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Immunology

Complete amino acid sequence of light chain variable regions derived from five monoclonal anti-*p*-azophenylarsonate antibodies differing with respect to a crossreactive idiotype

(lymphocyte hybridoma/idiotypy)

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ABSTRACT **The induced antibody response to the hapten *p*-azophenylarsonate in the A/J mouse has provided a model system for the detailed examination of a heritable crossreactive idiotype and its fine structural and serologic analysis. While earlier studies used the apparent homogeneity in the serum response for structural studies, a more complete understanding of the arsonate idiotypic system became possible with the development of monoclonal antibodies differing with respect to these determinants. Five monoclonal antibodies, four crossreactive idiotype positive and one crossreactive idiotype negative, were selected for complete amino acid sequence analysis. The sequences of the light chain variable regions of these molecules are presented here. The data indicate considerable sequence divergence of the monoclonal light chains from the serum light chains. However, there is a striking degree of homology among the monoclonal light chains regardless of the idiotype character of the parent molecule. Although minor variations are apparent throughout the variable regions, the joining regions are identical among light chains in all of these anti-arsonate antibodies. A particularly notable focus of variation is found at positions 92 and 93 in the third hypervariable region. The possible role of this region in the contribution of the light chain to the arsonate crossreactive idiotype is discussed. These data are consistent with the concept that the anti-arsonate monoclonal light chains originate from the joining of a specific J_κ gene segment to a single germ-line V_κ gene segment. These coding segments are likely further subject to a variety of somatic alterations that generate the modest sequence diversity found among the final protein products.**

While subserving its primary role of recognizing antigen, the antibody molecule can also display to an immune system its own array of antigens in immunogenic form. Those determinants, which are unique to that antibody and provoke an immune response, define an idiotype (1, 2). When, as first reported for cold agglutinins (3) and anti-gamma globulins (4), these unique determinants are found on antibody populations other than those produced by the individual that defined them, the anti-idiotype used defines a "crossidiotypic specificity" (3). A considerable body of data now supports the notion originally proposed by Jerne (5) that idiotypic determinants can serve as pivotal targets for the regulation of the immune response.

In the A/J mouse, the induced antibody response to the hapten *p*-azophenylarsonate on haptenated KLH has a predominant homogeneous component (20–70% of the serum response) bearing the serological determinants of the crossreactive idiotype (CRI) as defined by an appropriately absorbed rabbit antiserum (6). This homogeneity lends itself particularly well to both genetic and structural analysis. By using standard inbred strains of mice, the transmission of the arsonate idiotype behaves as a single gene inherited in simple Mendelian fashion and is linked, as other variable region markers, to the heavy (H) chain constant (C) region locus (7). Mapping to the H chain alone presented a paradox in view of our current understanding that the light (L) chain is also required for the expression of the idiotypic determinants. However, recent genetic studies using the relatively few mouse strains carrying a defined kappa light chain polymorphism (8, 9) demonstrate the requirement for an appropriate kappa chain gene complement for the formation of antibodies bearing the crossreactive idiotype (10, 11). Thus, complete understanding of the nature of the inherited crossreactive idiotype in the arsonate system will require detailed analysis of *both* H and L chain structures.

The definition of the structural correlate(s) of the CRI has been a major goal in our laboratory for several years. While initial studies centered on the serum molecules (12–16), a more detailed analysis of the range of the anti-arsonate response in both CRI^+ and CRI^- sets required clonal analysis, made feasible by somatic cell fusion techniques. NH_2-terminal studies on the resulting monoclonal anti-arsonate antibodies revealed a surprising degree of heterogeneity among CRI^+ molecules in both H and L chains, providing a striking contrast to the homogeneity of the serum sequences (17–20). This heterogeneity was corroborated by serological analyses demonstrating that, although each idiotype-positive hybridoma antibody expressed the CRI, albeit variably, each was also serologically unique (21). Accordingly, the interaction between a rabbit anti-idiotype to a monoclonal antibody, prepared in an analogous fashion to the serum anti-CRI (6), and the monoclonal antibody against which it had been prepared, generally could not be inhibited extensively by any other hybridoma antibody, whether CRI^+ in character or not, indicating the individuality of each of these molecules. In a heterologous system that measured the interaction between one CRI^+ hybridoma product and an anti-idiotype prepared against another, any monoclonal antibody defined as CRI^+ could inhibit (22). Thus, all CRI^+ antibodies share idiotypic determinants. Therefore, the capability of an inbred strain to produce different antibodies, constrained by the sharing of both specificity for antigen and idiotype, appears immense. The mechanism through which this capability is manifested remains a major dilemma.

To determine a structural basis for the CRI and the degree of variability within which this entity can fluctuate, the commonality among these heterogeneous molecules can be sought in the primary structure. To this end, the amino acid sequences

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Abbreviations: CRI, crossreactive idiotype; H and L, heavy and light (chain), respectively, and C, V, J, and D, constant, variable, joining, and diversity (region), respectively, of the immunoglobulin molecule.

of the variable (V) regions of the light chains derived from four CRI^+ hybridomas and one CRI^- hybridoma were determined.

MATERIALS AND METHODS

Preparation and Isolation of Hybridoma Antibodies. Hybridomas secreting A/J anti-arsonate antibodies were prepared as described (16–18). Ascites containing large amounts of monoclonal anti-arsonate antibodies (2–5 mg/ml) were purified by passage through an affinity column of arsonate-derivatized human gamma globulin conjugated to Sepharose 4B (23).

Idiotypic Characterization. The idiotypic characterization of these molecules has been described (18, 21, 22). Serological analysis of idiotypic determinants was performed as described (6) in a competition assay using ^{125}I-labeled A/J serum anti-arsonate antibodies as ligand for the rabbit anti-CRI and inhibiting with hybridoma supernatants. Those hybrid cells producing antibodies capable of inhibiting this interaction by at least 50% are classified as CRI^+. The amount of hybridoma required for this inhibition was 10 to 2900 ng. Subsequent characterization of these hybridoma antibodies included more extensive serologic studies (22, 23). Briefly, anti-idiotypic reagents were prepared in rabbits against several of the CRI^+ hybridoma antibodies. Absorption of these was performed as in the production of serum anti-idiotype (6). A competition assay was designed as described above but using labeled monoclonal antibody as ligand. Inhibition of the association of the ligand with anti-idiotype reagents prepared against any CRI^+ hybridoma was then measured by using hybridoma antibodies as competitors (18, 21, 22). In a representative experiment, CRI^+ monoclonal antibodies were added to inhibit the 93G7 anti-93G7 interaction. Only the homologous 93G7 appreciably inhibited, highlighting the individuality of 93G7. However, in an assay system using a heterologous combination (e.g., 93G7 as ligand and anti-123E6 as indicator) any CRI^+ hybridoma inhibited equally well, emphasizing the commonality among these molecules. Thus, these serological studies showed features that were *shared* and features that were *unique* to this set of antibodies.

Preparation of Isolated Chains and Peptides. Purified anti-arsonate antibodies were completely reduced, alkylated, and [^3H]carboxymethylated in the presence of 7 M guanidine·HCl. H and L chains were then separated by Sephadex G-100 gel filtration in 5 M guanidine·HCl.

L chain peptides were generated by tryptic digestion of citraconylated material or by chymotryptic digestion. Peptides were then purified by a combination of gel filtration chromatography on Sephadex G-50 or G-75 and high-voltage paper electrophoresis when appropriate (24).

Automated Amino Acid Sequence Analysis. Automated sequence analysis was performed using a Beckman 890C amino acid sequencer, modified by the addition of a cold trap (25), with dimethylallylamine or 0.1 M Quadrol programs and the nonprotein carrier Polybrene (26) as described (12). The phenylthiohydantoin amino acid derivatives were identified by gas chromatography (27), high-pressure liquid chromatography (26), and amino acid analysis after back hydrolysis with hydriodic acid (28).

RESULTS AND DISCUSSION

Amino acid sequence studies of serum antibodies induced in A/J mice with KLH-arsonate showed that, although the CRI^+ V_H region was entirely homogeneous, the CRI^+ L chain contained positions within framework segments at which minor heterogeneity of amino acids was evident, indicating that multiple V region frameworks could be associated with identical hypervariable regions (14). This apparent sharp restriction in the serum response implied that one or a very few antibodies bore the entire set of determinants making up the CRI. Therefore, the degree of heterogeneity found subsequently among monoclonal antibodies was unexpected.

Anti-Arsonate Hybridomas Are Serologically Distinct. The serological heterogeneity of the antibodies for which primary sequences are presented here is shown in Table 1 (18). Additional serologic characterization is presented elsewhere (21, 22). The amount of each hybridoma required to inhibit the interaction between radiolabeled A/J CRI containing anti-arsonate serum and its rabbit anti-CRI ranges from ≈10 ng for R16.7 and 93G7, comparable with the inhibition obtained by A/J anti-arsonate serum, to 2900 ng for 124E1. Hybridoma product 91A3 did not appreciably inhibit even in quantities in excess of 20,000 ng. Comparison of molecules at the extremes of scoring in the idiotype assay should provide maximum information toward the structural definition of these idiotypic determinants.

Anti-Arsonate Hybridomas Differ Dramatically from the Pooled Serum Sequence. The sequences of the L chain V regions derived from four CRI^+ and one CRI^- monoclonal antibody are shown and compared with the major sequence of the CRI^+ serum L chain in Fig. 1. The most striking feature of this comparison is the marked differences between the CRI^+ monoclonal L chains and their serum counterpart. These differences occur in hypervariable as well as framework regions. The total number of positions at which any differences from the serum sequence are found is 30 out of 108 V region residues. Of the nine positions previously found to vary in the serum sequence (14) only position 76, containing a serine in the minor sequence, corresponds to that found in the monoclonal chains. Thus, the sampling of L chains derived from hybridoma cell lines differs substantially from the L chains selected in response to antigen challenge *in vivo*. These apparent differences may be attributable to the various immunization regimens used to obtain the two sets of antibodies or to a selectivity in the fusion process itself.

Anti-Arsonate L Chains from CRI^+ Serum Antibodies and from Anti-Arsonate Hybridomas Possess Identical Joining (J) Regions Including a V/J Junctional Position Possibly Generated as a Result of Somatic Recombination. An important aspect of the *similarities* between the monoclonal and serum molecules is that, although consideration of the distribution of the differences between the two sets of molecules shows variation in frameworks 1, 2, and 3 and hypervariable regions 1, 2 and 3, framework 4, or the J region, is completely identical among all anti-arsonate L chains sequenced to date, including the L chain of the CRI^- molecule, 91A3. In addition, there is no other continuous 14-residue variable region stretch shared by both serum and monoclonal L chains. In this regard, it is

Table 1. Inhibition of binding of radiolabeled A/J anti-arsonate to its rabbit anti-idiotype by purified hybridoma products (18)

Unlabeled inhibitor	Amount giving 50% inhibition, ng	% inhibition by 2000 ng
A/J anti-arsonate	15	101
HP R16.7	9	94
HP 93G7	12	90
HP 123E6	50	58
HP 124E1	2900	48
HP 91A3*		4

Assays used 10 ng of ^{125}I-labeled specifically purified A/J anti-arsonate antibodies and slightly less than an equivalent amount of anti-idiotype.
* 50% inhibition not achieved with 20,000 ng.

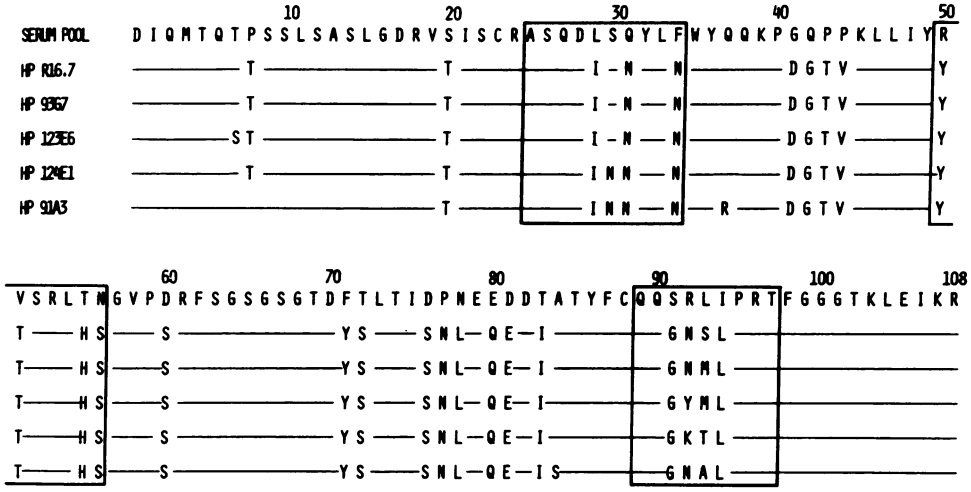

FIG. 1. Anti-arsonate hybridoma light chains. Comparison of amino acid sequences of light chains from CRI$^+$ and CRI$^-$ hybridomas with that of light chains from CRI$^+$ serum antibodies. —, Identical residues. Hybridoma light chains are given in order of relative capacity of their parent molecules to inhibit the reaction between A/J serum anti-arsonate and its rabbit anti-idiotype (see Table 1). Hypervariable regions are outlined. A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys, L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

relevant to note that the J region is encoded as a separate genetic element in the DNA (29, 30).

Evidence from DNA sequence analysis indicates that there are only four J_κ segments that can be used to assemble productive L chains (31). The four J_κ sequences found in BALB/c DNA and known to be represented in BALB/c antibodies are shown in Fig. 2*a*. The last line represents the amino acid sequence of the L chain J region of the anti-arsonate antibodies. This sequence shows identity with both J1 and J2 from positions 97–108. The A/J anti-arsonate L chains, therefore, could draw from one or both of these two J regions. Because new codons can be generated as a result of splicing at the junction of gene segments (31–34), which of these two J segments is actually used to form anti-arsonate L chains, can be deduced. Fig. 2*b* illustrates how an arginine codon can be generated from a somatic recombination event between the first base of the triplet just 3′ to the V region encoding sequences (CCC) and the second base of the 5′ triplet (TGG) of the J1 segment encoding its first amino acid (tryptophan) to yield CGG, a triplet for arginine. No recombination involving the first codon of J2 can give rise to an

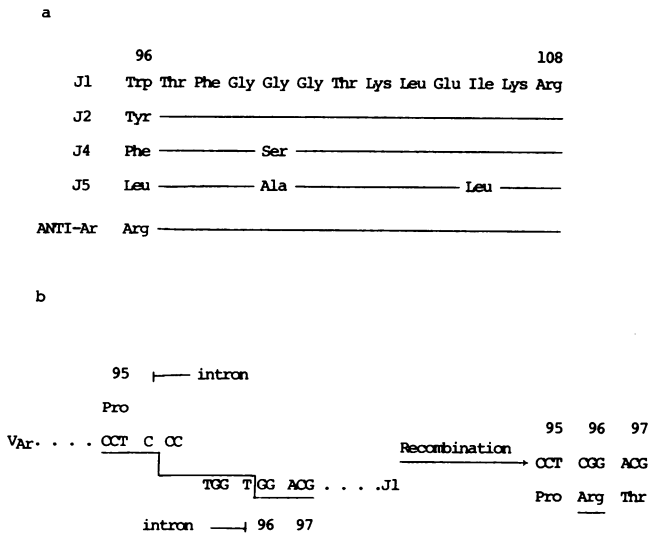

FIG. 2. Generation of anti-arsonate light chain J region. (*a*) Amino acid sequences of four germ-line BALB/c J_κ gene segments obtained from DNA sequences (29–31) and of all anti-arsonate (ANTI-Ar) J_κ regions. —, Identical residues. (*b*) Suggested mechanism for the formation of arginine at position 96 from a splicing event during V/J joining. Hypothetical V_{Ar} is taken from MOPC 41 DNA sequence (31). Numbers correspond to amino acid positions in mature light chains.

arginine at position 96. Thus, it is likely that J1 is used to assemble anti-arsonate L chains. This mechanism is hypothetical, particularly to the extent that, of necessity, it borrows from the known BALB/c DNA sequences, as no information regarding this DNA locus is available for the A/J mouse. Another explanation for the arginine at position 96 is that A/J has a polymorphism in one of its J segments. However, MOPC 173, derived from a BALB/c plasmacytoma (figure 4 of ref. 35), also has an arginine at position 96, arguing against this alternative and suggesting that a similar splicing mechanism may be operative in both strains.

The L Chains Derived from Anti-Arsonate Hybridomas Are Structurally Similar. In Fig. 3, the monoclonal L chains are compared with each other. Perhaps the most important aspect of these comparisons, when considered in contrast to the variation when they are compared with the serum sequences (Fig. 2), is their near identity, regardless of the history of the cell line producing the antibody. R16.7 originated in the laboratory of A. Nisonoff at Brandeis University, while the other four antibodies were produced in our laboratory (17). Among four independently derived arsonate-binding CRI$^+$ L chains, the V regions vary by as many as four amino acids (123E6 vs. 124E1) and as few as one (93G7 vs. R16.7). None of the sequences show complete identity, in spite of their strict restriction to a single κ subgroup, $V_\kappa 10$ (36).

There Is a Correlation Between L Chain Structure and Serological Characteristics of the Anti-Arsonate Hybridomas. It is noteworthy that the L chains originating from CRI$^+$ hybridomas rank roughly in their order of positivity for idiotype according to the number of amino acid differences found between them. The two most idiotype-positive molecules, 93G7 and R16.7 (Table 1), inhibit to the same extent in the idiotype assay at $\approx$10 ng. These V regions differ at only a single position, 93. 123E6, intermediate in CRI activity, requires 50 ng to inhibit the idiotype assay by 50% and differs from R16.7 and 93G7 at two and three positions, respectively. The CRI$^+$ 124E1 requires 2900 ng to inhibit the assay and differs from the other three L chains at three or four sites in the V region. Thus, the ordering of these L chains by the extent of amino acid differences follows precisely the ordering of idiotypic character of their parent molecules—i.e., R16.7, 93G7, 123E6, and 124E1. 91A3, a CRI$^-$ molecule also conforms to this hierarchy in that it varies from R16.7 at five positions. The distribution of these differences is dispersed throughout the V region. The most intriguing differences are found in the third hypervariable region. Positions 92 and 93 are the two successive sites in the V regions where variability is most striking. Three alternative amino acids

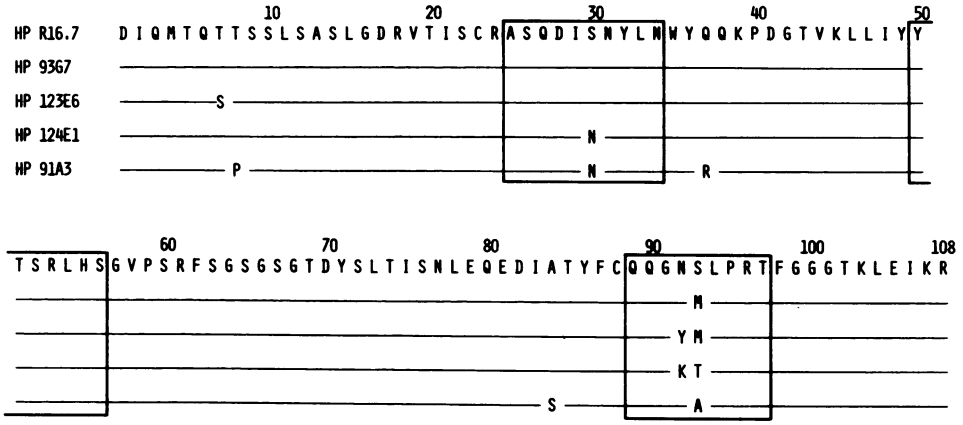

FIG. 3. Anti-arsonate hybridoma light chains. Comparison of amino acid sequences of the light chains from CRI$^+$ and CRI$^-$ antibodies with that of the light chain of the CRI$^+$ hybridoma R16.7. —, Identical residues. Hypervariable regions are outlined. For the single-letter code for amino acid residues, see the legend to Fig. 1.

are present at these two positions, contrasting with the other two variant positions, 7 and 30, where one molecule had a variant, the other three being identical. In addition, the argument made for the correlation of idiotype positivity and overall sequence variation holds for these two positions alone. That is, 93G7 and R16.7 have a single difference at position 93, 93G7 and 123E6 have a single difference at position 92, and 124E1 differs from the other three chains at both sites. This suggests that position 92 makes the most critical contribution to the idiotypic character in the L chain. The proteins 93G7 and R16.7 behave nearly identically in the idiotype assay and share in common an asparagine at position 92. 123E6 and 93G7 have a tyrosine-methionine exchange at position 92 but are identical at position 93; however, they vary substantially in their degree of idiotype reactivity. 124E1 differs from all other chains at both positions but at 92, lysine is distinctive from asparagine or tyrosine in that it has a longer side group and is positively charged. These observations can account for the more marginal idiotypic behavior of the 124E1 molecule. Thus, this condition is entirely consistent with a vital role for the third hypervariable region, particularly position 92, in the modulation of the idiotype in this system. It should be stressed, however, that much more structural data must be gathered for the H chains and for CRI$^-$ molecules. It is of related interest, though, that the diversity (D) segment, which contributes to the third hypervariable region of the heavy chain, is thought to control the private idiotype in dextran-binding antibodies (37).

These data imply that the 91A3 L chain, though derived from a CRI$^-$ molecule, is itself CRI$^+$ in nature. At the proposed crucial positions, 92 and 93, 91A3 has an asparagine-alanine which is similar to the strongly idiotype-positive R16.7, whose L chain sequence is asparagine-serine, or 93G7, which has the sequence asparagine-methionine at these positions. Since 91A3 contains an asparagine at position 92, characteristic of the L chains derived from the most CRI$^+$ molecules, the 91A3 L chain may very well be idiotype positive. This interpretation is consistent with recent experiments suggesting serologic similarity of L chains from CRI$^+$ and CRI$^-$ molecules (38).

When compared overall with the CRI$^+$ L chains, 91A3 differs from R16.7, 93G7, and 124E1 at five positions and from 123E6 at seven. Three of the 91A3 variations are framework changes that were invariant in the other monoclonal L chains. Though these cannot be entirely dismissed to account for the lack of idiotype reactivity of 91A3, it is unlikely that they could effect a total abrogation of this serologic property. Nor is the asparagine-serine interchange at position 30, which is shared with the CRI$^+$ 124E1, likely to be significant. The absence of a proper CRI$^+$ H chain is, therefore, the most probable explanation for the lack of the arsonate crossreactive idiotype on the 91A3 molecule. The increased number of framework differences in 91A3 suggests that these substitutions favor association of this L chain with a H chain with which it more readily combines—i.e., a CRI$^-$ H chain. Obvious predictions of these conclusions can be readily tested in chain recombination experiments.

A Novel Mechanism May Generate the Diversity in the Third Hypervariable Region of the Light Chain. In any case, it is clear that the third hypervariable region of the L chain has a high degree of variability. Junctional diversity generated at the actual splice site of V/J joining, the mechanism generally invoked to explain variation in this region of the L chain, cannot adequately account for the high degree of variation at positions 92 and 93, which are quite removed from that site. The situation is reminiscent of H chains, where the D segment is encoded separately in the germ line (39). However, at least in the BALB/c κ chain locus, there is no evidence for a separately encoded D-segment analogue. Unless the A/J mouse actually has a configuration in its L chain DNA similar to that found for H chains in BALB/c mice or the DNA sequenced in the BALB/c mouse represents a sequence that was preceded by a V/D rearrangement, another mechanism must generate this diversity. An alternative possibility is that this variation is generated as a consequence of joining V and J and that, during the splicing event, nicks in the DNA occur in the vicinity of the junctional event 5′ to that site. These may be subsequently repaired in an altered form. Finally, this area may simply be subject to a higher rate of somatic mutation. The concentration of variability in a complementarity-determining region may be suggestive of a role for somatic mutation in the generation of diversity among these L chains.

The Anti-Arsonate Hybridoma L Chains in A/J Mice May Derive from a Germ-Line Gene Similar to the MOPC 173 BALB/c Gene. Another feature of the monoclonal L chains comes from comparison with previously sequenced L chains. The CRI$^+$ monoclonal hybridomas were found to share remarkable homology with one particular mouse κ chain, MOPC 173 (35) derived from a BALB/c plasmacytoma that has no known antibody activity. The similarity of the anti-arsonate L chains with MOPC 173 becomes even more apparent when characteristic regions are compared. The clusters of amino acids at 41–44 and 55–60 are unique to MOPC 173 among published κ sequences, are distinct from those found in the anti-arsonate serum sequences, and are shared among all of the monoclonal L chains. This pattern is most evocative of a BALB/c germ-line V_κ gene and its A/J allelic counterpart in the CRI$^+$ monoclonal hybridomas. Considering the apparent extent of sharing of L chain repertoires between these two strains (8, 9), this suggestion seems particularly likely. A CRI$^+$ monoclonal antibody from a C.AL20 mouse, which is now available, should aide in clarifying whether these L chains are derived from a gene allelic to the MOPC 173 gene.

Of additional interest is that, with the exception of one NZB-derived κ chain, PC6684 (40), which is quite different from the L chains sequenced here, the MOPC 173 L chain is the only other known mouse L chain containing an arginine at position 96. This implies that, if this position is generated as a junctional event in V/J joining, the mechanism is very selective for the type of V region on which it operates.

Thus, L chains associated with arsonate specificity and originating as somatic cell fusion products show heterogeneity but in a sharply delineated fashion. The V_L regions so far examined, whether belonging to molecules bearing or lacking the cross-reactive idiotypic determinants, are 93–99% homologous. Much of the identity observed among these L chains, particularly the stringent conservation in complementarity determining regions, may be largely driven by the original selection of these antibodies by their ability to bind arsonate. It is nevertheless evident that additional selective forces act to generate these molecules. They are restricted to a single V region subgroup, $V_κ$ 10, suggesting derivation from an A/J counterpart to the BALB/c V_L region gene that gives rise to the MOPC 173 L chain. They vary from that germ-line sequence by a few scattered substitutions in the V gene segment in a manner suggestive of somatic mutation. In addition, positions 92 and 93 are exceptionally variable, which may be due to hypermutability or to a novel mechanism of DNA nicking and repair as a secondary consequence of V/J joining. Finally, the arginine at position 96 consistently found in these L chains may be formed as a reproducible somatic recombination between the germ-line V gene segment and a specific J region segment.

These data are consistent with the hypothesis that L chains from these arsonate-binding hybridoma antibodies originate from a single germ-line V gene segment and one J segment. The heterogeneity found among them is more likely the result of an array of somatic mechanisms than of a selection of similar germ-line genes.

We are grateful to Dr. Pila Estess for providing the 93G7, 123E6, 124E1, and 91A3 hybridomas and their serological characterization and to Drs. Al Nisonoff and Edmundo Lamoyi for their gift of the R16.7 hybridoma. It is a pleasure to acknowledge the skilled technical assistance of Sandy Graham, Martha Danhof, and Ana Garcia and the excellent secretarial skills of Ms. Kathy Able. This work was supported by the National Institutes of Health Grant AI-12127 and National Science Foundation Grant PCM 7923480.

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