

SOMATICALLY MUTATED FORMS OF  
A MAJOR ANTI-*p*-AZOPHENYLARSONATE ANTIBODY  
VARIABLE REGION WITH DRASTICALLY REDUCED  
AFFINITY FOR  
*p*-AZOPHENYLARSONATE

By-Products of an Antigen-driven Immune Response?

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The pivotal role played by antigen in the clonal selection of B cells for initial participation in an immune response is well established. Antigen selective mechanisms ensure that antigen-binding antibodies are produced during all stages of the immune response. However, antibodies that lack specificity for the immunogen might also be produced during the course of an antigen-driven immune response. It has been suggested that, through idiotype-antiidiotype network interactions within the immune system, production of antibodies that lack specificity for the immunogen but that share idiotopes with antigen-binding antibodies could result (1). In addition, data obtained by a number of investigators suggest that somatic mutation of antibody V region genes occurs at a rate of  $10^{-3}$ /basepair/cell division in B cells participating in an immune response (2, 3). One outcome of such V region structural alteration could be antibodies that lack, or have drastically reduced affinity for the immunogen. We sought to identify and characterize some of the antibody by-products of the antigen-driven immune response that are expected to be created by the mechanisms described above.

### Materials and Methods

*Immunizations and Hybridoma Formation and Characterization.* A/J mice were immunized intraperitoneally with 200  $\mu$ g *p*-azophenylarsonate–keyhole limpet hemocyanin (Ars-KLH)<sup>1</sup> emulsified in complete Freund's adjuvant (CFA) and either killed 13 or 21 d later or boosted with 150  $\mu$ g of Ars-KLH in saline intraperitoneally 30 d later and sacrificed 3 d after boost. Total spleen cells were fused to Sp2/0 and hybridomas were selected as

This work was supported by grants AI-23739 (to T. Manser), CA-24432 (to M. N. Margolies), and AI-13357 and CA-28900 (to M. L. Gefter) from the National Institutes of Health.

<sup>1</sup>Abbreviations used in this paper: Ars-KLH, *p*-azophenylarsonate–keyhole limpet hemocyanin; Id<sup>CR</sup>, crossreactive idiotype.

described (4). The fusion products were screened for  $V_H$  crossreactive idiotype ( $V_H Id^{CR}$ )-expressing hybridomas using a technique that detects  $V_H Id^{CR}$  mRNA in whole cell lysates immobilized on nitrocellulose filters (4). Supernatants from cultures containing  $V_H Id^{CR}$ -expressing hybridomas were tested for the presence of Ars-binding antibodies using an Ars-BSA plate-binding RIA. Supernatants from hybridomas that did not produce Ars-binding antibodies were then tested for the presence of antibodies bearing idiotopes characteristic of canonical combination (see text)-encoded antibodies that are recognized by four different antiidiotypic reagents (rabbit anti-crossreactive idiotype [CRI], mAb AD8, mAb 5Ci, and rabbit anti-45-49 [see references 5 and 6 for a detailed description of the specificities of these reagents]). Solid-phase competition RIA (using 36-65 as the labeled ligand) were used for this purpose.

*Nucleic Acid and Protein Sequencing Analyses.* Three strategies were used to determine the primary V region structure of antibodies: amino acid sequencing, direct antibody mRNA sequencing using oligonucleotide primers (7), and sequencing by base-specific chemical cleavage of V region cDNAs the synthesis of which was primed using  $^{32}P$  end-labeled oligonucleotides (8). For amino acid sequencing, hybridoma cell lines were grown as ascites tumors and antibodies were purified from ascites, and heavy and light chains were separated as previously described (9). The hVH65-110 antibody (see text) had a high enough affinity for Ars to be purified on Ars-KLH Sepharose columns. The hVH65-107 antibody (see text) would not bind to such columns, and was purified by chromatography on DEAE-cellulose. Light chain tryptophan peptides were prepared as described previously (10), and details of Edman degradation are as reported (11). Oligonucleotide primers that hybridize specifically to the 5' ends of the  $C_{\gamma 3}$ , the  $C_{\gamma 1}$ , and the  $C_{\kappa}$  genes, as well as two primers that hybridize specifically to sites within the  $V_H Id^{CR}$  and  $V_{\kappa} Id^{CR}$  genes were used.

## Results

In the advanced stages of the anti-Ars response (days 13–21 of the primary and day 3 of the secondary) a single combination of V region gene segments (the canonical combination) encodes a majority of the antibodies expressed by the responding B cell population (12). This canonical combination is composed of  $V_H Id^{CR}$ , a D segment apparently partially derived from the strain A DFL16.1 locus (13) and termed  $D^{CR}$ , the  $J_H 2$  gene segment, a member of the  $V_{\kappa} 10$  gene segment family termed  $V_{\kappa} Id^{CR}$  and the  $J_{\kappa} 1$  gene segment (12). Despite this homogeneity of combinatorial diversity, a variety of different Ars-binding V region structures are nevertheless expressed during the late stages of the anti-Ars response due to extensive alteration of these segments via somatic mutation (12).

Hybridomas were constructed from the spleen cells of Ars-KLH-immunized A/J mice, and were screened only for expression of  $V_H Id^{CR}$  mRNA (4). Mice undergoing a secondary or primary anti-Ars response were used. The times after immunization used (see Materials and Methods) were chosen to provide points representing different phases of the maturation of the response. The antibodies produced by  $V_H Id^{CR}$ -expressing hybridomas were subsequently screened for Ars-binding activity.

We routinely observe that a significant fraction of the hybridomas that express  $V_H Id^{CR}$  and are isolated from Ars-KLH immunized mice do not express either Ars, Ars-KLH, or KLH-binding antibodies (T. Manser, unpublished data). Table I shows that a large fraction of the  $V_H Id^{CR}$ -expressing hybridomas we isolated produce antibodies that do not bind to Ars-BSA. Some of these hybridomas are probably the result of fusion events between unactivated B cells and the myeloma

TABLE I  
*Characteristics of Hybridomas Isolated from Ars-KLH-immunized mice.*

Time of fusion	Total hybridomas	V <sub>H</sub> mRNA <sup>+</sup>	V <sub>H</sub> mRNA <sup>+</sup> , Ars <sup>-</sup>	V <sub>H</sub> mRNA <sup>+</sup> , Ars <sup>-</sup> , Id <sup>+</sup>
Day 13 of primary response	1,700	31	11	1
Day 21 of primary response	1,400	25	18	1
Day 3 of secondary response	1,450	36	15	0

The total number of hybridomas isolated at various times after immunization are indicated (total hybridomas) as are the numbers of these hybridomas that express RNA homologous to V<sub>H</sub>Id<sup>CR</sup> (V<sub>H</sub> mRNA<sup>+</sup>), antibodies that do not bind detectably to Ars (V<sub>H</sub> mRNA<sup>+</sup>, Ars<sup>-</sup>), and antibodies that bear idiotypic determinants characteristic of V<sub>H</sub>Id<sup>CR</sup>-encoded Ars-binding antibodies (V<sub>H</sub> mRNA<sup>+</sup>, Ars<sup>-</sup>, Id<sup>+</sup>). Four antiidiotypic reagents were used: two rabbit polyclonal antisera (anti-CRI and anti-45-49) and two antiidiotypic mAb (AD8 and 5Ci). The specificities of these antiidiotypic antibodies have been described elsewhere (see Materials and Methods). One hybridoma (hVH65-110) from the day 13 fusion bore an idiotypic determinant detected by the 5Ci mAb, and one antibody (hVH65-107) from the day 21 fusion bore idiotypic determinants recognized by the rabbit anti-45-49 polyclonal reagent.

fusion partner. To avoid the analysis of such cell lines we examined only V<sub>H</sub>Id<sup>CR</sup>-expressing hybridomas that produced Ars-nonbinding antibodies bearing idiotypic determinants characteristic of Ars-binding V<sub>H</sub>Id<sup>CR</sup>-encoded V regions. Because the precursor frequency of idiotype-bearing V<sub>H</sub>Id<sup>CR</sup>-encoded V regions in the naive B cell population is very low (14, 15), we would not expect to isolate hybridomas expressing such V regions as a result of fusion with unactivated B cells. Two cell lines expressing V<sub>H</sub>Id<sup>CR</sup> and idiotype-positive Ars-nonbinding antibodies were isolated 13 and 21 d after primary immunization. These hybridomas, designated hVH65-107 and hVH65-110, express IgG1 and IgG3 antibodies, respectively. Synthesis of antibodies of the IgG class is a property thought to be dependent on B cell activation (16), and suggests that these hybridomas were derived from B cells that were activated during the anti-Ars response. The frequency of this type of hybridoma among V<sub>H</sub>Id<sup>CR</sup>-expressing hybridomas isolated from the primary response was very low (3-4%), and none of the secondary hybridomas producing Ars-nonbinding antibodies expressed idiotype-positive V regions. The idiotypic crossreactivity of the hVH65-107 and hVH65-110 V regions was not extensive, as each reacted with only one of the four antiidiotypic reagents tested (see Table I).

The primary structures of the V regions expressed by hVH65-107 and hVH65-110 were determined and are shown in Figs. 1 and 2. The amino acid and nucleotide sequences are compared to the sequence of 36-65, an Ars-binding antibody encoded by the canonical combination of V gene segments in germline form. From this comparison, and Southern blotting analysis of the rearranged V<sub>H</sub> and V<sub>κ</sub> genes in hybridoma DNAs using V<sub>H</sub>Id<sup>CR</sup> and V<sub>κ</sub>Id<sup>CR</sup> probes (data not shown) we conclude that the V regions expressed by hVH65-107 and hVH65-110 are encoded by the canonical combination of gene segments but have been extensively altered by somatic mutation.

Figs. 1 and 2 also show that both antibodies contain serine at V<sub>H</sub> junctional position 99 and arginine at V<sub>κ</sub> junctional position 96, two junctional amino acids that have been shown to be important for Ars-binding activity in canonical



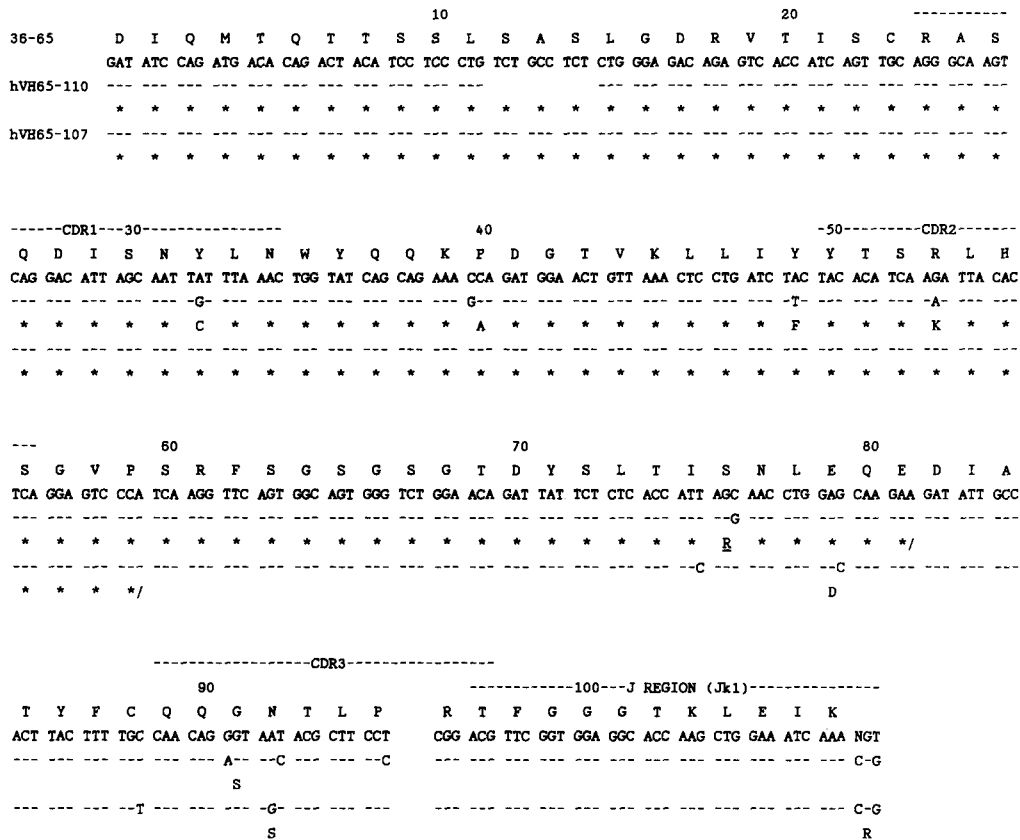
V<sub>k</sub> SEQUENCES

FIGURE 2. The sequences of the V<sub>k</sub> genes and their protein products expressed by hVH65-110 and hVH65-107 are displayed as described in Fig. 1 in comparison to the complete amino acid sequence (M. N. Margolies, manuscript in preparation) and nucleotide sequence (22) of the V<sub>k</sub> gene or protein expressed by 36-65. The finding of cysteine at position 32 in the hVH65-110 V<sub>k</sub> sequence was confirmed by amino acid sequence analysis of the hVH65-110 light chain after reduction and radioalkylation. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00637.

combination-encoded antibodies (17, 18). V<sub>H</sub> junctional positions 100 and 107 are characteristically variable among canonical combination-encoded Ars-binding V regions. The amino acids found at these positions in the hVH65-110 V region have been previously observed to occur in several canonical combination-encoded antibodies that have high affinity for Ars (14); those in the hVH65-107 V region have not.

While neither antibody binds detectably to Ars-BSA, lightly conjugated Ars-KLH, or KLH alone, we could detect binding to highly conjugated preparations of Ars-KLH. This allowed us to estimate the affinities of these antibodies relative to 36-65 ( $K_a = 5 \times 10^5/M$ ) and several other high-affinity anti-Ars antibodies ( $K_a$  of  $10^6$ – $10^7/M$ ) using a previously described plate binding assay (17). The values

obtained for relative association constants in this way were  $10^3/M$  for the hVH65-107 V region and  $10^4/M$  for the hVH65-110 V region.

### Discussion

Several hypotheses might account for our ability to isolate, from mice undergoing an anti-Ars response, hybridomas that express V regions the structures of which are characteristic of a major high-affinity Ars-binding V region but which have extremely low affinity for Ars. First, the B cells expressing these antibodies could have been stimulated by an antigen(s) unrelated to Ars. This seems highly unlikely, since private idiotypic determinants characteristic of this V region structure are not found in A/J nonimmune sera or in sera from A/J mice immunized with antigens (e.g. KLH) other than Ars (18 and T. Manser, unpublished data). Second, the B cells expressing these low-affinity V regions could have been stimulated at the onset of the anti-Ars response due to the high concentration of antigen (200  $\mu\text{g}$ ) administered in adjuvant. This idea, however, assumes that canonical combination-encoded, somatically mutated V region structures preexist in the Ars-nonimmune B cell population. This assumption is not supported by previous experiments indicating that most if not all somatic mutation occurs during the antigen-dependent stages of B cell differentiation (6, 12). Third, the B cells expressing these V regions may have been stimulated via idiotype-antiidiotype network interactions that were induced by Ars immunization but that are capable of Ars-independent B cell activation (e.g. stimulation mediated by idiotype-specific T helper cells). The fact that the two antibodies we isolated express few of the idiotypic determinants characteristic of canonical combination-encoded V regions and have low affinity for Ars does not support this idea.

Finally, the hypothesis that is most consistent with our previous observations is that random somatic mutations occur at a high rate in V genes during primary clonal expansion, resulting in the generation of antigen-nonbinding V regions. The B cells expressing such V regions may not continue to participate in the response. However, these cells might be "rescued" as hybridomas if the fusion event takes place soon after the "lethal" V region somatic mutation occurs. If this assertion is correct, then the B cells that gave rise to hybridomas hVH65-107 and hVH65-110 were directly clonally descended from cells that produced antibodies with high affinity for Ars. The location of most of the productive somatic mutations in the V genes expressed by these hybridomas in and around complementarity-determining regions supports this idea. Interestingly, several highly nonconservative mutational changes have occurred in these V regions (e.g. in hVH65-110  $V_{\kappa}$ : Tyr-32 to Cys, and Pro-40 to Ala; in hVH65-107  $V_H$ : Lys-38 to Ile). This latter class of mutations may be responsible for the Ars<sup>-</sup> phenotype of the V regions these genes encode.

The two antibodies we have characterized apparently represent a new class of antibody that is a by-product of the antigen-driven immune response. Further isolation of antibodies of this type may provide new insights into the mechanisms of regulation of V region expression during the immune response. In addition, the study of such antibodies will undoubtedly help to further elucidate the effects of somatic mutation on the structure and function of antibody V domains.

We thank Alan Greenspan and Rou-Fun Kwong for help with sequencing analyses, and Drs. Arnold Levine and Thomas Shenk, in whose laboratories some of the experiments were performed.

*Received for publication 30 June 1987 and in revised form 10 August 1987.*

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