridoma protein (40) that is not encoded in the corresponding germ-line gene (21) and may also represent a somatic point mutation. The pattern of mutation observed in the IgM proteins is consistent with that found in a cloned IgA cell line *in vitro* (22, 23) and indicates that the *in vitro* system may be a valid reflection of the *in vivo* process.

An interesting but difficult question, which cannot be answered at this time, is whether the mutations in the anti-galactan hybridomas occurred *in vivo* or *in vitro*. After fusion, cells are incubated for 24–48 hr (during which time little, if any, division occurs) prior to distribution in microtiter wells containing selective medium. Since HyGal 6, 8, and 10 each arose from a separate microtiter well, the phenylalanine-91 substitution shared by HyGal 6 and 8 would have had to occur prior to this time. After growth in selective medium (10–14 days), wells are tested for antigen binding and positive colonies are immediately cloned. These clones are grown a second time, retested, and cloned again. Thus, mutations other than phenylalanine-91 may have occurred at any point prior to the second cloning. An observation relative to this question is that HyGal 11 and 12, which meet all three criteria for clonality (and for which we have suggested such a relationship), have single identical substitutions in both V_H and J_H regions (26) when compared to prototype anti-galactan sequences. Thus, these two hybridomas, which express identical H- and L-chain V-region sequences, have not accumulated additional coding changes during the fusion and cloning process, suggesting that many of the mutations in HyGal 6, 8, and 10 may have occurred prior to fusion. It should be noted, however, that point mutations clearly can occur *in vitro* (22, 23), although it is not presently known whether the mutation rate is high enough to account for the number of substitutions observed in the present study.

While we are now beginning to dissect somatic mutational events, it is still unclear what the role of this process is in generating functional diversity (altering antigen-binding sites) as opposed to structural diversity (any change in protein sequence). Clearly, in this system, as well as in antibodies to phosphocholine (19, 41), the observed somatic mutations do not appear to significantly alter antigen-binding

specificity. It can be argued, however, that because these systems are positively selected by antigen, any somatic mutations that do alter specificity will be undetected, as these molecules are never identified. It is only in negatively selected systems, in which selection is based on loss or decrease in antigen binding (22, 23) or alteration in V-region antigenic structures (42), that such molecules would be defined. Two such examples have been reported for V-region somatic mutations effecting antigen binding (22, 23). Thus, one of the major biologically relevant questions of somatic mutation still unanswered is the role of this process in generating functional diversity and its contribution to the total antibody repertoire.

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