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Somatic mutation and clonal expansion of B cells in an antigen-driven immune response

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The variable (V) regions of three closely related monoclonal antibodies produced by hybridomas which had been isolated from a single mouse were sequenced at the level of the mRNA. The sequences and the restriction analysis of the immunoglobulin loci carried by the hybridoma cells indicate that the antibodies are derived from cells belonging to a single B cell clone. The sequence data imply a high frequency and stepwise occurrence of somatic point mutations in the expressed V region genes and substantial clonal expansion of B cells in the mouse. The mutations appear to be randomly introduced into heavy and light chain V region genes. Mutations are also seen in the complementarity determining regions which may thus have been involved in the selection of the cells producing the three antibodies.

Key words: V gene sequences / somatic antibody diversification/frequency of somatic mutations/clonal B cell expansion

Introduction

McKean *et al.* (1984) and Rudikoff *et al.* (1984) have sequenced variable regions of antibodies produced by different members of B lymphocyte clones activated by antigen *in vivo*. Their results indicate that somatic mutation of rearranged V region genes is an ongoing process in activated B cells in the course of which point mutations are introduced over many generations of clonal growth at a frequency close to 1×10^{-3}/bp/generation. Because of the profound implications of this result for the clonal expansion and selection of cells in the immune system we consider it appropriate to present here an additional analysis of the same type, in which we determine the nucleotide rather than the amino acid sequences of the entire V regions of three antibodies derived from a single B cell clone.

The main difficulty in identifying progeny of a single B cell clone in the immune response of an animal lies in the polyclonal nature of antibody responses. In the study of McKean *et al.* (1984) a serological marker of a certain group of light chain V regions was used as a clonal marker. In the present experiments we exploited the fact that antibody responses against antibodies of the animal's own repertoire (so-called isogeneic anti-idiotypic responses) are often weak and irregular, suggesting a limited number of appropriate precursor B cells.

This expectation is verified by the results presented below. Three out of four monoclonal anti-idiotypic antibodies isolated from an immunized mouse apparently originate from a single B cell precursor, but differ from each other by somatic point mutations. The fourth antibody has a different clonal origin (unpublished data). A genealogical tree can be constructed on the basis of the sequence data, indicating stepwise mutation and a mutation frequency identical to the one calculated by McKean *et al.* (1984).

Results

Hybridomas

The current investigation involves the sequence analysis of three anti-idiotope antibodies derived from a C57BL/6 mouse immunized three times with the Fab-fragment of the monoclonal anti-NP antibody S43 (Reth *et al.*, 1978) coupled to keyhole limpet hemocyanin (KLH). The nucleotide sequences of the variable region genes of two of these anti-idiotope antibodies (A20/44 and A8/4; both IgG1, κ) have been previously reported (Sablitzky and Rajewsky, 1984) and appeared to have diverged from the same B cell precursor. The nucleotide sequence of the third one (A2/69, IgG1, κ) described here, enables us to accommodate all three anti-idiotope antibodies into a genealogical tree.

Sequence comparisons

The developmental relationship of the anti-idiotope cell lines A2/69, A20/44 and A8/4 is inferred from the nucleotide sequence of the variable gene segments which they express (Figures 1 and 2). The three cell lines express strongly homologous V_H, V_κ and J_κ and the same D and J_H gene segments.

With respect to the heavy chain V region genes (Figure 1), all three cell lines have productively rearranged J_H3 but carry a point mutation at position 105, resulting in the substitution of the amino acid alanine by proline. This point mutation can be identified since the nucleotide sequence of the C57BL/6 J_H3 gene segment is known (Krawinkel *et al.*, 1983).

The D region sequences, encoding four amino acids, are identical in the three antibodies [two nucleotides of codon 100 remained unidentified (X), but the mRNA sequences look identical on the autoradiogram (data not shown)]. Comparing them with the known germ line D region sequences (Kurosawa and Tonegawa, 1982) one finds only five nucleotides (ATTAC at positions 100, 101 and 102) which could be of germ line origin. Thus, at least five of 12 nucleotides may be somatically derived N sequences, which are thought to be inserted in the joining process (Alt and Baltimore, 1982).

The V_H genes expressed in A2/69, A20/44 and A8/4 are strongly homologous and most likely derived from a previously identified germ line V_H gene, namely that expressed by the hybridoma cell line Ac38.205.12 (Dildrop *et al.*, 1984). A fourth, closely related V_H gene, expressed by yet another anti-idiotope cell line (A6/24), is probably also derived from the Ac38.205.12 V_H gene (Sablitzky and Rajewsky, 1984). In Figure 1 the consensus sequence of all five V_H genes is taken as a reference sequence and considered to be carried in the germ line. The V_H genes expressed in antibodies A2/69 and A20/44 differ from each other by only one nucleotide at posi-

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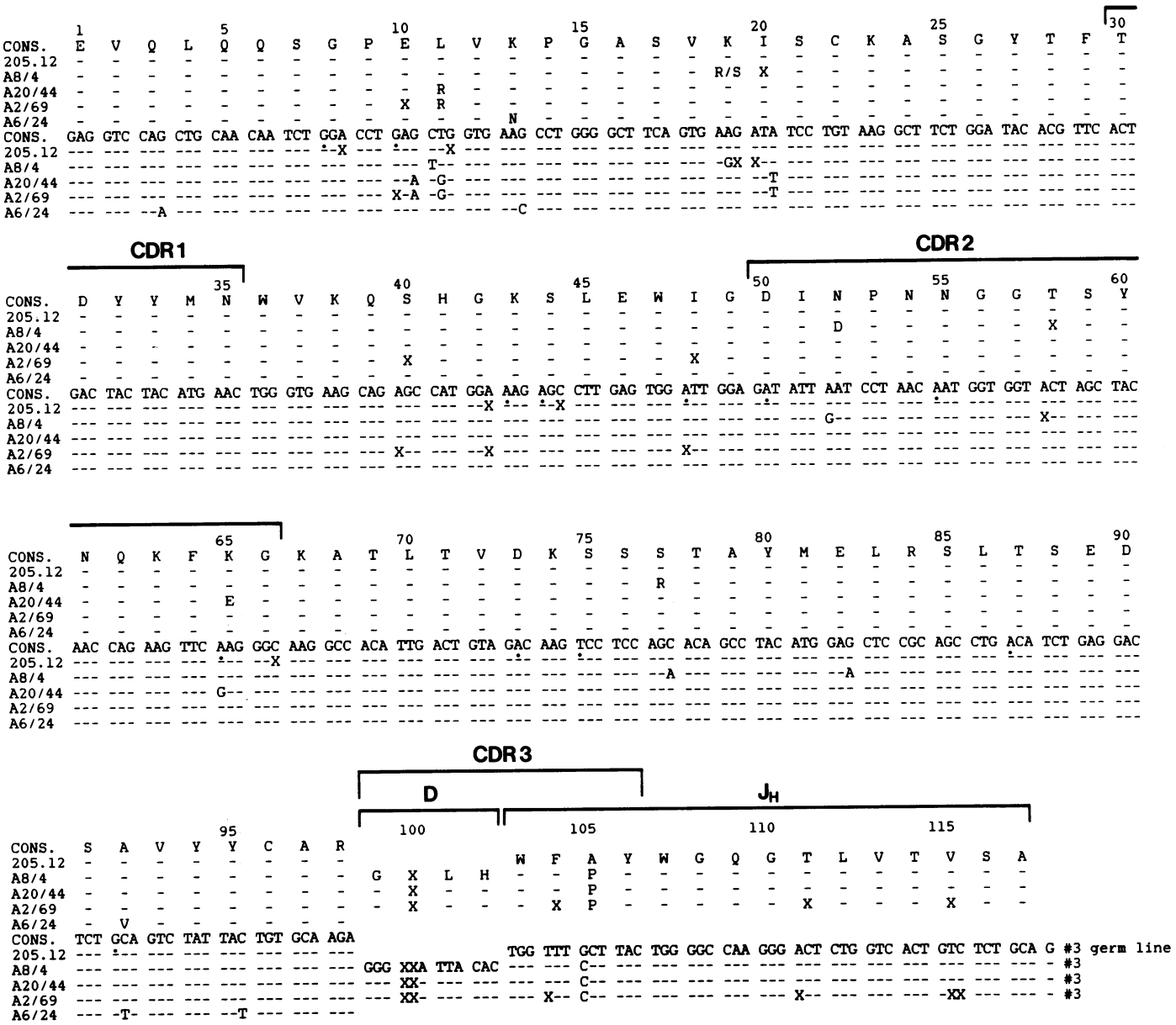

Fig. 1. Sequences of the V_H, D and J_H gene segments expressed in the anti-idiotope antibodies A8/4, A20/44 (Sablitzky and Rajewsky, 1984) and A2/69 are compared with the sequences of the V_H genes expressed by the hybridoma cell lines Ac38.205.12 (designated as 205.12) (Dildrop *et al.*, 1984) and A6/24 (Sablitzky and Rajewsky, 1984). The consensus sequence of all five V_H genes and the germ line sequence of J_H3 (Krawinkel *et al.*, 1983) is taken as a reference sequence. The same amino acid and/or nucleotides are indicated with dashes. X indicates ambiguities in the mRNA sequences. ∴ indicates ambiguities in the mRNA sequence of 205.12, which could be deduced of the previously determined amino acid sequence (Dildrop *et al.*, 1984).

tion 65. This leads to the replacement of the germ line encoded amino acid lysine (A2/69) by glutamic acid (A20/44). Both V_H gene sequences share the same nucleotide differences compared with the consensus sequence at positions 10, 11 and 20, resulting in an amino acid exchange at position 11 (leucine *versus* arginine). Comparison of the V_H gene A8/4 to the consensus sequence indicates five nucleotide substitutions (positions 11, 19, 52, 77 and 82), three of which (positions 19, 52 and 77) lead to amino acid replacements.

The sequences of the light chain V region genes expressed in the anti-idiotope antibodies are shown in Figure 2. For comparison, the V_χ gene expressed by yet another anti-idiotope cell line, As79 (IgG2b, $\varkappa$; Reth *et al.*, 1979) is also shown. The As79 V_χ gene is closely related to the V_χ genes expressed by the other anti-idiotope cell lines and, presumably, all four genes derive from the same V_χ gene in the germ line. Consequently, a consensus sequence is given in Figure 2 and considered to represent the V_χ gene which was rearranged to $J_\chi5$ in the precursor cell of the anti-idiotope cell lines A2/69, A20/44 and A8/4. The V_χ genes expressed in antibodies A2/69 and A20/44 are identical except for one nucleotide at position 49 where A2/69 encodes asparagine and A20/44 lysine. Both V_χ genes share replacement mutations at positions 74 (serine *versus* isoleucine) and 77 (serine *versus* asparagine). A common point mutation in $J_\chi5$ at position 99 results in the replacement of the germ line encoded amino acid glycine by alanine. An additional silent point mutation is present in the $J_\chi5$ gene segment expressed in antibody A2/69, at position 97. The $J_\chi5$ gene segment is also expressed in antibody A8/4, but is identical to its germ line counterpart (Sakano *et al.*, 1979). The nucleotide sequence of the V_χ gene A8/4 differs at four positions from the consensus

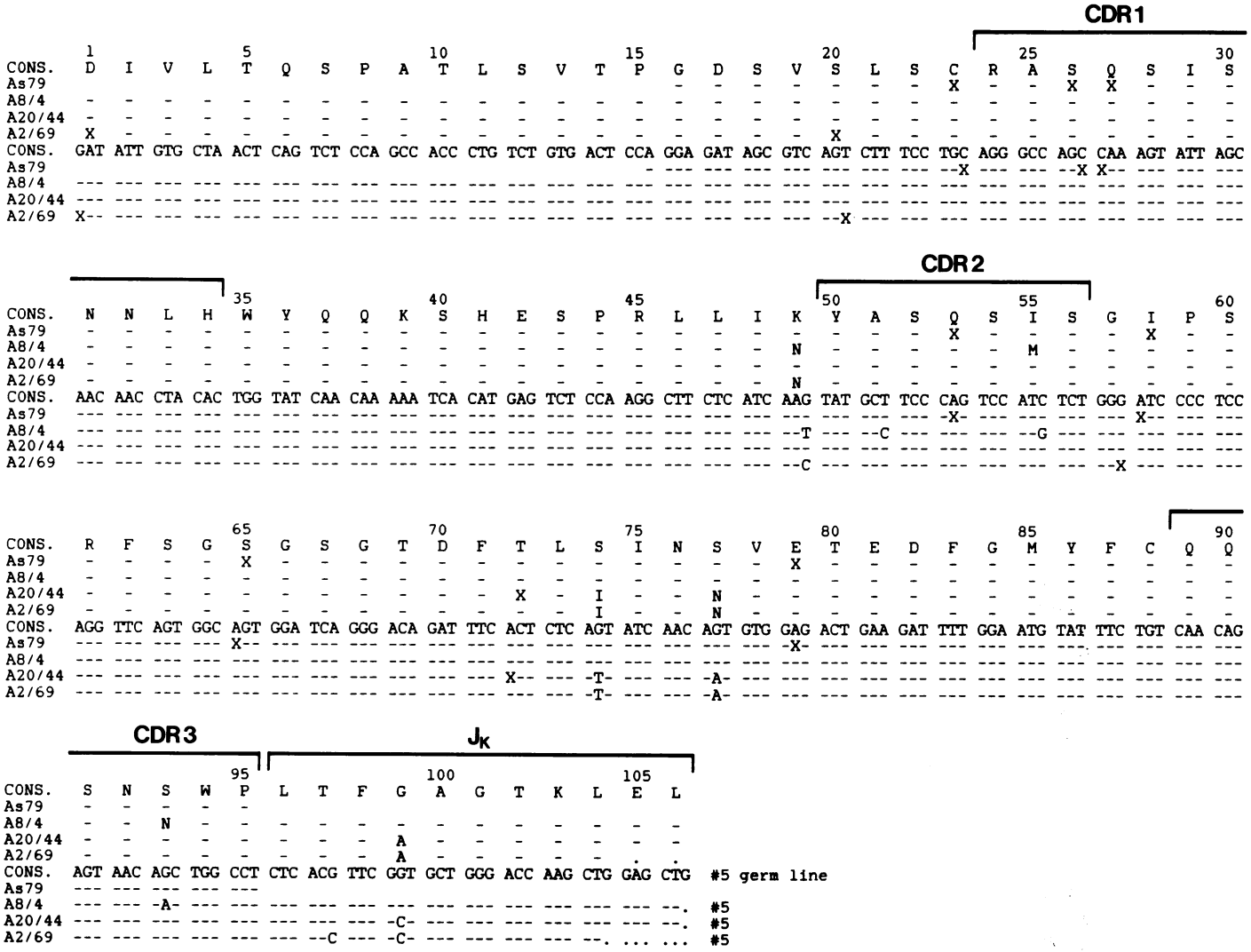

Fig. 2. The nucleotide as well as deduced amino acid sequences of the V_χ and J_χ gene segments expressed by the anti-idiotope cell lines A8/4, A20/44 and A2/69 are compared with the sequence of the V_χ gene expressed in another anti-idiotope antibody As79 (Reth *et al.*, 1979). The consensus sequence of the four V_χ genes and the germ line sequence of $J_{\chi 5}$ (Sakano *et al.*, 1979) is taken as a reference sequence.

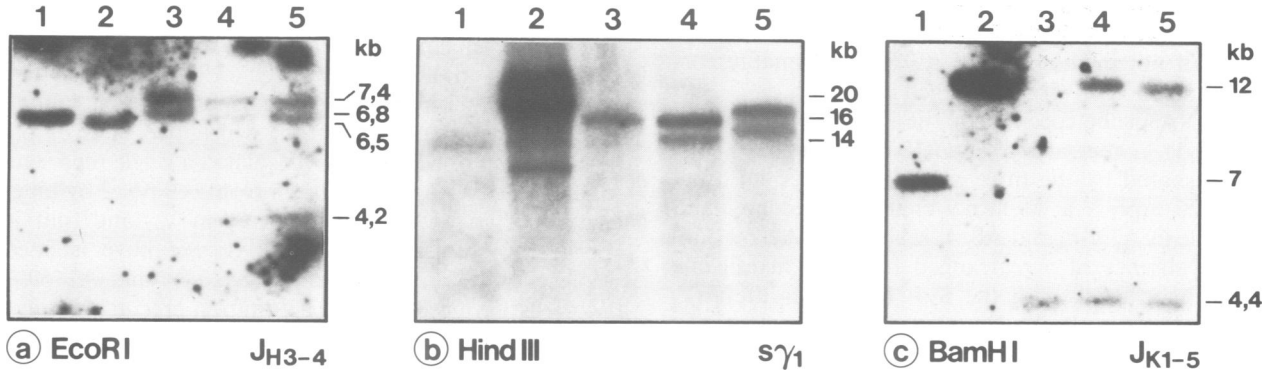

Fig. 3. Southern blot analysis of the anti-idiotope cell lines A2/69 (3), A20/44 (4) and A8/4 (5), the fusion partner X63.Ag8.653 (1) and C57BL/6-liver (2). Genomic DNA was either digested with *Eco*RI and hybridized with a J_H specific probe ($J_{H3,4}$ probe; kindly provided by M. Boersch-Supan) **(a)**, or with *Hind*III and hybridized with a DNA probe specific for the switch region 5′ of the Cγ1 genes (Sγ1 probe; Shimizu *et al.*, 1982) **(b)**, or with *Bam*HI and hybridized with a J_χ specific probe ($J_{\chi 1,5}$ probe, kindly provided by A. L. M. Bothwell) **(c)**.

sequence. Three of these nucleotide differences result in amino acid substitutions, namely at positions 49 (asparagine *versus* lysine), 55 (methionine *versus* isoleucine), and 93 (aspargine *versus* serine).

V gene rearrangements studied by Southern blot analysis

To confirm the developmental relationship between the anti-idiotope cell line A2/69, A20/44 and A8/4, genomic DNA

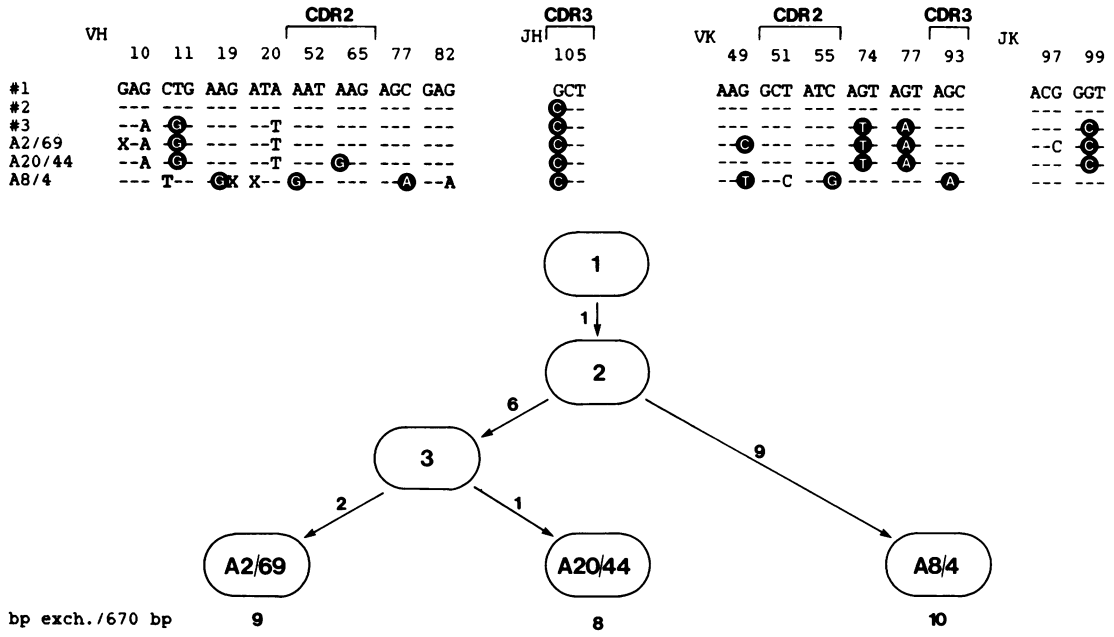

Fig. 4. One possible genealogical tree describing the clonal relationship between the anti-idiotope cell lines A2/69, A20/44 and A8/4. At the top only those codons of the nucleotide sequences are shown, in which the variable region genes expressed in the anti-idiotope antibodies differ from each other. Replacement substitutions are displayed. The generation of the anti-idiotope cell lines of a single B cell clone by accumulating somatic point mutations is shown in the lower part. Numbers next to the arrows indicate the amount of somatic mutations.

was isolated and specific probes, homologous to part of the J_H region (J_{H3-4} probe; kindly provided by M. Boersch-Supan), to the switch region 5′ to the Cγ1 gene ($S_{\gamma 1}$ probe; Shimizu *et al.*, 1982) and to the J_χ region ($J_{\chi 1\text{-}5}$; kindly provided by A. L. M. Bothwell) were used to detect rearrangements of the IgH and IgK loci by the Southern blot technique (Southern, 1975; Wahl *et al.*, 1980). As shown in Figure 3, similar rearrangements of the genomic DNA are found in the three anti-idiotope cell lines. Two *Eco*RI fragments (7.4 and 6.8 kb) hybridizing with the J_{H3-4} probe are seen in the case of all three anti-idiotope cell lines. The 6.8-kb fragment is derived from the fusion partner X63.Ag8.653. Since the 7.4-kb fragment is present in all three anti-idiotope cell lines, it must represent the actively rearranged J_H-region. A third fragment (4.2 kb), only present in the anti-idiotope cell lines A20/44 and A8/4, but not in the cell line A2/69, presumably corresponds to the non-actively rearranged J_H region.

Using a DNA probe specific for the switch region of the Cγ1 gene (Sγ1), a rearranged *Hind*III fragment (16 kb) is found to be shared by the three anti-idiotope cell lines (all three cell lines express a γ1 heavy chain). A second band (14 kb), visible in A20/44 and A8/4, is absent in A2/69 (Figure 3b). The absence of a rearranged fragment in cell line A2/69 after hybridizing with the Sγ1- as well as the J_{H3-4} probe most likely reflects the loss of the non-active chromosome no. 12 in this hybridoma.

Hybridization of *Bam*HI digested genomic DNA of the anti-idiotope cell lines to a J_χ specific probe ($J_{\chi 1\text{-}5}$) is shown in Figure 3c. One fragment (4.4 kb) representing the actively rearranged locus, is present in all three anti-idiotope cell lines. A second fragment (12 kb) is only present in the cell lines A20/44 and A8/4, but not in A2/69. The loss of the 12-kb fragment in the hybridoma cell line A2/69 could again be due to chromosome loss.

Discussion

Clonal relationship between the anti-idiotope cell lines

The A2/69, A20/44 and A8/4 cell lines express strongly homologous or identical V_H, D, J_H, V_χ and J_χ gene segments. In the case of J_H all three lines and in that of J_χ two of them express the same somatic point mutation. The restriction analysis demonstrates indistinguishable rearrangements of heavy and light chain loci on both active and non-active chromosomes, as far as the latter are still present in the hybridoma cells. Collectively these results indicate that the cells expressing the three anti-idiotope antibodies are derived from a single precursor B cell in the immunized mouse.

Accordingly, a genealogical tree can be constructed delineating the simplest clonal relationship between the three anti-idiotope producing cells (Figure 4). In this picture, the clone originates from a precursor B cell (cell no. 1) which expresses presumable germ line V region genes. A point mutation in J_H3 results in cell no. 2 from which two lines of progeny derive: from one line we have isolated a single representative, namely the cell A8/4. It differs from cell no. 2 by nine point mutations, five of which occurred in V_H and four in V_χ. From the second line of progeny we have isolated two representatives, namely the cells A20/44 and A2/69. These two cells have cell no. 3 as a common precursor from which they differ by one (V_H) and two point mutations (in V_χ and J_χ), respectively. Cell no. 3 is derived from cell no. 2 by three point mutations in V_H, two in V_χ and one in J_χ.

Clonal expansion of antibody producing cells

The data in Figure 4 and those of McKean *et al.* (1984) and Rudikoff *et al.* (1984) provide genetic evidence for the clonal expansion of antibody producing cells. Previous estimates of clone sizes were obtained from experiments in which B cells expanded in irradiated hosts. Under these conditions clone

sizes ranged from 2×10^4 to 5×10^4 (Bosma and Weiler, 1970), and in serial transfers a B cell clone could go through some 90 rounds of division (Williamson and Askonas, 1972). Our analysis of somatic mutation indicates a similar, substantial clonal expansion in unirradiated animals. The clone depicted in Figure 4 has in all likelihood undergone a minimum of some 17 divisions (see below) which would formally result in ~130 000 cells in the clone. We have picked three members of the clone by cell fusion. If we assume that every thousandth to tenthousandth activated B cell is successfully fused then the number of activated B cells of the clone, present in the spleen at the time of cell fusion, must have ranged between 3000 and 30 000. [We usually obtain roughly one hybridoma per 10^5 input spleen cells. However, antigen-activated B cells are known to fuse preferentially to the myeloma partner and are overrepresented in the fused population (Köhler and Milstein, 1975)]. This is only a fraction of the calculated total membership of the clone. In an attempt to explain this discrepancy one might think that the remaining cells had homed to lymphoid compartments other than the spleen or were not in an activated state at the point of fusion, which would reduce fusion efficiency. We might also simply have lost hybrids during the cloning procedures (see Materials and methods). However, an additional explanation of the discrepancy between the calculated clone size and the number of hybrids isolated comes from the analysis of somatic mutation itself. The frequency of somatic mutation is so high that the size of the clone is constantly reduced because of the generation of somatic mutants which either lost antigen binding, or express antibodies with reduced affinity for antigen and therefore not participating in further clonal expansion (see below).

Somatic diversification

From Figure 4 we draw the following conclusions concerning somatic diversification of antibody producing cells in the mouse: somatic mutations occur in a stepwise manner and are spread over the entire V region, including V_H, V_χ, J_H and J_χ. If we assume that we are dealing with point mutations only (and our data do not provide any indication for other types of mutation such as recombination which had previously been observed in a hybridoma cell *in vitro* (Dildrop *et al.*, 1982; Krawinkel *et al.*, 1983) then these mutations distribute almost equally between framework regions (13/19) and CDRs (6/19) which represent roughly 25% of the entire V region. The fraction of silent mutations is roughly 30% (6/19) and silent mutations are found both in CDRs (1/6) and frameworks (5/13). Thus, somatic mutations appear to be introduced in a random fashion.

In essence, the present analysis of clonally related V region genes confirms and extends the amino acid sequence data of Rudikoff *et al.* (1984) and McKean *et al.* (1984) except that the latter authors find a clear selection for amino acid substitutions in the CDRs. We do not think that this discrepancy represents a serious conflict. McKean *et al.* interpret their result as a demonstration of selection of cells by antigen. The same can be invoked in our case since we see replacement substitutions in CDRs. Significantly, the only mutation common to all three anti-idiotope antibodies is a replacement substitution in the CDR3 of the heavy chain (alanine to proline in position 105) which might dramatically affect antigen binding specificity. In addition, two independent (parallel) mutations in codon 49, close to the CDR2 of the V_χ genes expressed in antibodies A2/69 and A8/4 result in the replacement of the amino acid lysine by asparagine, again perhaps reflecting selection by antigen. The difference between our results and those of McKean *et al.* may thus only reflect differences in 'adaptability' (Manser *et al.*, 1985) of the particular binding sites operative in the two experimental systems.

Most importantly, our data confirm the high frequency of somatic mutation deduced by McKean *et al.* (1984) from their sequence data. If we assume as they do, that in our experiment somatic mutation was set in motion at the time of immunization, by stimulating a recently born B cell into proliferation, and that somatic mutation occurs at a constant rate in the expanding clone, then we calculate a mutation frequency of $7 \times 10^{-4} - 9 \times 10^{-4}$/bp/generation, allowing 17 generations within the 17 days between priming and cell fusion (Figure 4). This frequency is in perfect accord with the one calculated by McKean *et al.* (1984). It corresponds to roughly one mutation per cell division and implies, within an antigen-driven antibody response, the generation of substantial numbers of B cells either producing antibodies with a different antigen binding specificity or producing no functional antibody molecules at all. The fate of the former cells is of particular interest for those studying the diversification of the antibody repertoire in ontogeny. The existence of the latter mutants sets limits to the mutation frequency. A significant fraction of the point mutations must be expected to produce non-functional antibody molecules, so that increasing the mutation rate beyond one point mutation per cell division would soon lead to the collapse of the system. Thus, a mutation rate of 1×10^{-3} is close to the acceptable maximum, if the rate is constant. A higher rate could only be conceived if either mutation is non-random or the mechanism generating mutations operates only occasionally, in some cells of the proliferating clone.

The simple view outlined above sees somatic mutation essentially as a mechanism by which cells whose receptors bind the antigen with increased affinity are continuously generated and selected in an immune response. However, somatic mutation could also precede activation by antigen and seems indeed to occur in high frequency in an Abelson virus transformed pre-B cell line (Wabl *et al.*, 1985). If this were true in general, the clone depicted in Figure 4 could have undergone more cell divisions than we had assumed. Consequently, the mutation frequency would be lower, but, considering the age of the immunized mice in our experiment and that of McKean *et al.* (1984), by less than a factor of 10. Thus, there can be no doubt that a special mechanism introducing somatic mutations in rearranged antibody V region genes operates in B cells and contributes to the generation of the antibody repertoire. Whether this mechanism operates in all B cells and at all steps of B cell differentiation remains to be established. It is also still unresolved, to which extent somatic B cell mutants are accumulated in the immune system during the life time of the organism. In this respect the mouse with a life time of 2–3 years may not be the ideal experimental animal.

Materials and methods

Anti-idiotope cell lines

The hybridoma cell lines A2/69, A20/44 and A8/4, secreting monoclonal anti-idiotope antibodies (all three IgG1, κ), were isolated from a single C57BL/6 mouse. This animal had been primed with 44 μg of the Fab-fragment of the monoclonal anti-NP antibody S43 (Reth *et al.*, 1978) cross-linked with keyhole limpet hemocyanin, in Freund's complete adjuvant. The

material was injected i.p. and s.c. A similar injection of the same amount of antigen was given 7 days later, this time in Freund's incomplete adjuvant. On day 14, the same antigen dose was injected i.v. Three days later, i.e., 17 days after priming, the spleen cells of the animal was fused with the myeloma line X63.Ag8.653 (Kearney *et al.*, 1979). Growth of hybrid cells was observed in 240 tissue culture wells. Antibodies binding to antibody S43 (detected with a monoclonal anti-κ chain antibody) were initially detected in the supernatants of 16 wells, but only four S43-binding hybridomas could be isolated as cloned lines. The monoclonal anti-idiotope antibody As79, of SJL origin and specific for antibody B1-8 (Reth *et al.*, 1978), has been described previously (Reth *et al.*, 1979).

mRNA sequencing

Poly(A)$^+$ RNA of the hybridoma cell lines was isolated and directly sequenced by using synthetic oligonucleotide primers specific for certain regions of the mRNA to initiate reverse transcriptase reaction (Sablitzky and Rajewsky, 1984). The cDNA synthesis was specifically stopped by adding dideoxynucleotides and the nucleotide sequence determined (Hamlyn *et al.*, 1978).

Southern blot analysis

Genomic DNA isolated from the hybridoma and myeloma cell lines and from C57BL/6-liver was analysed by the Southern hybridization technique (Southern, 1975; Wahl *et al.*, 1980) using the restriction enzymes and probes described in Figure 3. The $J_{H3.4}$ probe was kindly provided by M. Boersch-Supan, the $J_{\chi 1.5}$ probe by A. L. M. Bothwell and the Sγ1 probe by T. Honjo.

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