EVOLUTION OF ANTIBODY STRUCTURE
DURING THE IMMUNE RESPONSE
The Differentiative Potential of a Single B Lymphocyte

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How an animal develops a state of antigen-specific, humoral immunity during the course of a primary immune response is arguably one of the most long-standing questions in the field of immunology. The clonal selection hypothesis (1, 2) explains this phenomenon by assuming that immunity is elaborated by an antigen-selective mechanism that draws from a large preexisting repertoire of B lymphocytes, each of which is committed to the expression of a single antibody structure. Immunity then results from a quantitative increase in antigen-specific antibodies via proliferation of the appropriate B lymphocyte, induced by antigen binding to the antibody this B cell expresses as a cell surface receptor. It is now clear that these basic tenets of the clonal selection hypothesis are correct. However, investigations on the expression of antibody diversity by hybridoma populations isolated at various times during immune responses have revealed that the development of humoral immunity involves not only quantitative but also extensive qualitative changes in preexisting antibody structures. These changes result from two processes: somatic mutation of antibody heavy and light chain variable region (V) genes, causing alterations in the structure and function of antibody V domains (3-7); and isotype switch recombination, resulting in replacement of one class of antibody constant (C) region for another (for reviews see references 8, 9).

Such insights place a much greater level of importance on the antigen-dependent differentiative capacity of a single B cell than is suggested by the original clonal selection hypothesis. Consequently, they have necessitated a reexamination of the validity of this hypothesis. We have previously suggested (10) that V gene mutation occurs as a function of cell division and concurrently with antigen selection for an extended period. Given these assumptions, V gene mutation can be easily incorporated into a “neo-Darwinian” form of the clonal selection theory that invokes a multistep process of mutation and cellular selection. Initial support for this model came from studies of the expression of antibody diversity among sets of hybridomas that were derived from many different B cell clones (3-5). While such analyses do provide insight into the differentiative and selective events that occur within the entire responding B cell population during an immune response, they suffer from a lack of resolution regarding the mechanistic nature of these events. Analyses of hybrid-
omas derived from individual clones of B cells (11–14) are more informative in this regard since they reveal not only the repertoire of antibody structures expressed by the clone at the time of immortalization, but also provide mechanistic insights into the ontogeny of this repertoire, since all of these structures share a common progenitor that was expressed by a single cell. Here I report the results of such an analysis on 26 hybridomas derived from two clones of B cells at an intermediate stage of the immune response of a single mouse.

Materials and Methods

**Immunizations and Hybridoma Formation and Screening.** Female A/J mice originally obtained from the Jackson Laboratories (Bar Harbor, ME) were immunized intraperitoneally at 8–10 wk of age with 100 µg IArs-KLH in CFA. The response of A/J mice to this hapten is similar to the response to p-azophenylarsonate (Ars) (15) in that a major idiotypic family of antibodies (IdCR) is reproducibly elicited (Fleming, M., S. Fish, J. Sharon, and T. Manser, manuscript submitted for publication). 1 wk after initial immunization the mice were immunized intraperitoneally three times in succession at 2-d intervals with 50 µg IArs-KLH in PBS. 3 d after the final immunization (16 d after the initial immunization) the mice were killed and their spleens were taken as a source of B cells for hybridoma formation. Hybridomas were generated and initially screened for expression of mRNA encoded by the VHIdcR gene segment as previously described (16). Hybridization-positive cell lines were then screened for production of antibodies reactive with the monoclonal antidiotype antibody E4 using solid-phase competition radioimmunoassays and 36-65 as the labeled ligand. We (17; Manser, T., unpublished observations) and others (18) have previously shown that E4 is specific for A/J anti-Ars or anti-IArs antibodies that are encoded by a single combination of V gene segments that includes VHIdcR (termed the canonical combination). The heavy chain isotypes expressed by hybridomas were determined using an ELISA isotyping kit obtained from Boeringer Mannheim (Indianapolis, IN).

**Nucleotide Sequencing and Southern Blotting Analyses.** The sequences of the VH and VK genes expressed by hybridomas were determined via oligonucleotide-primed direct mRNA sequencing as previously described (19). For Southern blotting analysis, hybridoma DNAs were purified as described (20) and digested with either Eco RI or Bam HI (New England Biolabs, Beverly, MA) according to the manufacturer's instructions. The digests were electrophoresed on 0.8% agarose gels and transferred to nylon membranes. After baking and UV crosslinking the membranes were hybridized according to the method of Church and Kieffer-Higgin (21) with DNA probes that had been 32P-labeled via nick translation. For the analysis of isotype switch recombination a single blot was hybridized many times in succession, also according to the method of Church, with a variety of probes specific for the various “switch” DNA regions. The probes used were: a restriction fragment containing a 133-bp internal region of the VHIdcR gene segment (22); a Hind III fragment containing the entire BALB/c JH locus; a restriction fragment encompassing a region 3' of JH4 and just 3' of the IgH enhancer; J14B (23), a restriction fragment that lies between the IgH enhancer and the μ switch region of BALB/c (obtained from Drs. S. Tilley and B. Birsbein, Albert Einstein College of Medicine, Bronx, NY); and the plasmids py3/BgH2.5, py7/EH10.0, py2b/E6.6, and py2a-1 which contain restriction fragments that span the γ3, γ1, γ2b, and γ2a switch DNA regions and pe/XH1.5, containing an internal region of the Ce gene of BALB/c, respectively (24). All were obtained from Dr. W. Dunnick (University of Michigan Medical School, Ann Arbor, MI); py2a-1 was originally from the laboratory of Dr. K. Marcu (SUNY at Stony Brook, Stony Brook, NY).

**Measurement of Intrinsic Affinities.** A tyrosine conjugate of IArs was prepared as described (25). mAbs were purified from hybridoma culture supernatants via arsonate affinity chroma-

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1 *Abbreviations used in this paper:* IArs, p-azophenyl-meta-iodoarsonate; CDR, complementary determining region; KLH, keyhole limpet hemocyanin.
tography and intrinsic affinities of the purified antibodies for the IArs-tyrosine conjugate were then determined using fluorescence quenching essentially as described previously (26).

Data Analysis. Fluorescence quenching antibody affinity data were analyzed using an iterative curve fitting program obtained from Dr. J. Sharon, Boston University Medical Center (Boston, MA) and Dr. A. Sharon, Massachusetts Institute of Technology (Cambridge, MA). In the case of some very high affinity antibodies, assignment of a \( K_a \) was not possible due to the small differences between antibody and hapten concentrations and data point scatter at large quenching values. In comparison with other quenching curves that gave rise to measurable affinities, the curves derived from these antibodies were indicative of affinities of \( 10^8 \) liters/M or greater.

The generation of "cellular lineages of mutation" (see text) using \( V_H \) and \( V_\kappa \) sequence data was carried out by minimizing the number of "steps" in a phylogeny (in this application the number of parallel \( V \) region mutations and the number of assumed reversion events in the phylogeny are minimized). Only shared mutations that had been unequivocally identified (see legend to Fig. 1) were used in the analyses. It was assumed that each mutant form of a \( V \) region (\( V_H + V_\kappa \)) was representative of a cell that had existed at one time in the immune response.

Results

Hybridomas were generated from three A/J mice during an intermediate stage (day 16) of the immune response to \( p \)-azophenyl-meta-iodoarsonate (IArs)-KLH. The hybridomas were screened for both expression of a \( V_H \) gene segment (\( V_{HIdIR} \)) that encodes a major fraction of the anti-IArs antibodies in this response and the expression of an idiootope (E4) characteristic of a particular \( V \) region that is reproducibly expressed in this response (see Materials and Methods). More than 90% of the \( V_H^+ \) hybridomas were also E4+. To determine whether the E4-expressing hybridomas from these mice were derived from single B cell clones, six hybridomas were chosen at random from each mouse and the sequences of the \( V_H \) genes they express were partially determined via oligonucleotide-primed direct mRNA sequencing. Nucleotides are added by apparently template-independent mechanism at the junctions of \( V_H \) gene segments during joining events ("N" regions, reference 20). These joining events take place during the antigen independent stages of B cell differentiation (27-29). Among B cells expressing identical \( V \) gene segments "N" region sequence can therefore be used as an indication of whether such cells were derived from the same or different "naive" B cell precursors. On the basis of "N" region sequences at the \( V_H-D \) and \( D-J_H \) junctions of the sequenced \( V_H \) genes it was concluded that hybridomas isolated from only one of the mice were largely derived from a single clone of B cells (hybridomas from the other two mice appear to have been derived from four to five different clones; data not shown). Subsequent analyses were therefore concentrated on hybridomas from this mouse.

Nucleotide Sequences of the \( V_H \) and \( V_\kappa \) Genes Expressed by the Clonally Related Hybridomas. The \( V_H \) and \( V_\kappa \) genes expressed by the 26 hybridomas derived from this mouse were sequenced by direct mRNA sequencing. This analysis revealed that all of the hybridomas express the same set of five \( V \) gene segments, which we have previously termed the "canonical" combination of segments since it is reproducibly and predominantly expressed in the anti-\( p \)-azophenylarsonate response of A/J mice (5). The sequences are therefore presented in Figs. 1 and 2 in comparison to the sequences of the germline gene segments that make up the canonical combination. \( V_H-D \) and \( D-J_H \) "N" region sequence suggests that the 26 hybridomas were derived from two different clones (the \( V_\kappa-J_\kappa \) junction is invariant among all canonical combination
encoded V regions). One B cell appears to have given rise to 20 of the hybridomas and another to six. The sequences are not identical to the germline sequences due to the presence of numerous somatic mutations (in Figs. 1 and 2 only the V gene segment codons in which at least one mutation is observed are shown). Since hybridomas do not mutate their expressed V region genes at a high rate (30), the mutations observed in these genes must have occurred in vivo before hybridoma formation.

Genomic $J_H$ and $J_k$ Rearrangements in the Clonally Related Hybridomas. In an effort to independently confirm the clonal relatedness of the two groups of hybridomas, Southern blots were done to examine the configuration of unexpressed $J_H$ and $J_k$ loci. Since gene segment rearrangements occur during the antigen-independent stages of B cell differentiation, both productive and aberrant rearrangements should be shared by members of a clone. It has previously been demonstrated that B cell-derived aberrant $V_k$ to $J_k$ and $V_H$ to $D$-$J_H$ or $D$ to $J_H$ rearrangements can indeed be used as an indication of clonal relatedness among hybridomas (11, 13, 14). Fig. 3 shows the results of examination of the $J_H$ locus using Eco RI. These data corroborate the "N" region data by revealing that, in addition to giving rise to a band of a size expected for the productively rearranged allele ($H^+$) 19/20 of the hybridomas in the large group (labeled with asterisks in Fig. 3), and 5/6 in the small group of "N" region identity (labeled with arrows in Figure 3) give rise to the same size aberrant $J_H$ band ($H^-$).

Fig. 4 presents the results of an examination of $J_k$ rearrangements in DNA from the hybridomas using Bam HI. The results of this analysis further corroborate the clonal relatedness of the large group of "N" region identity; in addition to sharing a band of the size expected for the productively rearranged allele ($K^+$) 15/20 of its members (labeled as described above) share a common aberrant $J_k$ rearrangement ($K^-$). The five members of the large group that lack the band containing the aberrant rearrangement may have lost the chromosome containing this locus during hybridoma formation or growth.

Affinities for IArs of the Antibodies Produced by the Clonally Related Hybridomas. The intrinsic affinities of the antibodies expressed by both the large and small clonally related sets of hybridomas for an IArs-tyrosine conjugate were determined (in IArs-KLH the majority of hapten is conjugated to tyrosine). The data presented in Table I show that all of the antibodies but one (HIP-19) have affinities for IArs-tyrosine that are comparable to or higher than three antibodies that are encoded by the canonical combination of V gene segments in unmutated form (36-65, P65D6-5, and 1PIC: these three antibodies differ only in single amino acid residues at the $V_H$-$D$ and $D$-$J_H$ junctions). The $V_H$-$D$ and $D$-$J_H$ junctional amino acids in the V regions expressed by the large clonally related set are histidine and probably aspartic acid, respectively. One of the unmutated canonical antibodies (P65D6-5) has these same
The nucleotide sequences of VH and DJH gene segments are shown as are the CDRs. These sequences are organized such that as many of the somatic mutations that are shared among the members of each clone are grouped. Sequence is presented in triplet form and the amino acid codons are numbered sequentially from the mature NH2 terminus. "N" region nucleotides present at the VH-D and D-JH junctions that differ among canonical V region genes are indicated by an "N". The nucleotides present at these positions in the expressed genes are shown explicitly. Ambiguity in sequence at these positions is indicated by a colon. The first codon in the D region of canonical antibodies is an invariant TCX serine codon that is of unknown origin (48, 49).
Figure 2. Nucleotide sequences of the V<sub>e</sub> genes expressed by IA,<sub>v</sub>-induced hybridomas. Sequences are presented as described in the legend to Fig. 1 in comparison to the unmutated sequences of the V<sub>K</sub> and J<sub>K</sub> gene segments that encode canonical V regions (50). The single amino acid codon generated during the joining of these gene segments is indicated (junction). This codon is invariant among all V<sub>e</sub> genes that have been sequenced to date that partially encode canonical V regions.
FIGURE 3. JH region rearrangements in the HIP hybridomas. Most independently isolated hybridomas and myelomas contain aberrant Jk and JH rearrangements that reside on restriction fragments of differing MW (27, 42, 51). Therefore, identity in molecular weight of restriction fragments containing such rearrangements is a good indication of clonal relatedness. The probe used in this Southern blotting analysis is a plasmid containing an Xba I–Eco RI restriction fragment that encompasses a region of 700 bp beginning ~700 bp 3' of the JH4 gene segment and ending 3' of the IgH enhancer. Hybridoma DNAs were digested with Eco RI. In both panels A and B, lanes containing digests of A/J kidney (germinal) DNA and Sp2/0 (the fusion partner used for hybridoma formation) DNA are indicated by “G” and “S,” respectively. The number of each HIP hybridoma is indicated above each lane, as are the hybridomas belonging to the large (indicated by an asterisk) and small (indicated by an arrow) groups of N region identity. The lanes labeled A–D correspond to HIR-6, HIR-2, HIQ-6, and HIR-2, four IAr-induced canonical combination expressing hybridomas isolated from two other mice. In B the germine lane contains a band of low molecular weight that is an apparent artifact (the G lane in panel A does not contain this band nor do any other germine Eco RI digests that I have previously hybridized with the JH probe). The positions corresponding to productive rearrangement of the VαHIdCR gene segment to JH2 are indicated by “H”+. The band at this position migrates only slightly faster than the Sp2/0-derived band. In one cell line (HIP-1) the size of the restriction fragment containing the productive VDJ rearrangement is not consistent with the flanking restriction maps of VαHIdCR and JH2. The reason for this discrepancy is under investigation. Bands corresponding to aberrant JH4 rearrangements are indicated by “H”−. In one case in each group (HIP-8 and 20) this band is smaller than expected. The possibility that additional D to JH or VαH to DJH rearrangements may have occurred in these cell lines is currently under investigation. An Eco RI digest of HIP-5 DNA is not present in this analysis but on other blots gave rise to an H− band of a molecular weight identical to that of the HIP DNA digests in panel A (data not shown).

junctions and so exactly represents the hypothetical unmutated precursor of all of the antibodies expressed by the large set of hybridomas. Many of the antibodies have affinities that are at least 20-fold higher than the affinity of the unmutated canonical antibodies. Some of these affinities were too high to measure accurately and so are indicated by “>10k” (see Materials and Methods).

Isotype Switch Recombination in the Clonally Related Hybridomas. All of the members
FIGURE 4.JK region rearrangements in the HIP hybridomas. The probe used in the Southern blotting is a Hind III restriction fragment that encompasses a 2.7-kb region of the JK locus that contains all of the JK gene segments. This fragment contains only 70 bp of sequence upstream of JK1. Identical hybridization patterns were obtained with a probe specific for the intron region between J5 and Ck. Hybridoma DNAs were digested with Bam HI. The data are presented as described in the legend to Fig. 3 except that the positions of productive and aberrant JK rearrangements are indicated by K+ and K−, respectively. Lanes A–C correspond to HIR-4, HIR-2, and HIQQ2, two IArs-induced canonical V region–expressing hybridomas isolated from different mice than the HIP hybridomas. All putative members of the small group of N region identity lack an aberrant JK rearrangement. In one case in the large group (HIP-19) the size of the restriction fragment containing the productive V,Jk rearrangement is not consistent with the flanking restriction maps of the VklJk gene segment and the Jk locus. The reason for this discrepancy is under investigation.

of the two sets of hybridomas express one of the four different IgG isotypic classes of antibody (see legend to Fig. 5), indicating that their B cell precursors underwent isotype switching. To investigate the isotype switch DNA rearrangements that occurred in the clones that gave rise to the hybridomas, a Southern blotting analysis was conducted on hybridoma DNAs using Eco RI and a probe specific for a region between the IgH enhancer 3′ of the JH locus and the μ “switch” DNA repeats 5′ of the Cμ constant region gene. Fig. 5 shows that the majority of these hybridomas give rise to two rearranged bands with this probe, indicating that both B cell–derived IgH alleles have undergone isotype switch recombination involving the μ switch region. Since isotype switching happens only at extremely low frequencies in hybridomas (9), these rearrangements must have taken place within the two B cell clones before hybridoma formation. Bound probe was eluted from these blots and they were rehybridized sequentially with probes specific for the γ3 switch region, the γ1 switch region, the γ2a and γ2b switch regions and the ε coding region. In all but a few cases a band detected with the μ probe was also detected by a probe specific
TABLE I

Intrinsic Affinities of mAbs for IArs-Tyrosine

| Antibody   | Affinity for IArs-Tyr (K_a 1/M) |
|------------|---------------------------------|
| HIP-1      | >10^8                           |
| HIP-2      | 9.8 x 10^6                      |
| HIP-3      | 1.7 x 10^7 (2)                  |
| HIP-4      | >10^8                           |
| HIP-5      | 1.4 x 10^8                      |
| HIP-6      | >10^8                           |
| HIP-7      | 1.4 x 10^7                      |
| HIP-8      | 2.2 x 10^7                      |
| HIP-9      | 8.7 x 10^6                      |
| HIP-10     | 8.7 x 10^6                      |
| HIP-11     | 1.8 x 10^7                      |
| HIP-12     | 1.4 x 10^7                      |
| HIP-13     | 2.4 x 10^6                      |
| HIP-14     | 2.7 x 10^7                      |
| HIP-15     | 5.4 x 10^6                      |
| HIP-16     | 1.5 x 10^7                      |
| HIP-17     | 2.7 x 10^7                      |
| HIP-18     | 5.7 x 10^6                      |
| HIP-19     | 1.8 x 10^8                      |
| HIP-20     | 3.6 x 10^7                      |
| HIP-21     | 1.0 x 10^7                      |
| HIP-22     | 9.0 x 10^6                      |
| HIP-23     | 5.1 x 10^6                      |
| HIP-24     | 4.0 x 10^6                      |
| HIP-25     | 3.6 x 10^5                      |
| HIP-26     | 1.0 x 10^5                      |
| 36-65 (V,Y)| 1.0 x 10^6 (3)                  |
| P65D6-5 (H,D)* | 5.2 x 10^6                   |
| 1PIC (R,Y) | 7.8 x 10^6                      |

Antibodies were purified and affinities were determined as described in Materials and Methods. The affinities are listed as association constants next to the names of the hybridomas from which the antibodies were derived. In some cases affinities were determined more than once and the average value presented. These cases are indicated by a number in parentheses, corresponding to the number of measurements that were done, next to the affinity value obtained. High affinities that could not accurately be assigned a K_a value (see Materials and Methods) are indicated as ">10^8". The first group of antibodies and affinities are from the large group of clonally related hybridomas. The second group is from the small group of clonally related hybridomas. The third group of antibodies are all Ars-induced antibodies that have unmuted canonical V regions and differ only at single V_H-D and D_JH junctional amino acid positions. The amino acid residues present at these junctions are indicated next to the hybridoma name using the one letter amino acid code. The member of this group labeled with an asterisk represents the hypothetical unmutated precursor of all antibodies expressed by the large group of clonally related hybridomas due to apparent identity of all of its V_H and V_J junctional amino acids with the antibodies expressed by this group.

for the switch region DNA adjacent to the C region gene encoding the isotypic class of antibody expressed by the hybridoma, in agreement with the deletion model for isotype switch recombination (31).
The efficiency of antibody V region somatic evolution

**Figure 5.** μ Switch region rearrangements in the HIP hybridomas. The probe used in this Southern blot analysis is a 750-bp Hind III restriction fragment that encompasses a region beginning ~1.1 kb 3' of the Jμ4 gene segment and ending ~500 bp 5' of the μ switch tandem repeat region (J14B). Hybridoma DNAs were digested with Eco RI. Eco RI sites are present in germline DNA of the BALB/c mouse ~1.7 kb 5' of the μ switch repeat region and ~2.4 kb 3' of the Cμ coding region, and are separated by ~11 kb. The size of the germline band obtained with A/J kidney DNA (lanes G) is, however, >20 kb. Since the 5' Eco RI site has been shown to be present in the same location in A/J and BALB/c DNA (22) the reason for this allelic difference may be due to differences in the length of the μ switch region or to differences in sequence in the 3' flanking region of the Cμ gene. Since most isotype switch recombinations take place between the probe region and the μ coding region (23), and these regions are on the same germline Eco RI restriction fragment, an analysis of this sort using DNA from hybridomas derived from B cells that have isotype switched will reveal a rearranged band. The data are presented as described in the legend to Fig. 3 and the lanes are in an order identical to those in panels A and B in Fig. 3. The isotypic classes of antibody expressed by the hybridomas are as follows: IglG3: HIQ-6 and HIQ-14; HIQ-23 and HIP-2; IglG2b: HIP-18 and HIP-3; IglG2a: HIP-25 and HIP-19; all other hybridomas express IglG1.

**Discussion**

The B cell clones from which the hybridomas analyzed here were derived clearly underwent a multitude of distinct differentiation events that altered the structure and function of the single antibodies they initially expressed. Examination of these changes has furnished a sensitive measure of the potential of a single antigen naive B cell to generate novel antibody structures; the data demonstrate that this potential
is enormous. In addition, this analysis has provided further insight into the mechanism and timing of isotype switch recombination and V region hypermutation, the two processes known to be capable of altering the structure and function of antibodies during the course of an immune response.

**Relative Timing of Isotype Switch Recombination and Somatic Mutation.** The configuration of the IgH loci in members of the two hybridoma clones (Fig. 5) demonstrates that extensive and diverse isotype switch recombination took place during the proliferation and differentiation of their B cell precursors. Among members of the large clone all four of the IgG isotypes are expressed (see legend to Fig. 5) and most members have distinct switch configurations at both alleles (Fig. 5). The results of the analysis of two IgG2b-expressing hybridomas (HIP-3 and HIP-18) from the large clone also provide direct evidence that somatic mutation can occur after isotype switching. Fig. 6 shows that the cell lines give rise to identical hybridization patterns with all the C region switch DNA probes. This is unlikely to be coincidental due to the diversity of switch region configurations observed among the other members of the clone (Fig. 5). This suggests that these hybridomas represent two members of a subclone of B cells that was derived from a single isotype switched precursor. Since the V genes expressed by HIP-3 and HIP-18 differ by at least seven mutations, these mutations must have occurred after a stable switch configuration was established in the subclone. Moreover, since only these two hybridomas of the 26 isolated from both clones have identical switch rearrangement configurations, isotype switch recombination

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**Figure 6.** Identical isotype switch configurations in two of the clonally related hybridomas. Southern blots on DNAs from hybridomas HIP-3 and HIP-18 (two members of the large clone that express IgG2b) were done using Eco RI and hybridized sequentially with different switch region specific probes (see legend to Fig. 5 and Materials and Methods). In the above autoradiograms germline bands are indicated with a "G" and bands derived from the hybridoma fusion partner (Sp2/0) are indicated by an "S". The switch region probes used are indicated by the appropriate isotype name above each panel. The analysis of these data suggests that the aberrantly rearranged allele in these hybridomas has undergone isotype switching to γ1. No bands were detected after probing with a IgG3 switch region probe, indicating that both allelic copies of the γ3 region have been deleted in these hybridomas.
appears to have been an ongoing process at the time these clones were immortalized as hybridomas. Since none of the hybridomas from either clone express V genes that share all mutations (see below), somatic mutation also appears to have been an ongoing process at the time of B cell immortalization. Taken together with previous data demonstrating that V regions expressed by B cells that have not isotype switched (i.e., express IgM) sometimes contain mutations (reviewed in reference 32), the data indicate that isotype switching and somatic mutation are completely independent processes that may, but need not, occur concurrently during the expansion of a clone. A more detailed analysis of switch rearrangements that have occurred in these clones, and the implications of these data for the mechanism and regulation of isotype switch recombination will be the subject of a future report (Manser, T., manuscript in preparation).

Somatic Mutation and Antigen Selection. The sequences of the V_H and V_K genes expressed by the two clones reveal that these genes are somatically mutated (Figs. 1 and 2). V_H and V_K genes have been mutated to similar extents, an average of 1% of the nucleotide positions in each have suffered mutations (see Table II). All of the mutations observed are nucleotide replacements. All of the hybridomas express V_H and/or V_K genes that contain unique mutations, indicating that mutation was ongoing at the time the clones were immortalized as hybridomas. The mutational diversity collectively expressed by the members of the large clone is enormous (88 distinct mutations are observed). These data demonstrate that a single naive B cell is capable of generating a vast assortment of mutationally distinct offspring. Since we (16, 17) and others (3, 7, 33) have conducted experiments that strongly suggest that all somatic mutation occurs during the immune response, this diversity must have been generated in a short period of time (16 d or less), implying a very high rate of somatic mutation with respect to cell division.

Not all of the somatic mutations observed are unique, some are shared among several V genes (the sequences in Figs. 1 and 2 are grouped to emphasize such shared mutations). Weigert (11) and Rudikoff (12) and their colleagues initially suggested that such a pattern of mutation among V genes expressed by clonally related sets of hybridomas could be used to construct "cellular lineages of mutation" reflective of the temporal order of somatic mutation during the growth of the progenitor B cell clone. This approach led to the calculation of a minimum rate of mutation of \(10^{-3}/V\) region base pair/cell division (11). Such an analysis assumes that mutations are fixed in a growing clone by cell division and that the degree to which a mutation is shared is directly related to the time at which it occurred during the expansion of the clone, the most shared mutations having occurred earliest and the least shared having occurred latest. In the case of many of the shared mutations present among the V genes expressed by the two clones analyzed here this rationale cannot be easily applied. Such mutations are present in the V genes expressed by members of both clones and in the V genes expressed by other IArs induced hybridomas isolated from other mice that express V_HID^CR and V_KID^CR (data not shown).

The most extreme example of this type of mutation occurred at codon 59 in CDR2 of the V_HID^CR gene segment. In more than half of the V_H genes an A to T transversion took place in the first nucleotide position of this codon. Remarkably, this mutation alone converts codon 59 from encoding lysine to an amber termination codon. This mutation presents a paradox. It is the most shared mutation among
both groups of clonally related hybridomas yet creates a translation termination codon that might be expected to terminate the proliferative capacity of a cell that suffered it.

Of the many "cellular lineages of mutation" that can be generated from data from the large clone, all have very high numbers of assumed parallel (independently occurring in different sublineages) mutational events (>20). Fig. 7 shows the two most parsimonious lineages that could be constructed, in which parallel mutations and reversions are lowest. During the construction of each lineage different assumptions were made concerning how mutational events had occurred at VH position 59. Lineage A assumes that the A to T mutation in codon 59 (indicated by a double asterisk) alone confers a severe proliferative disadvantage (or death), but in combination with other mutations amino acid codons can be generated at position 59 that confer a proliferative advantage to the cell, perhaps due to the action of antigen selection. In support of this idea, none of the VH genes actually contain an amber codon since in all VH genes that have the position 59 A to T transversion an additional mutation is located within codon 59. Further, other types of mutational events have taken place at codon 59 (indicated by single asterisks), and a total of 22 of the 26 VH genes expressed by the two clones have suffered mutations leading to amino acid replacements at this position (Fig. 1). In addition, the mutant codons present at position 59 encode a small number of related amino acids, 14/23 encode tyrosine and 19/23 encode aromatic amino acids. Finally, preliminary experiments suggest that a lysine

![Figure 7](image-url)

**Figure 7.** Two "cellular lineages of mutation" generated from the sequences of the V genes expressed by the large group of clonally related hybridomas. The lineages were constructed as described in Materials and Methods. In each case, the B cell from which each HIP hybridoma was derived is indicated by the appropriate number and the hypothetical precursor B cell expressing an unmutated V domain is indicated by a cross-hatched "P". B cells that define branch points in the lineages, and are inferred to have existed before the generation of hybridomas, are indicated by a "B". The occurrence of the A to T mutation at VH position 59 (or this mutation plus another mutation in the same codon) is indicated by a double asterisk and other mutations in this position are indicated by a single asterisk. Mutations in this position that result in reversion of the position 59 amber mutation (see text) are indicated by an exclamation point (lineage B). The length of a branch in either lineage is not proportional to the number of mutations that define that branch.
to tyrosine change introduced at \( V_H \) position 59 by site-directed mutagenesis increases the affinity for IArs-tyrosine of an otherwise unmutated canonical \( V \) domain five-fold (Sharon, J., and T. Manser, manuscript in preparation). However, lineage A is not supported by the observation that in no case is a mutation that is observed together with the A to T mutation in codon 59 also observed in isolation, despite the fact that several of the \( V_H \) genes do not contain mutations at position 59.

Lineage B assumes that the A to T mutation occurred only once (again indicated by a double asterisk) and despite causing a termination codon, progeny were produced following this event that subsequently suffered additional mutations in codon 59 (indicated by exclamation points). Thus, the assumption is made in this case that a cell that has suffered a “lethal” mutational event does not die immediately, but continues to divide and give rise to new mutant progeny for an extended period. While this lineage avoids the assumption, made during the construction of lineage A, that a large number of parallel mutations have occurred at \( V_H \) position 59, the total number of parallel mutations in CDR regions that must be invoked to construct this lineage is comparable to the total number of such mutations in lineage A.

Both lineages are difficult to reconcile with the idea that random mutations accumulated over large numbers of cell divisions influenced by antigen selection during the expansion of the clone. Lineage A requires that \( V_H \) position 59 is an extreme example of a mutational “hotspot” (34, 35). In addition, unless it is assumed that most of the additional mutations at position 59 occurred simultaneously with the A to T mutation, it must be concluded that a large number of cells suffered this mutation alone, leading to their “mutational death” (36). Simultaneous “mutations” might occur via gene conversion, as has been demonstrated to take place during the primary diversification of the chicken \( V_\lambda \) repertoire (37). However, to account for the data, it must be assumed that at least three conversion donors exist that contain the A to T mutation (see Fig. 1). While gene conversions involving mouse \( V \) genes have been observed to occur at a low frequency in hybridomas (38) there is no direct evidence that such a process occurs at high frequency during the immune response (39, 40).

Lineage B assumes that a single B cell can “survive” a mutation that destroys its \( V \) region. Antigen selection is a hallmark of both B and T cell immune responses. This process requires the expression of a functional antigen receptor. If the expression of the receptor ceases in a cell, that cell becomes as “invisible” to the antigen as a cell that expressed a receptor that cannot bind the antigen. Lineage B, however, assumes that a single cell that suffers an amber mutation can continue to produce large numbers of new mutant progeny, despite being incapable of binding antigen. The continued propagation of a “deleterious” mutation via clonal expansion is in conflict with the notion that antigen driven clonal selection takes place throughout the course of the immune response.

A comprehensive examination of the distribution and type of mutations in the \( V \) genes expressed by both sets of hybridomas is presented in Table II. It can be seen that the frequency of mutations observed in \( V_H \) framework regions is 10-fold lower than in CDRs. A process of random mutation and antigen selection occurring over long periods of cell division could yield such a distribution of mutations if many framework mutations destroy antibody function, i.e., lead to “mutational death.” However, this assumption also implies that while mutations that cause amino acid replace-
TABLE II

Distribution and Type of Somatic Mutations Present in the VH and VK Genes Expressed by Clonally Related Hybridomas

|      | Expected R/S ratio | Observed R/S ratio | Total mutations | Frequency of mutation |
|------|--------------------|--------------------|-----------------|-----------------------|
| VH   |                    |                    |                 |                       |
| F1   | 3.7                | NA                 | 2               | 0.2%                  |
| CDR1 | 3.5                | 3.3, 5.0           | 13, 10          | 3.3, 2.6              |
| F2   | 3.8                | NA                 | 1               | 0.1                   |
| CDR2 | 4.8                | 11.0, 4.9          | 67, 41          | 5.4, 3.3              |
| F3   | 3.1                | 4.0                | 10              | 0.4                   |
| CDR3 | 4.6                | 3.0                | 7, 5            | 1.1, 0.8              |
| F4   | 2.6                | NA                 | 1               | 0.1                   |
| All Fs | 3.3                | 6.0                | 14              | 0.25                  |
| All CDRs | 4.5               | 6.25, 4.7         | 84, 55          | 3.2, 2.3              |
| Total |                    |                    | 98, 69          | 1.3, 0.9              |

|      | Expected R/S ratio | Observed R/S ratio | Total mutations | Frequency of mutation |
|------|--------------------|--------------------|-----------------|-----------------------|
| VK   |                    |                    |                 |                       |
| F1   | 2.9                | 1.7, 2.0           | 8, 6            | 0.5, 0.4              |
| CDR1 | 5.6                | 5.0, 3.0           | 12, 7           | 1.5, 0.9              |
| F2   | 3.7                | 8.0, 7.0           | 9, 8            | 0.8, 0.7              |
| CDR2 | 3.2                | 4.3, 4.0           | 16, 10          | 3.2, 2.0              |
| F3   | 3.4                | 5.1, 4.4           | 25, 19          | 1.1, 0.8              |
| CDR3 | 3.1                | 9.0, 6.0           | 10, 7           | 1.6, 1.1              |
| F4   | 2.9                | 2.0                | 6               | 1.1                   |
| All Fs | 3.2                | 2.2, 2.2           | 48, 39          | 0.8, 0.7              |
| All CDRs | 3.9               | 4.8, 3.2           | 38, 24          | 1.9, 1.2              |
| Total |                    |                    | 86, 63          | 1.1, 0.8              |

In the case of each CDR and framework region (F) of the VH and VK genes expressed by the clonally related hybridomas a compilation of the number and type of mutations is presented. The first column presents the ratio of mutations resulting in amino acid replacements (R) to the ratio of translationally silent (S) mutations expected in each region if mutation were to occur randomly. The second column presents the actual ratios calculated from the mutations present in the V genes expressed by all the HIP hybridomas. The third column shows the total number of mutations in each subregion. The last column presents the frequency (per base pair) of mutation in each subregion. In cases where two values are presented in one column, the first value corresponds to the observed or calculated value assuming that all mutations in that subregion were independent events. The second value corresponds to the value derived from the assumption that all shared mutations in that subregion occurred only once in a clone.

In contrast, the data presented in Table II show that of the 14 mutations in VH framework regions only two are silent (R/S observed = 6), a number expected if mutation occurred randomly in these regions (R/S expected = 3.3). Further, the R/S ratio is significantly lower than expected for random mutation in only framework 3 of the expressed Vk genes.

Given only the data presented in Table II it might be concluded that antigen selection did not act on the two clones at all. This idea is not supported by the data presented in Table I. While there is considerable variation in the intrinsic affinities...
for IArs of the antibodies expressed by individual members of each clone, all but one of these affinities are comparable to or greater than the intrinsic affinity for IArs of three antibodies encoded by the canonical combination of V gene segments in unmutated form. In some cases the affinities are at least 20-fold greater than the affinities of the unmutated antibodies. Thus, either most of the mutations that canonical combination encoded V regions can suffer are likely to increase affinity for IArs, which seems highly unlikely (36), or antigenic selection has resulted in a skewed representation of mutations that result in increased affinity for the eliciting epitope. It is interesting to note that no single recurrent mutation (e.g., the A to T mutation at VH position 59), or subset of recurrent mutations, correlates with high affinity. This implies that a variety of distinct amino acid changes, or combination of changes in canonical V regions can result in substantially increased affinity for IArs.

Amendments to the "Neo-Darwinian" Model of the Somatic Mutation and Clonal Selection Process. An explanation of the data that is within the confines of a "neo-Darwinian" clonal selection model must assume that at least certain nucleotide positions in the CDR regions of the VH and VK genes expressed by the clones are mutational "hot-spots," and that the mutations that took place at these and other positions were subjected to a highly stringent process of antigenic selection. Lineage A in Fig. 7 assumes that the A to T mutation in the first position of VH codon 59 occurred seven independent times, and a G to C mutation occurred in the third position of this codon six times. Lineage B assumes that many other CDR mutations in both VH and VK have occurred independently multiple times. It should be emphasized that the data demonstrate that, irrespective of which lineage (if either) accurately reflects events that occurred in vivo, the degree of efficiency (as a function of cell division) in fixing mutations that increase affinity for antigen by this single clone of B cells was enormous. Moreover, the frequency of isolation of members of the clone via hybridoma technology was probably low (perhaps 0.1%), so that each of the independent mutation and selection events deduced from the hybridoma data must have actually occurred many hundreds or thousands of times in vivo. Further, the clone was immortalized as hybridomas only 16 days after initial immunization. Since we (16, 17) and others (3, 7) have obtained data suggesting that somatic mutation does not take place during the initial stages of an immune response, the time during which this clone sustained mutation was probably <16 d. Such remarkable frequencies of mutation and selection events imply that, if somatic mutation occurs as a function of cell division, the rate of B cell division in vivo is very high and the effects of antigen selection on this rate are large.

Alternatively, it may be that somatic mutation occurs independently of cell division. If this were true, a single B cell might be capable of sequentially expressing different mutant forms of its V region, each of which could be subjected to antigenic selection. In this case, the number of cell divisions necessary to create the mutant V region repertoire requisite for efficient affinity maturation might be small. Mechanistically, it is difficult to imagine how such a process might take place. However, since little is known about the properties of somatic mutation and antigenic selection, this seems a viable hypothesis at present. The appeal of this "neo-instructionist" model in light of the data presented here is that it proposes that the efficiency of the mutation-selection process is high and that mutations that destroy V region function (e.g., the A to T mutation in the first position of VH codon 59) can be temporary and need not automatically lead to cell death.
The hybridomas analyzed here were isolated relatively early in the immune response. In contrast, most of the mutant V regions previously analyzed by us and others are expressed by hybridomas that are isolated during the initial stages of secondary immune responses, at least one month after primary immunization (4-6, 13, 42-45). These secondary V regions display a large number of somatic mutations in CDR regions, some of which are recurrently observed and have been shown to encode amino acid substitutions that increase V region affinity for antigen. They also contain large numbers of framework region mutations; however, the R/S ratio of these framework mutations is lower than expected for random mutation, indicating that V regions that sustained certain framework R mutations were lost from the population. Thus, the CDR regions of the antibodies characterized here have secondary V region "quality," while most of their framework regions do not. Taken together with the points discussed above, these observations suggest that "somatic evolution" of V region structure may occur in two phases during the immune response to model antigens such as IArs-KLH. In the first phase, CDR mutations are generated and fixed by antigenic selection with high efficiency, resulting in affinity maturation. Positive antigen selective forces (46, 47) most influence the outcome of this phase of the response. In the second phase, mutation continues, but since most of the CDR mutations that can increase affinity have already been fixed, further affinity maturation is difficult. However, due to the extended period of mutation, mutations are eventually sustained in framework regions, and the V regions that suffer framework mutations that decrease affinity for antigen are selected against, resulting in a low observed R/S ratio among secondary V regions. During primary immune responses to natural pathogens, the "first" phase of somatic evolution may actually occur multiple times as the responding B cell population efficiently adapts to antigenic variants that arise in the pathogen population.

Summary

Changes in the structure and function of antibodies occur during the course of an immune response due to variable (V) region gene somatic mutation and isotype switch recombination. While the end products of both these processes are now well documented, their mechanisms, timing, and regulation during clonal expansion remain unclear. Here I describe the characterization of antibodies expressed by a large number of hybridomas derived from single B cell clones at an intermediate stage of an immune response. These data provide new insights into the mechanism, relative timing, and potential of V gene mutation and isotype switching. The data suggest that somatic mutation and isotype switching are completely independent processes that may, but need not, occur simultaneously during clonal expansion. In addition, the results of this analysis demonstrate that individual B cell clones are far more efficient than previously imagined at generating and fixing particular V region somatic mutations that result in increased affinity for the eliciting epitope. Models to account for this high efficiency are discussed. Taken together with previous data, the results of this analysis also suggest that the "somatic evolution" of V region structure to a single epitope takes place in two stages; the first in which particular mutations are sustained and fixed by antigen selection in the CDR regions of the V region genes expressed in a clone over a short period of clonal expansion, and the second in which these selected CDR mutations are maintained in the growing clone, delete-
rious mutations are lost, and selectively neutral mutations accumulate throughout the length of V genes over long periods of clonal expansion.

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1230 THE EFFICIENCY OF ANTIBODY V REGION SOMATIC EVOLUTION

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