

## STRUCTURAL CHARACTERIZATION OF ANTIIDIOTYPIC ANTIBODIES

Evidence that Ab2s Are Derived from the Germline  
Differently than Ab1s

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Idiotypic specificities were originally described by Kunkel et al. (1) and Oudin et al. (2) as markers that distinguished the V regions of specific antibody molecules. Jerne (3) later proposed that idiotypes might serve as regulatory targets of the immune system and that there was a coexistence within each individual's repertoire of complementary V region partners. According to this hypothesis, the network could provide a means of regulating available repertoires and controlling ongoing immune responses. In a more recent version, Jerne (4) proposed that the germline encodes "pairs" of antiself and anti-idiotypic antibodies that recognize the antiself set, and thus, establishing a series of interacting antibodies (Ab1, Ab2, Ab3, . . .).

We have addressed some of these issues by sequencing a group of syngeneic and allogeneic monoclonal Ab2s primarily in the antiarsonate system. In this antihapten response (unlike the response to more complex antigens) the resulting antibodies are relatively homogeneous involving distinct V, D, and J gene segments. We reasoned that if complementary pairs of V regions were encoded within the germline, then the syngeneic antiidiotypic response, like the antiarsonate response, should also be homogeneous. On the other hand, in an allogeneic (i.e., different Ig loci), the Ab2 response would resemble the response to any exogenous protein antigen; i.e., elicit a heterogeneous population of antibodies recognizing distinct epitopes and would be derived from a variety of different V region gene segments.

Jerne's hypothesis has been addressed by sequencing Ab2s in several other antigen systems (5-8). In the two most extensive studies reported, the structures of syngeneic antiidiotypic antibodies were homogeneous, supporting the view that complementary pairs of V genes exist in the germline. However, contrary to Jerne's hypothesis, the antibodies were not derived directly from germline genes; somatic

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processes were involved in their generation. Additionally, many of the Ab2s had structurally novel D segments. In this report, we expand the information available on Ab2s and propose that the origin of such antibodies differs in syngeneic vs. allogeneic systems. Further, our data, together with the available sequences of other Ab1 and Ab2 antibodies, suggests that the genetic origin (especially of the D segments) of these molecules may differ.

### Materials and Methods

*Hybridomas.* The derivation of hybridoma cell lines E3, E4, H8, 2D3, 12S18-1, 12S28-16, 12S84-3, 6C4, 6B1, N20, D8-3, GB4-10, B3682, B3675, and B3938 have been described previously (9-14).

*Isolation of Sequencing of mRNA.* Isolation of mRNA was by the guanidinium thiocyanate method as described by Chirgwin et al. (15). Sequence analysis of mRNA from hybridoma cell lines, using dideoxynucleotides, was done essentially by the method described by Kaartinen et al. (16). The H and L chain V region sequences of hybridomas H8, E4, 12S18-1, 12S28-16, and 12S84-3 were determined using this method exclusively. The partial H chain V region sequences presented in this report were also done by this method. The H and L chain V region sequences of hybridomas E3 and 2D3 were determined using mRNA sequence analysis, but were confirmed by specific cDNA cloning similar to the method described by Levy et al. (17).

*Isolation and Sequencing of Specific V Region cDNAs.* Total cellular RNA was used as a template for first strand synthesis, which was primed with a synthetic oligonucleotide complementary to the V-C bridge with the addition of an additional six nucleotides, creating an Xba I restriction enzyme site and a 2-bp spacer on the 3' end of the oligonucleotide. The sequence of the primer used for cloning H chain V regions was (5'AAATCTAGAGGGCCAGTGG-ATAGAC3'). The sequence of the primer used for cloning  $\kappa$  V regions was (5'AAATCTAGATGGATGGTGGGAAGATG3'). Second strand synthesis was accomplished by the addition of RNase H and DNA polymerase I. T4 DNA polymerase was used to blunt the ends, and the Xba I site (incorporated into the cDNA via the primer) was cleaved with the restriction endonuclease Xba I. The V region cDNA was ligated into the Sma I and Xba I sites of a suitable phagemid vector (generally PTZ18U or PTZ19U), and the DNA transformed into competent BSJ72 cells. Alternatively, Eco RI linkers were added after the T4 DNA polymerase step. cDNA was size selected on a low melt agarose gel. The resulting cDNA was cloned into the Eco RI site of the  $\lambda$ -Zap (Stragene, La Jolla, CA) phage vector. Bacterial colonies or phage plaques were screened with end-labeled synthetic oligonucleotides. Sequencing of the resulting cDNAs was done by preparing ssDNA from the phagemid, and sequencing via the method of Sanger (18).

### Results and Discussion

#### *The A/J Strain's Antiarsenate Response Is Associated with a Germline-encoded Idiotypic*

When immunized with the hapten para-azophenylarsenate (Ars), the antibody response of the A/J strain can be grouped into three families, Ars A, B, and C (19). This response is dominated by a crossreactive idiotype termed CRI<sub>A</sub>, which is known to be associated almost exclusively with antibodies in the Ars A family (20). With rare exception, all of the antibodies in this family are derived from the same germline V<sub>H</sub>, D, J<sub>H</sub>, V <sub>$\kappa$</sub> , and J <sub>$\kappa$</sub>  gene segments. Both the H and  $\kappa$  chain V region gene segments encoding these antibodies have been cloned and sequenced (21-23). Ab1s that express the CRI<sub>A</sub> most strongly are those that are closest to germline in structure, so the CRI<sub>A</sub> can essentially be termed a germline-encoded idiotype (24, 25).

*Seven Monoclonal Antiidiotypic Antibodies Recognize Public Idiotypes that Comprise a Portion of the CRI<sub>A</sub>*

The isolation and binding characteristics of the seven monoclonal anti-idiotypic antibodies described here have been previously reported (9, 10). Briefly, four allogeneic Ab2s were isolated from BALB/c mice immunized with polyclonal Ars A antibodies and then were boosted with a monoclonal Ars A antibody. BALB/c mice normally do not express this idio type presumably because they lack the appropriate H chain V region gene segment.

Table I shows the reactivities of the Ab2s with a panel of Ars A antibodies. The variability in reactivity with the Ab1s exhibited by each Ab2 implies that each of the antibodies recognizes a different determinant on the Ab1s. The Ab1s that express the CRI<sub>A</sub> most strongly (as defined with a polyclonal rabbit antisera) also express most of the idiotypes defined with these Ab2s. This implies that these Ab2s recognize structures defined as the CRI<sub>A</sub>.

The determinants on Ars A molecules that influence the binding of each anti-idiotypic have been mapped to specific regions of the V regions (26). The expression of three of the idiotypes (E3, E4, and H8) is dependent on amino acid residues located in the D region of Ars A molecules. The fourth Ab2 (2D3) recognizes a determinant encoded by the V<sub>H</sub> Ars gene, the expression of which is most heavily influenced by amino acid 59 in the second hypervariable region of the H chain. All Ars A antibodies that have a lysine in this position express the idio type while those that have other amino acids at position 59 do not.

The three syngeneic Ab2s described in this report were isolated from A/J mice immunized with A/J polyclonal CRI<sub>A</sub>-positive antiarsenate antibodies (10). The Ab1 specificities of two of these Ab2s is shown in Table I. The epitope that these antibodies recognize may be the same or similar to the epitope recognized by the allogeneic Ab2, E4, since their reactivity patterns are nearly indistinguishable. The serologic expression of syngeneic antiidiotypic antibodies in this system is very low, as has been reported in other idiotypic systems (27, 28).

*Four Allogeneic Antiidiotypic Antibodies Most Likely Derive from Different V<sub>K</sub> and V<sub>H</sub> Germline Genes*

*L Chain Analysis.* Figure 1 shows the nucleotide and deduced amino acid sequences of the L chain V regions from mAbs E4, H8, 2D3, and E3. Each is distinct from

TABLE I  
*Reactivity of Ab2s with Antiarsenate Ab1s*

Ab1	Ab2 reactivities						
	CRI	E4	E3	H8	2D3	12S18-1	12S28-16
3665	+	+	+	+	+	+	+
101F11	+	+	-	+	+	+	+
93G7	+	+	+	+	-	+	+
91A3	+	+/-	-	-	-	-	-
96B8	-	-	-	-	-	-	-
22B5	+	+	-	+/-	-	-	+/-
9A5	+	+	+	+	-	+/-	+/-

(+) Strong reactivity; (-) no detectable reactivity; (+/-) weak, but significant binding.

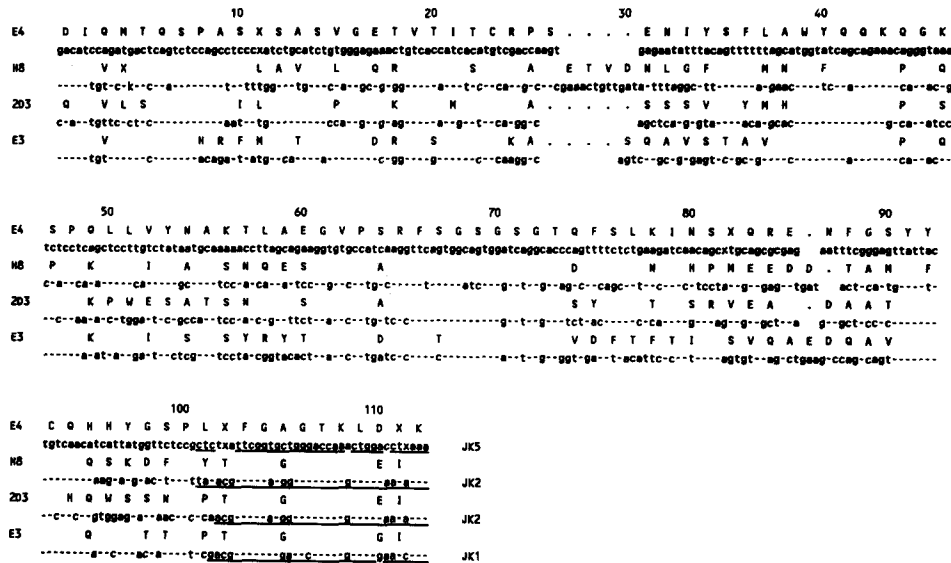


FIGURE 1. Nucleotide and deduced amino acid sequences of  $V_{\kappa}$  region sequences of allogeneic Ab2s compared with E4's  $V_{\kappa}$  sequence. -, Identical nucleotide; A, adenine; C, cytosine; G, guanine; T, thymine. Only amino acid residues differing from the top sequence are indicated. A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine. Under-scoring indicates homology with germline  $J_{\kappa}$  segment as indicated. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00794.

the rest bearing no more than 55% amino acid sequence homology, implying that all derive from different  $\kappa V$  region gene families. E4 derives from a member of the  $V_{\kappa}$  12-13 family and  $J_{\kappa}5$ ; H8 derives from the  $V_{\kappa}21$  family and  $J_{\kappa}2$ ; 2D3 derives from the  $V_{\kappa}4$  family and  $J_{\kappa}2$ ; and E3 derives from the  $V_{\kappa}19$  family and  $J_{\kappa}1$ .  $V_{\kappa}$  families are as delineated by Potter et al. (29). Since these molecules are not closely homologous (<95%) to any published germline  $\kappa V$  region gene segment, it is impossible to judge whether or not somatic mutation has contributed to their generation. However, somatic mutation is apparent in the  $J_{\kappa}$  segments of both E3 and E4. Since it is well documented that somatic mutation occurs in the  $V_{\kappa}$  segments of expressed antibodies at a similar frequency to that seen in  $J_{\kappa}$  (30), it is likely that moderate somatic mutation has occurred in these  $V_{\kappa}$  gene segments as well.

**H Chain Analysis.** Fig. 2 shows the nucleotide and deduced amino acid sequences of the H chain V regions of mAbs E4, H8, 2D3, and E3. Each is distinct from the others, even deriving from different H chain V region gene families. The E3 H chain is a member of the 3609 H chain V region gene family, whereas the H chains of the other three allogeneic Ab2s are members of the J558 family. Since the H chain V regions of E4, H8, and 2D3 are only 70–80% homologous to one another, it is certain that each derives from a separate member of the J558 gene family. Southern filter hybridization studies using  $J_H$  region probes confirms this conclusion (data not shown).

As in the L chain V region gene segments, none of these molecules is >95% ho-

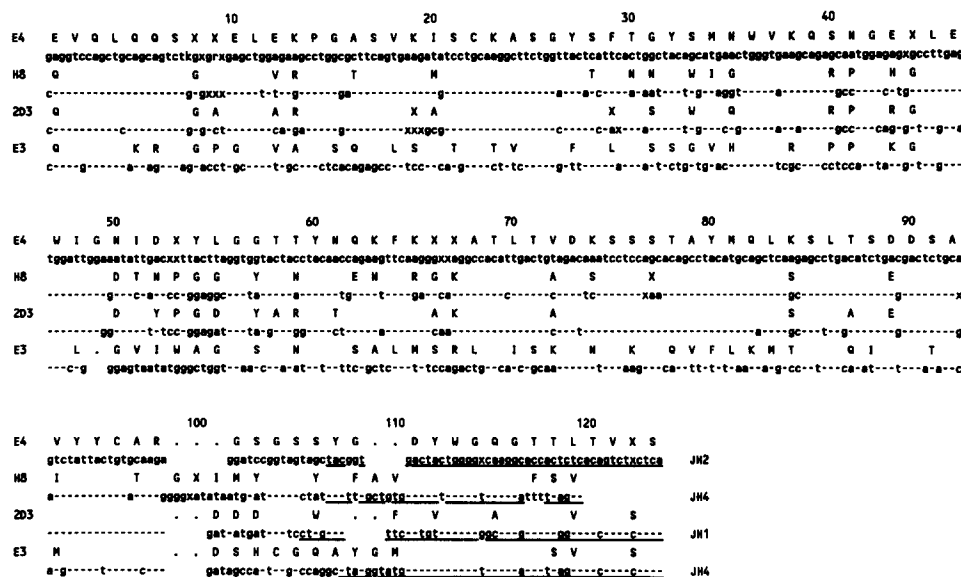


FIGURE 2. Nucleotide and deduced amino acid sequences of  $V_H$  region sequences of allogeneic Ab2s compared with E4's  $V_H$  sequence. (-), Identical nucleotide; A, adenosine; C, cytidine; G, guanosine; T, thymidine. Only amino acid residues differing from the top sequence are indicated. A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine. Under-scoring indicates homology with germline  $J_H$  segment as indicated. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00794.

mologous to any published germline  $V_H$  gene segment, making it impossible to judge the extent of somatic mutation in the  $V_H$  gene segments. H8 and E3 both use  $J_H4$ ; whereas E4 uses  $J_H2$  and 2D3 uses  $J_H1$ . Somatic mutation is apparent in all of the  $J_H$  segments, and so somatic mutation has probably occurred in the  $V_H$  gene segments of these Ab2s as well.

#### *The D Segments of Allogeneic Ab2s Do not Directly Derive From Germline D Segments*

The D segments of the four allogeneic Ab2s are depicted in Fig. 3. None can be completely explained by any of the known germline D segments. The core of E4's D segment probably derives from DFL16.1. The first three to five nucleotides are probably the result of N segment addition by terminal transferase during V-D rearrangement. H8's D segment may be the result of an inverted fusion between the noncoding strand of DFL16.2 (cDFL16.2 in Fig. 3) and the coding strand of DSP2.7. We have previously proposed that an inverted fusion may explain the conserved N segment seen in the majority of Ars A molecules (31). Ollier et al. (6) have proposed a similar mechanism in two GAT Ab2s. First, an inverted recombination would occur between the coding strand of DSP2.7, using the alternative signal sequences proposed by Kurosawa and Tonegawa (32), and the noncoding strand of DFL16.2 (cDFL16.2). Then, normal D to  $J_H$  joining between the usual signal sequences of DSP2.7 and  $J_H4$  would take place. Finally,  $V_H$  to D joining would occur between

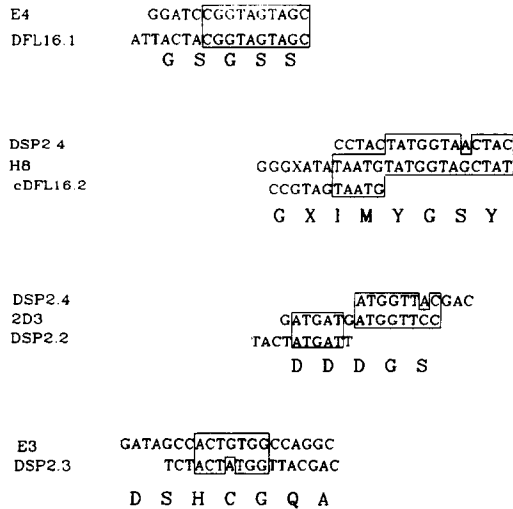


FIGURE 3. Nucleotide and deduced amino acid sequences of the D region of monoclonal allogeneic anti-idiotypic antibodies. Boxes have been placed around nucleotides identical to sequences of known germline D sequences. The reverse complement of a particular sequence is indicated by a *c* before the name. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00794.

the 5' portion of the noncoding strand of DFL16.2 (cDFL16.2) and the 3' portion of  $V_H$ . 2D3's D segment appears to be distinct from any D segment described to date. D-D fusion is again a possibility in that the first 5 bp could have been derived from one DSP2 type D segment, and then the next 6 bp derive from another. There are other possible explanations for these D segments, including  $V_H$  region replacement (33), yet-undiscovered D segments, and specific terminal transferases (34). E3's D segment may derive in part from DSP2.3.

In contrast to what is usually seen in Ig D segments, there are many hydrophilic residues (especially acidic residues) in these allogeneic Ab2 D segments. 2D3's D segment is acidic, having three aspartic acid residues in a row. The residue in the Ab1s that seems to most strongly affect the expression of the epitope recognized by 2D3 is the lysine in position 59 of the H chains (26). At physiologic pH, lysine is positively charged, whereas aspartic acid is negatively charged. Thus, a portion of the interaction between the antiidiotype and the primary antibody may be ionic in this instance.

### *Three Syngeneic Antiidiotypic Antibodies Are Structurally Virtually Identical*

**L Chain Analysis.** Fig. 4 shows nucleotide and deduced amino acid sequences of the  $\kappa$  chain V region of two of the three syngeneic Ab2s, 12S18-1 and 12S28-16. They are identical to one another except for two silent substitutions (amino acids position 86 and 88). Their  $J_\kappa$  segments are identical to the reported BALB/c  $J_\kappa 1$  germline (35). Southern filter hybridization analysis using  $J_\kappa$  probes confirms that the syngeneic Ab2 L chains derive from the same  $V_\kappa$  gene segment (data not shown), which is distinct from the  $V_\kappa$  gene segments used by the allogeneic Ab2s. The amino acid sequences of the L chain V regions are also quite distinct from the allogeneic Ab2s, the closest of which (E3's) is only 70% homologous. 12S18-1 and 12S28-16 are members of the  $V_\kappa 14$  family.

**H Chain Analysis.** Fig. 5 shows the H chain V region sequences of the three syn-

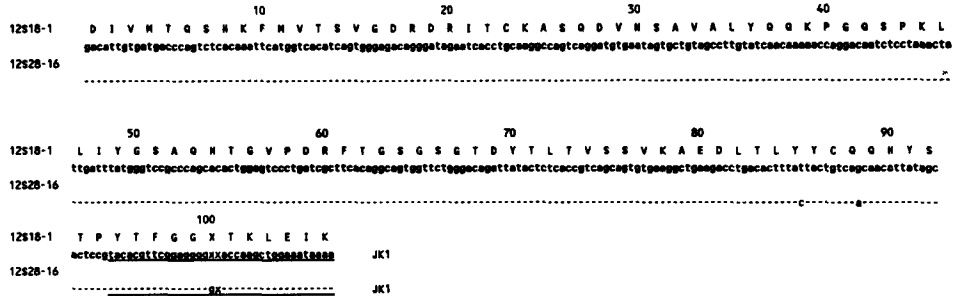


FIGURE 4. Nucleotide and deduced amino acid sequences of  $V_{\kappa}$  region sequences of syngeneic Ab2s compared with 12S18-1's  $V_{\kappa}$  sequence. -, Identical nucleotide; A, adenosine; C, cytidine; G, guanosine; T, thymidine. Only amino acid residues differing from the top sequence are indicated. A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine. Under-scoring indicates homology with germline  $J_{\kappa}$  segment as indicated. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00794.

genic Ab2s. They are identical to one another even in their V-D and D-J junctions. Southern filter hybridization analysis confirms that they derive from the same H chain V region germline gene segment (data not shown) that is a member of the J558 gene family. The J558 family can be divided into several subfamilies (23), and the allogeneic Ab2  $V_H$  sequences are most similar to NPb-like members of this family. This  $V_H$  gene segment is distinct from the  $V_H$  gene segments used by the

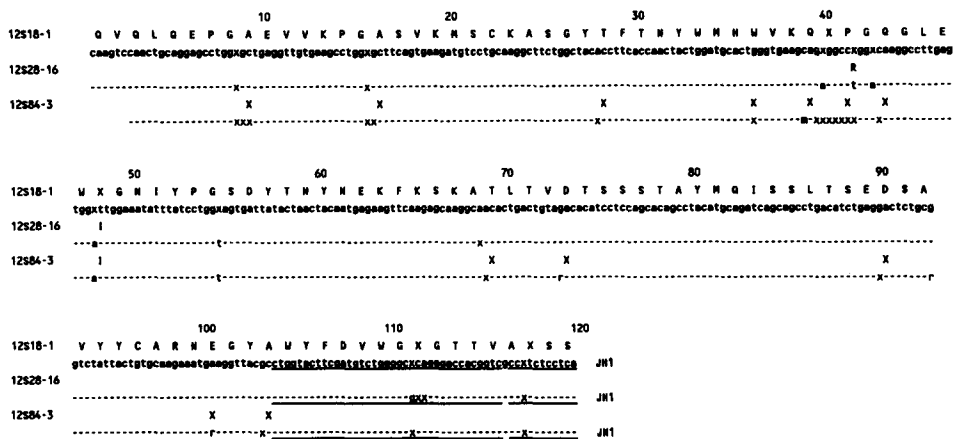


FIGURE 5. Nucleotide and deduced amino acid sequences of  $V_H$  region sequences of syngeneic Ab2s compared with 12S18-1's  $V_H$  sequence. -, Identical nucleotide; A, adenosine; C, cytidine; G, guanosine; T, thymidine. Only amino acid residues differing from the top sequence are indicated. A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine. Under-scoring indicates homology with germline  $J_H$  segment as indicated. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00794.

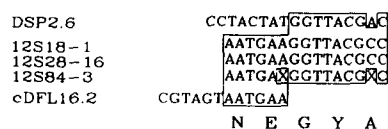


FIGURE 6. Nucleotide and deduced amino acid sequences of the D region of monoclonal syngeneic anti-idiotypic antibodies. Boxes have been placed around nucleotides identical to sequences of known germline D sequences. The reverse complement of a particular sequence is indicated by a *c* before the name.

four allogeneic Ab2s. While three of the syngeneic Ab2s (E4, H8, and 2D3) also derive their  $V_H$  segments from the J558 family, they are still only 75–80% homologous to this  $V_H$  segment. Although the allogeneic Ab2 E4 has a similar serologic reactivity pattern to the syngeneic Ab2s, its primary structure is no more homologous to the syngeneic Ab2s than are the other allogeneic Ab2s.

The complete identity of these three H chains (even at their VD and DJ joints) suggests that they may be clonally related. Southern filter hybridization studies using  $J_K$  and  $J_H$  probes could neither confirm nor rule out this possibility (data not shown). These molecules derive from 3 of 10 cell lines established after the fusion of the pooled spleens of three mice. The hybridomas were selected for study because of isotypic differences (10). One is a  $\gamma_1$ , one is a  $\gamma_{2a}$ , and the third is a  $\gamma_{2b}$ . The syngeneic response in the arsonate system is extremely weak serologically as in many of the systems studied to date (5). In this instance, a significant portion of the Ab2 response arose from either a single B cell precursor or from a small number of closely related clones using the same germline gene segments.

#### *The D Segments of Three Syngeneic AB2s May Have Derived from D-D Fusion*

Fig. 6 shows the D segments of the three syngeneic Ab2s. Each is 14 bp long. The first 6 bp appear to be derived from the noncoding strand of DFL16.2 (cDFL16.2); the next eight appear to be from the coding strand of DSP2.6 and are identical except for a 1-bp difference. Again, an inverted recombination would explain the origin of these D segments, but in this case, half of the D segment appears to have derived from the inverted fusion. Alternatively, this D segment may derive from an undiscovered germline D segment. It is possible that the A/J's repertoire of D segments is different from the BALB/c's, as limited D segment polymorphism has been reported previously (36, 37).

#### *Novel D Segments Are a Feature of Ab2s*

In three systems in which Ab2s have been structurally studied, a high proportion of the molecules appear to have D segments that are generated by novel mechanisms. In addition to the arsonate Ab2s, we have performed limited sequence analysis on several other Ab2s from other antigen systems (antiphosphocholine, antidextran, and anti-3 fucosyl lactosamine) to determine whether or not novel D segments are a common feature of Ab2s (Fig. 7). This also enabled us to determine the structural similarity in the  $V_H$  segments of these antibodies. The results have been compiled in Table II with other Ab2 structures already reported.

In the systems studied to date, V region gene usage is restricted in syngeneic Ab2s recognizing public idiotypes, but not in allogeneic Ab2s. In the anti-GAT and anti-NP systems, syngeneic Ab2s were strikingly similar in both their H and L chain V regions (5, 6). In the PC and 3 fucosyl-lactosamine systems, the  $V_H$  segments of syngeneic Ab2s are homologous to one another, but their D and  $J_H$  segments



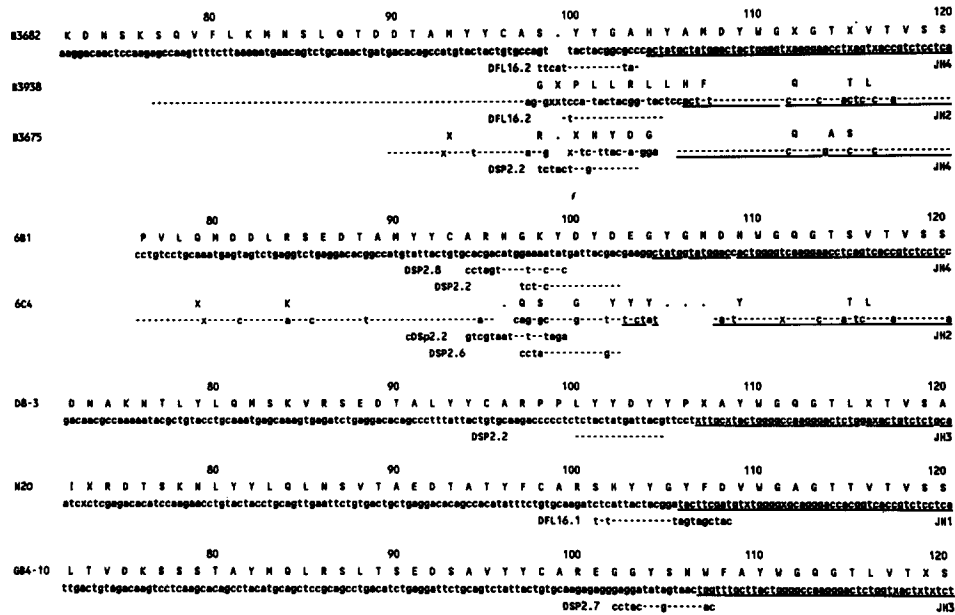


FIGURE 7. Partial nucleotide and deduced amino acid sequences of V<sub>H</sub> region sequences of Ab2s from other antigen systems. B3682, B3938, and B3675 are syngeneic antiphosphocholine Ab2s. GB4-10 is an allogeneic antiphosphocholine Ab2. 6B1 and 6C4 are syngeneic anti-3-fucosyl lactosamine Ab2s. N20 is an antidextran Ab2. D8-3 is anti-J558. -, Identical nucleotide; A, adenine; C, cytosine; G, guanosine; T, thymidine. Only amino acid residues differing from the top sequence are indicated. A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tryrosine. Germline D segments with maximum homology with expressed D segments are shown under each Ab2 sequence. Underscoring indicates homology with germline J<sub>H</sub> segment as indicated. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00794.

differ. In contrast, the allogeneic PC Ab2s use distinct H and L chain V regions (7). The only syngeneic Ab2s that derive from unique V<sub>H</sub> genes with respect to other Ab2s within that system are GAT Ab2s that recognize private idiotypes (HP9, HP12, and HP13) (6) and one NP Ab2 (5). These data support Jerne's idea of the coexistence of complementary sets of germline V region genes.

The present studies show that there is nothing particularly unique to the V regions of antiidiotypic antibodies. With the exception of the V<sub>κ</sub> segment from the reovirus Ab2 (8), these antibodies derive from germline H and κ chain V region genes that can easily be placed within already described V region families.

Previously unpublished Ab2 sequences are shown in Fig. 7. B3682, B3938, and B3675 are syngeneic PC Ab2s. GB4-10 is an allogeneic PC Ab2. 6B1 and 6C4 are syngeneic 3 fucosyl-lactosamine Ab2s. N20 and D8-3 are syngeneic dextran Ab2s. As can be seen, the D segments of several appear to have arisen via D-D fusion (most notably in antibodies 6B1 and 6C4). However, several others derive via conventional rearrangements. To date, a total of 32 antiidiotypic antibodies have been partially

TABLE II  
Summary of Available Ab2 Structures

Name	Syngeneic/ allogeneic	System	V <sub>H</sub> Family	Length of DH	Length of 5'N	Length of 3'N	Reference
A2597	S	NP	J558	29	3	7	5
A3160	S	NP	J558	29	1	9	5
A3940	S	NP	J558	30	9	8	5
A8/24	S	NP	J558	12	5	2	5
A6/24	S	NP	J558	18	2	3	5
HP22	S	GAT	3660	33	16	6	6
HP27	S	GAT	3660	33	16	6	6
HP20	S	GAT	3660	30	4	20	6
HP21	S	GAT	3660	30	4	9	6
HP25	S	GAT	3660	30	4	9	6
HP9	S	GAT	S107	21	10	0	6
HP12	S	GAT	3660	18	2	6	6
HP13	S	GAT	J558	36	9	6	6
4C11	A	PC	J558	11	0	1	7
F63	A	PC	J558	19	13	0	7
GB4-10	A	PC	J558	18	9	0	*
B3682	S	PC	3609	13	0	4	*
B3938	S	PC	3609	22	4	2	*
B3675	S	PC	-	15	1	3	*
D8-3	S	J558	X24	27	7	5	*
N2O	S	Dex	3660	15	2	1	*
87.92.6	S	Reo	J558	18	4	4	*
E3	A	ARS	3609	20	7	6	*
E4	A	ARS	J558	21	5	0	*
H8	A	ARS	J558	24	12	1	*
2D3	A	ARS	J558	14	7	1	*
12S18-1	S	ARS	J558	14	6	0	*
6C4	S	3FL	7183	18	6	3	*
6B1	S	3FL	7183	26	9	5	*

\* Sequence information from this report.

or completely sequenced. Since three in one case and two in another may have been clonally related, this represents 29 distinct Ab2s.

It is possible that novel D segments occur commonly in many types of antibodies. To differentiate whether or not novel D segments are a common structural feature of Ab2s (and therefore relevant to their specificity), we have compared D segments from Ab2s with those of Ab1s. For each antibody analyzed, the D segment was defined as the portion of the third hypervariable region that could not be accounted for by either the 3' portion of the V<sub>H</sub> segment or the 5' portion of the J<sub>H</sub> gene utilized. Then, the percentage of each D segment that could be explained by a known germline segment via a simple V<sub>H</sub>-D-J<sub>H</sub> assembly was determined (i.e., without N segment addition, somatic mutation, or D-D fusion). 100 Ab1 sequences were included in this analysis (reference 38, DNASStar, Inc., Madison, WI).

Since at least a portion of the D segment can usually be explained by one of the known germline D elements, we have also compared the N segments (39) of these Abs with those of Ab1s. We define the N segments as the 3' or 5' portion of the D segment not explained by the germline D segment. Since N segments are thought

TABLE III  
*D Segment Analysis of Antibodies with Various Specificities*

Specificity	No.	Length	Percent germline	Length of 5'N	N segments length of 3'N	GC/AT ratio
Hapten	34	18.9	67	2.7	2.4	1.7
Unknown	22	17.0	66	2.5	3.4	1.6
Protein	11	12.6	61	2.6	1.4	1.2
Self carbo- hydrate	4	14.7	60	3.5	2.0	3.4
Self protein	29	14.5	54	3.8	2.6	2.0
Ab2s	29	22.2	52	6.1	4.4	1.2
Ab3s	7	16.2	72	2.1	2.0	2.6

to be the result of terminal transferase additions of nucleotides during  $V_H$ -D and D- $J_H$  rearrangement, and this enzyme has a preference for C's and G's over A's and T's (40), we have compared not only the length of both the 5' and 3' N segments in these antibodies, but also the relative GC content. If N segment derivation in most antibodies is primarily due to terminal transferase nucleotide additions, but in other antibodies is the result of some other mechanism, this may be reflected in the relative GC content of their N segments. The results of this comparison are shown in Table III.

From this analysis, D segments in antiidiotypic antibodies are slightly longer than those of Ab1s and the difference in length is primarily due to the 3' and 5' N segment additions. This does not directly reflect the length of the third hypervariable region; it simply reflects the percentage of the third hypervariable region derived from the D segment or from N segment additions. Thus, Ab2s derive a larger portion of the third hypervariable region from D and N than do Ab1s. In addition, Ab2s are slightly more homologous to germline D segments than are Ab1s. The difference in relative GC/AT content is perhaps the most interesting result of this comparison. This ratio in most antibodies ranges from 1.6 to 3.4; whereas in Ab2s and anti-protein antibodies, it is 1.2.

Comparisons like these must be analyzed with caution in that the available Ig literature is somewhat biased towards antibodies with particular specificities. The number of Ab1s in several categories is relatively small. This comparison does not take into account the strong selective pressure the immune system itself applies regarding junctional diversity (41, 42). However, these results in addition to previous reports of conserved N segments (31) and D-D fusion (6) suggest that mechanisms other than those involving terminal transferase play a role in generating diversity during the rearrangement process. Whether or not this is most readily explained through novel mechanisms of rearrangement, undiscovered D elements within the germline, somatic mutation, or other mechanisms, remains to be determined.

It seems unlikely that novel mechanisms should play a greater role in the generation of antibodies of one specificity than another. If D-D fusion occurs normally during ontogeny, it probably occurs in many antibodies. There are certainly examples of Ab1s in which D-D fusion may have occurred. These may become more apparent as antibodies of more specificities are studied.

There is indirect evidence for D-D fusion in the following analysis. Of the 129

antibodies in this comparison (100 Ab1s, 29 Ab2s), a germline D element can be defined in 121. Of these, 70 use DFL16.1, DFL16.2, or DQ52. The other 51 use one of the members of the DSP2 family. There are seven known DSP2 D segments, two DFL16 D segments, and one DQ52 D segment. If D segment usage is random, DSP2 type D segments should be used most frequently. This is not observed in expressed antibodies. Antigenic selection could explain this discrepancy. DFL12 type D segments may generate antibodies that bind a broad range of antigens. This seems somewhat unlikely, since the amino acid sequence derived from all of the D segments are fairly similar. Relative position in the genome might explain this preferential usage as it does in H chain V region genes (43). DFL16.1 (the most frequently used) has been mapped most 5' closest to VH. Alternatively, Kurasawa and Tonegawa (32) proposed that D-D fusion may occur routinely during ontogeny. They reasoned that since the alternative heptamer-nonamer recombination signals are found within the coding region of the DSP2 D segments, only a short portion of the coding region would remain after a D-D fusion event. DSP2 D segments might effectively be lost during successive D-D joining events except for short parts of the coding sequence that they proposed as the germline source of what are now termed N segments. Since DFL16 and DQ52 type D segments do not have alternative recombination signals, they would be protected and their entire coding regions left intact. This could explain the overuse of DFL16 type D segments in murine Igs and would infer that D-D fusion is an important diversifying mechanism in all antibodies.

Alternatively, if unusual D segments continue to be observed primarily in antiidiotypic antibodies, this would imply that Ab2s are derived from the germline differently than Ab1s. One possible explanation for the relatively low frequency of syngeneic antiidiotypic antibodies in these systems could be that structurally novel D segments are required for their antiidiotypic specificity, and events leading to the generation of these D segments may be infrequent. There is some additional indirect evidence in support of the idea that Ab2s are structurally distinct. If the unusual D segments observed in Ab2s are in any way artifactual, it would seem logical that the same artifact would be generated when inducing Ab3s. There have been very limited numbers of Ab3s sequenced (44), but novel D segments have not been observed (Table III). If further studies support our suggestion that Ab2s may be derived from the germline differently than Ab1s, a revision in contemporary formulations of idiotypic network would be in order as Ab1 and Ab2 should not be considered strictly operational terms.

### Summary

We have found that syngeneic Ab2s in the antiarsonate system are serologically and structurally similar to one another. In contrast, the allogeneic Ab2 response is heterogeneous and derives from a large number of unrelated germline gene segments. The Ab2 response of the BALB/c strain to polyclonal A/J Ars A molecules can probably best be compared with a response to a foreign protein and might have been predicted in a strain that completely lacks the H chain V region gene from which the Ab1 derives. Partial variable region sequences of Ab2s from three other systems in addition to previously reported Ab2 structures indicates that this difference in allogeneic vs. syngeneic Ab2s may be a general phenomena. These data support Jerne's hypothesis of complementary V region genes existing in the germline.

However, there is good evidence that these antiidiotypic antibodies are not derived directly from the germline, as somatic processes most likely play an important role in their generation. The D segments of Ab2s in the arsonate system as well as in other systems, are novel in structure and cannot easily be explained by previously described germline D segments. D-D fusion may play a role in the generation of the third hypervariable region in these antibodies.

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