Parallel Evolution of Antibody Variable Regions by Somatic Processes: Consecutive Shared Somatic Alterations in \( V_H \) Genes Expressed by Independently Generated Hybridomas Apparently Acquired by Point Mutation and Selection Rather than by Gene Conversion

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Summary

We identified, in independently generated hybridoma antibodies, blocks of shared somatic alterations comprising four consecutive amino acid replacements in the CDR2s of their heavy chain variable regions. We found that the nucleotide sequences encoding the shared replacements differed slightly. In addition, we performed genomic cloning and sequencing analyses that indicate that no genomic sequence could encode the block of shared replacements in any one of the antibodies and thus directly serve as a donor by a recombinational process. Finally, in a survey of other somatically mutated versions of the same heavy chain variable gene, we found several examples containing one, two, or three of the shared CDR2 mutations in various combinations. We conclude that the shared somatic alterations were acquired by several independent events. This result, and the fact that the antibodies containing the four shared mutations were elicited in response to the same antigen and are encoded by the same \( V_H \) and \( V_K \) gene segments, suggests that an intense selection pressure has fixed the shared replacements by favoring the clonal expansion of B cells producing antibodies that contain them. The basis of this selection pressure is addressed elsewhere (Parhami-Seren, B., L. J. Wysocki, M. N. Margolies, and J. Sharon, manuscript submitted for publication).

An important but poorly understood process termed "somatic mutation" introduces sequence alterations in antibody V genes expressed by members of the B lymphocytic lineage (1–7). In the absence of an identified mechanism, somatic mutations are operationally defined as sequence differences between an expressed V gene and the corresponding germline V gene from which it was derived. In practice, somatic mutations are identified by sequences of V genes expressed by B cells that are immortalized by cell fusion with myeloma cell lines (reviewed in reference 8). This hybridoma approach allows one to estimate both the timing of the mutational process and the rate at which it occurs. Results of such studies have shown that V gene–specific somatic alterations are acquired at a high rate (9–11) \( (10^{10} \) to \( 10^{13} \) bp per generation) during the antigen-driven stages of B cell differentiation (11–18). These observations support the idea that somatic mutations are introduced by a differentiative process rather than simply being the accumulated products of spontaneous events.

The influence of selection on mutated V regions has been documented structurally by the high ratio of replacement to silent mutations in CDRs as opposed to framework regions (9, 19), and by the reproducible isolation of independently derived hybridomas producing antibodies with identical amino acid replacements (12, 20–23). These results are complemented by others demonstrating that a positive selection pressure favors the clonal expansion of B cells expressing mutant antibody V regions with increased affinity for the eliciting antigen relative to that of the germ-line antibody (12, 16, 20, 24–27).

Here, we identify two independently generated hybridomas producing anti-p-azophenylarsonate (Ars)\(^1\) antibodies that are encoded by mutated versions of the same germline \( V_{H} \) gene segment and that remarkably share four consecutive identical amino acid replacements within a stretch of eight amino acids in the second CDR. The shared mutations are not the

\(^1\) Abbreviations used in this paper: ARS, p-azophenylarsonate; TMACI, tetramethylammonium chloride.
consequence of a single-step introduction from the genome by a recombinational (gene conversion) mechanism, as revealed by genomic searching and nucleotide sequencing analyses. We conclude that the shared mutations are acquired by multiple independent events and are fixed by an intense selection pressure that favors the clonal expansion of B cells expressing the mutant V region products.

Materials and Methods

Cell Lines. Hybridomas 45-49 (28) and 91A3 (29) were prepared from splenocytes of A/J mice immunized with protein conjugates of Ars. The antibodies produced by these hybridomas bind Ars and are encoded by the V\(_\alpha\) gene segment (V\(_\alpha\) Id\(^{39}\)) that encodes V regions bearing the major cross-reactive idiotype (CRI\(_\alpha\) or Id\(^{39}\)) associated with strain A anti-Ars antibodies (30). Hybridoma 91A3 was provided by Dr. J. Donald Capra (University of Texas Health Science Center, Dallas, TX).

Preparation of Peptides and Amino Acid Sequence Analyses of 45-49. The A/J anti-Ars hybridoma protein 45-49 (\(\gamma_1,\kappa\)) was amplified in the ascites of CAF1 mice (The Jackson Laboratory, Bar Harbor, ME) and purified by affinity chromatography (28). Partial NH\(_2\)-terminal sequences for the 45-49 H (48 cycles) and L (42 cycles) chains were reported previously (28). The sequence of the L chain was completed using peptides produced by cleavage with \(\epsilon\)-iodosobenzoic acid and with trypsin, resulting in the identification of all residues spanning positions 35-75, 62-96, and 97-108. The complete sequence of the H chain was obtained largely using tryptic peptides derived from the citraconylated chains and from tryptic daughter peptides obtained after partial or complete decitraconylation. Methods for the partial reduction and alkylation of purified antibodies, chain separation, preparation of tryptic peptides from citraconylated, completely reduced and alkylated chains, preparation of CNBr peptides, and purification of peptides from tryptic daughter peptides obtained after partial or complete decitraconylation. Methods for the partial reduction and alkylation of purified antibodies, chain separation, preparation of tryptic peptides from citraconylated, completely reduced and alkylated chains, preparation of CNBr peptides, and purification of peptides by gel filtration and HPLC were performed as described (31-34). Automated Edman degradation was carried out using either a sequencer (890C; Beckman Instruments, Inc., Fullerton, CA) or a gas phase sequencer (470A; Applied Biosystems, Inc., Foster City, CA) as described (34). Selective sequence of prolyl-containing peptides contained in mixtures was performed using \(\epsilon\)-phthalaldehyde treatment (35). Phenylthiohydantoin amino acids were identified by HPLC.

Probes. A 24-base oligonucleotide (GTGAATATAACCTTTTCCAGGATG) was synthesized and provided by Dr. K.L. Ramachandran of Biogen Research Corp., Cambridge, MA. This sequence is complementary to the coding strand spanning codons 52-59 (consecutive numbering system) in the CDR2 of the V\(_\alpha\) region of 45-49 (see Fig. 1). The 5' and 3' nucleotides of this probe define the somatically altered boundaries encoding the region containing the four amino acid replacements (somatic mutations) that are shared with 91A3. A similar oligonucleotide (\(\Delta\)TGAATGTAACCTTTTCCAGGATG) defining the corresponding region of the V\(_\alpha\)Id\(^{39}\) gene expressed by 91A3 (36) was synthesized by the Molecular Resource Center at the National Jewish Center for Immunology and Respiratory Medicine. This sequence differs at two positions (underlined) from the 24mer of 45-49. For use in hybridization analyses, these oligonucleotides were labeled to a sp act of 1-1.3 \(\times\) 10\(^6\) cpm/\(\mu\)g with \(3^P\) using T4 polynucleotide kinase (37).

Southern Blots. Cellular and genomic DNAs were digested with EcoRI (20 \(\mu\)g DNA, 50 U enzyme), run on 0.75% agarose gels, and transferred to nitrocellulose in 20 \(\times\) SSC (1 \(\times\) SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7) (38) or to Nylon (Gene Screen plus; New England Nuclear, Boston, MA) in 0.4 N NaOH (39). Hybridizations were done at 45°C in a solution containing 60 ng (6-8 \(\times\) 10\(^7\) cpm) of end-labeled probe, 6x SSC, 10% dextran sulfate, 5x Denhardt's solution (1 \(\times\) is 0.02% [wt/vol] BSA, polyvinyl pyrrolidone, and ficoll \([M = 400,000]\), 500 \(\mu\)g/ml ssDNA, 1% SDS, 25 mM Tris, pH 7.5, and 20 mM EDTA. Figure legends indicate specific departures from this general protocol and give details concerning washing conditions, lengths of hybridizations, and film exposures. Phage DNAs were handled similarly, except that the amount of digested DNA loaded per lane was less (25 ng).

Cloning Genomic Sequences. A/J kidney DNA was partially digested with MboI and run on a 0.6% agarose gel. Fragments with an average length of 16 kb were electroeluted into dialysis tubing, purified using an Elutip column (Schleicher & Schuell, Inc., Keene, NH), and ligated to the left and right "arms" of phage \(\lambda\) cloning vector EMBL4 (40) (Promega Biotech, Madison, WI) that had been digested with BamH1 and SalI. Ligation products were encapsidated (41) using a commercially available packaging extract (Gigapack; Stratagene, La Jolla, CA), and unamplified phage were plated on the Escherichia coli strain NM539. Resulting recombinant phage plaques (1.5 \(\times\) 10\(^5\)) were lifted onto nitrocellulose filters (42) in duplicate and hybridized with a \(3^P\)-labeled 24-base probe (encoding the shared somatic mutations in the CDR2 of antibody 45-49) for 16 h at 45°C in the following solution: 6x SSC, 5x Denhardt's solution, 100 \(\mu\)g/ml ssDNA, and 0.5% SDS. The filters were washed at 50°C in a solution of 6x SSC and 0.5% SDS. X-ray film (Kodak XAR-5) was exposed to the washed filters at -70°C overnight with an intensifying screen. Plaques giving positive hybridization signals were picked and isolated by three rounds of subcloning and screening under the same conditions.

To identify the recombinant phage harboring inserts with sequences most related to the 24mers of 45-49 and 91A3, washes at increasing temperatures were performed in a solution of 3 M tetrramethylammonium chloride, 25 mM Tris (pH 8.0), 20 mM EDTA, and 0.5% SDS, as described (43). In this buffer, the difference in melting temperatures of AT vs. G-C bp is eliminated (44).

Nucleic Acid Sequencing. A 5.5-kb EcoRI fragment containing the V\(_\alpha\)Id\(^{39}\) gene expressed by 45-49 was provided by Dr. Thomas Gridley (Roche Institute, Nutley, NJ). From this, a 380-bp PstI fragment containing the first 92 codons of the V\(_\alpha\)Id\(^{39}\) and 5' flanking sequence was subcloned into plasmid pGEM 4 (Promega Biotech) and sequenced in both directions using T7 and SP6 primers in a dideoxy chain termination procedure (45), according to the manufacturer's (Promega Biotech) instructions. Total RNA was isolated from 45-49 and 91A3 by the method of Chirgwin et al. (46). V\(_\alpha\)Id\(^{39}\) mRNA was sequenced from each of these hybridomas using a universal IgG primer and reverse transcriptase as described (47).

Genomic DNA fragments containing sequences that hybridized to the 24-base probe were subcloned into phagemid M13 KS+/- (Stratagene), isolated as single-stranded circles after superinfection with helper phage R408 (Stratagene), as described (48), and sequenced using the M13 reverse and T7 primers (45). In all, 14 recombinant fragments were subcloned and sequenced: the six clones with sequences most identical to the 24mer of 45-49 were subcloned as 297-bp TagI-Sau3AI fragments; four of the clones that were determined to be second most identical to the 24mer of 45-49 were subcloned as 144-bp Sau3A fragments; two clones with sequences most identical to the 24mer of 91A3 were subcloned as 190-bp Sau3A fragments; and two clones with sequences second most identical to the 24mer of 91A3 were subcloned as 210-bp HaeIII fragments.
Figure 1. Sequences of the $\text{V}_\text{H}$ and $\text{V}_\text{L}$ regions of anti-Ar hybridoma antibodies 91A3 and 45-49 compared with the corresponding germline sequences that are represented by antibody 36-65. The vertical bars in the $\text{V}_\text{H}$ regions delineate the region containing the four consecutive shared amino acid replacements (somatic alterations). Lines indicate identity with the top (germline) sequence. Gaps indicated by a period are introduced to maximize homology. Codons are numbered consecutively, and the one letter code is used to designate amino acids (63).
Results

Consecutive Shared Mutations in Independently Isolated Antibody V\textsubscript{\text{\textgamma}} Regions. The hybridomas under consideration are designated 45-49 and 91A3 (28, 29). They were produced in fusions of splenic lymphocytes from strain A/J mice that were immunized with Ars conjugates of carrier proteins, and both secrete antibodies that bind Ars.

The two hybridomas are of independent origin: 45-49 was produced by Ann Marshak-Rothstein at the Massachusetts Institute of Technology (28), and 91A3 was produced at Brandeis University by Estess et al. (29). The antibodies they produce were a topic of interest in earlier studies, when it was observed by NH\textsubscript{2}-terminal sequencing analyses that they appeared to be encoded by the same V\textsubscript{\text{\textgamma}} (V\textsubscript{\text{\textgamma}}\text{IdcR}) and V\textsubscript{\text{\textdelta}} (V\textsubscript{\text{\textdelta}}\text{IdcR}) gene segments that encode the predominant population of A/J anti-Ars antibodies, even though both lacked the associated idiotype called CR\textsubscript{\textalpha} or IdcR (28, 33, 49). This supposition was confirmed by Southern blotting analyses using V\textsubscript{\text{\textgamma}} IdcR and V\textsubscript{\text{\textdelta}} IdcR probes (data not shown) and by the complete amino acid sequences reported here for 45-49, and previously (50) for 91A3 (Fig. 1).

One of us (M.N. Margolies) determined the complete amino acid sequences of the H and L chain V regions of 45-49 (see Materials and Methods), compared them with the published sequences for 91A3 (50) and to the corresponding germline sequences (51–53), and observed that both shared four consecutive identical amino acid replacements (somatic mutations) in CDR2 of their H chains.

The H and L chain V sequences, aligned with respect to the corresponding germline sequences of antibody 36–65, are shown in Fig. 1. The sequences reveal that both antibodies are encoded by the same V\textsubscript{\text{\textgamma}} gene segments that encode the predominant anti-Ars IdcR\textsuperscript{*} antibodies, with the exception of the D region, and both are heavily mutated. Antibody 45-49, in particular, is hypermutated: it contains 13 amino acid replacements in the H chain V region and four in the L chain V region. Outside of the shared set of four amino acid replacements in the H chain CDR2s, however, the antibodies share no other mutations.

Nucleotide Sequences Distinguish Regions with Shared Amino Acid Replacements. We sequenced the cloned V\textsubscript{\text{\textgamma}} IdcR gene expressed by 45-49 and compared it to the published nucleotide sequence of the V\textsubscript{\text{\textgamma}} IdcR gene expressed by 91A3 (36) and to the sequence of the germline V\textsubscript{\text{\textgamma}} IdcR gene segment (51). These comparisons are shown in Fig. 1 and reveal that: (a) the 45-49 V\textsubscript{\text{\textgamma}} gene contains 20 nucleotide substitutions (somatic mutations); (b) the two V\textsubscript{\text{\textgamma}} IdcR genes share no other nucleotide substitutions outside of the region of shared amino acid replacements, and, most important; (c) in the region of shared amino acid replacements, the two genes differ in sequence by two nucleotides, one of which is a silent substitution in the third position of the tyrosine 57 codon, and the other is in the third position of the histidine 59 codon.

The importance of the nucleotide sequence differences in the regions of shared amino acid replacements prompted us to confirm both sequences. We obtained from J.D. Capra (University of Texas Health Science Center, Dallas, TX) the 91A3 hybridoma, prepared RNA from it and from 45-49, and sequenced both V\textsubscript{\text{\textgamma}} IdcR mRNAs by using reverse transcriptase primed with a C region oligonucleotide in the deoxyoligonucleotide chain termination procedure (47). Both sequences were confirmed.

Isolation from the Genome of Potential Donor Sequences for the Shared Mutations. The differences in nucleotide sequence between 45-49 and 91A3 in the region of shared amino acid replacements is most consistent with a model in which point mutations followed by intense selection for particular amino acid replacements resulted in the parallel fixation of the four shared somatic alterations in the CDR2s of 45-49 and 91A3. This conclusion seemed surprising, particularly in view of the fact that the shared blocks of four replacements were the only somatic alterations that were shared by the two V\textsubscript{\text{\textgamma}} IdcR genes: the changes suggested to us that they might have been introduced from the genome by recombinations involving cassettes of sequence information. In principle, the nucleotide sequence disparities could be accounted for by recombinations involving two different but related germline donors belonging to a family. We were therefore prompted to provide more conclusive evidence for one model or the other.

To this end, we prepared a total genomic library of A/J DNA and screened it for potential donor sequences (for the shared somatic alterations) using a 24-base probe derived from the CDR2 of 45-49. The 5' and 3' nucleotides of the 24-base probe were defined by the first and last nucleotide substitutions (mutations) in the region of shared amino acid replacements (see Materials and Methods). We ligated MboI fragments with an average length of 16 kb from partially digested A/J kidney DNA to the arms of the phage \lambda vector EMBL4 (40). 1.5 × 10\textsuperscript{6} recombinant phage containing approximately eight genomic equivalents of A/J DNA were screened in duplicate. Of 63 primary plagues that were initially selected, 52 were eventually recovered after three rounds of subcloning.

The hybridizations and washes in the primary screen and subcloning screens were uniformly performed under nonstringent conditions. To identify the genomic clones most identical in sequence to the 24mer, we subjected filters containing hybridized DNA from the 52 selected recombinant phage to a series of washes at increasing temperatures. To ensure that clones were selected on the basis of sequence identity and not on the basis of sequence composition (i.e., AT vs. G-C bp), we performed the washes in a solution containing 3 M tetramethylammonium chloride (TMACl) in which the melting temperature of AT bp is the same as that of GC bp (43, 44). Six recombinant phage were found to harbor inserts with sequence information that was most identical to the probe, as revealed by the signal intensity at the more stringent washing temperatures. At a temperature of 63°C in a solution of 3 M TMACl, however, all of the hybridization signal was eliminated while insignificant reduction in the hybridization signal was observed for the positive control, which was provided by recombinant phage containing the rearranged V\textsubscript{\text{\textgamma}} IdcR gene of 45-49. On the basis of melting temperature, a second group of six phage were judged to harbor inserts...
that were slightly less identical than the first six, but more so than the remaining 41. Four of these were selected for further analyses.

To confirm that we had cloned the genomic sequences most identical to the 24mer from 45-49, we performed a genomic Southern blot with this probe and identified EcoRI fragments of 4.9 and 4.5 kb in length that were most identical to the probe because, following a high stringency wash, only signals at those locations were observed (Fig. 2, A and B). Phage DNA from the first six selected clones also produced EcoRI fragments of 4.5 kb in length that hybridized with the 24mer, and three of the four selected phage from the second group produced a signal at 4.9 kb (Fig. 2 C).

The nucleotide sequence differences between the two \( V_{\text{d}} \text{IdcR} \) genes in the region of shared substitutions were located near one end of the block (at relative positions 18 and 24; Fig. 1). This, together with the fact that the genomic library was screened under nonstringent conditions, suggested to us that the 52 selected recombinant phage that were screened with the 24mer of 45-49 might also harbor the genomic sequences most identical to the corresponding region of 91A3. Thus, we screened the selected 52 phage with a DNA probe of 24 nucleotides that defined the somatically altered region of interest in 91A3. Two clones were identified that hybridized more strongly to the probe than any of the remaining 50. These two clones produced EcoRI fragments of 5.2 kb in length that hybridized with the 24mer of 91A3 (Fig. 3, B and C). Two other clones were identified that hybridized with the 24mer less strongly than the first two, but more so than the remaining 48. These produced EcoRI fragments of 3.2 kb that hybridized with the probe. When genomic DNA was digested with EcoRI and hybridized with the 24mer of 91A3 under nonstringent conditions, hybridization signals were seen only at 5.2 and 3.2 kb (Fig. 3 A). It thus appeared that we had also cloned the genomic sequence most identical to the 24mer of 91A3 in our initial genomic search with the corresponding 24 mer of 45-49.

**No Obvious Genomic Donors for the Shared Mutations.** From the selected phage that harbored the genomic sequences most identical to the 24-base segments defining the regions of shared mutations, we subcloned and sequenced short DNA frag-

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**Figure 2.** Cloning of potential donor sequences for the shared somatic alterations in 45-49. Southern blots of EcoRI-digested DNAs were performed as described in the Materials and Methods using the 24-base probe derived from the CDR2 of the \( V_{\text{d}} \text{IdcR} \) gene encoding 45-49. Hybridization signals at 5.5 kb in A and B identify the rearranged and expressed \( V_{\text{d}} \text{IdcR} \) gene of 45-49. (A) Blot was hybridized for 3 d, washed nonstringently at a temperature of 55°C in TMACI buffer, and exposed to film for 3 d. (B) Blot was hybridized for 3 d, washed more stringently at a temperature of 60°C in TMACI buffer, and exposed to film for 3 d. (C) Lanes 1-6 contain EcoRI-digested DNAs from recombinant phage (H1-H6) harboring inserts that were judged to be most identical to the 24 mer of 45-49 (see sequences in Fig. 4). Lanes 7-10 contain digested phage DNAs (H7-H10) harboring sequences that were slightly less related to the probe (see sequences in Fig. 4 A).

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**Figure 3.** Cloning of potential donor sequences for the shared somatic alterations in 91A3. Southern blotting analyses of EcoRI digests were done using the 24-base probe derived from the CDR2 of the \( V_{\text{d}} \text{IdcR} \) gene encoding 91A3, as described in Materials and Methods. (A) Blot was hybridized for 3 d, washed nonstringently in 6× SSC, at a temperature of 45°C, and exposed to film for 3 d. Signal at 5.5 kb identifies the rearranged and expressed \( V_{\text{d}} \text{IdcR} \) gene of 91A3. (B and C) Lanes 1-4 contain phage DNAs (K1-K4) digested with EcoRI that harbored sequences related to the 24 mer of 91A3. The higher temperature wash (C) shows that K1 and K2 contain sequences most identical to the 24 mer of 91A3 (see sequences in Fig. 4 B).
None of the cloned segments demonstrated significant homology with the \( V_{\text{H}} \text{IdCR} \) gene beyond the 5’ and 3’ boundaries of the probes that were used in the search, and none was an Ig V gene, as revealed in computer searches of the Genbank database (release 59.0) rodent files. These two observations argue that if recombinational events played any role at all in the somatic alteration of the \( V_{\text{H}} \text{IdCR} \) gene segments encoding the shared amino acid replacements, the sequences cloned here were probably not involved, even though they are the genomic sequences most identical to the regions of shared mutations.

**Discussion**

Recombinational mechanisms, including gene conversion, have undoubtedly contributed on an evolutionary time scale to the generation of the mammalian germline repertoire of V gene segments (reviewed in reference 54). A somatic gene conversion mechanism apparently diversifies the repertoire of V regions expressed by avian B cells during their ontogeny in the bursa (55, 56). A somatic gene conversion-like process has been invoked to explain somatic alterations sustained in antibody V genes expressed by hybridoma cell lines that are grown in vitro (57, 58). Evidence in support of such mechanisms as the means by which somatic mutations are introduced into mammalian Ig V genes in vivo, however, is lacking.

The first argument against such a mechanism derived from the finding that specific somatic alterations within V genes could not be accounted for by the germline sequences of the nearest V gene relatives. These observations were first made for V genes (antiphosphorylcholine \( V_{\text{H}} \) genes [6] and \( V \lambda \) genes [59]) belonging to small families, the members of which were all cloned and sequenced. The argument was thus based on the assumption that any recombinational event should involve V genes with the greatest degree of homology.

This assumption became questionable when it was realized that somatic alterations are under the influence of selection pressure(s), one of which favors the clonal expansion of B cells expressing altered V regions with increased affinity for the immunizing antigen. This selection pressure is revealed in general by the fact that mutated hybridoma V regions tend to bind antigen with higher affinity than unmutated V regions (12, 16, 24), and more specifically by experiments demonstrating that particular somatic alterations that are reproducibly and independently isolated confer an increased affinity for the eliciting antigen (25, 26). The influence of selection, therefore, introduces a bias in hybridoma sampling procedures, favoring the isolation of hybridomas expressing V genes containing the somatic alterations that confer upon the encoded antibody an increase in the affinity for the eliciting antigen. Thus, more distantly related \( V \) genes might be the donors for the observed somatic alterations via recombination, provided they introduce altered codons that encode the structural changes producing affinity increases.

The possibility that more distantly related \( V \) genes might function as recombinational donors for somatic alterations was addressed by Chien et al. (60), who searched the genome for potential donors of somatic alterations by performing stringent Southern blotting analyses with oligonucleotide probes.
that defined segments of somatically altered $V_\mu$ genes. They found no evidence for donors of somatic alterations by this method. The experiments reported here, which support their conclusion, carry this type of analysis a step further by cloning from the genome the sequences that are most identical to somatically altered segments shared by two $V_\mu$ genes expressed by hybridomas of independent origin. Our results show that no single sequence nor combination of two sequences in the A/J genome could serve as the donor(s) for all of the consecutive shared alterations found in the $V_\mu$ genes expressed by the two hybridomas. The possibility that the shared alterations were derived from genomic sequence information contributed by the SP2/0 cell line post-fusion is also unlikely from three lines of evidence. First, SP2/0 and A/J DNAs produce identical signal patterns upon hybridization with the oligonucleotide probes that define the shared alterations, and both sets of signals disappear with washes performed at the same level of stringency (Fig. 2 and unpublished results), indicating the absence of additional potential donor sequences in the SP2/0 DNA. Second, the shared alterations confer an increased affinity for the eliciting antigen, Ars (unpublished results), so their independent acquisition in vitro, without the apparent benefit of an antigenic selection pressure, would be a monumental coincidence. Finally, if somatic alterations were introduced from the SP2/0 parent in vitro, we would expect to observe them regardless of the timing of the fusion event, which is inconsistent with the observation that somatic alterations are seen neither in $V$ genes of hybridomas generated by fusions (with SP2/0) performed during the early primary immune response to Ars (12), nor in $V$ genes of hybridomas generated by fusions of preimmune B cells (13).

While this manuscript was in preparation, a third example of a mutated $V_{\mu}Id^{CR}$ gene containing the four shared CDR2 somatic alterations was reported by Fish et al. (61). Anti-Ars hybridomas 8AS2A, C, and E (apparently members of a single lineage) express a $V_{\mu}Id^{CR}$ nucleotide sequence identical to that of 45-49 in the region of shared CDR2 amino acid replacements. In the same report are other sequences of expressed $V_{\mu}Id^{CR}$ genes that contain one, two, or three of the four alterations shared by 45-49, 91A3, and 8AS2, supporting the hypothesis that each of the alterations is derived independently, perhaps by a point mutation mechanism. Most supportive of this idea are two members of a common lineage, clones AS13F and AS13B, that, respectively, have two and three of the shared replacements. Their sequences in this region are aligned (Fig. 5) compared with those of 45-49 and a germline (36-65) clone.

An asparagine to histidine replacement at position 52 is found in only one of the two clones (8AS13B), and the asparagine to lysine replacements at position 55 are acquired by alternative base transversions (T to either G or A). These observations support the interpretation that the sharing of four consecutive mutations by three independently derived antibodies is more likely due to the influences of selection than to an extreme bias (62) in the mutation mechanism. This interpretation is further supported by the differences in nucleotide sequence between 45-49 and 91A3: histidine 59 replacements occurred by alternative base substitutions, and a silent nucleotide substitution distinguishes the tryptophan 57 codons.

The seemingly improbable isolation of multiple parallel somatic alterations can and does occur as shown here for three antibodies derived from different laboratories. Although the analyses and observations cited above provide significant evidence against recombinational involvement in somatic alteration, we cannot rule out the possibility that very short donor segments (<15 bp) generated the somatic alterations. Rather, the main conclusion of this report is that the observed blocks of shared somatic alterations cannot be explained as the products of one or two direct recombinational (conversion) events involving genomic sequences; the shared somatic alterations are apparently the products of multiple independent events. We interpret this as an indication that an intense selection pressure has fixed the parallel changes in the three antibodies.

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