COMPLETE AMINO ACID SEQUENCE OF HEAVY CHAIN VARIABLE REGIONS DERIVED FROM TWO MONOCLONAL ANTI-*p*-AZOPHENYLARSONATE ANTIBODIES OF BALB/c MICE EXPRESSING THE MAJOR CROSS-REACTIVE IDIOTYPE OF THE A/J STRAIN

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Over two decades ago, it was shown that antibodies possessed unique antigenic characteristics (1, 2). These determinants were found to be associated first with the Fab, and later the Fv portion of the antibody molecule, the portion eventually proven to be responsible for each antibody's antigenic specificity (3, 4). Some of these variable region markers, later termed idiotypes, were shown to be inherited, first in rabbits (5) and then in other species (6). These results were interpreted to mean that different individuals possessed different genetic repertoires for the generation of immune responses (7). Subsequently, when it was postulated that idiotypes might be instrumental in the regulation of immune responses, these markers became a major focus in immunology (8).

The cross-reactive idiotype (CRI)[1] of A/J mice has served as a model for inherited idiotypic systems in an inbred strain. The hapten, *p*-azophenylarsonate, when coupled to keyhole limpet hemocyanin (KLH-Ars), produces a strong anti-hapten response when injected into A/J mice (9). Structural and serologic studies using antibodies from mice inoculated in this manner have shown that this response is generated from only a very limited number of variable region genes which give rise to three families of anti-arsonate antibodies (10–12). These three families have been termed Ars A, Ars B, and Ars C. The cross-reactive idiotypes associated with the Ars A and Ars C families have been termed CRI_A and CRI_C, respectively.

The CRI defined by using a polyclonal rabbit antisera raised against A/J anti-

[1] *Abbreviations used in this paper:* Ars, *p*-azophenylarsonate; CRI, cross-reactive idiotype; CRI_A, CRI_C, CRI associated with the Ars A and Ars C families, respectively; HPLC, high performance liquid chromatography; KLH, keyhole limpet hemocyanin; KLH-Ars, Ars coupled to KLH; RIA, radioimmunoassay; SSC, 1.5 M NaCl, 0.15 M sodium citrate, pH 7.2; TBE, 0.089 M Tris; 0.089 M boric acid, 0.002 M EDTA, pH 8.0; V_H, heavy chain variable segment.

arsonate antibodies and then extensively absorbed on preimmune A/J immuno-globulin, has been exclusively associated with the Ars A family of antibodies (10). Furthermore, amino acid sequence studies, as well as chain recombination experiments comparing several anti-arsonate antibodies that vary in their degree of expression of the CRI, suggest that the structures responsible for this idiotype are located in the heavy chain (13, 14).

In contrast to A/J mice, when BALB/c mice are inoculated in precisely the same manner, this idiotype is generally undetectable. While BALB/c mice produce a strong anti-arsonate response, serologic and structural studies have shown that antibodies of the Ars A family are not produced (10). Instead, the bulk of the response is in the Ars-B and Ars-C families (15). Breeding studies first demonstrated that the expression of the CRI was a genetic trait inherited in a classical Mendelian manner as if it were an autosomal dominant gene linked to the *Igh* gene complex (16). Later, using the relatively few mouse strains carrying a defined kappa light chain polymorphism (17), the requirement for an appropriate kappa chain gene for the formation of antibodies bearing the CRI was demonstrated as well (18).

The gene encoding the V_H segment of the Ars A family of antibodies has recently been sequenced and evidence has been presented that this gene is absent in the BALB/c genome (19, 20). This provides a sound explanation for the absence of the CRI in BALB/c mice and provides strong evidence that the structural correlates of the Ars CRI_A are located in the variable segment of the heavy chain.

There have been, however, several disquieting reports that BALB/c mice express the CRI_A under certain experimental conditions (21–23). For example, it has been shown that idiotype-positive antibodies can be elicited in BALB/c mice that are first primed with anti-CRI antibodies and then inoculated with Ars-KLH (23). In fact, these idiotype-positive antibodies can occasionally be produced in BALB/c mice that are inoculated with Ars-KLH alone. Hybridomas derived from such mice have recently been constructed[2] and serologic analyses of these monoclonal antibodies have confirmed the results of previous cellular and serum studies.

This study presents the complete heavy chain variable region amino acid sequences of two of these CRI-positive BALB/c monoclonal antibodies. The results show that while their D segments are remarkably similar to the D segments of a number of Ars-A monoclonal antibodies previously sequenced in our laboratory, the V_H segments (1–98) are strikingly different from any of the Ars A family V_H segments. In addition, rather than the usual J_H2 gene segment used by most CRI+ A/J anti-Ars antibodies, one of the BALB/c hybridoma products uses a J_H1 gene segment, while the other uses a J_H4. These data imply that this D segment in the context of an appropriate light chain may, in itself, confer expression of the CRI in certain molecules that use totally unrelated V_H and J_H segments.

Materials and Methods

Purification of Hybridoma Products. Hybridoma 9A5 was prepared by the fusion of spleen cells from a BALB/c mouse immunized first with rabbit anti-CRI and then with Ars-KLH with the non-secreting hybrid plasmacytoma cell line Sp1-43 as previously described.[2] Hybridoma 22B5 was prepared similarly, but from a mouse receiving only Ars-KLH, as previously described.[2] These hybridomas were injected into CAF₁ mice for the collection of ascites fluid. Hybridoma products were purified from the resulting ascites fluid by affinity chromatography as previously described (24). Briefly, the ascites fluid was bound onto a Sepharose 4-B column that had been conjugated with arsonilic acid–derivatized human gamma globulin. Purified antibody was eluted with 0.2 M arsonilic acid, dialyzed against deionized, distilled H_2O and lyophilized.

Heavy and Light Chain Preparation. To dissociate heavy and light chains for amino acid sequencing, whole purified hybridoma products were completely reduced and alkylated by the addition of 2-mercaptoethanol and then iodoacetic acid. The heavy and light chains were then separated on a Biogel A5M column in 5 M guanidine HCl. Desalting was accomplished by dialysis against 0.25 M NH_4OH. The heavy and light chains were lyophilized and subjected to sequence analysis.

The heavy chains of both hybridoma products had a pyroglutamic acid as the amino terminal residue, thus leaving the amino terminus unavailable for the Edman reaction. The heavy chains were, therefore, citraconylated and deblocked using pyroglutamic aminopeptidase and again subjected to automated amino acid sequence analysis (25, 26).

Preparation and Sequencing of Heavy Chain CNBr Fragments. The two hybridoma products HP 9A5 and HP 22B5 were cleaved at methionine residues by incubation with cyanogen bromide in 100 M excess over lysine residues in 70% formic acid for 24 h at room temperature.

In the case of HP 9A5, the heavy chain variable region was cleaved at only one position, which resulted in two disulfide-bonded peptides. By gel filtration on a Sephadex G-100 column in 5 M guanidine HCl, these two peptides, extending from positions 1–48 and 49–142, eluted in one peak. After desalting and lyophilization, these two peptides were dissociated from each other by complete reduction and alkylation. Separation was achieved by gel filtration on a Sephadex G-75 column in 0.25 M NH_4OH. The peptide extending from position 49–142 was sequenced to position 84 in the automated sequencer.

In the case of HP 22B5, the heavy chain variable region was cleaved at four positions, resulting in five peptides. The peptide extending from position 109–265, which was disulfide bonded to several disulfide-bonded fragments of the light chain, eluted in the column's exclusion volume. By subjecting the resulting protein pool to complete reduction and alkylation, the heavy chain peptide was separated from the light chain peptides by using a Sephadex G-100 column in 5 M guanidine HCl. This peptide was sequenced in the automated sequencer from positions 109–125.

The two disulfide-bonded peptides extending from positions 1–34 and 92–108 co-eluted with two other peptides—one extending from position 396–437, and one extending from position 49–91. By subjecting this entire pool to complete reduction and alkylation, the peptide extending from position 92–108 and the peptide extending from position 1–34 could each be isolated by using a G-50 Sephadex column in 0.25 M NH_4OH. The other two peptides (49–91 and 396–437) co-eluted. The peptide extending from position 92–108 was sequenced entirely on the automated sequencer.

The peptide extending from position 35–48 was isolated from a separate CNBr digestion of the whole molecule on a Sephadex G-50 column in 0.25 M NH_4OH. This peptide was sequenced entirely in the automated sequencer.

Preparation and Sequencing of Tryptic Peptides. CNBr fragments 1–48 of 9A5 and 1–34 of HP 22B5 were digested with TPCK trypsin (Worthington Biochemical Corp., Freehold, NJ). The resulting peptides were isolated by reverse phase high pressure liquid chromatography (HPLC) on an RP-300 column, as previously described (11).

CNBr fragments 49–142 of HP 9A5 and 49–91 and 396–437 of HP 22B5 were digested with TPCK trypsin. The resulting peptides were separated by ion exchange chromatography on a Sephadex-SP column in 8 M urea and a 0 to 0.2 M KCl gradient

(11), or by HPLC on an RP-300 column. CNBr fragment 49–142 of HP 9A5 was also digested with chymotrypsin. The resulting peptides were similarly separated by HPLC on an RP-300 column.

Sequence analysis of the peptides resulting from tryptic and chymotryptic digestion of CNBr peptides made it possible to obtain the complete amino acid sequence of both molecules.

Sequencing Methodology. Automated amino acid sequence analysis was performed using a Beckman 890C, 890D (Beckman Instruments, Inc., Palo Alto, CA) or Applied Biosystems gas phase sequencer. 0.25 M or 0.1 M Quadrol programs were used with the Beckman instruments. The PROTFA and TFAV programs were used with the Applied Biosystems machine. Phenylthiohydantoin amino acid derivatives were identified by HPLC using Waters NovaPak RCM C-18 and Waters CN columns (Waters Associates, Inc., Milford, MA) for identification. The amino acid compositions of selected peptides were determined on a Beckman System 6300 amino acid analyzer.

Measurement of Antigen Binding. Ars binding was quantitated in two solid-phase radioimmunoassays (RIAs). The first was a standard, indirect-binding RIA in which the binding of serial dilutions of hybridoma products was detected with ^{125}I-labeled rabbit anti-mouse Ig. The second was a competition RIA. Serial dilutions of unlabeled hybridoma products (HPs) were first added to microtiter wells that had been coated previously with either Ars-BSA or Ars-KLH and were allowed to compete for binding to antigen with a standard quantity (10 ng) of a ^{125}I-labeled anti-Ars hybridoma 93G7, added to the wells shortly thereafter.

Chain Recombination. Chain recombinations were performed by the method of Giles et al. (27). Briefly, heavy and light chains were dissociated by partial reduction with 0.1 M 2-mercaptoethanol in 0.5 M Tris, pH 8.3, and alkylated with 0.15 M iodoacetamide. After dialysis against Tris-buffered saline, pH 8.2, to remove the excess reducing and alkylating reagents, heavy and light chains were separated by gel filtration on a Bio-Gel A-5M column (Bio-Rad Laboratories, Richmond, CA) equilibrated with 5 M guanidine-HCl. The completeness of separation was verified by SDS-polyacrylamide gel electrophoresis. Homologous and heterologous recombinations were performed by mixing the heavy and light chain pools in 1:1 molar ratio and dialyzing against Tris-buffered saline. Molecules in the molecular weight range 300,000 to 100,000 daltons were isolated by gel filtration on a column of Sephacryl S-300 (Pharmacia, Piscataway, NJ) equilibrated with phosphate-buffered saline, pH 7.4.

Southern Filter Hybridization. The method for Southern filter hybridization (28) of mouse liver and hybridoma DNA was modified from that previously described by Sims et al (19). DNA was digested with *Eco* RI, *Sac* I, or *Xba* I and fractionated on a 0.6% agarose gel in TBE (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0). The DNA was denatured in the gel for 2 h in a solution of 0.5 N NaOH in 1.5 M NaCl. It was then neutralized for 2 h in 0.5 M Tris buffer, pH 7.5, containing 1.5 M NaCl, before transfer of the DNA to nitrocellulose in 20× SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.2). The filters were baked under vacuum at 80°C and prewashed at 42°C for 24 h in a solution containing 5× SSC, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin, 100 µg/ml herring sperm DNA, and 50% formamide. Hybridization was carried out at 42°C for 48 h in the same solution with the addition of 50 µg/ml poly (rA) and the nick-translated (29) probe at a final concentration of 1 × 10^6 cpm/ml. After a 48-h hybridization, the filters were washed sequentially with 3× SSC at room temperature, then 1× SSC and 0.1× SSC for 1 h each at 42°C. Kodak XAR-5 film was exposed at −70°C. A *Bam* HI/*Eco* RI fragment containing the J_H3 and J_H4 genes was isolated from a 0.6% agarose gel after electrophoresis of a subclone of BALB/c J_H genomic DNA kindly provided by Dr. P. Tucker (Univ. of Texas Health Science Center, Dallas).

Results and Discussion

BALB/c CRI+ Anti-Ars Antibodies Employ Light Chains Homologous to Those of the Ars A Family but Their V_H Segments Are Vastly Different. Amino terminal

sequence analysis of the light chains of both hybridoma products showed that the first 25 residues of both light chains are identical to A/J, CRI+, Ars-A family light chains (30) with the exception of one residue at position 17 in HP 22B5 where an alanine is substituted for the aspartic acid found in most Ars-A family light chains. This light chain has not been observed in other BALB/c anti-arsonate antibodies, but has been seen in BALB/c anti-oxazolone antibodies and in anti-arsonate antibodies of the congenic strain C.AL 20, which carries the A strain V_H segment genes on a BALB/c background (31).

The complete heavy chain variable region amino acid sequence of these two molecules is compared to that of the CRI+, Ars-A prototype molecule HP 93G7 in Fig. 1. The dissimilarities between these molecules and HP 93G7 are quite apparent. Of the 98 amino acid residues in the heavy chain variable segment (positions 1–98), HP 9A5 differs in 44. HP 22B5 differs in 43. This represents ~55% homology, which is no more homology than would be expected between any two randomly selected murine heavy chain variable segments. These data suggest that the BALB/c proteins derive from germline V_H gene(s) that are clearly different from the germline V_H segment that gives rise to the Ars-A family. On the other hand, the homology between the V_H segments of these two molecules is substantial, with only 16 differences in 98 residues (84% homology).

Expression of Idiotypic Determinants and Antigen Binding on Recombined Molecules. The recombined molecules were tested for the presence of idiotypic determinants as defined by rabbit anti-CRI. When heavy and light chains from 93G7 and 9A5 were recombined, the extent of idiotypy was dependent on the choice of heavy chain (Fig. 2A), a result consistent with previous observations in the Ars-A family (14). The light chains of 93G7 and 9A5 were interchangeable, however, suggesting that the light chain used by 9A5 is functionally identical (at least in its idiotypic properties) to those used by the Ars-A family. Additionally, as shown by the antigen binding data in Fig. 2B, the light chains of 93G7 and 9A5 were interchangeable in their ability to restore antigen binding to a given heavy chain. In this series of experiments, as in those reported previously for HP 101F11, an A/J CRI+ hybridoma with diminished antigen binding (32), the heavy chain determines the avidity for arsonate.

Two Similar BALB/c V_H Segments Are Encoded by Separate Germline Genes. In order to ascertain whether the two BALB/c hybridomas are derived from the same or different germline V_H gene segments, a series of Southern filter hybridizations were performed in which a J segment probe was used to detect rearranged genes. Fig. 3 illustrates the results obtained after *Eco* RI digestion. The probe revealed a single 6.7-Kb component in BALB/c liver DNA (lane *A*) that represents the germline configuration of the J_H cluster. In previous studies, this germline component has been observed in about half of the anti-arsonate hybridomas studied. The BALB/c fusion partner, (lane *B*), does not contain the 6.7-Kb germline component, but does contain a 5.8-Kb component that hybridizes to the probe. It has been previously shown that this represents a nonproductive rearrangement of a V_H gene segment. The hybridoma 22B5 (lane *C*) gave two restriction fragments of 10.8 Kb and 5.8 Kb. The latter fragment (5.8 Kb) co-migrated with, and presumably represents, the Sp2 restriction fragment. The former (10.8 Kb), therefore, represents the productively rearranged V_H

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                    10          20          30           40           50            60
93G7    E V Q L Q Q S G A E L V R A G S S V K M S C K A S G Y T F T|S Y G I N|W V K Q R P G Q G L E W I G|Y I N P G N G Y I N Y
U9A5    < I — — — V — — P — — K K P — E T — — — I — — |— — N F - V|— — — — — — — — — — A — — K A - M - A|W — — Y — — E P T H
U22B5   < I — — — V — — P — — K K P — E T — — — I — — |— — N — — M|— — — — — — — — — — A — — — K - M - A|W — — T Y T - E P T -

                    70          80          90            100          110           120
93G7    N E K F K G|K T T L T V D K S S S T A Y M Q L R S L T S E D S A V Y F C A R|S H Y Y G G S Y D F D Y|W G Q G T P L T V S S
U9A5    A D D — — — R F A F A L — T - A — — — — — I - N N — — K - S Y Y — — |— — Y — — — A — — Y|— — — A — — T V
U22B5   A D D — — — R F A F A L E T - G A — — — — L - I - N N - S N — — T — — |— — Y — — — S — — M|— — — S V — — G A
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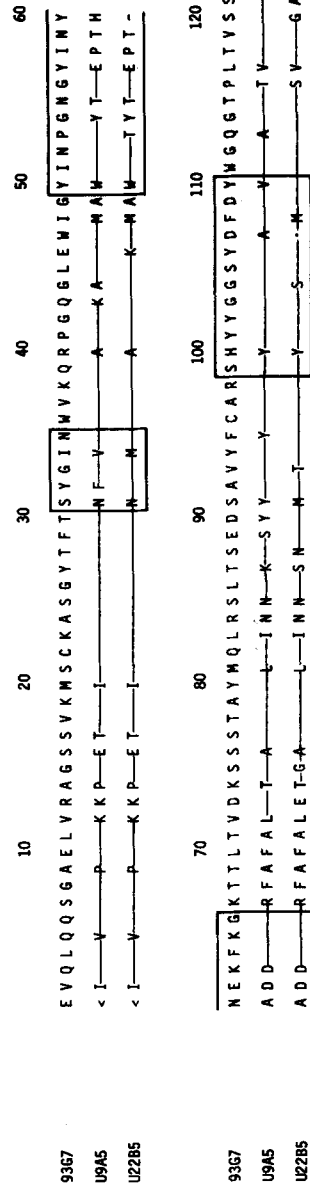
FIGURE 1. Comparison of the amino acid sequences of the heavy chain V regions of two BALB/c hybridomas (9A5, 22B5) bearing the cross-reacting idiotype of the A/J strain to the A/J anti-arsonate hybridoma 93G7 (19). (—) identical residues; (.) gap introduced to maximize homology. Numbering is sequential. Complementarity-determining 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; <, pyroglutamic acid. Positions 47 and 48 in the 9A5 molecule are probably but not definitively EW, and should be considered tentative.
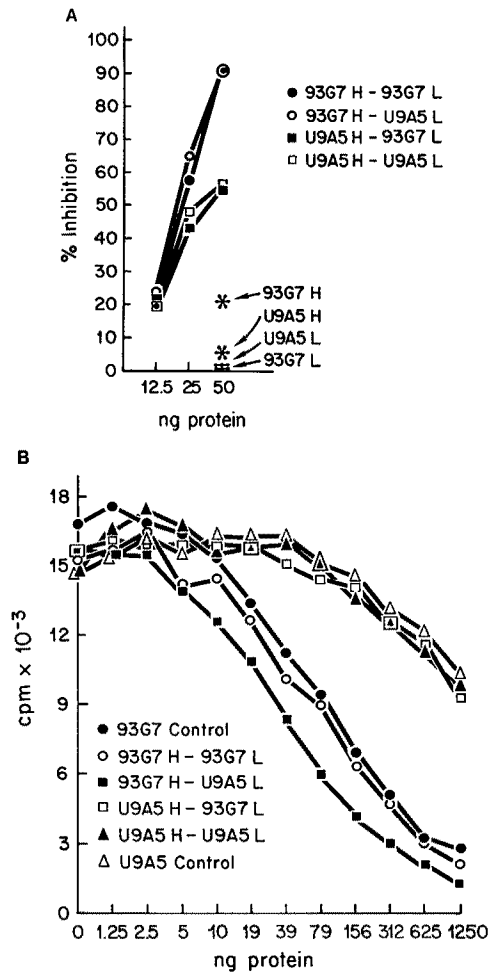
FIGURE 2.   Expression of the CRI (A) and antigen binding (B) by anti-Ars antibodies generated in chain recombination experiments. Homologous and heterologous combinations of light and heavy chains from HP 93G7 (the prototype A/J CRI+ anti-arsonate antibody) and HP 9A5 (one of the BALB/c CRI+ anti-arsonate antibodies) are compared using the recombined hybridoma products and isolated light and heavy chains as controls. (A) Inhibition by recombined molecules of a fixed quantity (10 ng) of ^{125}I-labeled HP 93G7 by slightly less than an equivalent amount of rabbit anti-CRI antiserum. 50 ng amounts of isolated chains were used as controls. (B) Competition between recombined molecules of ^{125}I-labeled HP 93G7 (10 ng) for binding to Ars-BSA-coated polyvinyl plates. Isolated chains corresponding in molar amounts of 1,250 ng of whole immunoglobulin were used as controls all of which showed negligible competition.

gene segment of hybridoma 22B5. Lane D shows the results with hybridoma 9A5. Three restriction fragments are noted, one at 22 Kb, one at 6.7 Kb, and one at 5.8 Kb. Initially, the 6.7-Kb fragment was thought to represent the germline J$_H$ cluster (see lane A) while the 5.8-Kb fragment was thought to represent the Sp2 restriction fragment. Thus, the 22-Kb fragment in hybridoma 9A5 most likely represents the productively rearranged V$_H$ segment gene. Since the difference between the sizes of the restriction fragments in hybridomas 9A5
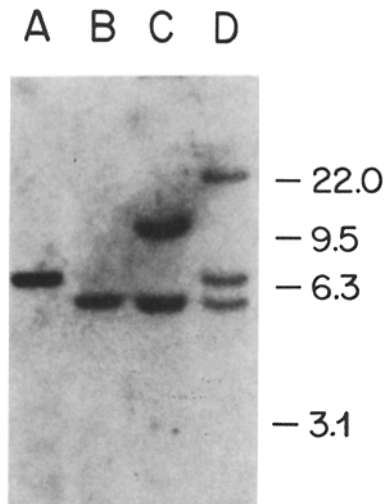
A   B   C   D

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                    — 22.0
                    — 9.5
                    — 6.3


                    — 3.1
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FIGURE 3.   Southern filter hybridization of anti-Ars hybridoma DNA digested with *Eco* RI. Digests of DNA from BALB/c liver (*A*), SP2/0-Ag-14 (*B*), 9A5 (*C*), and 22B5 (*D*) are blotted with a J_s-J_4 probe prepared from BALB/c genomic DNA. The positions of molecular weight markers (linear oligomers of lambda dv21 DNA, Boehringer-Mannheim, Indianapolis, IN) are indicated.

and 22B5 is greater than the difference between J_H1 and J_H4 (the two J_H segments utilized by these hybridomas (Fig. 1), these data support the view that these two hybridomas are encoded by different V_H gene segments.

This interpretation was supported by two further experiments (data not shown). A digestion with the restriction enzyme *Sac* I again revealed a single restriction fragment in the BALB/c germline (4.4 Kb), a single restriction fragment in the Sp2 DNA (4.5 Kb), two restriction fragments in hybridoma 22B5 (13.0 and 4.5 Kb), and three in hybridoma 9A5 (11.5, 4.8, and 4.5 Kb). These data supported the interpretation that both the BALB/c hybridomas contained the Sp2 nonproductively rearranged gene (4.5 Kb) but did not support the initial conclusion that hybridoma 9A5 had retained the BALB/c germline gene, as the 4.4-Kb germline restriction fragment was not seen. However, since the difference between the restriction fragments in the two BALB/c hybridomas was greater (and in the wrong direction) than would be anticipated from the J1-J4 usage, the interpretation of the *Eco* RI experiment, that two different V_H gene segments were being utilized in the BALB/c hybridomas was supported fully.

Experiments done with the enzyme *Xba* I also confirmed this conclusion. Both of the BALB/c hybridomas contained a restriction fragment that co-migrated with the Sp2 nonproductively rearranged V_H gene segment, and again the productively rearranged V_H gene segments in the two BALB/c hybridomas were not consistent with J1/J4 usage of the same germline V_H gene segment. Rather, the results were more consistent with the conclusion that different germline V_H gene segments had been utilized by the two hybridomas.

Areas Within the V_H Segments Bearing Structural Homology with Ars A V_H Segments Probably Do Not Affect Idiotype Expression. Within the V_H segments there are

three portions of these two molecules which bear significant structural similarity to HP 93G7. The three molecules are all fairly homologous between positions 18-30, 35-47, and 92-98. However, these areas are framework regions and contain the cysteine residues responsible for the V_H internal disulfide bond (18-30, 92-98) or make contact with light chains (35-47). The amino acid sequence in these areas tends to be highly conserved throughout all families of V_H segments. Furthermore, in extensive comparisons of A/J Ars-A CRI+ and CRI− molecules, differences in these areas have never correlated with presence or absence of the CRI (11, 32). It is, therefore, unlikely that any amino acid residues within these areas contribute significantly if at all to idiotype expression.

Classically, one would expect structures responsible for idiotypy to be confined to hypervariable regions. Interestingly, both the first and second hypervariable regions of HP 9A5 and HP 22B5 are quite distinct from those of the prototype molecule, 93G7. In the second hypervariable region, these molecules all share an isoleucine and asparagine in positions 51 and 52. But again, comparisons of A/J CRI+ and CRI− molecules have never correlated differences in these positions to idiotype expression. Likewise, these molecules share phenylalanine, lysine, and glycine at positions 64-66, but similarly there is no compelling evidence that these residues affect idiotype expression (11, 32).

V_H Segments of the BALB/c CRI+ Anti-Arsonate Antibodies Probably Represent a Separate V_H Subgroup. When trying to assign these antibodies to one of the three anti-arsonate families, it became apparent that these molecules were markedly dissimilar from all of the families. Serologically, they are different from Ars-A in that they lack the 91A3 marker.[2] They are also serologically different from Ars-B and Ars-C in that they are strongly positive in the classic CRI assay. Structurally, they are obviously different from Ars-A, as has already been discussed, but they are just as dissimilar in this regard to both the Ars-B and Ars-C families. In fact, the only anti-arsonate antibody bearing significant homology to these molecules is HP 45-165, an A/J CRI− anti-arsonate antibody described by Margolies et al. (33). Only the amino terminal amino acid sequence has been determined for this antibody's heavy chain, but there is only one difference between it and HP 22B5, and three differences between it and HP 9A5 in the first 35 positions. Unlike the BALB/c CRI+ antibodies, the light chain of 45-165 is unrelated to the Ars-A family of light chains (33).

Furthermore, assignment of the V_H segments of these BALB/c antibodies to one of the five V_H subgroups is also extremely difficult because of the dissimilarity of these molecules with any murine V_H segments sequenced to date. The structure of the V_H segments are so dissimilar to any previously sequenced V_H segments that it is probable that DNA probes made to either 9A5 or 22B5 will detect a new family of germline V_H gene segments. Therefore, we suggest that these heavy chains may represent a separate V_H subgroup, which we have termed $V_H VI$.

BALB/c, CRI+ Anti-Ars Antibodies Employ the Same or Similar D_H Segment as Their A/J Ars A Counterparts but Use Different J_H Segments. Fig. 4 compares the D segments of these two BALB/c, CRI+ anti-Ars antibodies with three A/J Ars-A, strongly CRI+ antibodies, to the germline sequence of the BALB/c D segment FL16.1 sequenced by Kurosawa et al. (34). The homology of the D segments of

Dμ16.1   Y   Y   Y   G   S   S   Y

HP 93G7   H_____G_____

HP 16.7   N_____G_____

HP 36-65   V_____G_____

HP 9A5   G_____

HP 22B5   _____

FIGURE 4. Comparison of the amino acid sequence of the D segments of three CRI+ hybridomas of the A/J strain [93G7(19), 16.7(11), 36–65(29)] and the two BALB/c hybridomas that bear the A/J cross-reacting idiotype (9A5, 22B5). A straight line indicates identical residues. Note that four of the five sequences differ in position 5 from the BALB/c FL16.1 D segment.

these two BALB/c CRI+ antibodies to the D segments of the A/J antibodies is striking. HP 22B5's D segment matches the germline sequence FL16.1 exactly. The D segment of HP 9A5 has a single substitution, a glycine for the germline residue serine at position 104. However, an identical substitution exists in most of the A/J CRI+ antibodies. An attractive explanation for this particular high frequency substitution, as has been suggested, is that the A/J strain has in its germline glycine in place of serine at that position in its counterpart of the FL16.1 D segment (11). The serine-glycine interchange in HP 9A5 probably represents a somatic mutation and may contribute to the CRI+ nature of this molecule (see below). In terms of homology to the prototype molecule HP 93G7 (discounting V/D and D/J junctional positions) HP 9A5's D segment is 100% homologous and HP 22B5's is 83% homologous. There is also no difference in D segment length between these two molecules and HP 93G7.

In contrast to the D segments, the J_H segments of these BALB/c hybridoma products have little in common with their A/J counterparts. The majority of A/J CRI+ antibodies employ J_H2 (11), whereas J_H4 is utilized by HP 22B5, and HP 9A5 utilizes J_H1.

The Structure Imparting Expression of the CRI in HP 22B5 and HP 9A5 Is Most Likely Located in the D_H Segment. The most plausible conclusion from these data is that the structure responsible for the heavy chain's contribution to the expression of this idiotype is located in the D segment. The other two possible explanations for these results are (a) that the structural correlate of the idiotype is actually in the Ars-A light chain, or (b) that some portion of the V_H segments of 22B5 and 9A5 mimic three-dimensionally a portion of the Ars-A V_H segment. Overwhelming evidence from chain recombination experiments (13, 14) makes the first of these alternatives highly unlikely. The second of these possibilities is not attractive considering the degree of differences between the first and second hypervariable regions of 22B5 and 9A5 and the Ars-A family (only 40% homology), but nevertheless, cannot be entirely ruled out.

From computer-derived three-dimensional models of Ars-A antibodies, it has been suggested that the majority of the D segment is on the surface of the molecule and, therefore, may be exposed well enough to solvent to be antigenic in itself (30). The residues comprising this particular D segment are extremely

hydrophobic, which may also contribute to antigenicity. We propose that the V_H segments we have described, like their Ars-A counterparts, along with an appropriate light chain, can place this D_H segment into a specific three-dimensional configuration that is most probably the structure primarily contributing to idiotype expression in HP 22B5 and HP 9A5, and probably CRI_A antibodies, as well. This interpretation is in direct contradiction to results obtained not only in our laboratory but also from several other laboratories that have mapped the gene responsible for idiotype expression specifically to the V_H gene segment. One of these studies, using recombinant inbred strains that had BALB/c variable region genes to the left of V_{DEX} but A/J V_H gene segments to the right of V_{DEX}, showed conclusively that mice lacking the gene encoding the Ars-A V_H segment but possessing the J_H, C_H, and likely D as well as the light chain of A/J mice, did not express the CRI (20). This implies either that (a) this D segment is also encoded to the left of V_{DEX} or that (b) the second V_H appropriate for CRI expression in conjunction with the appropriate D segment was also deleted in these mice so that no appropriate V_H segment gene was available for use in the anti-arsonate response, or that (c) in an anti-arsonate response, clones producing anti-arsonate antibodies with the appropriate D segment and the appropriate light chain for CRI expression are generally only expanded if the Ars-A V_H segment is present as well.

The first of these explanations is feasible, but there is no evidence at this time to support the hypothesis that D gene segments are interspersed among the V_H gene segments. This explanation also does not address the issue that CRI^+ antibodies similar to HP 9A5 and HP 22B5 have not been seen in A/J mice even though both strains presumably have the genetic information to produce them. The second explanation is not attractive since the V_H segments of HP 9A5 and HP 22B5 have been observed in both the A/J and BALB/c strains, so it is highly unlikely that a recombinant inbred strain derived from them would lack this V_H gene. This leaves the third explanation as the most feasible, but the question remains: Why are molecules like HP 9A5 and HP 22B5 not regularly employed in either the A/J or BALB/c response to KLH-Ars?

Under Normal Circumstances B Cell Clones Producing These Antibodies May Not Be Expanded Possibly Because of Their Weaker Antigen Binding Characteristics.   The production of this second type of CRI+ antibody may be down regulated either at the T cell level or through gene regulation. This would explain reports showing that B cell clones producing CRI+ anti-Ars antibodies are present in classically CRI− strains, but these clones may not be expanded and their products not generally expressed in the serum anti-Ars response (21, 22). The reason for this preferential selection is not understood, but there are obviously several possibilities. One would be that these antibodies may not bind arsonate as well as the Ars-A family in the A/J strain or the Ars-C family in the BALB/c strain and, therefore, cannot compete with these strong arsonate binders for antigenic stimulation. In fact, neither HP 9A5 nor HP 22B5 binds arsonate as well as HP 93G7 in a standard inhibition assay (Fig. 2B). By first expanding CRI+ clones in the BALB/c strain with an initial injection of anti-CRI, these clones, now in greater numbers, may favorably compete for antigen during an anti-Ars response, thus explaining the expression of the CRI in BALB/c mice in this instance. The

fact that CRI+ antibodies similar to HP 22B5 and HP 9A5 have not been identified in the A/J strain even though A/J mice presumably have the genetic capability to produce them, is consistent with the idea that clones producing these antibodies are suppressed because of weak antigen binding and not because of presence or absence of the idiotype. However, more data are needed to address this issue. It is likely that other factors in addition to antigen binding characteristics govern the expression of molecules like HP 22B5 and HP 9A5 during an immune response to arsonate.

Idiotypes Are Not Necessarily Subgroup Markers. These data also create another parodox in that they contradict the notion that antibodies which share unique serologic characteristics are almost always very similar to one another structurally as well and, therefore, are generally of the same subgroup. These data suggest that idiotypy may in some instances be determined by only a small fraction of an entire molecule, in that antibodies employing entirely different V_H subgroups express the major CRI of the A/J strain. This idea is not new, as it was actually postulated even before the discovery of the molecular mechanisms of antibody synthesis which, as we now know, allow for the utilization of different D or J_H segments with the same V_H segment or vice-versa as is demonstrated in this case. Indeed, in the early 1970's, the observation that similar idiotypes could be found in antibodies of differing V region subgroup led to some of the early notions of gene interaction (35–40).

Anti-Anti-Idiotype Antibodies (Ab3) Are Not Necessarily Equivalent to the Original Antibody (Ab1) and Have Implications Concerning the Modulation of the Immune Response. These data also broaden our understanding of the idiotype cascade phenomenon. Briefly, it has been well documented that an immunologic response is induced by immunization with anti-idiotypic (Ab2) antibodies. The resulting antibodies (Ab3) may or may not express the idiotype of the original idiotype-bearing antibody (Ab1) and may or may not share the antigenic specificity of the original antibody. It has been postulated that Ab3 that are equivalent to Ab1 in antigenic specificity and idiotypy would be closely related structurally to Ab1 as well. This study shows that this is not necessarily true—and again, raises the question of why certain antibodies are expressed when the immune system is manipulated, but are not generally expressed during a normal immune response.

Other implications arise from these data as well. In envisioning an idiotypic regulatory system, one must now take into account the fact that antiidiotypic antibodies may have the capability to react with and affect antibodies of different V_H subgroups. One could theorize from this the possibility that a specific antiidiotypic antibody may have the potential to regulate antibodies with entirely different binding specificities. This would imply that the number of regulatory antibodies required for modulation of any possible immune response may be much smaller than the number of specific antibodies in an individual's antibody repertoire. Also, if one ascribes to the idea that antiidiotypic antibodies provide internal selective pressure for the maintenance of certain variable region genes (41), this example of idiotype sharing between antibodies employing different V_H segments would imply that a single antiidiotypic antibody could maintain antibodies of multiple binding specificities.

Similarly, this idea that idiotype expression may result from the presence of a

very small part of a molecule's structure may help in the understanding of the structural moiety that imparts idiotypic specificity to T cell populations. Furthermore, one could surmise that any one specific T cell clone may very well be able to interact with more than one B cell clone. In fact, this observation of shared idiotypy between vastly different molecules is consistent with recent reports showing that idiotype bearing T cells do not transcribe the corresponding V_H gene segment responsible for antibodies bearing the same idiotype (42). Since the structures imparting idiotypy to T cells are different gene products from their B cell counterparts, these structures are most likely significantly different structurally even though they may behave similarly serologically. As the structures of T cell idiotypes are determined, it would seem most probable that the structural equivalent of these idiotypes will represent only a very small percentage of an entire molecule, which is very likely considerably different from an antibody molecule bearing a serologically indistinguishable idiotype.

This difference between T cell and B cell idiotypes would imply separate evolutionary paths for the development of T and B cell functions and would suggest less interdependence between the two. Therefore, the sharing of serologic determinants between B and T cells might be construed as coincidental and not important in the modulation of the immune response. However, the majority of functional studies indicate that idiotypes do, indeed, modulate immune responses (41), so it is possible that serologically similar, but structurally dissimilar molecules, may actually evolve and function separately, but in coordination with one another, each having the capability to affect the other's expression.

Structural Basis of the CRI. Fig. 5 represents a schematic summary of the gene segments used in the construction of anti-arsonate hybridomas in the A/J and BALB/c strain, all of which bear the CRI. In several previous studies, the light chains of most of these hybridomas have been interchanged without any detectable variation in either the expression of the CRI or the binding to the hapten, p-azophenylarsonate. Thus, the degree of idiotype expression and the strength of interaction with hapten, are directly attributable to variations in the heavy chain. As can be seen from Fig. 5, two different V_H subgroups (gene segments) are employed. The typical Ars A hybridomas of the A/J strain utilize the $V_H V$
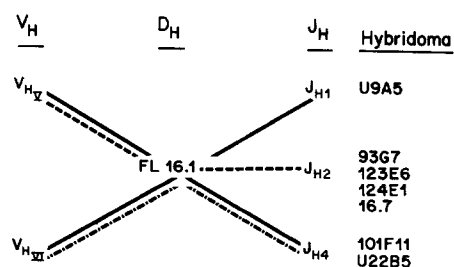

FIGURE 5. Schematic summary of gene segments used in the construction of anti-arsonate hybridomas of the A/J or BALB/c strain, all of which bear the cross-reacting idiotype. In each instance, where they have been tested, the light chains of these hybridomas appear interchangeable. The $V_H V$ and $V_H VI$ subgroups differ in ~45% of their sequences. J_H1, J_H2, and J_H4 differ in ~40% of their positions. The only apparent common feature of these molecules is the FL16.1-like D segment.

germline gene segment. In all instances studied, these derive from the same germline V_H segment. The BALB/c hybridomas derive from two closely related gene segments that we have termed V_HVI. They differ from the V_HV sequence in over 45% of their amino acid positions. A single hybridoma has been identified in the A/J strain which is idiotype negative, but that likely derives from a similar V_HVI germline gene. All hybridomas appear to utilize a variant of the FL16.1 D gene segment, but three different J_H gene segments have been detected. One BALB/c hybridoma (9A5) uses J_H1, another (22B5) uses J_H4. The typical A/J hybridomas (93G7, 123E6, 124E1, and 16.7) utilize J_H2, but an occasional A/J hybridoma utilizes J_H4 (101F11) (41). In all three instances in which a J_H segment other than J_H2 is used (9A5-J_H1, 101F11, and 22B5-J_H4), binding to hapten is considerably weaker than when a J_H2 gene segment is utilized. However, all of these molecules are positive for the major cross-reacting idiotype of the A/J strain. Since their V_H gene segments are in toto over 45% different, and their J_H gene segments are varied and are over 40% different, their only apparent common feature is the "variant" FL16.1 D segment. Thus, these data provide strong evidence that the FL16.1 D segment, in the context of an appropriate light chain, is the structure primarily responsible for the expression of the major cross-reacting idiotype of the A/J strain.

Summary

The primary structure of A/J anti-*p*-azophenylarsonate (anti-Ars) antibodies expressing the major A-strain cross-reactive idiotype (CRI_A) has provided important insights into issues of antibody diversity and the molecular basis of idiotypy in this important model system. Until recently, this idiotype was thought to be rarely, if ever, expressed in BALB/c mice. Indeed, it has been reported that BALB/c mice lack the heavy chain variable segment (V_H) gene that is utilized by the entire family of anti-Ars antibodies expressing the A/J CRI. Recently, however, it has been possible to elicit CRI_A+, Ars binding antibodies in the BALB/c strain by immunizing first with anti-CRI and then with antigen. Such BALB/c, CRI_A+ anti-Ars antibodies can be induced occasionally with antigen alone.

V_H region amino acid sequences are described for two CRI_A+ hybridoma products derived from BALB/c mice. While remarkably similar to each other, their V_H segments (1–98) differ from the V_H segments of A/J CRI_A+, anti-Ars antibodies in over 40 positions. Rather than the usual J_H2 gene segment used by most A/J CRI_A+ anti-Ars antibodies, one BALB/c CRI_A+ hybridoma utilizes a J_H1 gene segment, while the other uses a J_H4. However, the D segments of both of the BALB/c antibodies are remarkably homologous to the D segments of several A/J CRI_A+ antibodies sequenced previously, as are the amino terminal amino acid sequences of their light chains.

These data imply that BALB/c mice express the A/J CRI_A by producing antibodies with very similar, if not identical, light chain and heavy chain *D* segments, but in the context of different V_H and J_H gene segments than their A/J counterparts. The results document that molecules that share serologic specificities can have vastly different primary structures.

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