Complete Amino Acid Sequence of the Heavy-Chain Variable Region from an A/J Mouse Antigen-Nonbinding Monoclonal Antibody Bearing the Predominant Arsonate Idiotype†

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ABSTRACT: The 1F6 hybridoma protein, exhibiting the predominant cross-reactive idiotype (CRI) associated with the immune response to *p*-azophenylarsonate in A/J mice but failing to bind the hapten arsonate, was elicited following immunization with rat anti-CRI [Wysocki, L. J., & Sato, V. (1981) *Eur. J. Immunol. 11*, 832–839]. The dissociation of idiotype and antigen binding in this hybridoma provides an opportunity to determine structural features involved in antigen binding and idiotypic sites. The complete heavy-chain variable region (V_H) amino acid sequence was obtained by automated Edman degradation of the intact chain and fragments due to CNBr cleavage, trypsin digestion, mild acid hydrolysis, and carboxypeptidase A digestion of a CNBr fragment. Comparison of the CRI^+ arsonate-nonbinding 1F6 sequence with the CRI^+ germ-line V_H gene sequence reveals that the 1F6 heavy chain differs from the germ-line-encoded amino acid sequence at seven positions within V_H [Siekevitz, M., Gefter, M. L., Brodeur, P., Riblet, R., & Marshak-Rothstein, A. (1982) *Eur. J. Immunol. 12*, 1023–1032]. The 1F6 V_H appears to arise from the CRI^+ germ-line V_H by somatic mutation at at least seven amino acid residues, each of which could be due to a single nucleotide base change. The diversity (D) gene-encoded segment of 1F6 is similar to that of the CRI^+ antigen-binding hybridoma 36-65 except for two amino acid substitutions. Further, the idiotype (CRI) is preserved despite use of a J_{H4} gene segment in 1F6 as compared to J_{H2} in all CRI^+ arsonate-binding hybridomas examined to date. The lack of antigen binding in the 1F6 hybridoma may be correlated with the presence of a J_{H4}-encoded sequence, although alterations in the light-chain variable region, or elsewhere in the heavy-chain variable region, cannot be excluded.

Dominant idiotypes in inbred strains of mice are serologically defined phenotypic markers that reflect germ-line genes encoding antibody variable regions. Such idiotypes are ordinarily defined following conventional immunization with antigen and are thought to be related to structural determinants in or near the antibody combining site. The dominant idiotype CRI,[1] associated with the immune response to *p*-azophenylarsonate–protein conjugates in A/J mice (Kuettner et al., 1972), is an example of such a heritable variable region marker. However, when manipulations other than conventional immunization are used, it is possible to produce Ig molecules bearing a predominant idiotype which no longer bind antigen. Wysocki & Sato (1981) produced three such antigen-nonbinding idiotype-bearing hybridomas in A/J mice by immunization with a rat monoclonal antiidiotypic antibody. The amino acid sequences of the amino-terminal portion of the V_H and V_L regions of these anti-(anti-CRI) antibodies proved indistinguishable from those of arsonate-binding idiotype-bearing hybridomas produced following immunization with antigen (Margolies et al., 1983b). Thus, Ars-binding (Ars^+) CRI^+ and Ars-nonbinding (Ars^-) CRI^+ immunoglobulins are derived from similar or identical V_H and V_L genes. The availability of these two groups of monoclonal antibodies, obtained by means of somatic cell fusion, led to a further examination of the structural changes rendering CRI^+ monoclonal antibodies antigen nonbinding.

We report here the complete heavy-chain variable region amino acid sequence of the CRI^+, Ars^- hybridoma protein 1F6. The sequence differs from a CRI^+, Ars^+ prototype by substitution of seven amino acids in the V_H gene-encoded region and two amino acids in the D gene-encoded region and by the use of a J_{H4} sequence, rather than a J_{H2} gene-encoded sequence (Sakano et al., 1980; Kurosawa & Tonegawa, 1982).

Materials and Methods

Hybridoma Cell Lines. The derivation of the cell line 1F6 secreting a CRI^+, Ars^- hybridoma protein has been described in detail previously (Wysocki & Sato, 1981). Briefly, A/J mice were immunized intraperitoneally with a rat anti-CRI monoclonal antibody. Spleen cells from immunized mice were fused with the Sp2/0-Ag14 myeloma cell line. Fusion products were screened for Ars binding (Marshak-Rothstein et al., 1979) and CRI (Marshak-Rothstein et al., 1980). Three hybridoma cell lines bearing CRI but not binding Ars were subcloned. One of these (1F6) was amplified in the ascites form in pristane-primed F1 (Balb/c × A/J) mice obtained from Jackson Laboratories, Bar Harbor, ME.

Purification of Hybridoma Protein. Hybridoma protein was precipitated from ascites by 40% $(NH_4)_2SO_4$ at 4 °C for 24 h. After centrifugation (1000*g*), the supernatant was discarded; the pellet was dissolved in a minimum volume of 10

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[1] Abbreviations: Ars, *p*-azophenylarsonate; CDR, complementarity-determining region; CRI, a predominant cross-reacting idiotype in the A/J strain of mouse defined by rabbit antisera; D, heavy-chain diversity gene-encoded region; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; GAB, 5 M guanidine hydrochloride/0.1 M sodium acetate, pH 5.5; HPLC, high-performance liquid chromatography; Ig, immunoglobulin; J_H, heavy-chain joining gene-encoded region; OPA, *o*-phthalaldehyde; Pth, 3-phenyl-2-thiohydantoin; SCMC, *S*-(carboxymethyl)cysteine; TFA, trifluoroacetic acid; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; V, variable; V_H, heavy-chain variable gene-encoded region; V_L, light-chain variable gene-encoded region; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; LPS, lipopolysaccharide.

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mM potassium phosphate buffer (pH 7.4) and dialyzed against this buffer for 24 h. The 1F6 protein was purified by ion-exchange chromatography on a DEAE-cellulose column (2.5 × 50 cm) with a linear gradient (1-L total volume) from 10 to 30 mM (potassium phosphate buffer, pH 7.4). Fractions obtained by ion-exchange chromatography were screened for the presence of antibody by electrophoresis on cellulose acetate strips. The yield of purified antibody was 3–4 mg/mL ascites.

Preparation of Heavy Chains. Antibodies eluted from ion-exchange columns were dialyzed against water. Following concentration by lyophilization, antibody was partially reduced in 0.5 M Tris-HCl (pH 8.2) containing 5 mM EDTA and alkylated with iodo[^{3}H]acetic acid (New England Nuclear, 25 μCi) as described (Novotny & Margolies, 1983). Separation of heavy and light chains was carried out on an AcA 44 (LKB) column (2.5 × 97 cm) as described (Novotny & Margolies, 1983). The purity of the isolated heavy and light chains was determined by SDS–PAGE (Laemmli, 1970).

CNBr Cleavage. Partially reduced and alkylated heavy chains (1 μmol) were dissolved in 2–3 mL of 70% formic acid (Aldrich). Fifty milligrams of CNBr (Eastman) was added, and cleavage proceeded for 24 h at room temperature. The mixture was lyophilized, dissolved in 5 M guanidine hydrochloride/0.1 M sodium acetate buffer, pH 5.5 (GAB), and applied to an AcA 44 (LKB) column (2.5 × 97 cm) in the same buffer. The column effluent was monitored by the absorbance at 280 nm. Fractions of 4.0 mL were collected. Pooled fractions were freed from salt by dialysis against water in Spectropor H tubing or by gel filtration on columns of Sephadex G-10 in 0.03 M NH_4OH and lyophilized.

Complete Reduction and Alkylation. Complete reduction and alkylation with iodo[^{14}C]acetic acid (New England Nuclear, 25 μCi) of intact heavy chain and CNBr-cleaved heavy-chain fragments were performed as described (Novotny & Margolies, 1983; Juszczak & Margolies, 1983). Fully reduced and alkylated CNBr-cleaved heavy-chain fragments were dissolved in GAB and applied to an AcA 54 (LKB) column (2.5 × 82 cm) in the same buffer. The column effluent was monitored by the absorbance at 280 nm. Fractions of 3.5 mL were collected. Pooled fractions were desalted by dialysis vs. water in Spectropor H tubing or by gel filtration on a Sephadex G-10 column (2.5 × 96 cm) in 0.03 M NH_4OH and lyophilized.

Modification of Lysine Residues and Trypsin Digestion. Completely reduced and alkylated heavy chains (1 μmol) or AcA 54 purified, CNBr-cleaved, heavy-chain fragments (200 nmol) were dissolved in GAB (3 mL). Solid succinic anhydride (Sigma) was added in portions until at least a 50-fold excess relative to lysine residues was obtained. The pH was maintained at 8.0 by addition of 2 M NaOH. The sample was dialyzed in Spectrophor F (heavy chains) or H (CNBr fragments) dialysis tubing overnight against 1% ammonium bicarbonate (pH 8.0). Trypsin (TPCK treated) (Worthington) digestion of heavy chains was performed as previously described (Cannon et al., 1978).

The heavy-chain tryptic peptides were lyophilized, dissolved in GAB (1–2 mL), and subjected to gel filtration on an AcA 54 column (2.5 × 82 cm) in the same buffer. Pooled fractions were desalted on columns of Sephadex G-10 (2.5 × 96 cm) in 0.03 M NH_4OH and lyophilized.

Carboxypeptidase A Digestion. An aliquot (2.5 nmol) of succinylated, CNBr-cleaved heavy-chain fragments was dissolved in 5% triethylamine (Pierce) for 10 min at room temperature and lyophilized in order to convert the lactone form to the homoserine peptide. Carboxypeptidase A (Worthington) digestion was in 0.2 M NH_4HCO_3 for 3 h at room temperature with an enzyme to substrate ratio of 1:50 (w/w). The digest was lyophilized twice after the addition of 0.5 mL of 1 M acetic acid and submitted for amino acid analysis (Ambler, 1972). An identical sample was acidified immediately after addition of enzyme and served as a zero-time control. An additional control, containing enzyme but no peptide, was include in each experiment for determining background amino acids.

Mild Acid Hydrolysis. An aliquot (25 nmol) of succinylated, completely reduced and alkylated CNBr heavy-chain peptide was cleaved with 0.5 mL of 0.03 M HCl for 2 h at 130 °C, based on the method described by Tsung & Fraenkel-Conrat (1965). The sample was lyophilized prior to further purification.

High-Performance Liquid Chromatography of Tryptic and Mild Acid Cleaved Peptides. High-performance liquid chromatography (HPLC) on μBondapak C_{18} (0.39 × 30 cm) (Waters) using 0.1% trifluoroacetic acid (TFA) (Pierce) (Bennett et al., 1977) and a linear gradient of 0–60% acetonitrile (Baker) over 30 min (1 mL/min) was used to isolate peptides from heavy-chain tryptic peptides and mild acid hydrolyzed CNBr fragments with detection at both 214 and 280 nm. Chromatographic peaks from several separations were collected manually, pooled, and lyophilized.

Amino Acid Analysis. Amino acid compositions were determined by using a Dionex D-500 analyzer after hydrolysis of samples in sealed, evacuated tubes at 110 °C for 24 h in constant-boiling HCl.

Amino Acid Sequence Methods. Automated Edman degradation was performed in a Beckman 890C sequencer equipped with a Beckman cold trap and a Sequemat SC-150 sequential controller (Sequemat, Inc., Watertown, MA). A 0.1 M QUADROL program (Brauer et al., 1975) which employs a single acid cleavage (3 min) was used. At cycles N-terminal in proline, the program was altered to include two successive cleavages. Polybrene (Tarr et al., 1978; Klapper et al., 1978) was added to the sequencing cup prior to degradation of peptides. A Sequemat P-6 autoconverter was used for the conversion of 2-anilino-5-thiazolinone amino acids to the Pth derivatives by using methanol/HCl (Margolies et al., 1982). All the Pth-amino acids, as well as the Pth derivatives of *S*-(carboxymethyl)cysteine (SCMC) and ε-succinyllysine, were identified by high-performance liquid chromatography on analytical cyanopropyl columns (IBM) using a phosphate buffer system, based on the method described by Hawke et al. (1982). At cycles where proline was N-terminal, *o*-phthalaldehyde (Pierce Chemical Co.) was added to the cup in butyl chloride from a separate reagent bottle (R_4) (Spiess et al., 1983; Brauer et al., 1984). The OPA blocking method was used also to analyze peptide mixtures as described (Brauer et al., 1984). Each residue was identified in at least two sequencer degradations.

Results

DEAE-cellulose chromatography resulted in the purification of hybridoma protein (yield 3–4 mg/mL ascites) free of transferrin, the major contaminating protein eluting at low ionic strength. The degree of purity of the 1F6 hybridoma was indistinguishable from that of arsonate-binding monoclonal antibodies purified by affinity chromatography. Partially reduced and alkylated heavy chains, separated from light chains by gel filtration on an AcA 44 column, were subjected to automated Edman degradation (30 nmol). As a proline residue was identified at cycle 41, *o*-phthalaldehyde treatment was used in a subsequent degradation at this cycle prior to

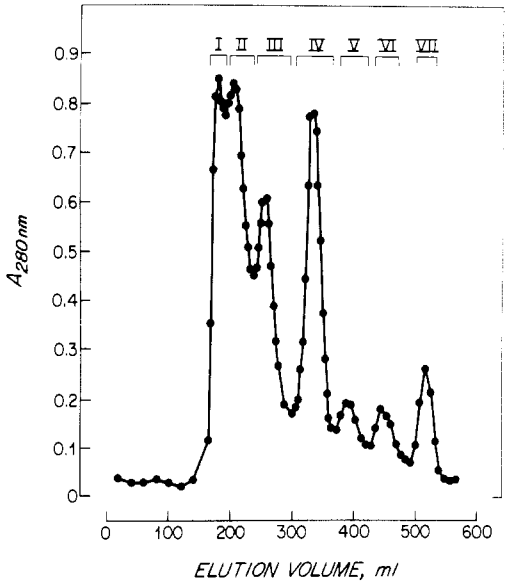

FIGURE 1: Gel filtration of CNBr peptides of the 1F6 heavy chain (1 μmol) on a column of AcA 44 (2.5 × 97 cm). The column was equilibrated in 5 M guanidine hydrochloride/0.1 M sodium acetate, pH 5.5. Fractions of 4 mL were collected. Fractions contained within the brackets were pooled, dialyzed against water, and lyophilized.

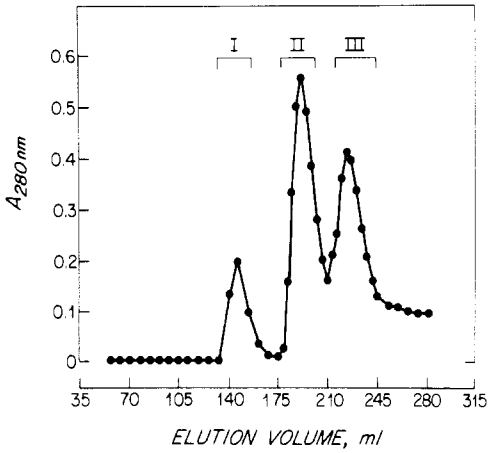

FIGURE 2: Gel filtration of variable region CNBr peptides on a column of AcA 54 (2.5 × 82 cm) in 5 M guanidine hydrochloride/0.1 M sodium acetate, pH 5.5. The CNBr peptides contained in pool IV (Figure 1) from partially reduced and alkylated heavy chains were subjected to complete reduction and alkylation to cleave the disulfide bridge between peptide CB1 (pool II, residues 1–80) and peptide CB2 (pool III, residues 81–137; see Figure 5). Fractions (3.5 mL) contained within the brackets were pooled, dialyzed against water, and lyophilized.

coupling, followed by two successive acid cleavages (6 min). This treatment reduced the background sufficiently to permit positive identification of Pth-amino acids for 52 successive cycles (Figure 5). The initial yield was 15 nmol, and the repetitive yield was 94%.

Partially reduced and alkylated heavy-chain CNBr fragments (1 μmol) were separated on an AcA 44 column in GAB (Figure 1). The peptide contained in pool IV was freed from salt by dialysis and lyophilized. An aliquot of pool IV (20 nmol) was subjected to Edman degradation, revealing two Pth-amino acids at each cycle in approximately equal yield. These results were consistent with one sequence beginning at the N-terminus of the heavy chain (E-V-Q-L-Q...) and a second sequence beginning at position 81 (Q-L-R-G-L...) on the basis of homology with known CRI^+ sequences (Figure 6) (Capra et al., 1982; Siekevitz et al., 1982). As the first constant-region methionine residue in IgG1 (Adetugbo et al., 1977) is located at position 137 [numbering according to Kabat et al. (1983)], the sequence results are consistent with two peptides in pool IV (1–80 and 81–137) linked by a disulfide bond, accounting for the entire variable region (Figure 5, CB1 and CB2, respectively). Fractions from the remaining chromatographic peaks were pooled. Some of these were screened by Edman sequence analysis, revealing peptides originating from the constant region; the remaining pools were not examined further.

Following complete reduction and alkylation, the two variable-region CNBr peptides in pool IV (Figure 1) were separated on an AcA 54 column in GAB (Figure 2). The peptides in pools I, II, and III (Figure 2) were dialyzed extensively against water and lyophilized. Pool I was screened by Edman sequence analysis and revealed two amino acids at each degradative cycle identical with residues identified in the parent pool IV (Figure 1), indicating that pool I (Figure 2) contained incompletely reduced peptides. Sequence analysis of pool II (Figure 2) revealed a single sequence (E-V-Q-L-Q...) corresponding to peptide CB1 (Figure 5). Because the N-terminal sequence of the complete heavy chain had been determined for 52 degradative cycles, corresponding to the first 52 residues of this peptide, separate aliquots of succinylated peptide CB1 were subjected to carboxypeptidase A digestion, trypsin digestion, and mild acid hydrolysis to derive the remaining sequence of CB1.

Amino acid analysis of the carboxypeptidase digest of CB1 yielded homoserine (0.46 nmol), tyrosine (0.58 nmol), alanine (0.25 nmol), and threonine (0.05 nmol) and established the carboxy-terminal sequence of CB1 as Thr^{77}-Ala-Tyr-Hse^{80} (Figure 5). Amino acid analysis had revealed the presence of four arginine residues in peptide CB1, but the arginine at residue 40 was followed by a proline residue and was not expected to be susceptible to tryptic cleavage (Carnegie, 1969). An aliquot of the tryptic digestion mixture was analyzed by automated Edman degradation. Three residues were detected at each degradative cycle tentatively attributable to three peptides (A-G-S-S-V..., residues 14–38; Q-R-P-G-Q..., residues 39–73; and S-S-S-T-A..., residues 74–80). The N-terminal peptide (residues 1–13) (Figure 5) was succinylated and was thus refractory to Edman degradation. The first two sequences were known from the prior degradation on the intact heavy chain. The last of these three peptides overlapped the sequence (76–80) (Figure 6) determined by carboxypeptidase A digestion. The mild acid hydrolyzed peptides were separated by HPLC; an aliquot (6 nmol) was subjected to Edman sequence analysis and revealed a sequence corresponding in part to residues 74–80, due to mild acid cleavage at aspartyl residue 72. Although Pth-Arg, Pth-Ala, and Pth-Tyr were identified at cycles 1, 5, and 6, respectively, Pth-serine and Pth-threonine were not identified for this peptide, possibly due to exposure to trifluoroacetic acid, used as the HPLC buffer. The C-terminal homoserine lactone was presumably extracted from the spinning cup and not identified. The partial sequence of this peptide (MA1, Figure 5) provides an overlap between residues 73 and 74 (Figure 5). The remainder of the sequence of peptide CB1 was completed by using a tryptic fragment from the succinylated heavy chain (vide infra).

Initial sequence analysis of an aliquot (10 nmol) of pool III (Figure 2) revealed the presence of a peptide (CB2) corresponding to residues 81–137. Automated Edman degradation of 40 nmol of the peptide in the presence of Polybrene permitted the identification of 48 successive cycles corresponding to residues 81–113, with an overlap of 8 residues into the IgG1 constant region (Adetugbo et al., 1977) to Ala-125 (Figure

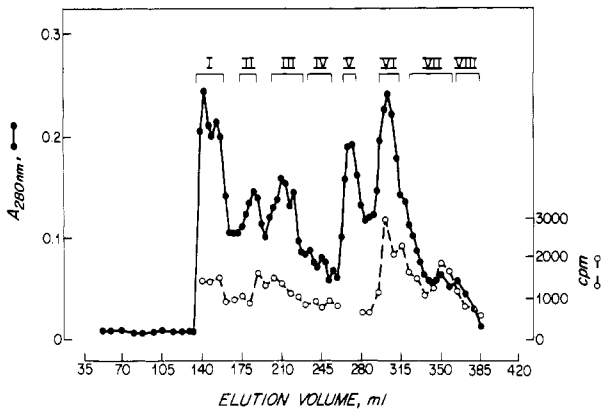

FIGURE 3: Gel filtration of a tryptic digest of completely reduced and alkylated 1F6 heavy chain. The digest (600 nmol) was applied to a column of AcA 54 (2.5 × 82 cm) in 5 M guanidine hydrochloride/0.1 M sodium acetate, pH 5.5. Fractions (3.5 mL) were pooled as indicated by the brackets. Pools V and VIII were desalted by gel filtration on G-10 (2.5 × 96 cm) in 0.03 M NH_4OH and lyophilized. Pool V contained fragment R1 (residues 39–73; see Figure 5). Pool VIII contained fragment R2 (residues 74–82a; see Figure 5) contaminated by another variable region fragment (residues 82b–94). Fragment R2 was further purified by high-performance liquid chromatography (see Figure 4).

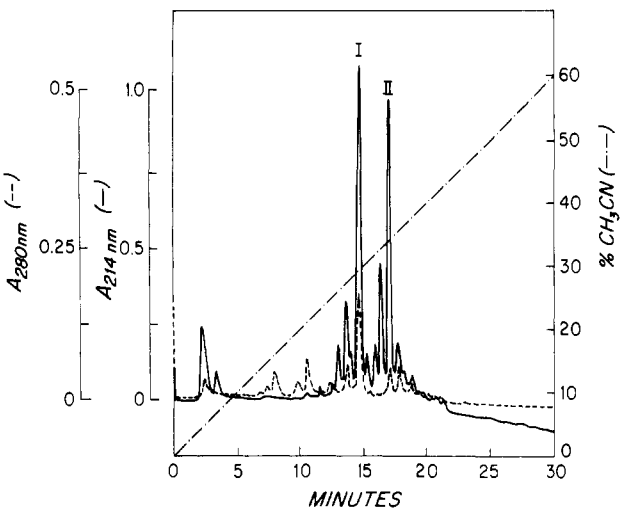

FIGURE 4: High-performance liquid chromatography of heavy-chain tryptic peptides. A mixture of two variable region peptides (pool VIII, Figure 3) (25 nmol) was applied to a μBondapak C_{18} column (0.39 × 30 cm) in 0.1% trifluoroacetic acid, and the peptides were separated with a linear gradient of acetonitrile (0–60%, 30 min) (flow rate 1 mL/min). Pool I contained peptide R2 (residues 74–82a; see Figure 5).

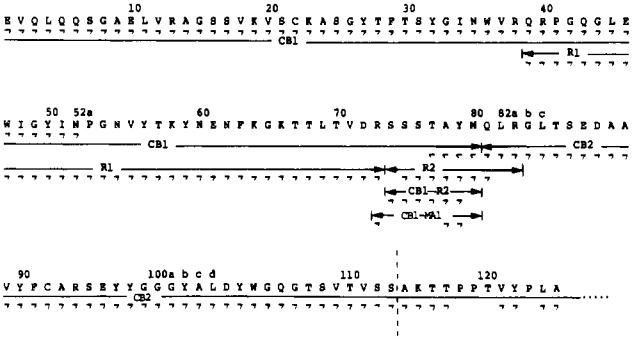

FIGURE 5: Variable region amino acid sequence of the CRI^+, Ars^- hybridoma heavy-chain 1F6. The major peptides used to establish the sequence are indicated: CB, R, CB-R, and CB-MA are used to denote peptides obtained following cleavage by CNBr, trypsin (following succinylation), trypsin following succinylation of a CNBr fragment, and mild acid hydrolysis of a CNBr fragment, respectively. Results obtained by sequence analysis of individual fragments are indicated by arrows (pointing right) below the designated fragments. Results obtained by carboxypeptidase A digestion are indicated by arrows (pointing left) below the designated fragment. Numbering is according to Kabat et al. (1983). The variable and constant regions are separated by a dashed vertical line. The sequence of the IgG1 CH1 domain is from Adetugbo et al. (1977). A one-letter code for amino acids is used (IUPAC–IUB Commission on Biochemical Nomenclature, 1968).

5). The initial yield was 19 nmol, and the repetitive yield was 95%.

In order to provide an overlap between peptides CB1 and CB2 (Figure 5) and to complete the sequence of CB1, heavy chains were completely reduced and alkylated, succinylated, and trypsin cleaved, and the resultant arginine peptides were separated on an AcA 54 column in GAB (Figure 3). Automated Edman sequence analysis revealed that pool V contained the fragment corresponding to positions 39–73 in the variable region (Figure 5) and contaminating constant-region peptides. Using an aliquot of pool V (20 nmol) and *o*-phthalaldehyde treatment at cycle 3 (i.e., a proline residue in the peptide R1) reduced the background due to contaminating peptides to a level that allowed complete sequence analysis of the peptide (Figure 5, R1). The initial yield was 12 nmol, and the repetitive yield was 95%. Sequence analysis of an aliquot (5 nmol) of pool VIII (Figure 3) revealed two amino acids at each cycle corresponding to peptides encompassing residues 74–82a and 82b–94 (Figure 5). These peptides (5 nmol) were separated by HPLC on a μBondapak C_{18} column using 0.1% TFA and a linear gradient of 0–60% acetonitrile (30 min) (Figure 4). Pool I contained peptide 74–82a (R2, Figure 5) and was nonradioactive, while pool II contained ^{14}C counts as expected (Pth-SCMC at position 92). Sequence analysis of peptide R2 (2.5 nmol) provided an overlap between CB1 and CB2 at residues 80 and 81 (Figure 5, R2). Residue 74 was not identified, possibly due to destruction of this residue by trifluoroacetic acid and the presence of contaminants at cycle 1. The sequence of peptide R2 completed the sequence of the variable region of the 1F6 heavy chain.

Discussion

The immunoglobulin molecules expressing the predominant idiotype in A/J mice associated with the response to *p*-azophenylarsonate comprise a large set of closely related, but nonidentical, variable-region structures. Amino acid sequence studies on idiotype-bearing (CRI^+), arsonate-binding (Ars^+) monoclonal antibodies indicate that the variable regions in both heavy and light chains are highly homologous (>95%) but contain substitutions both in binding site (complementarity-determining) regions and in nonbinding site (framework) regions (Marshak-Rothstein et al., 1980a; Estess et al., 1979, 1980; Margolies et al., 1981; Siegelman & Capra, 1981; Slaughter & Capra, 1983).

The localization of the structural determinants common to the members of this family which confer shared idiotypy is unknown. The sequence variability among them is reflected in the finding that CRI^+ hybridoma proteins exhibit distinct private idiotypes (Marshak-Rothstein et al., 1980b; Lamoyi et al., 1980a) and may also be distinguished serologically by monoclonal antiidiotypic reagents (Marshak-Rothstein et al., 1981; Rothstein et al., 1983). Although it is possible to thus distinguish individual CRI^+ antibodies serologically, this family of monoclonal antibodies shares conserved determinants or "public" idiotype (Marshak-Rothstein et al., 1980; Lamoyi et al., 1980b; Nelles et al., 1981).

The heavy chains of CRI^+ A/J immunoglobulins are each derived by somatic mutation from a single germ-line gene

present at the V_H locus of A/J mice (Siekevitz et al., 1983). The number and identity of the V_L genes encoding the light chains are not known, but likely are analogous to the results for the heavy chains.

In addition to the group of A/J CRI$^+$ Ars$^+$ antibodies that have been produced by immunization with antigen, an unusual set of monoclonal antibodies in which idiotypy and antigen-binding functions are not coordinately expressed has been produced. Wysocki & Sato (1981) observed that when immunization was carried out with a hapten-inhibitable rat monoclonal anti-CRI antibody, a significant increase in the Ars-nonbinding population as well as in the Ars-binding population occurred, even though Ars-nonbinding CRI is not increased following immunization with Ars–protein conjugates. These results are inconsistent with the coordinate expression of related idiotypes postulated by Jerne (1974). In order to examine the molecular basis of idiotype–antiidiotype interactions in the arsonate system, three hybridoma proteins possessing CRI but failing to bind arsonate were produced from an A/J mouse immunized with the monoclonal rat anti-CRI antibody (Wysocki & Sato, 1981).

The results of partial amino acid sequence analysis of these three CRI$^+$ Ars$^-$ monoclonal antibodies (3D10, 1F6, and 3A4) are shown in Figure 7. The heavy chains are each compared to the sequence of the CRI$^+$ Ars$^+$ hybridoma protein 36-65 (Marshak-Rothstein et al., 1980; Siekevitz et al., 1982), which is the unmutated product of the A/J V_H germ-line gene sequence (Siekevitz et al., 1983). The light-chain sequences are compared to the most common V_L sequence of CRI$^+$ Ars$^+$ monoclonal antibodies (Siegelman & Capra, 1981). In both instances, the CRI$^+$ Ars$^-$ partial V-region sequences are indistinguishable from those of the set of conventional CRI$^+$ Ars$^+$ antibodies (Margolies et al., 1983b). These sequence results and the results of DNA hybridization studies of Siekevitz and co-workers for antibody 3D10 (Siekevitz et al., 1982, 1983) and Wysocki and co-workers (L. J. Wysocki et al., unpublished results) for antibody 1F6 indicate that the CRI$^+$ Ars$^-$ and CRI$^+$ Ars$^+$ hybridoma proteins are derived from the same germ-line V_H gene. For the light chains, it is also likely that both sets are derived from very similar or identical V_L germ-line gene(s). Thus, immunization using a monoclonal antiidiotypic antibody can lead to the production of an anti-antiidiotype that is nearly indistinguishable in V_H- and V_L-encoded structure from the original idiotype. For definition of the structural differences which result in the lack of antigen binding in the CRI$^+$ anti-antiidiotype antibodies, sequence analysis of the 1F6 heavy- and light-chain variable regions is necessary. The complete heavy-chain variable region amino acid sequence of 1F6 is reported here.

The amino acid sequence of the heavy-chain V region of the CRI$^+$ Ars$^-$ hybridoma protein 1F6 is compared to that of the CRI$^+$ Ars$^+$ hybridoma protein 36-65 (Siekevitz et al., 1983) in Figure 6. Antibody 36-65 is identical in sequence with the germ-line V_H gene sequence (residues 1–94) (Siekevitz et al., 1983). The 1F6 heavy chain differs from the V_H-encoded germ-line sequence at seven residues (positions 20, 38, 55, 62, 73, 82b, and 87). Using nucleic acid hybridization and sequence analysis methods identical with those reported previously for the CRI$^+$ Ars$^-$ antibody 3D10 (Siekevitz et al., 1982), Wysocki and co-workers (L. J. Wysocki et al., unpublished results) have demonstrated that the CRI$^+$ Ars$^-$ 1F6 hybridoma protein utilizes the 36-65 germ-line V_H gene. Thus, the 1F6 V_H-encoded region (residues 1–94) is derived from the V_H CRI$^+$ germ-line gene by somatic mutation at a minimum of seven positions. Each of these mutations could arise from a single nucleotide base change.

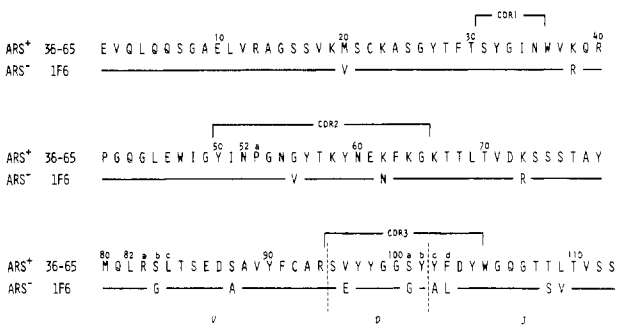

FIGURE 6: Amino acid sequences of heavy-chain variable regions of A/J monoclonal antibodies bearing a predominant idiotype. The sequence of the arsonate-binding antibody 36-65 was reported previously (Marshak-Rothstein et al., 1980; Siekevitz et al., 1982) and is identical in its V_H-encoded portion (residues 1–94) with the germ-line gene sequence for the CRI$^+$ V_H gene (Siekevitz et al., 1982, 1983). The sequence of the arsonate-nonbinding hybridoma 1F6 is reported here. A horizontal line in the 1F6 sequence indicates identity with the residues in the 36-65 sequence. Complementarity-determining regions (CDR) are indicated in brackets. Regions encoded by the V_H, D, and J_H genes are separated by dashed vertical lines. The numbering is according to Kabat et al. (1983).

Two of the seven mutations occur in CDR2 and might be responsible for the lack of antigen binding. However, the substitution at position 55 (valine) has been observed (Slaughter & Capra, 1983) in the CRI$^+$ Ars$^+$ hybridoma protein R16.7. Similarly, the asparagine substitution at position 62 in 1F6 has been reported in the CRI$^+$ Ars$^+$ hybridoma protein 124E1 (Slaughter & Capra, 1983). These observations make these mutations less likely to be related to failure to bind arsonate, although it is not possible at present to assess the contributions of mutations at both position 55 and position 62 in the same V_H combined with a CDR3 sequence in 1F6 that is different from that of the hybridoma proteins R16.7 and 124E1.

The CRI$^+$ Ars$^-$ 1F6 heavy chain differs from the CRI$^+$ Ars$^+$ heavy-chain 36-65 at two positions (96 and 100a, Figure 6) in the putative D gene-encoded segment. It is not known whether the amino acid differences are the consequence of junctional diversity, somatic mutation, or similar but distinct germ-line D genes. The germ-line D gene sequences corresponding to these hybridoma protein amino acid sequences in the A/J strain are not known. However, the core of the D gene sequence in several CRI$^+$ A/J hybridoma proteins (Y-Y-G-G-S-Y, Figure 6) corresponds to a combination of the D genes FL 16.2 and Sp 2.3 reported for BALB/c mice by Kurosawa & Tonegawa (1982). Slaughter & Capra (1983) have suggested instead that the A/J D gene sequence is derived from an FL 16.1 like D segment which has a substitution at position 100 compared to BALB/c. Although antibodies 1F6 and 36-65 utilize the same V_H gene, similar D sequences, and likely the same V_L genes, they differ in their J_H gene-encoded sequence. The CRI$^+$ Ars$^+$ hybridoma protein 36-65 utilizes the J_{H2} gene while the sequence of the CRI$^+$ Ars$^-$ hybridoma protein 1F6 corresponds to that of the BALB/c J_{H4} gene (Sakano et al., 1980) with a single somatic mutation at position 100d. Siekevitz et al. (1983) demonstrated that a single germ-line V_H gene rearranges with the J_{H2} gene segment in all of the CRI$^+$ Ars$^+$ hybridoma cell lines examined. However, among CRI$^+$ Ars$^-$ hybridomas, rearrangement occurs instead to any J_H gene segment (Gefter et al., 1984). Thus, the predominant idiotype is preserved irrespective of the J_H segment utilized.

In chain recombination studies using rabbit antiidiotypic antisera, both heavy and light chains appear to contribute to

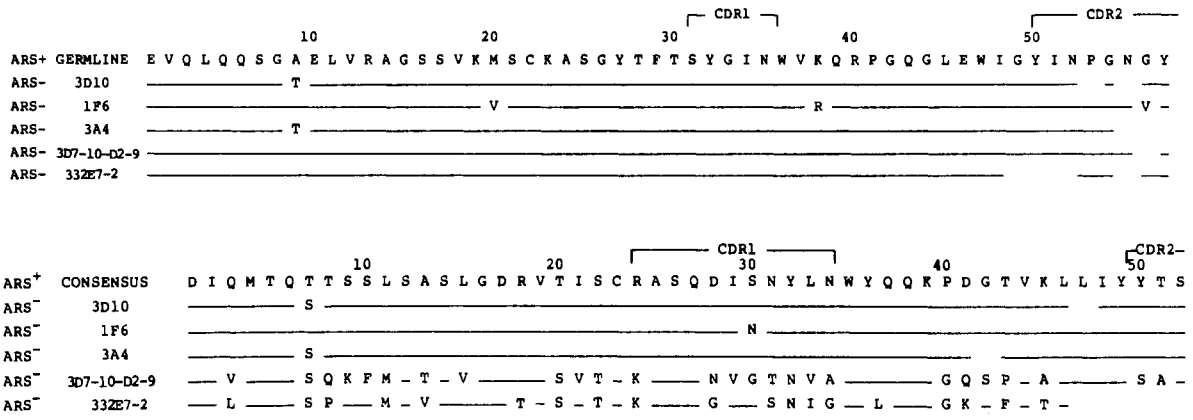

FIGURE 7: Amino acid sequences of murine A/J hybridoma proteins bearing a predominant cross-reacting idiotype (CRI). Partial sequences of heavy chains (top panel) of five arsonate-nonbinding antibodies are compared to the CRI V_H germ-line gene encoded sequence shown at the top (Siekevitz et al., 1982, 1983). Hybridomas 3D10, 1F6, and 3A4 were produced by immunization with monoclonal antiidiotype (Wysocki & Sato, 1981; Margolies et al., 1983b). The A/J IgM hybridoma 3D7-10D2-9 was a result of fusion following LPS stimulation, and the CAL 20 IgM hybridoma 332E7-2 results from fusion following immunization with arsonate linked to *Brucella abortus* (Haber & Margolies, 1984; L. J. Wysocki et al., unpublished results). The corresponding light-chain sequences of these five arsonate-nonbinding hybridomas (bottom panel) are compared with the most common V_L sequence of a set of CRI^+ Ars-binding hybridomas (Siegelman & Capra, 1981; Margolies et al., 1981).

the idiotypic determinants, although the appropriate heavy-chain donor dominates (Rothstein et al., 1983; Milner et al., 1983). However, when monoclonal antiidiotypic antibodies of murine or rat origin are used, including some that are hapten inhibitable, idiotypic determinants may be detected on isolated heavy chains (Cannon & Woodland, 1983). The results of these serologic studies are supported by the amino acid sequence results shown in Figure 7 (Haber & Margolies, 1984). The CRI^+ Ars^- hybridoma protein 3D7-10D2-9 was the result of fusion with LPS-stimulated splenocytes from an unimmunized A/J donor (D. Wechsler and V. Sato, personal communication). The CRI^+ Ars^- hybridoma protein 332E7-2 is from a CAL-20 mouse immunized with arsonate coupled to *Brucella abortus* (D. Nemazee and V. Sato, personal communication). Both of these IgM monoclonal antibodies demonstrate an unmutated CRI^+ germ-line partial V_H sequence but use light chains entirely different from the typical CRI^+ Ars^+ associated light chains. Thus, not only may the predominant idiotype be preserved despite use of a different J_H segment (1F6) but also it may be detected in these two hybridoma proteins (3D7-10D2-9 and 332E7-2) despite the use of different light chains. Although the lack of Ars binding in the latter two antibodies may be due to "incorrect" light-chain pairing, it is also known that they differ in their J_H regions from the CRI^+ Ars^+ antibodies. Antibody 3D7-10D2-9 utilizes a J_{H1} gene segment while antibody 332E7-2 utilizes a J_{H3} gene segment (Gefter et al., 1983). In the case of the CRI^+ Ars^- 1F6 protein, however, the partial light-chain sequence (Margolies et al., 1981) does not reveal differences from those of CRI^+ Ars^+ molecules. The loss of antigen binding in 1F6 as in other CRI^+ Ars^- hybridoma proteins might be correlated with the use of a different J_H segment (and/or the resultant J_H–D junctional changes) other than those used in CRI^+ Ars^+ monoclonal antibodies. In the anti-phosphorylcholine system, Cook et al. (1982) demonstrated that marked alterations in antigen binding in a mutant myeloma were due to a single J_H amino acid difference. The 1F6 sequence (Figure 6), however, demonstrates substitutions elsewhere in V_H which could also account for the lack of binding to arsonate. In this connection, Rudikoff et al. (1982) described a variant myeloma derived from the anti-phosphorylcholine protein S107 which, despite preservation of idiotypy, has lost antigen binding owing to a single amino acid interchange in the heavy-chain CDR1. Although we have thus far demonstrated a correlation between lack of binding and use of a J_{H4} gene segment, we cannot exclude the possibility that changes in the light chain and/or V_H somatic mutations account for the lack of Ars binding. Sequence analysis of the 1F6 light chain and of the variable domains of similar molecules will help settle this issue.

The existence of antigen-nonbinding molecules with preserved idiotype, as well as the occurrence of CRI^- Ars^+ molecules derived from the CRI^+ V_H gene (Margolies et al., 1983a,b; Haber & Margolies, 1984) which have lost idiotype, is a further reminder that the variable region amino acids forming idiotypic determinants and those contacting antigen are not necessarily synonymous and do not occupy the same sites in the variable region (Saul et al., 1978). Structure–function relationships are unlikely to be elucidated in systems in which positive selection by antigen is employed. In contrast, "negative" selection provides antigen-nonbinding monoclonal antibodies (Cook et al., 1982; Rudikoff et al., 1982) for analysis of the structural changes resulting in significant alterations in binding. In the arsonate system, the production of antigen-nonbinding idiotype-bearing hybridomas, through manipulations that do not involve conventional immunization with antigen (Figure 7), should permit the assignment of those structural features necessary for specific antigen recognition.

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Registry No. Immunoglobulin G1 (heavy chain variable region mouse *p*-azophenylarsonate nonbinding), 91384-54-2; *p*-azophenylarsonate, 7334-23-8; immunoglobin G1 (mouse D region), 91385-24-9; immunoglobin G1 (mouse J region), 91385-25-0.

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