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Immunology

Hybridoma proteins expressing the predominant idiotype of the antiazophenylarsonate response of A/J mice

(protein sequence/monoclonal antibodies/isotype/germline gene)

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ABSTRACT **Hybridoma cell lines that secrete monoclonal antiazophenylarsonate antibodies were isolated from the fusion of A/J splenic lymphocytes with a myeloma cell line. A small percentage of these hybridoma proteins were recognized by rabbit antisera that detect the crossreactive idiotype characteristic of the antiazophenylarsonate response of A/J mice. The isotype, pI value, and amino-terminal sequences of four independently derived idiotype-positive hybridoma proteins were determined. These proteins were either of the IgG1 or IgG2a heavy chain class. For two mice tested, the majority of the idiotype in the immune serum was shown to be of the same isotype as the fusion-derived monoclonal antibodies. The pI values of the hybridoma proteins differed from one another and ranged from 6.9 to 7.6. Amino acid sequences of the heavy chains showed a significant degree of homology with each other, but each chain was unique in the framework or the first complementarity determining region (or both). A comparable pattern of sequence variation was evident for the light chains. The azophenylarsonate idiotype, therefore, appears to consist of a family of nonidentical but closely related molecules that are the product of more than one germline gene or the result of somatic mutation of a single germline gene.**

A curious aspect of the immune response is the occasional finding that certain inbred strains of mice, when immunized with particular defined antigens, respond with a characteristic predominant idiotype; that is, a significant proportion of the antibodies elicited in all members of the strain appear identical by criteria such as serological crossreactivity, fine specificity, or isoelectric focusing (1–4). The crossreactive idiotype (Id) associated with the hyperimmune response of A/J mice to the azophenylarsonate (Ars) hapten is an example of this type of restricted response (5). Because idiotype-positive anti-Ars antibodies bind hapten with relatively low affinity (6), factors other than avidity would appear to govern their expression.

It has been suggested that a particular idiotype may dominate a response because it is identical or closely related to the product of a germline gene (7). This distinction becomes important when considering the relationship between the genotype and the generation of the antibody repertoire. Capra and coworkers have recently determined the sequence of the heavy and light chains from preparations of Id^+ anti-Ars antibody purified by combined affinity chromatography and isoelectric focusing. They reported that the heavy chains were homogeneous throughout the entire variable domain (8). The light chain sequences were identical within the complementarity determining regions (CDRs) but varied in occasional framework residues (9). These results support the hypothesis that either the entire variable region or at least the CDRs are directly encoded by a single germline gene (10).

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In the present study we report the isolation and characterization of hybridoma cell lines that secrete proteins serologically crossreactive with the Ars idiotype. These lines were initially identified as Id^+ by their ability to interact with a rabbit anti-Id antiserum. In contrast to the Id^+ antibodies described previously, the sequences of the heavy and light chains of the hybridoma proteins differ both within the framework region and the first CDR, indicating that idiotype consists of a family of molecules. The present studies also revealed that the isotype associated with idiotype varies among individual mice.

MATERIALS AND METHODS

Immunizations. A/J mice were initially obtained from the Jackson Laboratory and subsequently maintained in our own colony. All mice used for fusions were first immunized intraperitoneally with 400 μg of Ars-coupled keyhole limpet hemocyanin (Ars-KLH) emulsified in complete Freund's adjuvant and then boosted 2–3 months later with either 400 or 40 μg of Ars-KLH in phosphate-buffered saline. Spleens were removed 3 or 4 days later.

Hybridoma Cell Lines. Splenic lymphocytes were fused to the cell lines P3/X63Ag8 (P3) (11) and the nonproducing variant SP2/O-Ag (SP2) (12) as described (13). Approximately 2 weeks after fusion, hybrid clones were screened for anti-Ars activity by hemolytic spot test analysis (13) on sheep erythrocytes that had been coupled with Ars by a modification of the procedure of Sherman *et al.* (14). Areas of lysis were detected after a 1-hr incubation at 37°C in the presence of phosphate-buffered saline containing 10% guinea pig serum and 1% rabbit anti-mouse Ig as an enhancing serum. Culture supernatants from wells containing anti-Ars activity were screened for idiotype in the radioimmunoassay described below. Cloning of cell lines and production of large quantities of ascitic fluid containing antibody have been described (13). Anti-Ars hybridoma proteins were purified by affinity chromatography with Ars-bovine gamma globulin-conjugated Sepharose 4B. Specific antibody was eluted with 2 M KSCN.

Isotype Determination of Hybridoma Proteins. The heavy chain isotype of anti-Ars-producing hybridomas was initially determined by spot test analysis in which class-specific reagents obtained from Melloy Laboratories (Springfield, VA) served as enhancing sera. Assignments were later confirmed with purified hybridoma proteins in Ouchterlony precipitin tests.

Production of Rabbit Antiserum. Our first Id^+ hybridoma cell line (16-46) was identified by Robert T. Woodland (University of Massachusetts Medical School, Worcester, MA) by radioimmunoassay (15). The anti-idiotype reagents prepared

Abbreviations: Id, crossreactive idiotype; Ars, azophenylarsonate; KLH, keyhole limpet hemocyanin; P3, P3/X63Ag8; SP2, SP2/O-Ag; PTH, phenylthiohydantoin; CDR, complementarity determining region.

in our laboratory came from a rabbit that had first been immunized with A/J anti-Ars serum antibody complexed with Ars-rabbit IgG and emulsified in complete Freund's adjuvant. Nine weeks later it was boosted with 1 mg of affinity-purified 16-46 protein emulsified in complete Freund's adjuvant followed by a second injection of 16-46 protein in incomplete Freund's adjuvant. Serum was obtained 9 days after the last immunization. The anti-Id reagent used to screen for subsequent anti-Ars-secreting hybridomas was prepared by passing this immune rabbit serum through a column of Sepharose 4B coupled with normal A/J IgG. The anti-Id reagent used in the other radioimmunoassays was rendered specific by passage through a BALB/c IgG-Sepharose 4B column followed by passage through a column coupled with an A/J anti-Ars Id^- IgG2a hybridoma protein (36-60).

An antiserum specific for the P3 MOPC 21 (IgG1) myeloma protein was similarly prepared by successive absorption of a rabbit anti-MOPC 21 antiserum with normal A/J IgG and MOPC 70A (BALB/c IgG1) myeloma protein.

Radioimmunoassays. Idiotype levels were compared in two separate assay systems: the "serum idiotype" system and the "hybridoma idiotype" system. In both cases, hybridoma proteins and immune A/J serum competed for the binding of ^{125}I-labeled idiotype by a rabbit anti-idiotype serum. In the serum idiotype system, affinity-purified serum idiotype was used both for immunization of the rabbit and for the radiolabeled protein. The reagents and protocol were provided by Robert Woodland and have been described (15). In the second or hybridoma idiotype system, the anti-Id antiserum prepared from the rabbit immunized with 16-46 was used with affinity-purified 16-46 Fab fragments as radiolabeled ligand.

The isotype distribution of serum idiotype was determined in a three-step assay. Polyvinyl microtiter dishes (Dynatech Laboratories, Alexandria, VA) were first incubated with 1.0 μg of rabbit anti-Id antiserum, followed by dilutions of serum or hybridoma proteins. The plates were incubated for the last time with one of the following ^{125}I-labeled affinity purified antisera. Rabbit anti-mouse IgG serum was the gift of Michael Bevan (Center for Cancer Research, Massachusetts Institute of Technology); it was purified by absorption to A/J IgG-Sepharose 4B followed by elution with 3 M KSCN. Anti-IgG2a antibody was affinity purified from the serum of a BALB/c mouse immunized against the hybridoma protein 16-46. ^{125}I-Labeled rabbit anti-IgG1 antibody was generously supplied by Patricia Gearhart (Carnegie Institute of Washington).

The concentration of anti-Ars antibody in serum samples was determined in the radioimmunoassay system described by Klinman *et al.* (16), with 16-46-4-8 as a protein standard.

All iodinated proteins were prepared as described (15).

Sequence Analysis. Heavy and light chains were separated as described (17) except that Ultrogel ACA-44 (LKB) was used for gel filtration. Isolated chains (20–70 nmol) were subjected to automated Edman degradation in a Beckman 890C sequencer with a 0.1 M Quadrol program (18). Phenylthiohydantoin (PTH)-amino acids were identified by gas/liquid chromatography, high-pressure liquid chromatography (19, 20), and thin-layer chromatography (21). Criteria used for identification and quantitation of PTH-amino acids have been reported (22). The sequences reported here are based on two or more sequencer experiments or at least two methods for PTH-amino acid identification (or both).

Other Methods. Hybridoma proteins were focused in 5% polyacrylamide slab gels, pH range 5–11, as described (23) and stained with bromphenol blue. Fab fragments were prepared as described (24). Protein concentrations were determined by absorbance at 280 nm assuming $E^{1\%}_{1\,cm} = 14$.

RESULTS

Isolation of Hybridoma Cell Lines. The derivation and characteristics of cell lines obtained from five individual animals are presented in Table 1. The ratio of Id^+ to Ars^+ wells among the last four fusions listed in Table 1 differed despite the fact that the donor mice involved were littermates immunized in identical fashion with the same preparation of antigen. The isotype of Id^+ antibodies derived from these fusions also differed. An additional 347 clones containing anti-Ars antibody from four other fusions were screened for idiotype, but no other Id^+ wells were found.

Isotype of Serum Idiotype. The isolation of hybridoma cell lines secreting Id^+ antibody of the IgG2a class was unexpected because the majority of studies had found that idiotype was restricted to IgG1 molecules (8, 25, 26). To determine whether this association was an aberration resulting from the hybridization process or actually reflected serum idiotype components, we examined the isotype distribution of serum samples taken from 31b and 36a mice on the day of fusion. The concentration of anti-Ars antibody in both sera was approximately 200 μg/ml. If the proteins secreted by hybridomas actually reflect the distribution of isotypes within the idiotype, then the serum from fusion 31b, which produced an IgG2a Id^+ cell line, might be expected to contain predominantly IgG2a-associated idiotype, and the serum from fusion 36a, which resulted in IgG1 Id^+ cell lines, should similarly contain IgG1-associated idiotype. The results (Fig. 1) are consistent with this postulate. Id^+ hybridoma protein and the Id^+ fraction of serum antibody were bound by immobilized anti-idiotypic antibody and quantitated with ^{125}I-labeled anti-mouse IgG (Fig. 1*A*), anti-IgG1 (Fig. 1*B*), and anti-IgG2a (Fig. 1*C*). A comparison of the amount of ^{125}I-labeled anti-mouse Ig bound by the purified Id^+ hybridoma proteins to that bound by Id^+ antibody in the serum samples shows that fusion 31b and fusion 36a sera contain 20 and 40 μg/ml of idiotype, respectively, a substantial fraction (10–20%) of the total anti-Ars antibody in each sera. Both of the hybridoma proteins, 36-65 (IgG1) and 16-46-4-8 (IgG2a), titrated identically, regardless of isotype. An idiotype-negative hybridoma protein, 36-60 (IgG2a), was not detected by this assay.

As shown in Fig. 1 *B* and *C*, 36-65 was recognized by the anti-IgG1 serum but not by the anti-IgG2a serum. The opposite was true of 16-46-4-8. Both sera contained IgG1 Id^+ molecules. It is evident that virtually all of the idiotype in the fusion 36a sample is IgG1. Likewise, a major proportion of the idiotype in the fusion 31b serum sample bound by the ^{125}I-labeled anti-mouse IgG (Fig. 1*A*) is also bound by the ^{125}I-labeled anti-IgG2a reagent (Fig. 1*C*).

Table 1. Derivation and characteristics of hybridoma cell lines

Fusion no.*	Myeloma parent	$Id^+/Ars^{+\dagger}$	Isolated cell lines		
			Designation	Id	IgG
16	P3	1/82	16-46‡	+	2A
31a	SP2	0/59	31-41	−	1
31b	SP2	2/22	31-62	+	2A
36a	SP2	19/69	36-65	+	1
36b	SP2	1/3	36-60	−	2A
			36-71	+	1

* Each entry in this column represents a fusion conducted with lymphocytes from an individual animal.

† Total number of Id^+ wells relative to the total number of wells containing anti-Ars antibody.

‡ Two variants of 16-46, 16-46-4-8, and 16-46-6-46, which had lost the capacity to produce the MOPC 21 (P3) IgG1 heavy chain, were identified by their inability to react with a rabbit anti-MOPC 21 idiotype specific antiserum.

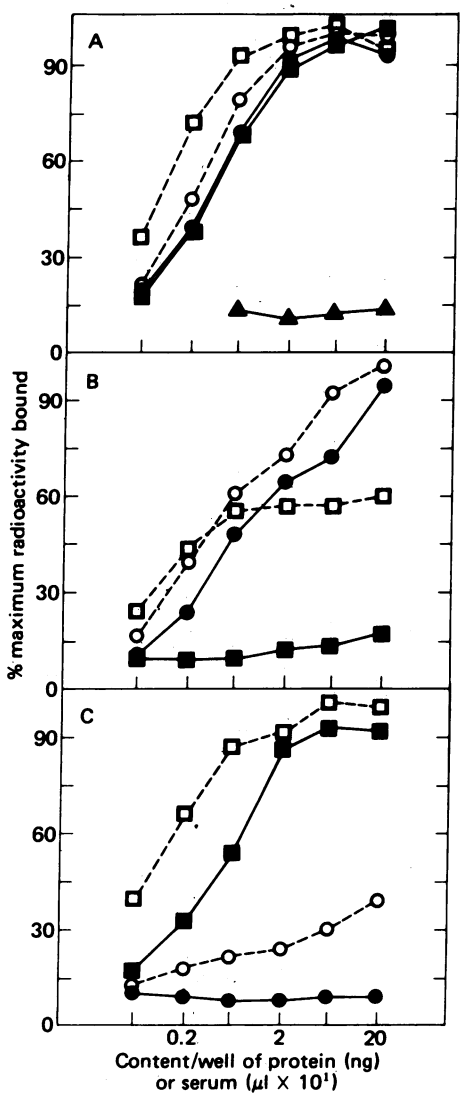

FIG. 1. Isotype distribution of serum idiotype. Sera or hybridoma proteins bound to rabbit anti-Id-coated polyvinyl microtiter plates were overlaid with ^{125}I-labeled anti-mouse IgG (*A*), anti-IgG1 (*B*), and anti-IgG2a (*C*). □---□, Fusion 31b serum; ○---○, fusion 36a serum; ■—■, 16-46-4-8; ●—●, 36-65; ▲—▲, 36-60.

Comparison of Rabbit Anti-Hybridoma Idiotype and Rabbit Anti-Serum Idiotype Assay Systems. Because the IgG2a Id$^+$ cell lines were detected with a rabbit antiserum made against 16-46 protein, it could be argued that these lines did not express the idiotype marker originally described by Nisonoff and coworkers (5). To resolve this issue, we compared the ability of the hybridoma proteins to compete for the binding of 16-46 Fab by our rabbit anti-hybridoma reagent (Fig. 2*A*) with their ability to compete for the binding of a serum idiotype ligand by a rabbit anti-serum idiotype reagent (Fig. 2*B*). In both systems, the hybridoma proteins and an immune serum standard titrated over the same relative concentration range. However, the systems differed in detail with regard to the absolute level of inhibition.

In the hybridoma system (Fig. 2*A*), 16-46-4-8 and 36-65 competed as effectively as immune serum; 31-62 and 36-71 did not quite compete to base line. Identical results were obtained if the anti-idiotypic antibody used for assay was absorbed to an immobilized Id$^+$ hybridoma protein and eluted with hapten. The original anti-idiotypic serum was 50–60% hapten inhibitable; the latter was 100% inhibitable. Anti-Ars idiotype-negative hybridomas showed an insignificant level of inhibition

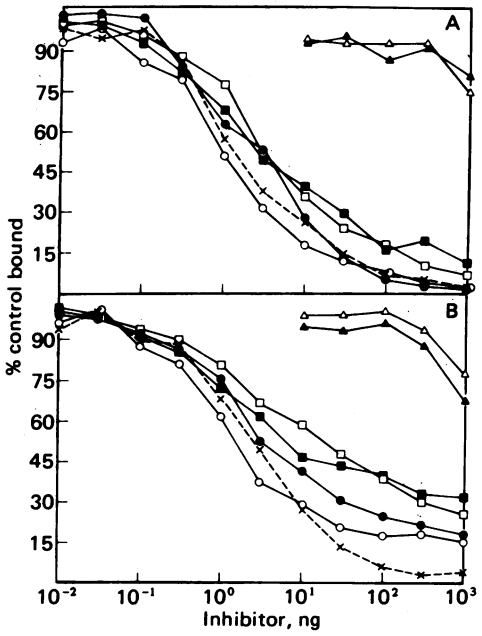

FIG. 2. Comparison of hybridoma proteins and immune serum in the hybridoma and serum idiotype systems. (*A*) Inhibition of the binding of ^{125}I-labeled 16-46 Fab by an anti-Id antibody from a rabbit immunized with the hybridoma protein 16-46. (*B*) Inhibition of the binding of ^{125}I-labeled Id$^+$ antibody by anti-Id antibody from a rabbit immunized with serum idiotype. ●, 16-46-4-8; ■, 31-62; ▲, 36-60; ○, 36-65; □, 36-71; △, 31-41; X, A/J anti-Ars-KLH. (For units on the horizontal axis equal μl × 10^{-3}.)

in this assay. Because the inhibition curves of the hybridoma proteins and immune sera exhibited comparable slopes, it was possible to quantitate the amount of idiotype present in inmmune sera by using 16-46-4-8 as a standard. Examination of 14 A/J Ars-KLH immune sera in this assay system indicated that 15–99% of the anti-Ars antibody was idiotype positive. Neither the BALB/c nor the B10.A mouse strain produced idiotype as defined by this assay system.

In the serum idiotype assay system (Fig. 2*B*), none of the hybridoma proteins attained the same degree of inhibition as the immune serum; 16-46-4-8 and 36-65 reached approximately 85% of the serum activity whereas 31-62 and 36-71 reached about 70%. A mixture of the four hybridoma proteins did not compete to a greater extent than did 36-65 alone. Either the hybridoma proteins lack certain "idiotypic determinants" or the rabbit antiserum idiotype reagent is slightly contaminated with activity against Id$^-$ serum components.

Isoelectric Focusing Patterns of Hybridoma Proteins. Most A/J serum idiotype molecules have pI values of 6.7–6.9 (25). In contrast, three of the hybridoma proteins focused outside this range (Fig. 3); 16-46-4-8, 31-62, and 36-65 had pI values of 7.6, 7.5, and 7.1, respectively. Only 36-71, one of the less effective competitors in the serum idiotype assay, focused at pH 6.9.

Amino Acid Sequences. The differences in the inhibition curves of the various hybridoma proteins and their dissimilar isoelectric focusing patterns implied that each clone secreted a unique protein. To determine if the differences in physical and serological behavior were due solely to light chain heterogeneity, we prepared heavy and light chains from each of the affinity-purified hybridoma proteins.

The results of amino-terminal sequence analysis of hybridoma heavy chains are shown in Fig. 4, where they are compared to the previously reported idiotype-positive antibodies obtained from immune mice (8, 29, 30). A single sequence was found for each heavy chain. All the hybridoma heavy chains

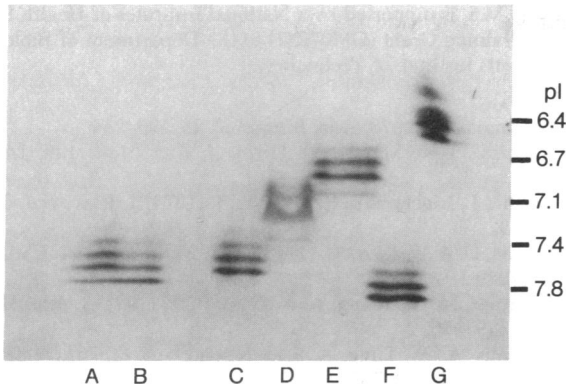

FIG. 3. Isoelectric focusing patterns of hybridoma proteins. Lane A, 16-46-4-8; lane B, 16-46-6-46; lane C, 31-62; lane D, 36-65; lane E, 36-71; lane F, 36-60; lane G, MOPC 21.

differ from each other and from the earlier sequences in the framework or first CDR (or both) although they appear to belong to the same or to a closely related subgroup.

The amino acid sequences of the hybridoma light chains are also presented in Fig. 4, where they are compared to the two dominant light chain sequences reported previously (9). The hybridoma light chains differ from each other in both the framework and first CDR. They appear to belong to the same subgroup as the nonhybridoma chains, although, once again, there are differences throughout the sequence.

DISCUSSION

The original studies by Nisonoff and his collaborators on the A/J anti-Ars response have described the appearance of a predominant idiotype whose major properties are as follows: (*i*) serological crossreactivity; (*ii*) dominance (20–70%) of the anti-Ars antibody in hyperimmune serum; (*iii*) IgG1 subclass; (*iv*) pI value ranging from 6.7 to 6.9; (*v*) lower than average affinity for hapten; and (*vi*) homogeneous sequence of the complete heavy chain variable region and of the light chain hypervariable regions (5, 6, 8, 9, 25). The nature of the idiotype associated with the anti-Ars response, as revealed by the hybridoma proteins in the present report, although similar by the first two criteria, differs in several respects.

The Id^+ hybridoma cell lines were initially identified with an antiserum specific for serum idiotype, but they did not compete quite as effectively as immune serum antibody in the serum idiotype radioimmunoassay. One explanation for the difference might be contamination of the anti-Id reagent with antibodies against "minor" shared idiotypes. Gill-Pazaris *et al.* (31) have recently reported the isolation of A/J hybridoma cell lines that are recognized by a fraction of the antibody contained in a rabbit anti-Id antiserum and would constitute such a set of minor idiotypes. Another interpretation is that the "major idiotype" is actually a family of sequences, and no single monoclonal protein expresses all the determinants recognized by an anti-"serum idiotype" reagent. This "family" model is supported by the similar, but distinct, sequences of the hybridoma heavy and light chains (Fig. 4). A comparable family of interrelated hybridoma proteins has been derived from the C57BL/6 primary anti-nitrophenyl response (32).

The inability to detect a similar degree of heterogeneity in earlier studies, even when examining antibody from individual mice (30), may be due to the methods used for protein purification. Proteins were obtained from the ascites of hyperimmunized mice, purified by affinity chromatography, and then subjected to isoelectric focusing. Proteins that focus outside the pH range 6.7–6.9 would be missed in such an analysis. In addition, minor structural variants are difficult to detect in sequence analysis of pooled material, a complication that is avoided by the use of monoclonal hybridoma proteins. However, it is difficult to reconcile the fact that each of the hybridoma proteins appears to be more closely related to one another than they are to the sequence previously reported by Capra *et al.* (8, 9). The possibility exists that the amino acid sequence of the majority of Id^+ molecules in immune serum does correspond to those previously reported and that for some unknown reason these particular Id^+ B cells either did not fuse successfully or were not detected by our assay systems. The proportion of Id^+ clones we identified was low relative to the total number of anti-Ars-secreting clones, despite high levels of idiotype in the serum. Gill-Parzaris *et al.* (31) have claimed a higher proportion of Id^+ clones in fusions that used A/J mice that had been comparably immunized.

The sequence variation among the hybridoma proteins raises the question of what is the structural basis of idiotype. Because the pattern of reactivity of our hapten-eluted anti-idiotypic antisera is the same as the original anti-idiotypic reagent with

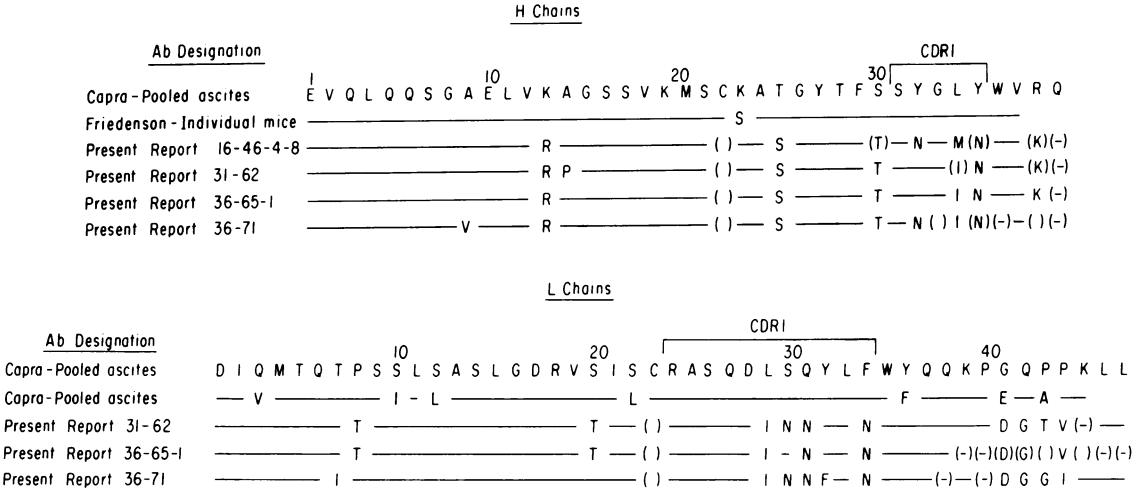

FIG. 4. Amino acid sequences of murine A/J anti-Ars hybridoma heavy and light chains carrying Id. Amino acids are indicated in the one-letter code (27). A line indicates identity with the topmost sequence. Numbering of residues and designation of complementarity determining regions is according to Kabat *et al.* (28). All residues were identified by at least two independent methods or in two separate sequencer runs (or both) except for residues in parentheses. A blank within parentheses indicates failure to identify a PTH-amino acid (heavy chains were not further reduced and alkylated after separation from light chains).

regard to both serum idiotype and the hybridoma proteins, it would appear that the idiotypic marker is at least partially associated with the binding site. Nevertheless, because the sequences of the hybridoma proteins differ within the first CDR, the assumption that idiotype is dependent on identical primary sequences within all three CDRs can be excluded. The present sequence data are consistent with the following possibilities. (*i*) Idiotype is dependent on homologous sequences in portions of the variable region domain other than the segment reported above (second or third CDR or J region); (*ii*) idiotype is dependent on only a few relevant residues contributed by one or more of the CDRs from one or both chains; or (*iii*) idiotype may result from alternative sets of residues which together satisfy the requirements for reaction with an anti-idiotypic antiserum. The nonidentity of the competition curves of the hybridoma proteins (Figs. 2 and 3) indicate that "idiotype" is not a unique structure. Instead, idiotype may be viewed either as a set of determinants, some of which are shared by the hybridoma proteins, or as a single structure subject to a minor degree of degeneracy. This issue, in principle, will be clarified when the complete hybridoma variable region sequences and monoclonal anti-idiotype antibodies become available.

In contrast to the earlier sequence study (9), all the hybridoma light chains appear to belong to the same subgroup. Previous genetic studies have shown that the light chain locus contributes to the inheritance of the idiotype marker (33); the similarity of the hybridoma light chain sequences may reflect the requirement for the expression of a particular allele(s) of the light chain locus.

Another unexpected property of the hybridoma proteins was the frequent occurrence of IgG2a Id^+ molecules. Most previous studies had shown idiotype to be associated with the γ_1 constant region (8, 25). Marchalonis *et al.* (26) reported that the serum Id^+ antibody isolated and sequenced in their laboratory was IgG2a. They suggested that the observed difference could be attributed to the breeding history of their mice or to the use of a different Ars-protein carrier. These arguments are not sufficient to explain the results of the present study. The IgG2a nature of our hybridoma proteins was shown to be representative of the IgG2a Id^+ serum components of certain mice. Because both the γ_1 and γ_{2a} cell lines were derived from littermates immunized with the same preparation of antigen, the regulatory network controlling the association of variable and constant region genes appears to be a complex phenomenon.

The existence of cell lines that secrete a predominant idiotype affords the unique opportunity to address a number of questions concerning the expression of the antibody repertoire. Is the structural information for idiotype only present in A/J mice or are regulatory genes involved? Does the existence of a family of sequences indicate the presence of a family of genes or is there somatic diversification of a single germline gene? The use of V_H and V_L nucleic acid probes prepared from these cell lines should permit the resolution of these issues at the molecular level.

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