STRUCTURAL STUDIES ON INDUCED ANTIBODIES
WITH DEFINED IDIOTYPIC SPECIFICITIES

IX. Framework Differences in the Heavy- and
Light-Chain-variable Regions of Monoclonal
Anti-p-azophenylarsonate Antibodies from A/J Mice
Differing with Respect to a Cross-Reactive Idiotype*

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Homogeneous antibody populations from a variety of sources have proven extraordinarily useful in deducing the structure of immunoglobulin molecules. Serological studies of myeloma proteins, in particular, have been invaluable in delineating many aspects of normal immunoglobulin structure, and both myeloma proteins and induced antibody populations in a variety of species have been used extensively in defining and localizing individual antigenic specificities or idiotypes (1-4). Subsequently, Williams et al. (5) and Kunkel et al. (6) demonstrated shared cross-idiotypic specificities among cold agglutinin and anti-gamma globulin antibody populations isolated from genetically unrelated individuals, thus suggesting a relationship between idiotype and antibody specificity.

Amino acid sequence analysis of homogeneous antibodies has also provided investigators with a much clearer understanding of antibody molecules, particularly with regard to structure/function relationships. For example, the domain hypothesis of antibody structure was only formulated when complete primary structures could be compared (7). Compilation of sequence data from several laboratories yielded the concept of subgroups (8-10) and hypervariable regions (11-13). Affinity-labeling studies of purified antibodies have subsequently demonstrated that the hypervariable regions are those portions of the molecule that make contact with an antigenic determinant and are responsible for antigenic specificity (14, 15).

Efforts in our laboratories over the past 10 yr have been devoted to the serological and structural dissection of an induced antibody system in an attempt to understand its molecular and genetic implications. All A/J mice, when immunized with the hapten p-azophenylarsonate (Ar)1 coupled to keyhole limpet hemocyanin (KLH),

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1 Abbreviations used in this paper: anti-id(93G7), rabbit anti-idiotype against HP93G7; Ar, p-azophenylarsonate; CRI, cross-reactive idiotype; GAT, copolymer of glutamic acid, alanine, and tyrosine; HP, hybridoma product; KLH, keyhole limpet hemocyanin; PTH, phenylthiohydantoin; V, variable.
make a restricted anti-Ar response, 20–70% of which bears a cross-reactive idioype (CRI) detected by an appropriately absorbed rabbit antiserum (16). The CRI is linked to the immunoglobulin heavy-chain locus (17), and has been one of the variable (V)-region markers used to demonstrate linkage of variable and constant regions of immunoglobulin heavy chains in mice. Recently, linkage of the CRI to the kappa chain locus has been demonstrated as well (18, 19).

All structural analyses done to date indicate that the serum anti-Ar response is extremely restricted. Thus, IgG_1 CRI-positive heavy chains isolated from conventional anti-Ar antibodies exhibit a homogeneous sequence that includes their hypervariable regions (20–22). CRI-negative heavy chains are homogeneous and identical to the CRI-positive chains with the exception of their hypervariable regions, which are quite heterogeneous. CRI-positive light chains appear to be drawn from at least three different V_k-subgroups, but also have homogeneous and identical hypervariable region sequences (23–25). The CRI-negative light chains were too heterogeneous to sequence.

These results suggest that the CRI-positive anti-Ar response is under strict genetic control and that the cross-idiotypic specificity measured serologically is comprised of amino acids in the hypervariable regions.

To further explore the relationships between the CRI-positive and CRI-negative molecules and to verify the presumed homogeneity of the serum anti-Ar response, we undertook the generation of monoclonal anti-Ar antibodies by somatic cell fusion. Serologic analysis of the hybridoma antibodies indicates heterogeneity in their ability to react with a conventional anti-idiotypic reagent raised against serum anti-Ar, and suggests that each hybridoma product may possess in addition to the cross-reactive idiotypic determinant(s), unique antigenic specificities (19, 26). We have previously reported on the amino terminal amino acid sequences of two such monoclonal anti-Ar hybridomas (27, 28) and here extend those findings to an additional six molecules.

Materials and Methods

**Immunizations.** 8- to 10-wk-old female A/J mice were immunized intraperitoneally with 0.5 mg of Ar coupled to KLH in complete Freund's adjuvant (16, 27). Animals were boosted intravenously with antigen in saline on day 28, and spleens were removed for fusion 72 h later.

**Hybridizations.** Hybridizations were done by the method of Kennett et al. (29) as previously described (27). Briefly, cells from one or two spleens were pelleted together with 1 × 10^7 Sp2/0-Ag14 cells (30) in the presence of 0.2 ml of 30% polyethylene glycol 1,000 (J. T. Baker Chemical Co., Phillipsburg, N. J.) for 6 min. After dilution and washing, the cells were suspended in 30 ml of HY-HT medium (29) that contained 15% fetal bovine serum and were distributed into microplates (Costar, Data Packaging, Cambridge, Mass.) at 50 µl (~1 × 10^6 cells)/well. 24 h later, an additional 50 µl of HY-HT medium that contained twice Littlefield's concentration of aminopterin was added to each well to make a HAT selective medium (31). Fresh HY-HAT medium (29) was added at 5-d intervals, and culture supernates were changed at least twice before assaying for anti-Ar activity.

**Antisera.** Rabbit anti-mouse immunoglobulin, A/J anti-Ar, rabbit anti-CRI, and goat anti-rabbit Fc were prepared as previously described (16, 32). Anti-idiotypic antiserum against HP93G7 was prepared in a rabbit by injections of specifically purified hybridoma product. The antiserum was adsorbed by passage over two columns of Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) to which a crude fraction of normal A/

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J globulins had been conjugated by using cyanogen bromide (33). Subclass-specific rabbit anti-mouse immunoglobulin reagents absorbed for cross-reacting specificities were purchased from Litton Bionetics Laboratory Products (Kensington, Md.) for Ouchterlony analyses.

Assays. Culture supernates were assayed for anti-Ar activity by a solid-phase radioimmunoassay with polyvinyl Cooke microtiter plates (Cooke Engineering Co., Alexandria, Va.) coated with bovine serum albumin-Ar as previously described (26, 27). Anti-Ar antibodies were detected with 125I-labeled rabbit anti-mouse immunoglobulin. A competition assay for the CRI was carried out as previously described (16) with 10 ng of specifically purified 125I-labeled serum anti-Ar, rabbit anti-CRI, goat anti-rabbit Fc, and various unlabeled inhibitors, including Ar binding culture supernates and A/J serum anti-Ar. 4 of 19 anti-Ar isolates were positive for the CRI. A second competition assay was carried out with 10 ng of radiolabeled purified HP93G7, adsorbed rabbit anti-idiotype against hybridoma product (HP)93G7 [anti-id(93G7)], goat anti-rabbit Fc, and unlabeled inhibitors.

Isolation of Monoclonal Anti-Ar Antibodies. Anti-Ar-producing hybridoma cells were grown to mass culture, and aliquots were stored in liquid nitrogen. Cells from each line were injected intraperitoneally into Pristane-primed CAF1 mice at 5 × 10^6 cells/mouse. 10-14 d later, ascites fluids were drained, and anti-Ar antibodies were purified on a column of human gamma globulin-Ar coupled to Sepharose 4B (32, 33). Bound material was eluted with 0.2 M arsanilate, dialyzed exhaustively against Tris-buffered saline (pH 8.2), then against distilled water, and lyophilized.

Sequencing Methods. Purified anti-Ar antibodies were completely reduced and alkylated in 5 M guanidine HCL/0.5 M Tris (pH 8.2). Heavy and light chains were separated in the same buffer on a Ultrogel AcA34 column, 1 × 100 cm. Each peak was pooled, dialyzed against distilled water, and lyophilized. Chains were assessed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Laemmli (34). Because the hybridomas were generated by fusion with a nonsecreting cell line, no further separations were necessary. Amino terminal sequence analysis was performed on the intact individual chains as previously described with a Beckman automated sequencer (model 890C, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) (20, 22–25, 35). Phenylthiohydantoin (PTH) amino acids were identified with high-pressure liquid chromatography, gas chromatography, and back hydrolysis with subsequent amino acid analysis.

Results

Production and Characterization of Anti-Ar-producing Hybridomas. The eight monoclonal anti-Ar antibodies subjected to amino terminal amino acid sequence analysis were generated in two separate fusion experiments. In the first experiment, 21 of 54 hybrids were positive for anti-Ar activity. Because the frequency of positive wells was high, HP93G7, HP91A3, HP92D5, and HP94B10 were cloned by limiting dilution to insure their monoclonality. In the second experiment, only 39 of 268 potentially positive hybridomas exhibited anti-Ar activity. Because the frequency of anti-Ar-positive hybridomas in this experiment was only 15%, the cells were not cloned; and four of the hybrids, HP121D7, HP123E6, HP124E1, and HP123E4 were chosen for further study. Sequence analyses indicate that in terms of the anti-Ar-producing population of cells, the hybrids are monoclonal. With respect to other parameters, the hybrid cells may be heterogeneous, and each culture may contain cells derived from more than one fusion event. This should not interfere with studies performed on an isolated, specific product.

Supernates from the eight cell lines, as well as purified anti-Ar antibody from CAF1 ascites, were tested for their ability to inhibit the binding of radiolabeled, purified, CRI-positive antibodies from A/J mice to the rabbit anti-CRI reagent. As indicated in Table I, four of the hybridoma products effectively inhibit the binding of the 125I-labeled CRI-positive antibodies by 50%, although marked quantitative differences in
their ability to do so are evident. One hybridoma product, HP93G7, appears almost as effective as serum anti-Ar as an inhibitor of the CRI/anti-CRI reaction. The remaining four anti-Ar antibodies are unable to inhibit the reaction at all, even in very large amounts. By Ouchterlony analysis, all eight hybridoma products are of the IgG1 subclass.

The ability of the four CRI-positive hybridoma products, one CRI-negative hybridoma product, and A/J serum anti-Ar to inhibit the reaction between radiolabeled specifically purified HP93G7 and its autologous anti-idiotype is shown in Table II. The failure of a very large amount, 2,000 ng, of each of the other CRI-positive hybridoma products to cause 50% inhibition of the binding of anti-id(93G7) to HP93G7 indicates that all of the idiotypic determinants of protein 93G7 are not present on any other individual anti-Ar hybridoma. In addition, although serum anti-

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**Table I**

Displacement of Labeled A/J Anti-Ar from Its Rabbit Anti-Idiotype Antibodies By Unlabeled A/J Anti-Ar Antibodies or Hybridoma Products with Anti-Ar Activity *

| Unlabeled inhibitor | Nanograms required for 50% inhibition | Percent inhibition by 2 µg |
|---------------------|---------------------------------------|--------------------------|
| Serum anti-Ar       | 11                                    | 97                       |
| HP93G7              | 12                                    | 90                       |
| HP121D7             | 300                                   | 71                       |
| HP123E6             | 1,900                                 | 51                       |
| HP124E1             | 2,900                                 | 47                       |
| HP91A3              | †                                     | 4                        |
| HP123E4             | †                                     | 2                        |
| HP94B10             | †                                     | 0                        |
| HP92D5              | †                                     | 4                        |

* Each assay used 10 ng of 125I-labeled, specifically purified A/J anti-Ar antibodies and slightly less than an equivalent amount of anti-idiotype. † 50% inhibition never achieved.

**Table II**

Displacement of Labeled HP93G7 from Its Autologous Anti-Idiotype Antibodies By Unlabeled A/J Anti-Ar Antibodies or Hybridoma Products with Anti-Ar Activity *

| Unlabeled inhibitor | Nanograms required for 50% inhibition | Percent inhibition by 2 µg |
|---------------------|---------------------------------------|--------------------------|
| Serum anti-Ar       | 1,600                                 | 55                       |
| HP93G7              | 80                                    | 97                       |
| HP121D7             | >2,000                                | 19                       |
| HP123E6             | >2,000                                | 14                       |
| HP124E1             | >2,000                                | 15                       |
| HP91A3              | †                                     | 7                        |

* Each assay used 10 ng of 125I-labeled, specifically purified HP93G7 and slightly less than an equivalent amount of anti-id(93G7). † 50% inhibition never achieved.
Fig. 1. Anti-Ar hybridoma heavy chains. Comparison of the amino acid sequences of the CRI-positive and CRI-negative hybridoma heavy-chain-variable regions with the sequence of CRI-positive heavy chains found in serum. Identical residues are indicated by a line and only differences are noted.

Fig. 2. Anti-Ar hybridoma light chains. Comparison of the amino acid sequences of the CRI-positive and CRI-negative hybridoma light chains with the sequence of CRI-positive light chains found in serum. Identical residues are indicated by a line and only differences are noted. The major framework sequence of pooled antibody is listed on top with the minor sequence noted below for positions 3, 10, 12, and 22.

Ar antibodies were capable of causing 50% inhibition, a very large amount, 1,600 ng, was required. Thus, one or more idiotypic determinants of HP93G7 are present only at a very low concentration, or are absent, from the pooled serum antibodies.

Amino Terminal Amino Acid Sequence Analysis. Eight monoclonal anti-Ar antibodies were selected for amino acid sequence analysis. Four of these molecules bear the A/J anti-Ar CRI as defined by their ability to inhibit by at least 50% the reaction between serum or ascites CRI-positive molecules and a rabbit anti-CRI reagent. The other four molecules are negative for the idiotype. Isolated heavy and light chains from each of the eight molecules were subjected to automated sequence analysis. Each PTH amino acid was subjected to three analytical procedures, and unambiguous assignments could be made at every position. The results are shown in Figs. 1 and 2,
where the first framework segments are compared with the previously reported structures of anti-Ar heavy and light chains isolated from hyperimmunized A/J mice.

Discussion

The amino terminal framework sequences of eight individual anti-Ar antibodies obtained by somatic-cell fusion of A/J spleen cells with a nonimmunoglobulin-secreting tumor-cell line have been analyzed. The 16 chains (8 heavy and 8 light) of these hybridoma antibodies are compared with the previously sequenced heavy and light chains of anti-Ar antibodies isolated from hyperimmunized A/J mice (20, 22–25, 35). In the previous studies, the only detectable sequence differences between anti-Ar antibodies with and without the CRI lay within the hypervariable regions of the heavy chains (CRI-negative light chains were too heterogeneous to sequence). Except in mice suppressed for the CRI, CRI-positive and CRI-negative heavy chains gave a homogeneous and identical framework sequence. In addition, the heavy- and light-chain hypervariable regions of CRI-positive antibodies were homogeneous, whereas those of CRI-negative antibodies were very heterogeneous. The slight framework heterogeneity found in CRI-positive light chains was attributable to the presence of at least three different framework structures associated with identical hypervariable regions (23–25). Because the only detectable structural difference between CRI-positive and CRI-negative anti-Ar antibodies lay within the hypervariable regions, and these two types of molecules were known to differ serologically with respect to a CRI, it was of interest to us to further investigate the possible structural relationships between them.

CRI-Positive Anti-Ar Hybridoma Heavy Chains Are Similar but Not Identical to CRI-Positive Anti-Ar Heavy Chains Isolated from Serum or Ascites. Four different CRI-positive anti-Ar hybridoma heavy chains have been sequenced, and the first 30 residues are compared in Fig. 1 to the CRI-positive heavy-chain sequence previously reported (20, 22). Whereas antibodies isolated from hyperimmune A/J serum consistently gave a nearly homogeneous sequence (20, 21), clear differences from this sequence exist in the heavy chains of the hybridoma antibodies. With the exception of the Lys-Ser interchange at position 13 in HP123E6, all the CRI-positive heavy-chain substitutions could have arisen from a single base change in the DNA encoding the major (serum) sequence.

CRI-Negative Anti-Ar Heavy Chains May Either Be Very Similar to CRI-Positive Heavy Chains or Be Completely Different. The four CRI-negative heavy chains can be divided into two groups according to their amino terminal framework sequences (Fig. 1). Two of the molecules, HP91A3 and HP123E4, are clearly as related to the serum heavy-chain sequence as are the CRI-positive hybridoma heavy chains. That is, they belong to the VHI-subgroup of mouse immunoglobulin heavy chains (20). Again, however, definite differences from the serum sequence are noted in each of the chains. As with the CRI-positive chains, substitutions are found at several different positions, but appear to be more random with respect to the variant amino acid.

In sharp contrast to the serum-like sequences are two anti-Ar, CRI-negative heavy chains that bear no resemblance to any of the A/J anti-Ar molecules thus far reported (20, 21, 35, 36). Comparison with all mouse heavy chains reported to date (37) indicates that they are most like the MOPC-315 heavy chain (38). Several points are worth noting with respect to these unusual chains. First, the two chains are identical to position 30. Second, because the light-chain sequences (see below and Fig. 2) are
entirely different, the two hybridoma products arose independently and, thus, may reflect a germ-line heavy-chain sequence. Third, MOPC-315 is an IgA myeloma of BALB/c origin (39), and the finding of a strikingly similar variable region in a heavy chain from an A/J mouse may indicate a sharing of V_H repertoires between the two mouse strains.

**Variability in Anti-Ar Hybridoma Light Chains Parallels That Found in the Heavy Chains.** The sequences of both CRI-positive and CRI-negative anti-Ar hybridoma light chains are illustrated in Fig. 2, along with the sequences found in serum A/J anti-Ar antibodies that are positive for the CRI. Several striking features are apparent. First, with respect to the V_k-subgroup, the hybridoma molecules are more restricted than the CRI-positive molecules in A/J serum. Although only one heavy-chain V-region subgroup is present in the serum CRI-positive molecules, it is found in association with at least three different light-chain V-region subgroups (23–25). Six of the eight hybridoma light chains (four with the CRI and two without) clearly belong to the same V_k-subgroup as the major population of serum anti-Ar light chains, whereas none belong to the two other subgroups. As in the monoclonal heavy chains, amino acid substitutions are found in the framework portions of all of the hybridoma light chains. Whereas the framework substitutions are not as extensive as those found in the heavy chains, a preponderance of Ser and Thr is again apparent.

Perhaps the most striking observation is the Ser-Thr interchange at position 20 in all six of the light chains that belong to this subgroup. It must be emphasized that the sequence previously reported for the CRI-positive light chain was derived from antibodies isolated from a single mouse (25). When pools of CRI-positive anti-Ar light chains from several mice were analyzed, Ser and Thr were equally represented at position 20 (23). It was only in the individual animal that the major (80%) residue at this position was a Ser. Therefore, whereas it is not unexpected to find a Thr at position 20 in anti-Ar light chains, it is somewhat surprising to find it in all six hybridoma chains. A survey of all mouse kappa light chains sequenced to date indicates that all have either a Ser or a Thr at position 20, with Thr being by far the more common (70%). There are several possible interpretations for these findings. One is that all V_k germ-line genes encode a Thr (or Ser) at position 20 and that there is a directed (programmed) somatic alteration to (but only to) the alternate form. Thus, most kappa light chains will have a Thr, whereas all others a Ser. Another possibility is that the hybridoma and serum anti-Ar light chains reflect the existence of another germ-line gene that is extremely similar but not identical to that which encodes the major CRI-positive light-chain-variable region. This then would be a fourth light-chain framework associated with this cross-idiotype specificity in addition to the three previously reported (25).

A similar situation is seen at positions 7 and 8 in two of the CRI-positive hybridoma light chains. Again, when anti-Ar light chains isolated from pooled material were analyzed, Ser was found at the 15% level in position 7 and Thr at the 60% level in position 8. However, in chains from a single mouse, no Ser was found, and only 15% Thr. If each of these additional differences reflects additional germ-line genes, the number of light chains associated with CRI must be increased again. Unlike most of the substitutions found in the heavy chain, the light-chain framework differences are clearly not random, as each one is present to some extent in the serum anti-Ar antibodies of at least some mice.
In contrast to the CRI-negative serum antibodies, which are extremely heterogeneous, two of the CRI-negative hybridoma light chains appear to be drawn from the same repertoire as the CRI-positive chains. The substitutions at positions 25 and 27 in HP91A3 may represent the beginning of the first hypervariable region. The HP92D5 and HP94B10 light chains, like their corresponding heavy chains, are strikingly different from the other six light chains and from each other as well. Neither chain bears any resemblance to A/J anti-Ar light chains. In particular, neither chain has anything in common with the MOPC-315 light chains. Their origin and relevance are unknown.

Implications of the Amino Terminal Amino Acid Sequence Analysis of Anti-Ar Hybridoma Antibodies. In light of the work that has been done on the arsonate system, these results have important implications with regard to our thinking on what constitutes an idiotypically homogeneous antibody population as well as on the general concept of idiotypic inheritance. It is clear that, in the absence of trivial explanations, the CRI-positive anti-Ar antibody population in the A/J mouse, although appearing homogeneous when serum or ascites are analyzed, is not. To some extent, the microheterogeneity found in the hybridoma chains is not inconsistent with the previously reported sequences. Thus, the substitutions at positions 7, 8, and 20 in the light chains and positions 16 and 25 in the heavy chains can be found in pools of anti-Ar antibodies derived from several animals (20, 23). It is interesting to note that the Ser at position 25 in the heavy chain of the CRI-positive hybridoma product HP123E6 was only seen in the CRI-negative serum pool (20). The other substitutions found in the hybridoma heavy chains, however, have never been detected in either serum or ascites pools. As repeatedly stressed previously (20, 22–25), it is important to realize that sequencing methodology cannot detect variations of <5%. It may well be that if 100 such monoclonal antibodies were sequenced, one might find some degree of variation at virtually every position. How such molecules might arise and their relationship to one another can only be speculated upon. It does not seem unreasonable to hypothesize that, at least for the CRI-positive molecules, each derives from one or a few germ-line genes and that somatic mutation can and does operate on the framework portions of antibody molecules. This is in contrast to the situation with the variable regions of mouse myeloma lambda chains, where differences from the supposed germ-line sequence are found exclusively in the hypervariable regions (40).

Another possibility is that each of the hybridoma chains reflects a different germ-line gene. If so, there are at least two possibilities that might explain why they were not detected in earlier sequencing studies. As already mentioned, each gene product may be present at too low a level to detect; but, with differences as slight and apparently random as many of those seen here, could result in an overall homogeneous appearance. Second, it may be that whereas such minor clones exist in the A/J mouse, their expression is regulated against. When spleen cells are subjected to somatic-cell fusion, it may be that such nonexpressed clonal precursors are rescued from their regulatory environment. At this time, there is no way to discriminate between somatic vs. germ-line origins, although the situation with the light-chain sequences suggests the presence of multiple germ-line genes.

Table I indicates that whereas four of the hybridoma anti-Ar antibodies can inhibit the CRI/anti-CRI reaction, they do not do so to the same extent. This is reminiscent of the findings of Ju et al. (41), who have isolated hybridoma anti-copolymer of
glutamic acid, alanine, and tyrosine (GAT) antibodies, each of which express only a fraction of the common (C)GAT idiotypic specificities as well as having individual idiotypic specificities. A fundamental difference between the two systems, however, is the ability in the arsonate system of several monoclonal antibodies to cause >90% inhibition of the reaction between CRI and anti-CRI (19). None of the anti-GAT antibodies could inhibit serum anti-GAT >60%, probably because of the presence of at least two sets of idiotypic specificities in the serum antibody. Because of the monoclonality of the HP93G7 molecules, their ability to inhibit serum CRI 90% suggests that nearly all of the idiotypic determinants detectable by the rabbit anti-CRI can exist on a single molecule. One explanation then for the quantitative differences between the hybridoma products in the inhibition assay is that each monoclonal antibody only possesses a portion of the cross-reactive determinant(s), i.e., some, but not all, of the CRI-associated hypervariable regions. This would be consistent with the concomitant finding, indicated in Table II, that HP93G7 possesses additional idiotypic determinants not shared by any other hybridoma product tested thus far, and present at only very low levels, if at all, in serum anti-Ar antibodies. Data obtained with anti-idiotypic antisera raised against other CRI-positive anti-Ar hybridoma products indicate that many possess such individual antigenic determinants and that there is considerable serological as well as structural heterogeneity within the anti-Ar antibody population when analyzed at the monoclonal level (19). In addition, data to be published elsewhere (19) have demonstrated that the CRI may be associated with immunoglobulins of any class or subclass. In the case of CRI-positive anti-Ar antibodies found in induced A/J ascites, a large proportion of CRI-positive molecules are of the IgG1 subclass, and the IgG1 population can be purified by isoelectric focusing (33).

The finding of both shared and unique idiotypic determinants amongst monoclonal anti-Ar antibodies, combined with probable first hypervariable region identity between CRI-positive serum A/J antibodies and many hybridoma products (data not shown) plus the possibility of several heavy- and light-chain frameworks being associated with idioype-positive molecules suggests very strongly that either the germ-line anti-Ar repertoire is far greater than previously imagined or that somatic rearrangements have occurred yielding molecules with some, but not all, of the characteristics of the major CRI-positive molecules. Whether such rearrangements might occur by gene interaction (42), via more massive DNA rearrangements or posttranscriptionally, can only be speculated on at this time. Experiments are currently in progress to analyze the complete V-region structures of several of these molecules to probe the extent of the framework variation and perhaps answer these questions.

It might be argued that the amino acid substitutions in the framework segments of monoclonal anti-Ar antibodies are an artifact of the hybridoma system. The issue of somatic mutants arising in culture is one of concern, although it can tentatively be ruled out by some preliminary results. First, sequence analysis of material passaged continually in CAF1 mice and isolated 6 mo apart has given identical results. Second, repeated cloning and resequencing of myeloma proteins in the absence of any kind of selection for variants has not indicated that such mutants arise to any detectable extent (B. Birshtein. Personal communication.). It is important to note that such mutations probably do occur, but that in the absence of any selective advantage in culture, it is unlikely that such a mutant would become the dominant population. It
cannot be formally excluded that such a somatic event took place immediately subsequent to the fusion event at the one- or two-cell stage. These possibilities can and are being tested by recloning and resequencing of several independent clones.

A final point of interest concerns the two extremely unusual CRI-negative hybridoma products, HP92D5 and HP94B10. These two antibody molecules bear little resemblance to any of the induced A/J anti-Ar antibodies or to that population that arises when A/J mice are suppressed for the CRI (20–22, 35). Their origin in the A/J repertoire is unclear, although the heavy chains resemble those of the dinitrophenyl binding BALB/c myeloma MOPC-315. It may be that these two molecules are products of anti-Ar clones that are normally suppressed in A/J mice and that have been rescued by fusion.

In conclusion, it seems clear that the CRI-positive A/J anti-Ar antibodies that have appeared to be a restricted population by both the criteria of idiotypic cross-reactivity and amino acid sequence analysis may be, in fact, very heterogeneous. The extent of this microheterogeneity and its implications for the origins of antibody diversity are being explored by additional sequencing studies combined with serological analysis of the individual and cross-reacting idiotypic determinants on the monoclonal anti-Ar antibodies. These studies should lead to the precise chemical definition of both the CRI and the individual antigenic specificities associated with these molecules.

Summary

Amino terminal amino acid sequence analyses have been performed on the heavy and light chains of induced monoclonal antibodies with specificity for the hapten p-azophenylarsonate. Four of the eight antibodies react with conventional antisera to the previously described A/J anti-arsonate cross-reactive idiotype (CRI). Of the 16 chains analyzed, all but one contain sequence differences in their first framework segment (residues 1–30) that distinguish them from the heavy- and light-chain sequences found in anti-arsonate antibodies isolated from A/J serum or ascites fluid. The presence of such framework differences appears to be independent of whether or not the hybridoma antibodies bear the CRI. In spite of the framework substitutions, all four of the CRI-positive hybridoma antibodies have variable (V)-region frameworks that are very similar to each other and to the CRI-positive molecules found in A/J serum. Two of the four CRI-negative molecules are also structurally similar to the serum antibodies. Two others, however, are strikingly different from any serum anti-arsonate antibody thus far described and appear to reflect a completely separate repertoire of anti-arsonate antibodies in the A/J mouse. In addition, serological analyses with an anti-idiotypic antiserum generated against a CRI-positive hybridoma product suggest that each monoclonal antibody may possess individual antigenic specificities different from the determinant(s) detected with the conventional rabbit anti-CRI. The consistent appearance of framework substitutions in what has been thought to be a homogeneous antibody population has important implications for our understanding of the generation of antibody diversity and for the precise chemical definition of an idiotype.

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