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Immunology

Generation of antibody diversity in the immune response of BALB/c mice to influenza virus hemagglutinin

(antibody sequences/antibody genes/somatic mutation)

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ABSTRACT **We have examined the amino-terminal sequence of the κ light chains of a set of monoclonal antibodies specific for one of the major antigenic determinants (Sb) on the influenza virus PR8[A/PR/8/34(H1N1)] hemagglutinin molecule. This set was believed to be structurally related from earlier serological analysis that typed these κ chains as members of the variable (V) region $V_κ21$ group [Staudt, L. M. & Gerhard, W. (1983) *J. Exp. Med.* 157, 678–704]. Our sequence analysis confirms and extends this conclusion; all examples of this set belong to a subgroup of the $V_κ21$ group, $V_κ21C$. A special feature of this set of κ light chains is that all examples were derived from the same mouse (designated H36). This sequence analysis along with the characterization of gene rearrangements at the κ light chain loci of these hybridomas is consistent with the idea that certain members of this set are the progeny of one or two lymphocytes. Because of this potential clonal relationship, we can reach several conclusions about the diversity observed among these κ light chains: (*i*) the diversity is due to somatic mutation, (*ii*) somatic mutations occur sequentially and accumulate in the first complementarity-determining region, and (*iii*) the extent of somatic variation in this sample is high, suggesting a somatic mutation rate of about 10^{-3} per base pair per generation.**

Antibody diversity arises from several sources. Individuals inherit multiple variable (V) region gene segments for both heavy (V_H) and light ($V_κ$, $V_λ$) chains, joining (J) gene segments (J_H, $J_κ$, $J_λ$), and diversity (D) gene segments (D_H). The initial antibody repertoire of an individual is a product of the combinatorial joining of these gene segments, i.e., $V_κ$s with $J_κ$s or different V_H, D_H, and J_H combinations, that form complete $V_κ$ or V_H genes. Errors committed during the process of joining contribute additional diversity to this repertoire (reviewed in ref. 1). Finally, that somatic mutation further amplifies this germ-line repertoire seems to be established (2). The original evidence for somatic mutation favored a model by which point mutations accumulate sequentially during cell division (3). Other models link somatic mutation with specific events during lymphocyte differentiation (4, 5) and propose cataclysmic mechanisms of mutagenesis that introduce multiple amino acid substitutions in one step (6, 7). These models are based on the comparison of V region sequences of independently induced plasmacytoma and hybridoma antibodies to their putative germ-line counterparts. Hence, little can be concluded about the time course of somatic mutation.

A better understanding of the nature of somatic mutation can be reached by comparisons of the V genes of a cell lineage. Scharff and colleague have analyzed certain mutants and revertants of the cell line S107 and conclude that the *in vitro* rate of mutation at the V_H gene expressed in this plasmacytoma is significantly higher than that of nonimmunoglobulin genes (8). A possible *in vivo* analogy is described here: we have initiated a structural comparison of hybridomas derived from a single mouse and have identified a set or sets, the members of which may be clonally related. The pattern of variability observed so far suggests that somatic mutations accumulate sequentially and that *in vivo* somatic mutation occurs at a high rate.

MATERIALS AND METHODS

Anti-Hemagglutinin (HA) Hybridomas. Twenty-four days prior to fusion, a BALB/c mouse, H36, was primed by intraperitoneal injection of 1000 hemagglutinating units of PR8. Three days prior to fusion, an intravenous injection of the same dose of virus was administered. The procedures for fusion, *in vitro* growth, and serological characterization of the H36 panel of hybridomas has been described (9). The fusion partner was Sp2/0-Ag14 (10).

Protein Purification and Sequence Analysis. Hybridomas were grown in (BALB/c × NZB)F_1 mice that had been Pristane-primed (Aldrich). Immunoglobulins were isolated from ascitic fluid by using protein A-Sepharose (Pharmacia) (11). IgG1 hybridoma antibodies were isolated by twice precipitating with 50% saturated ammonium sulfate and chromatographing on a Bio-Gel A-1.5m column (Bio-Rad) in 0.01 M sodium phosphate/0.9% sodium chloride/0.01% NaN_3, pH 7.2. Heavy and light chains were separated by Sephadex G-100 gel filtration in 6.0 M urea/1.0 M acetic acid after a 90-min reduction with 15 mM dithiothreitol and a 60-min alkylation with 35 mM iodoacetamide in 0.2 M Tris·HCl (pH 8.0) at 5.0 mg/ml of protein.

The strategy for the amino acid sequence determination of the $V_κ21C$ light chains has been reported (12). Briefly, the amino-terminal 42–50 residues of each light chain were determined from sequence analysis of the intact light chain. Light chains (H36-15 and H36-18) that contained methionine at residue 33 were cleaved with cyanogen bromide, the fragments from residue 34 to residue 171 were purified on G-50 Sephadex, and the amino-terminal 40 residues were identified by sequence analysis. The light chains H36-5 and H36-7 were cleaved at tryptophan residues with cyanogen bromide (13), the fragment from residue 36 to residue 144 was purified on G-50 Sephadex, and the amino-terminal 40 residues were identified by sequence analysis. The tryptic fragments from residue 69 to residue 100 were isolated from light chains H36-5, H36-7, H36-15, and H36-18 on G-50 Sephadex and were sequenced completely.

DNA Hybridization Analysis. The DNA probes were isolated from a cloned 12.7-kb fragment containing the constant

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Abbreviations: V, variable; C, constant; D, diversity; J, joining; HA, hemagglutinin; kb, kilobase(s).

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(C) region κ light chain locus (C_κ) (14). From this cloned *Bam*HI fragment, a 0.9-kilobase (kb) *Eco*RI fragment (designated pR1) was subcloned (14), and a 1.1-kb *Xba* I/*Hin*dIII fragment (designated IVS) was isolated from a low-temperature-melting agarose (Sigma) gel. The physical maps of these probes are shown in Figs. 2 and 3 *Lower*. DNA was isolated from hybridomas, plasmacytomas, or 12- to 14-day mouse embryos (15). The DNA samples (usually 10 μg) were digested to completion with the appropriate restriction enzyme(s) (Bethesda Research Laboratories), electrophoresed through 0.7% agarose, transferred to nitrocellulose paper (Schleicher and Schuell), and hybridized to ^{32}P-labeled DNA probes. Hybridizations were performed as originally described by Southern (16) with the modifications of Wahl *et al.* (17). After a final wash in 45 mM NaCl/4.5 mM sodium citrate/0.1% sodium dodecyl sulfate for 1 hr at 65°C, filters were exposed to x-ray film at −70°C.

RESULTS

The focus of this study is the structure of the κ light chains from seven hybridomas (H36-1, -4, -5, -7, -15, -17, and -18) derived from an individual adult BALB/c mouse, H36. In particular, these antibodies bind to the same antigenic region, Sb, on the HA molecule of influenza virus but can clearly be differentiated from each other by paratypic and idiotypic analysis. Tissue culture fluids from six of these hybridomas were serologically positive for the V_κ21C subgroup of κ chains (9). Subsequent serological analysis with purified antibody showed that the H36-4 light chain is also cross-reactive with V_κ21C-specific antisera (18). The heavy chain isotype of each H36 antibody is shown in Fig. 1.

A survey of 45 V_κ21 amino acid sequences from NZB and BALB/c plasmacytomas has so far divided the V_κ21 light chain group into eight subgroups, each subgroup being defined as a set of V_κ21 chains that share certain amino acid residues between positions 1 and 96. The closely related V_κ21B and -C subgroups are defined by the residues indicated in Fig. 1, and V_κ21C in turn can be distinguished from V_κ21B by its own characteristic set of amino acid residues (12, 21, 22). All of the H36 V region sequences completed so far contain the V_κ21C-specific residues as well as the residues shared by the V_κ21B and V_κ21C subgroups.

These sequence data confirm the serological results in demonstrating that the H36 V_κ regions are members of the V_κ21C subgroup. The prototype sequences of the V_κ21 subgroups were originally defined by recurrent sequences. For example, the V_κ21C prototype sequence ($V_\kappa 21C^0$) is the sequence shared by 6 of the 12 V_κ21C light chains from plasmacytomas and the anti-HA hybridoma light chain H2-6C4 (Fig. 1). Prototype sequences are thought to be encoded by the *V_κ21* germ-line genes, a conclusion confirmed by the DNA sequence of several *V_κ21* genes, including the authentic *$V_\kappa 21C^0$* (20). The H36 V_κ sequences each differ at multiple residues from the $V_\kappa 21C^0$ prototype sequence. Since each of the H36 V_κ sequences share the V_κ21C subgroup-specific residue(s), the substitutions found in the H36 sequences must have resulted from somatic mutation of the *$V_\kappa 21C^0$* gene. The only other possibility, namely that these sequences are mutants of a second "*$V_\kappa 21C$*-like" germ-line gene, is unlikely, as none of the *V_κ21* germ-line genes defined to date (20, 21) resembles such putative *$V_\kappa 21C$*-like gene products. Furthermore, independently isolated *$V_\kappa 21C$* genes in either the unrearranged or aberrantly rearranged form code exactly for *$V_\kappa 21C^0$* (20, 23). Such surveys, being independent of light chain expression and hence free of constraints due to selection, should have yielded such *$V_\kappa 21C$*-like genes. Hence, we believe the H-36 κ chains are somatic mutants of the *$V_\kappa 21C^0$* germ-line gene.

κ Light Chain Gene Rearrangements of the H36 Hybridomas. A relationship between certain H36 hybridomas has been established from the nature of rearrangements at the κ light chain locus of these cell lines. These rearrangements are detectable by Southern blot analysis of hybridoma DNA digests using as probes segments of DNA near J_κ (IVS and

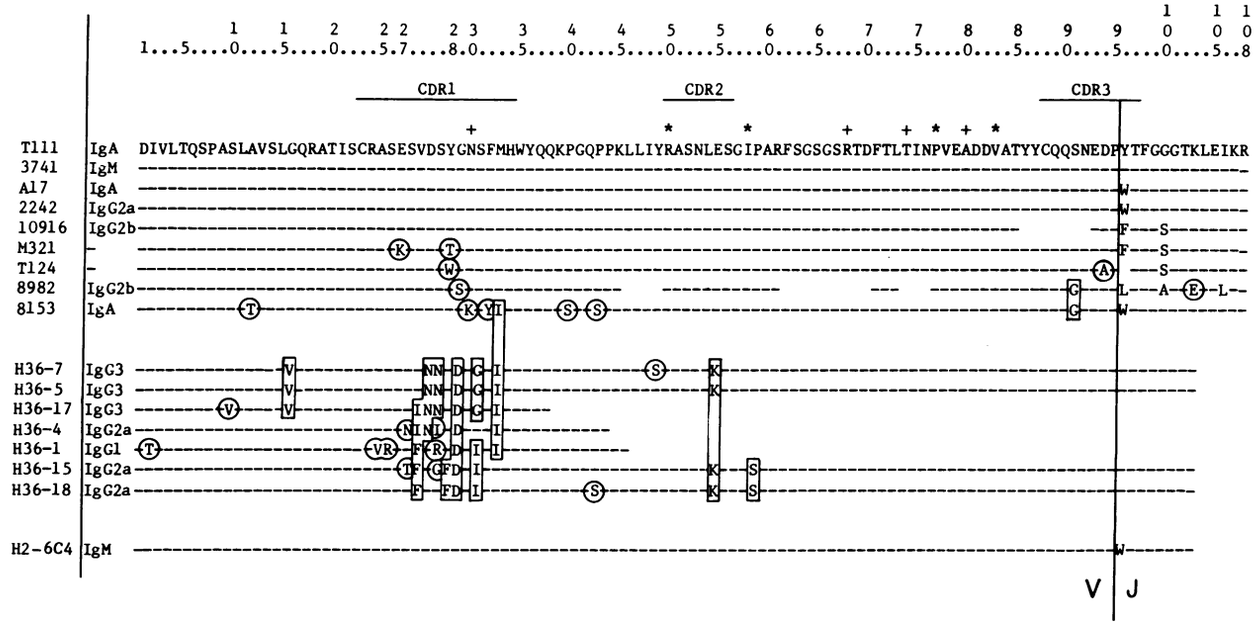

FIG. 1. Amino-terminal sequences of the κ light chains from H36 anti-HA (Sb) hybridomas and V_κ21C-expressing plasmacytomas. The T111 sequence (12), noted by the one letter code of Dayhoff (19), is identical to the V region sequence encoded by the *$V_\kappa 21C$* germ-line gene (20). Dashed lines represent amino acid sequence identities with the V_κ21C prototype sequence. Overbars delineate the regions of the κ light chain complementarity-determining (hypervariable) regions. Residues 30, 68, 74, and 80 (+) are the set of residues that is uniquely shared by the closely related V_κ21C and V_κ21B subgroups (21); residues 50, 58, 76, and 83 (*) are the set of residues that together defines the members of the V_κ21C subgroup. Encircled residues are substitutions unique to a given V_κ21C light chain; residues in boxes are substitutions shared between the light chains. Plasmacytoma sequences are taken from refs. 12, 21, and 22. M321 and T124 are Bence–Jones proteins. The PC8153 amino acid sequence is translated from the V_κ gene expressed in this plasmacytoma (unpublished data). The sequence of H2-6C4 is from ref. 12.

pR1, Figs. 2 and 3). The rearrangement bearing the productive κ light chain allele (κ^+) has been examined in *Bam*HI/*Hin*dIII double digests of H36 hybridoma DNA. Since the $V_\kappa 21C$ gene contains two *Bam*HI sites that map to amino acid residues 60 and 95 of this subgroup (20), the κ^+ rearrangement is seen as a 3- to 4-kb fragment by using the IVS probe (Fig. 2). Most of the H36 hybridomas have κ^+ rearrangements that are indistinguishable from that of the V_κ21C ($J_\kappa 2$) plasmacytoma PC3741 and clearly distinguishable from the $V_\kappa 21C$ gene rearranged to the other J_κ genes (Fig. 2). This analysis further supports the premise that the $V_\kappa 21C$ gene codes for the H36 light chains. That all examples are rearranged to $J_\kappa 2$ also supports the relatedness of this set of antibodies since $J_\kappa 2$ *per se* is not a prerequisite for HA(Sb) binding (i.e., the V_κ21C hybridoma, H2-6C4; Fig. 1). However, the *Bam*HI/*Hin*dIII rearrangements of two H36 hybridomas are not exactly the same: H36-17 is slightly larger and H36-7 is slightly smaller than the characteristic $V_\kappa 21C$-$J_\kappa 2$ rearrangement. The reason for the larger size of H36-17 is a point mutation that obliterates the *Bam*HI site coding for residue 95 (unpublished data). The reason for the smaller size of H36-7 is not known.

This analysis (Figs. 2 and 4) also identifies the silent κ light chain allele. Typically this allele is either unrearranged (κ^0) or aberrantly rearranged (κ^-). Three of the H36 hybridomas have indistinguishable κ^- alleles (H36-15, -1, and -4). Two examples (H36-5 and H36-18) have unique κ^- alleles, and two examples (H36-7 and -17) may have κ^0 alleles or may have lost the κ^- chromosome. κ^- alleles in hybridomas and plasmacytomas of independent origin are usually on different-size IVS-positive fragments (24, 25). That the κ^- rearrangements are of the same size in the H36-15, -1 and -4 hybridomas is significant because, in a survey of 75 plasmacytomas and hybridomas of independent origin, no three examples chosen at random had the same size κ^- fragment (unpublished data).

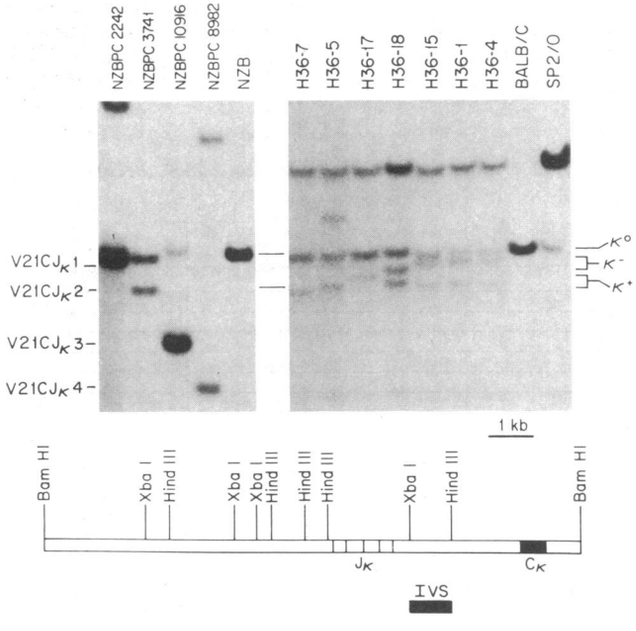

FIG. 2. κ light chain rearrangements in the H36 hybridomas and plasmacytomas. Southern blots of *Bam*HI/*Hin*dIII-digested DNA were hybridized with the IVS probe. (*Left*) Comparison of V_κ21C plasmacytomas in which each plasmacytoma $V_\kappa 21C$ gene has been shown to be joined to a different J_κ gene by amino acid sequence (21, 22). (*Right*) Comparison of the H36 hybridomas. The κ^+ allele from H36-1, -4, -5, -17, and -18 was identified by the DNA sequence of this rearrangement (unpublished data). The κ^+ rearrangement usually corresponds in size to the $V_\kappa 21C$-$J_\kappa 2$ rearrangement of the plasmacytoma, NZPC 3741. The κ^0 fragment is identified by comparison to the BALB/c embryo DNA digest. The κ^0 fragment is observed in all DNAs in part because of host tissue contaminating these *in vivo* grown hybridomas and plasmacytomas.

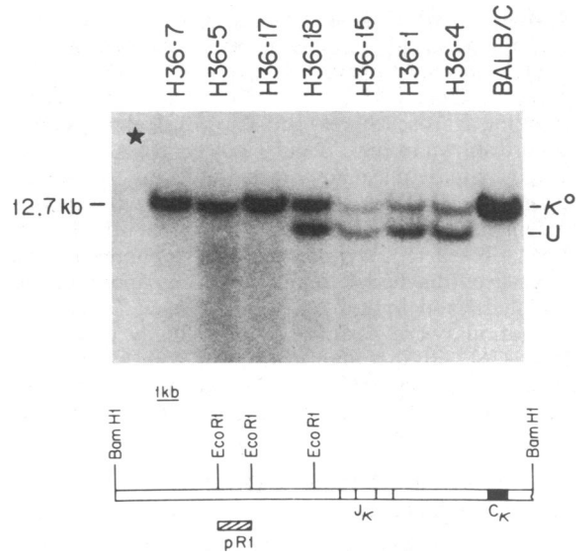

FIG. 3. Detection of upstream (U) κ light chain gene segments in the H36 hybridomas. Southern blot of *Bam*HI-digested DNA from hybridomas hybridized with the pR1 probe. All hybridomas share a 12.7-kb band corresponding in size to the κ^0, *Bam*HI fragment that includes upstream DNA. This could originate from the H36 hybridomas and/or contaminating host tissue in these *in vivo* grown cell lines. H36-1, -4, -15, and -18 share a unique band (labeled U) hybridizing with the pR1 probe. The star denotes a faint, reproducible fragment in the H37 hybridoma.

The DNA upstream of J_κ in the H36 hybridomas was analyzed with the pR1 fragment as a probe (Fig. 3). This region of the κ locus is often retained in hybridomas and plasmacytomas and is found in *Bam*HI fragments of a unique size relative to the germ-line fragment (24). Four of the H36 series (H36-18, -15, -1, and -4) have indistinguishable upstream elements. H36-7 has a unique upstream element. This is seen as a faint band (marked by a star in Fig. 3). Because the hybridization to this fragment is weak, we believe this may be due to a contaminating subpopulation of cells in the H36-7 line. As in the case of κ^- fragments, the equivalent size of upstream elements is a significant feature of this set of hybridomas as compared to plasmacytomas and hybridomas of independent origin.

For comparison, other PR8 HA-specific hybridomas have been analyzed (unpublished data). These include examples typed as V_κ21C but from different fusions and non-V_κ21C examples from the H36 fusion. The size of the κ^+ rearrangements of these V_κ21C examples is consistent with a $V_\kappa 21C$ rearrangement to J_κ gene segments other than $J_\kappa 2$, whereas non-V_κ21C examples do not coincide with any $V_\kappa 21C$-J_κ rearrangement. κ^- and upstream fragments are usually of different sizes in independently derived examples. Certain hybridomas derived from the same fusion do share κ^- and upstream fragments (H37-63 and H37-77 or H36-101, -12 and -6; ref. 9) as in the case of the examples from the H36 V_κ21C series.

DISCUSSION

In adult BALB/c mice, the secondary antibody repertoire to the influenza virus HA is large. Based on the repeat frequency of isolation of paratypically indistinguishable hybridoma antibodies from different mice, one can estimate that BALB/c mice are able to produce at least 300 different anti-

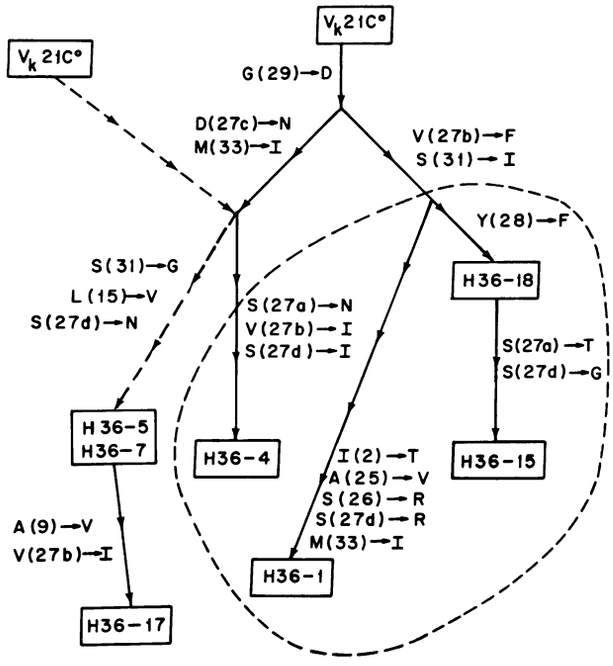

FIG. 4. Genealogical relationship of H36 hybridoma V_κ21C light chains. This tree shows the descent of the V_κ21C chains from the germ-line *V_κ21C* gene. The distances are proportional to the number of amino acid replacements from this germ-line sequence. Only the sequence to residue 33 (Fig. 1) has been used; hence, H36-5 and H36-7 are not separated. Further amino acid sequence analysis does separate this pair. This tree requires the fewest independent parallel replacements. In this case, valine-27b (V_κ21C^0) to isoleucine (H36-4 and -17) and methionine-33 (V_κ21C^0) to isoleucine (H36-1 and -4) are assumed to have occurred independently. It is interesting to note that the former substitution has occurred twice and the latter has been observed once in V_κ21 light chains from plasmacytomas. None of the other H36 substitutions have been seen in other V_κ21 variant chains. The set encircled are hybridomas thought to be related because all four have an indistinguishable upstream element and three of these (H36-1, -4, and -15) have an indistinguishable κ^- rearrangement. H36-5, -7, and -17 may be of independent origin as indicated by the dashed lines.

bodies binding to the antigenic site, Sb, on the HA (9). To examine how this diverse repertoire is generated, we have initiated a structural analysis of HA (Sb)-specific hybridoma antibodies that had been generated from a single donor mouse, H36. The seven H36 hybridomas were chosen on the basis of a previous serological analysis, which indicated that these antibodies used a κ light chain of the V_κ21C subgroup and formed a closely related set whose individual members, however, could be differentiated from each other by paratypic and/or idiotypic analysis (9). The amino acid sequences of these κ chains confirms that all belong to the V_κ21C subgroup. Further, the size of the DNA endonuclease fragment bearing the productive V_κ gene shows that all examples have rearranged the *V_κ21C* gene to the *J_κ2* gene segment. However, the V_κ sequences of the H36 κ light chains differ from each other and from the gene product of the *V_κ21C* germ-line gene. Hence, we believe that the diversity within this set arises by somatic mutation.

The extent and pattern of amino acid replacements is remarkable. The H36 V_κ21C regions that are nearly completely sequenced have either seven or eight replacements compared to the V_κ21C germ-line sequence. This is more than the average number of substitutions (*ca.* 1) observed in a survey of λ light chains or V_κ21 chains produced by mouse plasmacytomas (3, 21). The pattern of variability is also unusual in that many of the amino acid substitutions are shared between the members of this set. Such parallel replacements are infrequent among V_κ21 or V_λ variants. This large number of common replacements could result from independent antigen selection for particular V_κ21C light chain sequences that are required (in conjunction with certain V_H regions) for the formation of HA (Sb)-specific antibody combining sites. However, similar substitutions have not been observed so far in V_κ21C chains of anti-HA (Sb) antibodies from hybridomas of independent origin (H2-6C4, Fig. 1; unpublished data). Nevertheless, we cannot formally exclude this possibility because the isolated H chain of H36-15 could be shown to physically associate with an isolated V_κ21C^0-J_κ2 chain (PC 3741), yet failed to produce HA (Sb)-binding antibodies (unpublished data).

An alternative explanation for the common replacements is that these H36 hybridomas originated from just one or two lymphocyte precursors and that the shared substitutions represent the sequential accumulation of mutations in their progeny. One possible genealogical relationship of the H36 κ light chains to the ancestral *V_κ21C*^{0} germ-line gene is shown in Fig. 4. In this model the H36 sequences initially diverged from V_κ21C^0 by the glycine-to-aspartic acid substitution at position 29. The branches of this tree represent substitutions acquired at early generations, and each κ chain has a different terminus because all H36 κ chains have one or more unique substitutions. By this model, the number of independent, parallel substitutions is reduced to two examples.

That at least some of these H36 fusants may stem from a common precursor is suggested by the nature of rearrangements at the κ locus other than the productive *V_κ21C-J_κ2* rearrangement. Two sorts of rearrangements have been analyzed. One type is of the DNA upstream of the J_κ locus. This DNA is often retained in lymphocyte lines but in a unique context compared to germ-line DNA. As such rearranged DNA is the result of V_κ-to-J_κ recombination, the size of upstream fragments is diverse among plasmacytomas and hybridomas of independent origin (24). The second type of κ light chain rearrangement inspected is at the silent κ allele. Typically this allele is either unrearranged (κ^0) or aberrantly rearranged (κ^-) (25). Since the κ^- rearrangement often results from abortive V_κ rearrangements (23), these too are of diverse sizes in cell lines of independent origin. Hence, these types of rearrangements, when of identical size in cell lines, may have been inherited from a common ancestral lymphocyte.

Four hybridomas (H36-1, -4, -15, and -18) have indistinguishable rearranged upstream DNA; of these, three (H36-1, -4, and -15) have indistinguishable κ^- rearrangements. H36-18 has a slightly smaller κ^- rearrangement; this may be a different κ^- form or a secondary deletion of the κ^- shared by H36-1, -4, and -15. The other H36 hybridomas (H36-5, -7, and -17) do not share these upstream DNA or κ^- rearrangements. Thus, by these criteria, we believe that at least H36-1, -4, -15, and -18 may be related; H36-5, -7, and -17 may be of independent origin(s), but their relatedness to each other is strongly suggested by shared amino acid substitutions. It is also possible that all seven examples are related through a common precursor but that mutations at restriction sites or rearrangements affecting upstream DNA or the silent κ allele occurred during subsequent cell divisions.

From this evidence of relatedness between at least two subsets of the H36 hybridomas, we favor the hypothesis that amino acid substitutions shared by the V_κ regions of a subset represent the sequential accumulation of somatic mutations. By this interpretation certain features of somatic mutation emerge:

(*i*) Somatic mutation is an ongoing process occurring over many generations and during different stages of lymphocyte differentiation. Since the lineage comprising antibodies H36-1, -4, -15, and -18 (Fig. 4) includes examples with the same

heavy chain isotype (γ2a) (Fig. 1), the mutational differences between these must have occurred after the switch from IgM to Ig2a expression. This lineage also includes examples with different isotypes (γ1 or γ2a, Fig. 1). As the switch from IgM to distinct isotypes appears to occur in a single step (μ to γ1 and μ to γ2a) (26), the mutations shared by these examples must have occurred prior to the isotype switch.

(*ii*) The rate of somatic mutation seems to be high. If we assume that a precursor to these H36 mutants was selected during the primary immunization with PR8 because of the common glycine-to-aspartic acid substitution at position 29, then 6 to 7 replacements accumulated at the time of fusion (24 days after primary immunization). If these lymphocytes were dividing continuously with a generation time of 18 hr, the mutation rate would be in the range of 10^{-3} per base pair per generation. This estimate of a high mutation rate is consistent with the observations of Scharff and co-worker on the V_H of the plasmacytoma S107 (8).

(*iii*) The somatic mutations leading to amino acid replacements are clustered and are found mainly in complementarity-determining regions. In this respect the pattern of variability is similar to that found in V_λ and V_κ21 plasmacytoma light chains, but the number of replacements is more extensive. To explain the pattern of variability in V_λ, it was proposed that antigen may act in the selection (selective expansion) of B cells expressing mutated immunoglobulin receptors that fit the antigen (3). The marked clustering of replacement mutations in complementarity-determining regions of the light chains of the H36 hybridoma antibody set agrees with this proposal and further suggests that antigen selection acts on sequentially arising single-point mutations throughout the development of a B-cell lineage. The few mutations in framework regions also may play a role in modifying antibody specificity or else may have been coselected along with complementarity-determining region replacements. The latter could occur if the rate of mutation of these genes is high.

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