MOLECULAR ANALYSIS OF ORIGINAL ANTIGENIC SIN

I. Clonal Selection, Somatic Mutation, and Isotype Switching During a Memory B Cell Response

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A primary humoral immune response to most antigens results in the development of a long-lasting state of immunity. In addition to influencing the outcome of a secondary immune response to the priming antigen, this state of immunity may also alter subsequent immune responses to antigens that are structurally related to this priming antigen, a phenomenon that has been termed "original antigenic sin" (1–3). This crossreactive property of immunity results in the "immune history" of an animal having a direct bearing on its "immune status" to antigens that may be encountered in the future.

Molecular analyses of hybridomas isolated at various stages of humoral immune responses indicate that both the clonal composition of the responding B cell population and the structure and function of the antibodies expressed by this population vary with time (4). Isotype switch recombination results in changes in antibody constant (C) region structure and somatic hypermutation results in alteration of variable (V) region structure. A progressively stringent process of antigen selection also acts on the responding B cell population, resulting in the affinity maturation of expressed antibodies (5). It has been proposed that such "somatic evolution" of antibody structure is requisite for the formation of the memory B cell population (6). Moreover, it has been suggested that the longevity of humoral memory may result from the structural constancy of the antibodies expressed by memory B cells, as well as the clonal stability of this population of cells (7, 8). If this is true, then the "immune status" of an animal to a given antigen may be strongly influenced by the antibody structures that have been generated during previous immune responses to other antigens.

To directly examine how the memory B cell population elicited to one epitope might be used during subsequent immune responses to other, structurally related epitopes, we have explored the phenomenon of original antigenic sin at a molecular level. During the immune response of A/J mice to *p*-azophenylarsonate (Ars)[1] a family of antibodies encoded by a single V_H gene segment (V_HId^{CR}) is reproducibly

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[1] *Abbreviations used in this paper:* Ars, *p*-azophenylarsonate; CDR, complementarity determining region; KLH, keyhole limpet hemocyanin; Sulf, *p*-azophenylsulfonate.

and predominantly elicited (9). The structural analogue of Ars p-azophenylsulfonate (Sulf) is incapable of eliciting antibodies encoded by V_HId^{CR} in naive A/J mice but does elicit such antibodies in mice previously immunized with Ars. Characterization of the structure and function of antibodies expressed by hybridomas derived from the Ars-primed original antigenic sin response to Sulf has provided new insights into how the memory B cell population elicited to one epitope participates in the immune response to a structurally related epitope, and thus, how the immune history of an animal influences its immune status.

Materials and Methods

Preparation of Antigens and Immunization of Mice. Arsanilic acid (Aldrich Chemical Co., Milwaukee, WI) was recrystallized two times from hot water before use, while sulfanilic acid (Aldrich) was used without further purification. Both haptens were conjugated via a diazonium reaction to KLH as previously described (10) at conjugation ratios of ~40 mg of hapten to 1 g of KLH. For primary immunization, the conjugates were emulsified in CFA at a final concentration of 1 mg/ml and 100 μl of the emulsion was injected intraperitoneally. After varying periods of time, 100 μg of antigen was injected intraperitoneally in PBS to induce conventional secondary, or original antigenic sin responses. All mice were 8–10-wk-old, female strain A/J originally obtained from the Jackson Laboratories, Bar Harbor, ME.

Hybridoma Formation, Screening, and Analysis. Hybridomas were generated using Sp2/0 and screened for expression of mRNA homologous to the V_HId^{CR} gene segment as previously described (11). The hybridomas that gave rise to hybridization signals in the initial V_HId^{CR} expression screening were subcloned by limiting dilution, and the resulting subclones were further characterized. Production of Ars and Sulf binding antibodies was measured using solid-phase radioimmunoassays and Ars-BSA or Sulf-BSA as previously described (12). The expression of idiotopes was measured using solid-phase competition radioassays and ^{125}I-labeled 36-65, a mAb encoded by the canonical combination of V gene segments (see text) in unmutated form as the labeled ligand.

Nucleotide Sequencing and Southern Blotting Analysis. Total cellular RNA was purified from subcloned hybridoma lines as previously described (11) and used for determination of expressed V region sequence via the oligonucleotide primed, dideoxynucleotide chain termination method (13). DNA was purified from hybridoma lines as previously described (14), digested with restriction enzymes according to the manufacturer's instructions, and submitted to Southern blotting analysis using nylon transfer membranes as described (15). The probes used in J_H and J_κ rearrangement analyses were a fragment from the intron region between the J_H4 gene segment and the IgH enhancer, and a Hind III fragment that encompasses the entire J_κ locus, respectively. For isotype switch rearrangement analysis, probes specific for the μ (J14B and J14C), γ3 (pγ3/BgH2.5), γ1 (pγ1/EH10.0), γ2b (pγ2b/E6.6), γ2a (pγ2a-1), and α (p64-101) "switch DNA" regions, and the Cμ (pGGμ1), Cε (pε/X1.5), and Cγ3 (pγ3/B6.0) coding regions were used. All of the isotype probes except the J14 probes were obtained from Dr. Wes Dunnick and are described in reference 16. The J14 probes were obtained from Drs. Shirmain Tilley and Barbara Birshtein and are described in reference 17. All probes were labeled with ^{32}P via nick translation.

Antibody Purification, Isotyping, and Affinity Determination. mAbs were purified from culture supernatants using affinity chromatography on Ars-bovine gamma globulin Sepharose as previously described (18). The isotypes of these antibodies were determined using an ELISA mouse isotyping kit obtained from Boehringer Mannheim (Indianapolis, IN) according to the manufacturer's instructions. The intrinsic affinities of antibodies for tyrosine conjugates of Ars and Sulf were determined via fluorescence quenching also as previously described (18).

B and T Cell Purification and Adoptive Transfer. B cells were purified by complement-mediated lysis of spleen cells using an anti-Thy-1.2 mAb (HO13-4). An enriched fraction of T cells was obtained by depletion of B cells by panning of spleen cells on plates coated with goat anti-mouse Ig. Cells were injected intravenously into naive recipients that had received 650 rad γ-irradiation (Gammacell-40; Atomic Energy of Canada, Kanata, Ontario) 3 h earlier, and 1 h after cell transfer these mice were immunized intraperitoneally with 100 μg of antigen in PBS and bled 10 d later.

Results

Previous studies have shown that an original antigenic sin response requires that the boosting epitope be structurally related to the priming epitope (1–3). We tested a number of Ars analogues for their ability to elicit serum antibodies in A/J mice that bore idiotopes characteristic of antibodies encoded by the V_HId^{CR} gene segment. Only one of the analogues tested, *p*-azophenylsulfonate (Sulf), failed to elicit V_HId^{CR}-encoded antibodies in naive mice despite eliciting high levels of anti-hapten antibody, but did so in Ars immune mice. A substantial increase in the level of idiotopes characteristic of V_HId^{CR}-encoded antibodies was observed in sera 10 d after Ars-KLH immune mice were boosted with Sulf-KLH. In addition, naive irradiated A/J recipients that received spleen cells from Ars-KLH immune A/J mice produced large amounts of such idiotopes after an injection of Sulf-KLH in PBS. Further, naive irradiated A/J recipients that received purified Ars-KLH primed A/J B cells and KLH-primed A/J T cells also produced large amounts of V_HId^{CR}-encoded idiotopes after boosting with Sulf-KLH. In contrast, naive or KLH immune A/J mice did not produce detectable amounts of V_HId^{CR}-encoded antibodies in primary, secondary, or hyperimmune anti-Sulf-KLH responses (data not shown).

Hybridomas were produced 3 d after Sulf-KLH immunization (in PBS) of seven A/J mice that had been immunized with Ars-KLH (in CFA) either 1, 5, or 8 mo earlier. The hybridomas were screened for expression of mRNA homologous to the V_HId^{CR} gene segment, and the antibodies produced by cell lines that gave rise to hybridization signals were further analyzed for hapten binding specificity and idiotypy. A large fraction of the hybridization positive cell lines obtained produce antibodies that bind both Ars and Sulf and bear idiotopes (CRI and E4, see Table I for data on 5-mo hybridomas) characteristic of the V_HId^{CR}-encoded V regions that predominate the normal A/J anti-Ars secondary response. For the sake of brevity, we will refer to these hybridomas as "sin" hybridomas. Sin hybridomas were obtained at all times after Ars priming at frequencies similar to those expected from conventional secondary anti-Ars-KLH response fusions, indicating that the sin and secondary anti-Ars responses are quantitatively similar in terms of the degree of clonal selection of V_HId^{CR}-expressing B cells. We use a descriptive nomenclature to denote hybridomas isolated at the three different times; AS hybridomas were isolated at one month, 5AS hybridomas were isolated at 5 mo, and 8AS hybridomas were isolated at 8 mo after initial immunization with Ars-KLH.

The nucleotide sequences of the V_H and V_κ genes expressed by representative members of the sin hybridoma panels were determined and are shown in Figs. 1 and 2. These analyses indicate that these hybridomas express the V_HId^{CR} gene segment in somatically mutated form. This conclusion was corroborated via Southern blotting of hybridoma DNAs using J_H and V_HId^{CR} probes (see Table II). In addition, these data show that most of the hybridomas express V regions encoded by the single combination of V gene segments (termed the "canonical combination" and composed of V_HId^{CR}, DFL16.1^e, J_H2, a $V_\kappa10$ gene segment termed $V_\kappa Id^{CR}$, and $J_\kappa1$) that encodes the antibodies that predominate conventional anti-Ars secondary responses (19). Somatic mutations are evident not only throughout V_HId^{CR} but within the other V gene segments that encode the sin V regions as well. Among the sin hybridomas isolated from individual mice it was often possible to deduce that several hybridomas had been derived from the same B cell clone. Clonal assignments were made on the basis of: (*a*) identity of the component germline V gene

TABLE I

Frequencies of Various Types of Hybridomas Isolated from Anti-Ars, Anti-Sulf, and Sin Immune Responses of A/J Mice

Condition	Number of Mice	Number of Colonies	V_H^+	Ars binders	CRI^+	$E4^+$
1' Sulf-KLH in PBS	2	2,200	11 (0.5%)	2 (18%)	0	0
2 ' Sulf-KLH	3	3,300	13 (0.4%)	0 (0%)	ND	0
KLH→Sulf-KLH	4	14,500	131 (0.9%)	14 (11%)	0	0
Ars-KLH→ Sulf-KLH (5 mo)	3	5,750	**99 (1.7%)**	**40 (40%)**	**22 (22%)**	**19 (19%)**
1' Ars-KLH (6 wk)	3	6,300	24 (0.4%)	3 (12%)	0	0
1' Ars-KLH (5 mo)	2	8,700	41 (0.5%)	2 (5%)	0	0
2' Ars-KLH			**— (2.5%)**	**— (50%)**	**— (50%)**	**— (25%)**

The immunization conditions and number of mice from which fusions were done are indicated, as are the total number of hybridoma colonies obtained and screened from these fusions. The number of these colonies that gave rise to hybridization signals with the V_HId^{CR} probe in lysate hybridization screening (11) is indicated (V_H^+) as are the number of the V_H^+ hybridomas that produce antibodies that bind Ars (Ars binders) in a solid-phase assay, and the number of V_H^+ hybridomas that produce antibodies that bear the idiotypes CRI (44) or E4 (45) characteristic of V_HId^{CR}-encoded anti-Ars V regions. The corresponding percentage values for each of these classes of hybridoma are shown in parentheses. For comparison, the frequency of each of these classes of hybridoma that we routinely obtain from conventional secondary anti-Ars-KLH responses (19) are also shown (2' Ars-KLH). Several of the V_H genes expressed by V_H^+ hybridomas obtained from anti-Sulf-KLH responses that produce antibodies that weakly bind Ars were sequenced. In all cases these hybridomas were found to express V_H genes that appear to be encoded by germline J558 gene segments other than V_HId^{CR}. The expressed V_H and V_κ genes expressed by two of the hybridomas obtained 6 wk after Ars priming were also sequenced. All of the V gene segments expressed by one such hybridoma were not "canonical." The other hybridoma did express the V_HId^{CR} gene segment but in combination with "non-canonical" D, J_H, and V_κ gene segments.

segments that make up the expressed V genes; (*b*) identity of nucleotides present at the V_H-D and D-J_H junctions (recombination sites and "N" [20] regions) of these genes; and (*c*) identity in the size of restriction fragments derived from hybridoma DNA that contain the B cell derived aberrantly rearranged J_H locus (assayed by Southern blotting). The clone assignments (indicated by numbers from 1 to 19), and the data substantiating such assignments are summarized in Table II. Many of the clonally related sets of sin hybridomas can be further subdivided into groups that share all observed somatic mutations in both the sequenced regions of their expressed V_H and V_κ genes. These "sublineage" assignments are indicated in Table II by a letter following the clone assignment number (e.g., 1-A).

Our findings that Sulf fails alone to elicit serum antibodies bearing idiotopes characteristic of V_HId^{CR}-encoded V regions, and that Ars-KLH-primed B cells but not Ars-KLH-primed T cells are sufficient to adoptively transfer the serum sin response, strongly suggest that the sin hybridomas were derived from Ars-induced memory B cells that responded secondarily to Sulf. Further, as shown in Table I, panels of hybridomas generated during the Sulf-KLH responses of naive or KLH immune A/J mice contain cell lines that hybridized with the V_HId^{CR} probe only at low frequencies. Only a small fraction of these hybridomas produce antibodies that bind

Ars, and none of these hybridomas produce antibodies bearing idiotopes (CRI, E4) characteristic of the V_HId^{CR}-encoded, Ars-binding antibodies expressed during the anti-Ars response. Several of the V_H genes expressed by these Sulf-induced, V_H hybridization-positive hybridomas were sequenced (data not shown) and found not to be encoded by V_HId^{CR} but by previously characterized germline J558 V_H gene segments that are homologous to V_HId^{CR} (21). Table I also shows that fusions done 6 wk or 5 mo after only a primary injection of Ars-KLH in CFA do not give rise to a high frequency of hybridomas producing Ars-binding or idiotype-positive antibodies, making it unlikely that any of the sin hybridomas were derived from B cells responding primarily to Ars. In toto, these data demonstrate that the antigen driven clonal selection of V_HId^{CR}-expressing B cells in A/J mice by Sulf is completely dependent on prior priming of these cells with Ars.

The affinities for Ars-tyrosine and Sulf-tyrosine of antibodies produced by sin hybridomas isolated at 1, 5, and 8 mo after Ars priming, and representative primary or secondary mAbs induced by Ars-KLH alone are shown in Table III. In general, the affinities of the sin antibodies are similar to conventional secondary Ars antibodies. Namely, the Ars association constants range from 5×10^5 to 10^7 and the Sulf affinities are ~10-fold lower, ranging from $<5 \times 10^4$ to 5×10^5. Two of the sin antibodies (5AS1W and 8AS2I) have a substantially higher affinity for Sulf than for Ars. With the exception of these two antibodies, the sin antibodies produced by hybridomas 5 and 8 mo after Ars immunization have an average ratio of affinities that is three- to five-fold greater than the sin antibodies produced by hybridomas isolated 1 mo after Ars priming. This suggests that the specificity for Ars of these 5- and 8-mo antibodies are higher than are the 1-mo antibodies.

As shown in Table IV, a major fraction of the sin hybridomas were found to express IgG3, an isotype that is expressed only at low frequency among conventional Ars-induced secondary hybridomas and among secondary serum anti-Ars antibodies (12). Due to this unexpected observation, the isotype switch configurations of the B cell-derived IgH loci in the sin hybridomas were further characterized via Southern blotting.

According to the deletion model for isotype switching (22), when an IgM-expressing B cell switches to the expression of another isotype, a DNA deletion event occurs between two "switch" regions that removes the $C\mu$ gene and juxtaposes the C gene encoding the newly expressed isotype next to the expressed V_H gene. Thus, in the DNA of a B cell that has isotype switched a hybrid switch region is usually generated (17) that is revealed by a Southern blotting analysis in which two switch region specific probes (and/or a switch region probe and a coding probe) are seen to hybridize to the same size restriction fragment. Table IV shows that among the sin hybridomas such hybrid switch regions can often be detected in Eco RI-digested hybridoma DNAs. In addition, this analysis reveals that among most of the hybridomas both B cell-derived IgH alleles have undergone rearrangements involving C region genes (23–25). The analysis summarized in Table IV also reveals that: (*a*) switching events to different isotypes have occurred within a single clone of B cells; (*b*) sequential switching from $C\mu$ to $C\gamma3$ to $C\gamma1$ occurred in several of the precursor B cell clones, as revealed by hybrid switch regions that hybridize to the three relevant switch probes (e.g., 5AS2M); and, (*c*) in many cells lines rearrangements of switch regions that

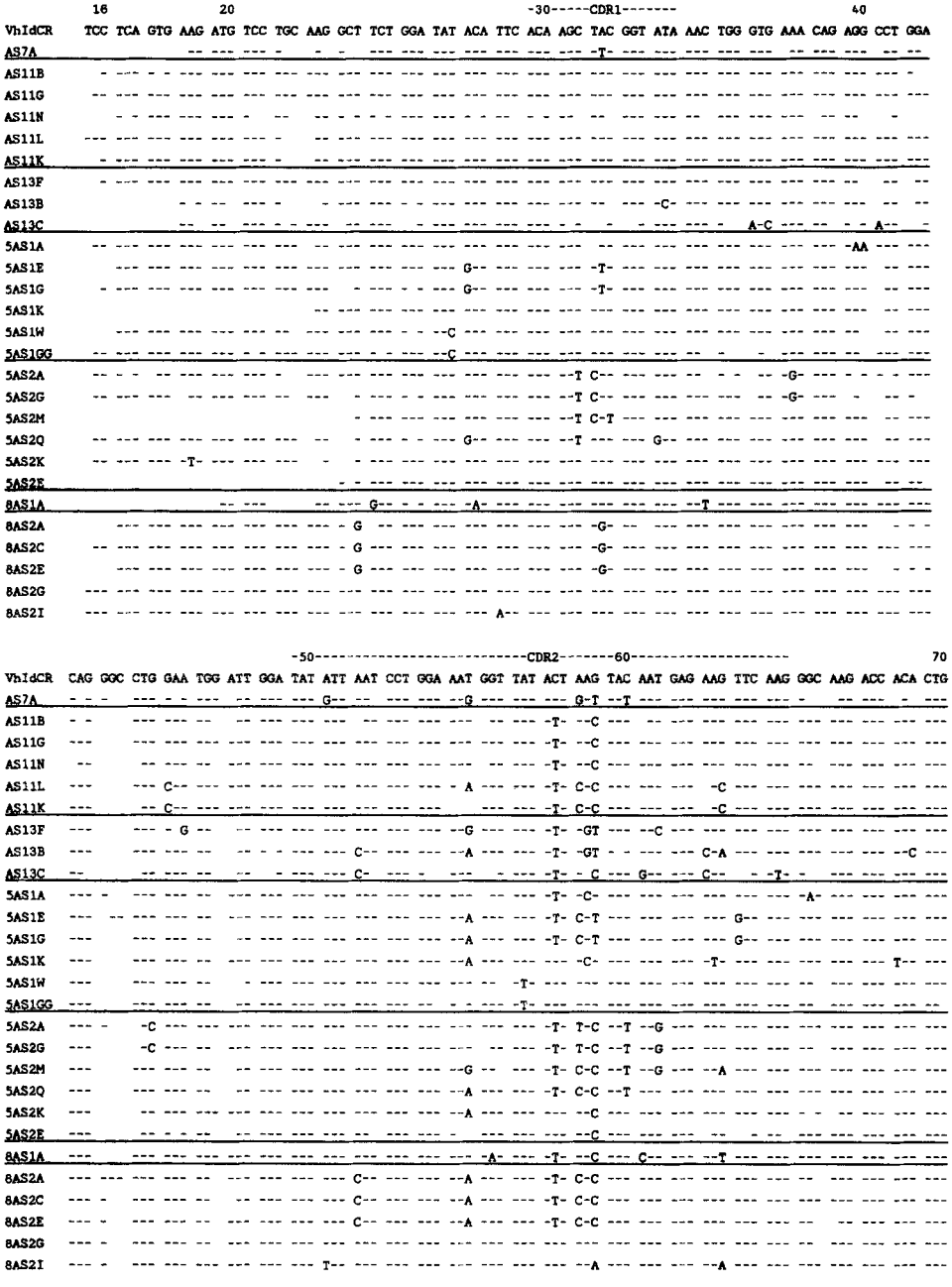
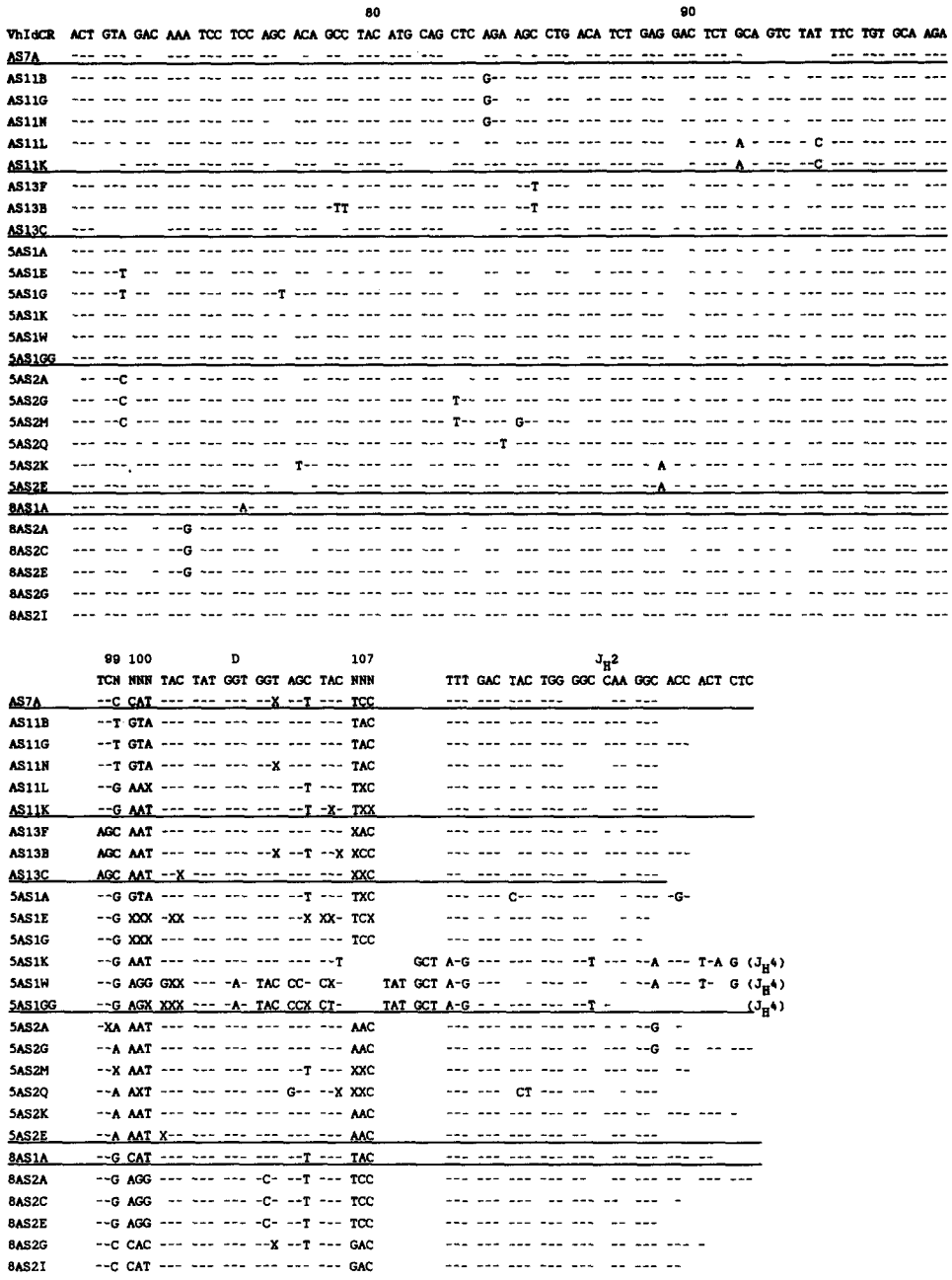


FIGURE 1. Nucleotide sequences of the V_H genes expressed by sin hybridomas. Sequences were determined as described in Materials and Methods and are presented in comparison to the sequences of the germline V_HId^{CR} (9), DFL16.1[c] (46), and J_H2 (47) gene segments. Nucleotide identity to these reference sequences is indicated by a dash. Amino acid codons are numbered starting from the mature NH_2 terminus. In the region encoded by the V_HId^{CR} segment, nucleotide positions that are uncertain or undetermined are indicated by a blank. Nucleotide differences are shown explicitly. The sequences derived from hybridomas isolated at different times



after Ars priming and/or different mice are separated by solid lines, and within these groupings the sequences are further organized to juxtapose the sequences that share somatic mutations. In the V-D-J region of the reference sequence, the nucleotides that vary among canonical V_H genes are indicated by an "N". In this region, uncertain or undetermined nucleotides in the sequenced genes are indicated by an "X". In most canonical V regions a TCN codon is present at position 99 that is of unknown origin (49).

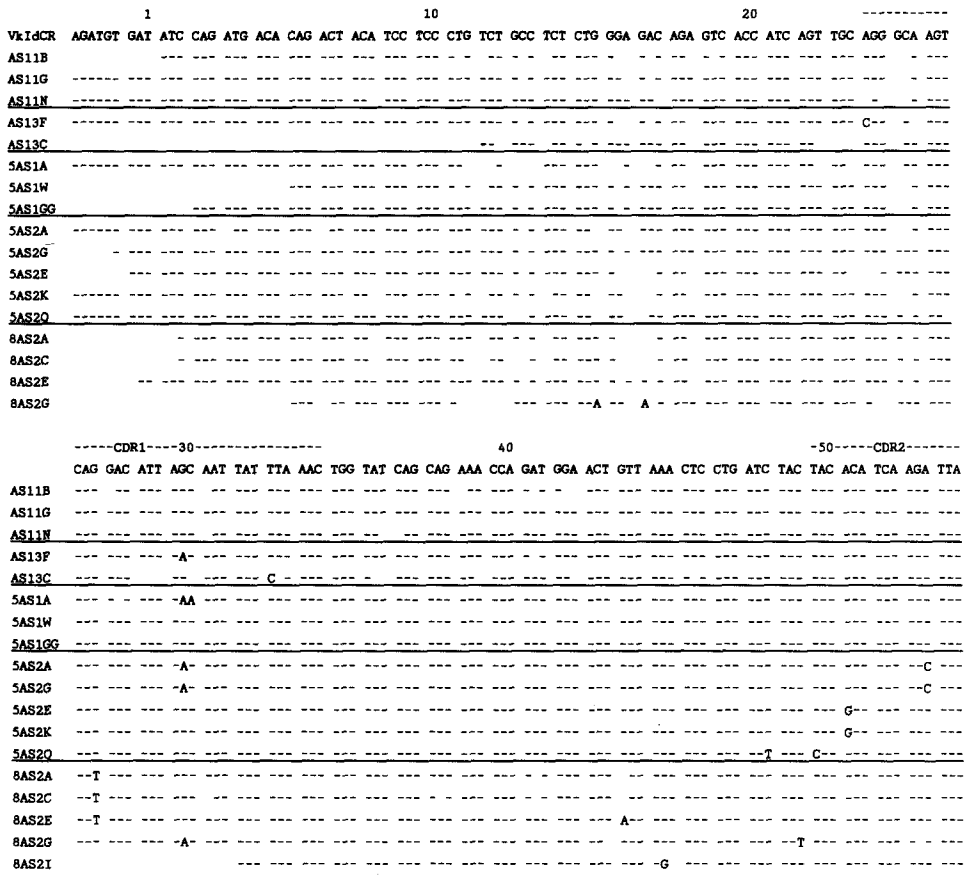


FIGURE 1. *Continued.*

are 3′ of the C gene corresponding to the expressed isotype have occurred. In cell lines containing this latter type of switch configuration most of the additional rearrangements involve the γ1 switch region. The nature of these downstream rearrangements is currently under investigation and will be the subject of a future report.

Discussion

The repertoire of V regions expressed by sin hybridomas is largely analogous to that expressed by conventional Ars-induced secondary hybridomas. V regions encoded by the canonical combination of gene segments predominate, and most sin V regions have suffered numerous somatic mutations, some of which are recurrently observed both among previously characterized secondary anti-Ars V regions (19) and the sin V regions characterized here (e.g., in V_H codons 55, 58, and 59 and V_κ codon 30; see Figs. 1 and 2). In addition, the ratio of mutations causing amino acid replacements (R) to those that do not (S) in the CDR regions of sin V genes is, in general, higher than expected from random mutation (R/S = ~3), and this ratio is lower than expected in many framework regions, implying that antigenic selection acted on the somatically mutated precursors of sin V regions (26). Further,

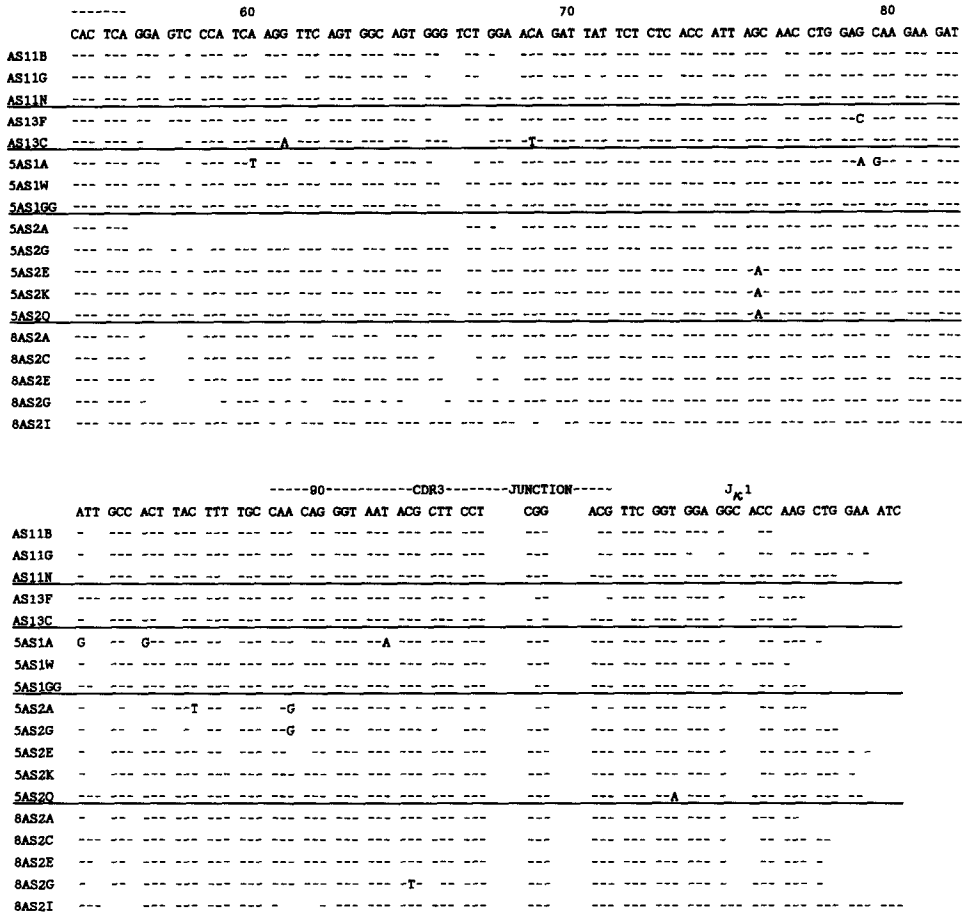


FIGURE 2. The sequences of the V_κ genes expressed by sin hybridomas. The sequences are presented as described in the legend to Fig. 1 in comparison to the germline sequence of the $V_\kappa Id^{CR}$ and $J_\kappa 1$ gene segments (50). The junctional codon present in canonical V_κ genes is an invariant CGG present at amino acid codon 96 (numbered sequentially from the mature amino terminus). The first six nucleotides in the reference sequence are part of the leader region of the V_κ gene.

the majority of sin V regions have greater affinity for Ars than for Sulf, and the most recurrent mutation among all the sin V regions (ACT to ATT at $V_H Id^{CR}$ codon 58 causing a Thr to Ile change) has been previously shown by site-directed mutagenesis to result in a threefold increase in affinity of a canonical V region for Ars (27). All these characteristics of the sin V region repertoire are made evident by the sin hybridomas isolated 1, 5, and 8 mo after Ars priming, attesting to the stability of the clonal composition and expressed antibody repertoire of the Ars-induced memory B cell population over long periods of time. These data provide a molecular explanation for the observations first made by Davenport et al. (1) and Fazekas de St. Groth and Webster (2) during their serological studies of the sin response to various serotypes of influenza, that sin antibodies have secondary response "character,"

TABLE II

Summary of the Structure of the Productively and Aberrantly Rearranged V Region Loci in Sin Hybridomas and the Deduced Clonal Origins of these Hybridomas

Mouse no.	Month rest	Hybridoma name	Canonical V_HDJ_H	Canonical V_KJ_K	V_H junctional group	J_H probe (kb)	Clone assignment
1	1	AS11B	Y	Y	1	5.5, 6.95	1-A
		AS11G	Y	Y	1	5.5, 6.95	1-A
		AS11N	Y	Y	1	5.5, 6.95	1-A
		AS11A	Y	Y*	1	5.5, 6.95	1
		AS11D	Y	Y*	1	5.5, 6.95	1
		AS11E	Y	Y*	1	5.5, 6.95	1
		AS11P	Y		1′		
		AS11Y	Y	Y*	1′		
		AS11L	Y	Y*	2	5.5, 6.1	2-A
		AS11K	Y	Y*	2	5.5, 6.1	2-A
		AS11M	Y	Y*	3	5.5, 6.1	2
		AS11F	Y	Y*	4′	5.5, 6.1	
		AS11C	Y		4		
		AS11R	J4	Y*	5	4.5, 5.35	5
		AS11W	J4	Y*	5	4.5, 5.35	5
		AS11S	J4	Y*	5	4.5, 5.35	5
		AS11J		Y*			
2	1	AS13F	Y	Y	6′	5.5, 2.1	6
		AS13B	Y	Y*	6′	5.5, 2.1	6
		AS13C	Y	Y	6′		6
		AS13G	Y	Y*	7′	5.5, 8.8	7
		AS13Z	Y	Y*	7′	5.5, 8.8	7
3	1	AS7A	Y	Y	8		
4	5	5AS1E	Y	Y*	9′	5.5, 5.8	9-A
		5AS1G	Y	Y*	9′	5.5, 5.8	9-A
		5AS1A	Y	Y	1′	5.5, 2.85	
		5AS1K	J4	Y*	10	4.4	
		5AS1W	D#, J4	Y	11	4.4, <2.0	11-A
		5AS1GG	D#, J4	Y	11′	4.4, <2.0, 2.75	11-A
		5AS1Q	D#, J3		12		
		5AS1EE	D#, J1		13		
5	5	5AS2A	Y	Y	14	2.35, 5.2	14-A
		5AS2G	Y	Y	14	2.35, 5.2	14-A
		5AS2M	Y	Y*	14′ or 16′	5.2, (5.5)	14
		5AS2E	Y	Y	14	5.5	14-B
		5AS2K	Y	Y	14	5.5	14-B
		5AS2C	Y	Y*	15	5.5	
		5AS2I	Y	Y*	16	5.5, 3.45	
		5AS2O	Y	Y*	16′	5.5	
		5AS2Q	Y	Y	14′ or 16′	5.5	
6	8	8AS1A	Y		17		
		8AS1C	Y		18		
		8AS1D	D#				

continued

TABLE II (*continued*)

Mouse no.	Month rest	Hybridoma name	Canonical V_HDJ_H	Canonical V_KJ_K	V_H junctional group	J_H probe (kb)	Clone assignment
7	8	8AS2A	Y	Y	19	5.5	19-A
		8AS2C	Y	Y	19	5.5, 6.3	19-A
		8AS2E	Y	Y	19	5.5, 6.3	19-A
		8AS2G	Y	Y	20	5.5, 4.75	
		8AS2I	Y	Y	21	5.5, 2.85	
		8AS2K	D^+		22		

The names of the sin hybridomas are shown, grouped according to the donor mouse and the time after initial Ars-KLH priming from which the hybridomas were obtained. A summary of the gene segments that encode the V regions expressed by the hybridomas appear in the next columns according to whether a particular cell line expresses the "canonical" combination of gene segments or not. If expression of this combination of segments was confirmed by sequencing and Southern blotting this is indicated by a "Y" (Yes). If confirmation was obtained by partial sequencing and/or northern and Southern blotting using V_HId^{CR} and $V_\kappa Id^{CR}$ probes, this is indicated by a "Y*". If the hybridoma does not express the canonical combination of gene segments, the gene segment(s) that differs is shown explicitly. D regions that appear to be encoded by gene segments other than DFL16.1^c (46) are indicated by a "D#". D regions that appear to be encoded by DFL16.1^c, but that differ in length from the canonical D region, are indicated by a "D^+". If segments were not characterized by either of these approaches this is indicated by a blank. The hybridomas are also grouped according to their deduced clonal origins. These clone assignments were made on the basis of identity of the V gene segments used, on nucleotides present at V_H-D and D-J_H junctions (V_H junction group), and on the size of Eco RI restriction fragments containing the B cell-derived aberrantly rearranged J_H locus (47) in these hybridomas. In the column labeled "V_H junction group," the hybridomas with identical V_H junctional nucleotides (see Figs. 1 and 2) are indicated by numbers, and in the cases where one or more of these nucleotides could not be determined this number is followed by an apostrophe. In the column labeled "J_H probe," the size in kilobase pairs of the Eco RI restriction fragments, derived from B cell DNA, that hybridize with a probe specific for the intron region between J_H and the IgH enhancer are shown. The size of the fragments that hybridize with both this probe and a V_HId^{CR} probe (the productive rearrangement, 5.5 kb when V_HId^{CR} joins to J_H2 or 4.5 kb when V_HId^{CR} joins to J_H4, are underlined. The size of the Eco RI fragment generated from germline A/J DNA is 5.8 kb. In the next column, the hybridoma clone assignments, based on all of the data summarized in this table, are indicated by numbers. If the V genes expressed by a set of clonally related hybridomas were found to share all somatic mutations (see Figs. 1 and 2) this is indicated by a letter (A or B) after the clone assignment number. Southern blots done to characterize B cell-derived J_κ loci were also done, but revealed that only a few of the sin hybridomas contained aberrantly rearranged loci. Two of the hybridomas gave rise to restriction fragments containing the productive rearrangement that was not consistent with the flanking restriction maps of the germline V_HId^{CR} and J_H loci. In both of these cases, further restriction mapping has indicated that either novel Eco RI sites or deletions have been generated in the 5′ flanking regions of the expressed V_H genes. The V region loci in these cell lines are currently being examined in more detail. In one of the cell lines (5AS2M) two bands were observed that hybridized with both the J_H and V_HId^{CR} probes and in another cell line (5AS1GG) three bands were observed with the J_H probe. The reason for these unusual patterns of hybridization is currently under investigation.

i.e., bind the eliciting antigen with greater affinity and specificity than the crossreactive antigen.

Two of the sin antibodies (5AS1W and 8AS2I), however, have much greater affinity for Sulf than for Ars. One of these (8AS2I) is encoded by the canonical combination of V gene segments. Examination of the types of somatic mutations present in these V regions shows that they lack the recurrent mutations characteristic of Ars-induced antibodies. These V regions may have fortuitously acquired high affinity for Sulf via somatic mutation that occurred during the primary anti-Ars response, could have been derived directly from the naive B cell population during the sin response, or may represent memory cells that were stimulated primarily with an environmental

TABLE III

Intrinsic Affinities of mAbs for Ars-Tyrosine and Sulf-Tyrosine

Antibodies	Mouse no.	Month rest	Hybridoma name	Affinity for Ars	Affinity for Sulf	Ars/Sulf ratio
Sin	1	1	AS11B*	4.3×10^5	3.6×10^4	12
			AS11G*	6.3×10^5	$<3.4 \times 10^4$	>19
			AS11A*	6.6×10^5	9.2×10^4	7
			AS11D*	4.6×10^5	$@7.4 \times 10^4$	6
			AS11E*	5.6×10^5	4.0×10^4	14
			AS11L*	9.8×10^5	1.1×10^5	9
			AS11M*	2.7×10^6	1.1×10^5	24
			AS11F*	1.9×10^6	$<4.2 \times 10^5$	>5
			AS11C		1.3×10^5	
	4	5	5AS1E*	9.0×10^6	$<3.7 \times 10^4$	>243
			5AS1G*	3.0×10^6	$@3.7 \times 10^4$	81
			5AS1A*	2.5×10^6	3.5×10^5	7
			5AS1K	3.6×10^5	$<3 \times 10^4$	>12
			5AS1W	1.4×10^5	$>1 \times 10^7$	<0.014
	5	5	5AS2A*	2.1×10^6	$@6.4 \times 10^4$	32
			5AS2G*	1.4×10^7	$@2.1 \times 10^5$	64
			5AS2E*	2.1×10^6	$@2.7 \times 10^4$	76
			5AS2K*	2.5×10^6	$<6.7 \times 10^4$	37
			5AS2I*	5.8×10^6	$<3.2 \times 10^4$	>181
			5AS2O*	$>1.4 \times 10^7$	5.9×10^5	>24
			5AS2Q*	1.2×10^6	$<5.7 \times 10^4$	>21
	7	8	8AS2A*	1.3×10^6	2.8×10^4	46
			8AS2G*	$<5 \times 10^5$		
			8AS2I*	1.4×10^6	$>1 \times 10^7$	<0.14
Primary Ars						
			36-65*	6.8×10^5	$<5 \times 10^4$	>14
			P65D6-5*	6.3×10^5	$<5 \times 10^4$	>12
			ABA5-1	2.1×10^5	$<5 \times 10^4$	>4
			ABA8-1	3.0×10^4	$<5 \times 10^4$	>1
Secondary Ars						
			44-10*	1.0×10^6	2.0×10^4	50
			45-223*	4.3×10^6	2.0×10^4	200
			hVH65-210*	6.6×10^5	$<5 \times 10^4$	>12
			hVH65-212*	3.6×10^6	2.4×10^5	15
			ABA2'-6*	1.0×10^7	3.0×10^5	30
			2P2C	5.6×10^5	3.6×10^5	1

* "Canonical" V regions.

mAbs were purified and their intrinsic affinities for tyrosine conjugates of Ars and Sulf were determined as described in Materials and Methods. The table presents these affinities as association constants (K_a in 1/M). The sin antibodies are grouped according to the time after initial Ars priming, and the mouse from which the hybridomas that produce these antibodies were derived. Affinities of a representative number of conventional anti-Ars primary and secondary mAb are also shown. Affinities below 5×10^4 were often difficult to measure accurately due to an inability to obtain a maximum fluorescence quenching value. Such affinities are indicated by "<some number $\times 10^4$." Likewise, affinities $>10^7$ were sometimes difficult to assign accurate values due to small errors in functional antibody concentration. Such affinities are indicated by ">some number $\times 10^7$." Affinity values that could only be approximated due to data point scatter are indicated by an "@." Antibodies encoded by the canonical combination of V gene segments are indicated with an asterisk.

antigen. We have never observed such V regions among the antibodies expressed by secondary anti-Ars hybridomas, and sera from Ars nonimmune A/J mice are devoid of idiotopes characteristic of $V_H Id^{CR}$-encoded, Ars-binding antibodies. This suggests that 5AS1W and 8AS2I represent B cells that were members of the Ars-induced memory B cell population, indicating that memory B cells generated to one epitope may harbor somatically mutated V regions that are not efficiently elicited during a secondary response to this same epitope, but that can be used in immune responses to other epitopes (7).

The intrinsic affinities of the sin V regions for Sulf are, on average, very low ($<10^5$). We present data elsewhere suggesting that such a low affinity is below the "affinity cutoff" of naive B cell surface Ig for antigen necessary for reproducible clonal selection in the primary immune response (Fish, S., M. Fleming, J. Sharon, and T. Manser, manuscript submitted for publication). While several of the sin antibodies we have characterized clearly have affinities for Sulf well above such a "cutoff," this is not the general case. These data suggest that clonal selection of memory B cells requires a lower affinity sIg-antigen interaction than does clonal selection of naive cells, an idea previously discussed by Klinman and his colleagues (28). Due to their low "affinity cutoff" for stimulation, the participation of memory B cells in immune responses to antigens other than those that initially elicited them may be a rather common occurrence.

Table III shows that the Ars specificity of the Ars-induced memory B cell population appears to increase with time after immunization, suggesting that antigen affinity-based selection is operative on this population long after primary immunization. This conclusion is in accord with previous observations demonstrating that the affinity of antibodies continues to increase for an extended period after immunization (5, 29). The increased specificity for Ars of the 5-mo sin antibodies appears to correlate with differences in the frequency and distribution of somatic mutations in the V genes expressed by sin hybridomas isolated at 1 vs. 5 mo, particularly in and around CDR1 of V_H and throughout V_κ (the same trend is observed among 8-mo hybridomas).

Perhaps the most striking observation regarding the structure of the V genes expressed by sin hybridomas is the high frequency in which cell lines derived from the same B cell clone express V regions that share all observed somatic mutations. Table II and Figs. 1 and 2 show that of the nine groups of hybridomas that appear to be derived from single clones, six contain two or more members that share all observed mutations in the sequenced regions of V_H and V_κ. Others have made similar observations among clonally related hybridomas isolated during the secondary anti-PC-*Proteus* response (8); the NP-primed, antiidiotype boosted adoptive secondary response (7); and the autoimmune response of MRL/lpr mice (30). In comparison, we have shown that among two groups of clonally related hybridomas isolated at an intermediate stage of the immune response (day 16 after initial immunization) mutationally identical V regions are never observed (31). These data suggest that somatic mutation does not occur at a rate close to that estimated for B cells undergoing a primary immune response (10^{-3}/V gene base pair/cell division [32] or approximately one mutation/V region/cell division) during the initial stages of the sin response. That is, memory B cells whose precursors have undergone V gene somatic mutation appear to be capable of clonal expansion in the absence of such mutation.

TABLE IV

The Configuration of Antibody Constant Region Loci in Sin Hybridomas

Hybridoma name	Expressed isotype	μ switch probe	IgM coding probe	γ3 switch probe	IgG3 coding probe	γ1 switch probe	γ2b switch probe	γ2a switch probe
AS11B	γ3	4.3, 14			14	7.35	*	G
AS11G	γ3	14, >23			14	7.35	*	G
AS11N	γ3	4.35, 14			14	8.2	*	G
AS11A	γ3	4.0, 14			14		*	G
AS11D	γ3	1.9, 14			14	12	*	G
AS11E	γ3	4.3, 14			14	7.3	*	G
AS11Y	γ1	4.4, 7.35					*	G
AS11L	γ1	4.3				4.3	5.5	G, 9.4
AS11K	γ1	3.95, 10				3.95	*	G
AS11M	γ3	15		15	15	G, 9.6	*	G
AS11F	γ1	3.75, 21, (6.5)	6.5, 21	G, 6.5	G, 6.5	G, 3.75, 6.5	*	G
AS11R	γ1	3.1, 9.4	G	20	20	3.1, 9.4, >23	*	G
AS11W	γ1	3.1, 9.4	G	20	20	3.1, 9.4, >23	*	G
AS11S	γ1	3.1, 9.4	G	G	G	3.1, 9.4, >23	*	G
AS11J	γ3	14			14	9.4	*	G
AS13B	γ1	3.5, 7.5				3.5, 7.5	*	G
AS13G	γ2b	3.75, 20	20	G	G	G	3.75	G
AS13Z	γ3	6.5, 14		8.1	14	6.5, 7.1	*	G
5AS1E	γ1	6.8, 12		17	17	6.8, 12, 17	*	G, 8.6
5AS1G	γ1	6.8, 12		17	17	6.8, 12, 17	*	G, 8.6
5AS1A	γ3	15, 21	6.7, 21	G, 6.7	G, 6.7, 15	6.7, 10, 12	*	G
5AS1K	γ2b	6.8				17	5.4	G
5AS1W	μ	5.1, 21	21	7.3	16	G	*	15

5AS1GG	γ3	15, 21, G	G, 21	G	G	G	*	G
5AS2A	γ3	8.5, 16		16	16	G, 8.5	*	G
5AS2G	γ3	8.5, 16		16	16	G, 8.5	*	G
5AS2M	γ1	7.75		7.75		7.75	4.8	G
5AS2E	γ3	16		16	16	13	*	G
5AS2K	γ3	16, 21	21	G, 16	16	13	*	G
5AS2C	γ3	5.7, 14	8.0		14	5.7, 8.0	8.1	G
5AS2I	γ1	8.5, 21	21	G	G	8.5, 16	*	G
5AS2O	γ3	17, 22	22	G, 17	G, 17	G	*	G
5AS2Q	γ1	6.7				6.7	*	G
8AS2A	γ1	3.95				3.95	*	G
8AS2C	γ1	3.95, 4.2				3.95, 4.2	*	G
8AS2E	γ1	3.95, 4.2				3.95, 4.2	*	G
8AS2G	μ	22	22	G, 15	G, 15	G	*	G
8AS2I	γ3	3.0, 14			14	G	2.5, 3.0	

The data obtained from sin hybridomas derived from different times after initial Ars priming and/or from different mice are grouped. In the case of each hybridoma the expressed isotype (determined serologically) is shown as are the size (in kilobase pairs) of the Eco RI restriction fragments derived from hybridoma DNA that hybridize with a variety of DNA probes specific for the IgM and IgG3 constant region genes and various "switch DNA" regions (see Materials and Methods). Restriction fragments of germline size (determined by comparison with the hybridization patterns obtained using A/J kidney DNA) are indicated by a "G". The sizes of hybridizing restriction fragments derived from the Sp2/0 fusion partner are not shown. An Sp2/0-derived restriction fragment(s) that hybridizes with the γ2b switch region probe comigrates with the germline fragment, making it impossible to directly determine whether the B cell–derived γ2b switch regions in the hybridomas are in germline configuration. Therefore, in the γ2b probe column the presence of a band of germline size only is indicated by an asterisk, and if a band of a different size was detected, only the size of this rearranged band is indicated. In the case of several of the cell lines, a band(s) was seen using a given probe that did not correspond to a band derived from a μ switch region or J_H probe, indicating that either: (*a*) rearrangements other than simple "switch" deletions had occurred in B cell derived DNA; (*b*) novel restriction sites had been generated via switch recombination in B cell derived DNA; or, (*c*) Sp2/0-derived IgH loci underwent secondary rearrangement during hybridoma formation or growth. The last possibility is rendered unlikely by the observation that these cell lines revealed a normal pattern of Sp2/0-derived bands with all the probes. Further characterization of the IgH loci with "switch" configurations that are inconsistent with a simple deletion event is ongoing and will be the subject of a future report. The sizes of the germline bands detected with the various probes were: μ switch probe, ~23 kb; IgM coding probe, ~23 kb; γ3 switch probe, ~20 kb; IgG3 coding probe, ~20 kb; γ1 switch probe, 14 kb; γ2b switch probe, 6.5 kb; and γ2a switch probe, 5.8 kb. The blots were also hybridized with probes specific for the ε coding and α switch regions. These probes revealed no bands that differed from the germline.

The high frequency of clonally related sin hybridomas and shared mutations in the V genes expressed by such hybridomas indicates that the sin response is generated from a small number of B cell clones that have undergone mutational diversification prior to the sin response. This observation is concordant with the previous conclusions of others that conventional secondary responses are generated from a small number of memory B cell clones that express somatically mutated V genes (33, 34). Thus, it appears that only a small fraction of all the mutationally distinct offspring generated during clonal expansion in the primary response enter the memory B cell population. The parameters that govern such limited entry into this population remain to be determined.

Another striking observation regarding the composition of the sin antibody repertoire is the prevalence of antibodies of the IgG3 isotype. The isotypic spectrum of anti-Ars antibodies expressed during the secondary anti-Ars-KLH response (predominantly IgG1; 12, 19) is, therefore, not an accurate reflection of the isotypic class(es) of antibody expressed by the Ars-KLH-induced memory B cell population. Obviously, the original surface isotype(s) expressed by the memory B cells whose clonal progeny gave rise to the sin hybridomas cannot be determined. However, the data in Table IV suggest that the configuration of the C region loci in many of these memory cells must have been either germline or switched to $C\gamma 3$. Previous examination of the isotypes expressed by the memory B cell population has yielded discordant results (35–41). Our data suggest that while a subset of the Ars-KLH-induced memory B cell population may be committed to the expression of IgG1, a large fraction of this population is not, and must give rise to progeny that undergo further switching to yield the spectrum of isotypes normally observed among secondary anti-Ars antibodies.

The discrepancy between the isotypic profiles observed among conventional secondary anti-Ars hybridomas isolated 3 d after boosting (12, 19) and the sin hybridomas characterized here indicates that isotype switching is a slower process during at least the initial stages of the sin response. This could be related to the overall lower affinity of the Ars-induced memory B cell population for Sulf than for Ars. Perhaps this lower affinity results in lower levels of the type of T cell help (e.g., Th2) required for isotype switching to IgG1 due to less efficient B cell antigen capture, resulting in lower levels of antigen presentation to Th cells. Alternatively, a significant fraction of the memory Th population may have been primed to Ars (42, 43), or an epitope that consists of an Ars-modified peptide subfragment of KLH. If this were true, the Sulf-KLH boost may not cross-stimulate the Ars-KLH-induced memory Th population as efficiently as it does the Ars-KLH-induced memory B cell population, perhaps leading to lower levels of isotype switching.

Summary

To determine how the memory B cell population elicited to one epitope might be used in immune responses to other, structurally related epitopes, we explored the phenomenon of original antigenic sin. Strain A/J mice reproducibly respond to immunization with *p*-azophenylarsonate (Ars) by production of anti-Ars antibodies encoded predominantly by a single V_H gene segment (V_HId^{CR}). The structural analogue of Ars *p*-azophenylsulfonate (Sulf) fails alone to elicit such V regions, but can do so in A/J mice previously immunized with Ars, providing a means to specifically

examine B cells capable of responding secondarily to a crossreactive antigen (i.e., memory cells). V_HId^{CR}-expressing hybridomas were derived from the Ars-primed, Sulf-boosted original antigenic sin response of A/J mice at various times after Ars priming, and the properties of the antibodies they express and the structure of the genes encoding these antibodies were characterized. The data obtained support the following conclusions: (*a*) The Ars-induced memory B cell population capable of being crossreactively stimulated by Sulf is largely formed from a small fraction of all B cells participating in the anti-Ars primary response that express somatically mutated V regions; (*b*) the antibody repertoire and clonal composition of this population are stable over long periods of time; (*c*) memory B cells are capable of clonal expansion in the absence of a high rate of V gene somatic mutation; (*d*) the activation requirements for clonal selection of memory, versus naive B cells appear to differ; and (*e*) a major fraction of Ars-induced memory B cells express either IgM or IgG3 prior to and during the initial stages of the sin response.

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