Random Mutagenesis of Two Complementarity Determining Region Amino Acids Yields an Unexpectedly High Frequency of Antibodies with Increased Affinity for Both Cognate Antigen and Autoantigen

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Summary

To gain insight into the mechanism and limitations of antibody affinity maturation leading to memory B cell formation, we generated a phage display library of random mutants at heavy chain variable (V) complementarity determining region 2 positions 58 and 59 of an anti-p-azophenylarsonate (Ars) Fab. Single amino acid substitutions at these positions resulting from somatic hypermutation are recurrent products of affinity maturation in vivo. Most of the ex vivo mutants retained specificity for Ars. Among the many mutants displaying high Ars-binding activity, only one contained a position 58 and 59 amino acid combination that has been previously observed among monoclonal antibodies (mAbs) derived from Ars-immunized mice. Affinity measurements on 14 of the ex vivo mutants with high Ars-binding activity showed that 11 had higher intrinsic affinities for Ars than the wild-type V region. However, nine of these Fabs also bound strongly to denatured DNA, a property neither displayed by the wild-type V region nor observed among the mutants characteristic of in vivo affinity maturation. These data suggest that ex vivo enhancement of mAb affinity via site-directed and random mutagenesis approaches may often lead to a reduction in antibody specificity that could complicate the use of the resulting mAbs for diagnostic and therapeutic applications. Moreover, the data are compatible with a hypothesis proposing that increased specificity for antigen, rather than affinity per se, is the driving force for formation of the memory B cell compartment.

During the course of a T cell–dependent antibody response, the V regions of antibodies expressed by B cells differentiating to memory are structurally altered because of hypermutation of the genes encoding them. Working in concert with hypermutation, a stringent process of antigen selection results in the affinity maturation of the antibody response. Frequent end products of the mutation–selection process are V regions containing recurrent amino acid changes at particular positions (1–3). Site-directed mutagenesis studies have shown that many of these recurrent mutations individually result in increased affinity for the eliciting antigen (2–4).

Affinity for antigen is only one of the factors that could influence the composition of the somatically mutated antibody repertoire expressed by the memory B cell compartment, however. Somatic mutation takes place throughout the length of V region genes, but is not a random process; hotspots and hot areas for mutation clearly exist (1, 5–7). In some responses, mutants with altered idiotypy (8) or antigen binding on-rate (9) may be differentially selected. It has also been suggested that somatic mutation will create autoanti-

bodies de novo (10–13), and that peripheral tolerance mechanisms must eliminate B cells expressing these autoreactive antibodies (14).

Secondary anti-p-azophenylarsonate (Ars)[1] antibodies from A/J mice are largely encoded by a single combination of V gene segments (termed "canonical"). Recurrent amino acid substitutions due to somatic mutation are observed alone or together among these antibodies at two positions in V_H CDR2: Thr-58 → Ile, and Lys-59 → Thr (based on an antibody sample representing >35 independent events; references 1, 15). These mutations individually and additively result in increased affinity for Ars, and they account for most of the affinity increase displayed by secondary anti-Ars antibodies (4). A high resolution x-ray crystal structure of a canonical anti-Ars antibody suggests that V_H residues 58 and 59 are not Ars contact residues (16) and so must alter affinity in-

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[1] *Abbreviations used in this paper:* Ars, p-azophenylarsonate; Ars-Tyr, Ars-N-acetyl-L-tyrosine; cpIII, coat protein III; PC, phosphorylcholine.

directly. To test whether the recurrent nature of the Thr-58 → Ile and Lys-59 → Thr amino acid substitutions is due entirely to the action of antigen affinity–based positive selection, libraries of a canonical antibody Fab that contain random amino acid substitutions at V_H positions 58 and 59 were generated.

Materials and Methods

Construction of Phage Display Fab Mutant Libraries. The pComb3 Fab expression vector (17) was modified via standard techniques to include a chloramphenicol resistance gene and to allow "drop in" cloning of V_H region PCR products, all as illustrated in Fig. 1. Site-directed, oligonucleotide-templated, PCR-based mutagenesis of V_H codons 58 and 59 was carried out as described (18) with the modification that *Pyrococcus furiosus* (Pfu) (Stratagene, La Jolla, CA) or Vent (New England Biolabs, Inc., Beverly, MA) thermostabile DNA polymerases were used. To generate more defined mutations, a primer containing the 58 and 59 antisense sequence CNT AAT (sense = ATT ANG) was used. The resulting plasmids were transformed into XL1-Blue (Stratagene), and colonies that grew on chloramphenicol plates were purified. Single colonies were picked into 96-well dishes containing luria broth. Replicas of these stock plates were created by inoculation into superbroth and were grown at 37°C to an OD₅₉₅ of 0.3, induced with 1 mM isopropyl β-D-thiogalactoside, superinfected with R408 phage at a multiplicity of infection of 10, and incubated for 18–36 h at 37°C.

ELISA Screening and Nucleotide Sequencing of Antibodyphage Clones. Supernatants obtained from superinfected phagemid-containing bacterial colonies were screened by direct binding ELISA assays using 96-well plates (Immulon-4; Dynatech Laboratories, Inc., Chantilly, VA) coated with either Ars-BSA, a rat anti–mouse κ chain mAb, or the monoclonal antiidiotypic antibody E4. Bound antibodyphage were detected using a sheep anti-M13 antiserum (5 Prime → 3 Prime, Inc., Boulder, CO), followed by an alkaline phosphatase conjugate of a donkey anti–sheep Ig antiserum (The Binding Site, Inc., San Diego, CA). Plates were developed with *p*-nitrophenylphosphate and read on an ELISA plate reader at 405 nM. Supernatants obtained from superinfection of colonies containing the wild-type phagemid and a phagemid containing a V_H frameshift mutation were used as positive and negative controls on every plate.

The V_H and κ chain V region ($V_κ$) genes present in various phagemids were asymmetrically PCR amplified and sequenced by a modification of the dideoxynucleotide chain termination method using Taq DNA polymerase (19).

Expression, Characterization, and Purification of Fabs. Phagemids from the libraries were modified to allow the expression of free Fabs. The cpIII sequences were deleted as described (17) and illustrated in Fig. 1, and the modified phagemids were transformed into XL1-Blue. The transformants were grown in 2× YT (1.6% tryptone, 1% yeast extract, 0.5% sodium chloride, 0.2% glycerol, 50 mM potassium phosphate, pH 7.2) at 30°C to an OD₅₉₅ of 0.1, induced with 1 mM isopropyl β-D-thiogalactoside, and further incubated for 18–36 h. Periplasmic contents were extracted as described (20). Fabs were affinity purified by chromatography on Ars-bovine gamma globulin Sepharose 4B (Pharmacia Biotech, Inc., Piscataway, NJ) as previously described (21). Representative preparations of affinity-purified Fabs were analyzed for purity on denaturing PAGE. Preparations were also analyzed by quantitative ELISA on Ars-BSA, rat anti-κ, or E4 plates before further use.

Measurement of Ars Affinity and DNA Binding. Purified Fabs were used in fluorescence quenching analyses with Ars-*N*-acetyl-L-tyrosine (Ars-Tyr) as previously described (22). Association constants were calculated by fitting the data to the equation $y = ax/(1 + bx)$. The difference between the measured and calculated quench values was minimized with a reiterative method programmed in Excel (Microsoft, Inc., Redmond, WA). DNA binding was assayed by direct binding ELISA essentially as described (23) using a biotinylated form of the monoclonal rat anti-κ antibody, followed by streptavidin-alkaline phosphatase (Sigma Chemical Co., St. Louis, MO). Fabs that gave significant signals at 10 μg/ml (relative to plates coated without DNA) were further analyzed. Binding to plate-immobilized heat-denatured calf thymus or mouse spleen DNA was competed with either soluble heat-denatured DNA, total yeast RNA, B cell hybridoma native DNA, or Ars-Tyr at various concentrations.

Computer Modeling and Graphics. The program Sybyl (Tripos Associates, St. Louis, MO) was used to generate graphic images of the 36-71 combining site using an Indigo 2 workstation (Silicon Graphics, Mountain View, CA) and coordinates deposited in the Brookhaven Protein Data Bank (Brookhaven National Laboratories, Upton, NY). The arsonate hapten was located in this binding site using the docking and energy minimization facilities of Sybyl, in accordance with a previously published model (16). Particular attention was paid to the hydrogen bond interactions with Arg-96L, Asn-35H, and Ser-99H. In addition, in the deposited coordinates, an Ala rather than a Thr was built at V_H position 59 because of truncated side-chain density. Since Thr is the residue found at this position in the 36-71 sequence, the Ala side chain was replaced with the Thr side chain followed by local energy minimization using Sybyl.

Results

As illustrated in Fig. 1, the phagemid vector pCombfab10 was constructed from the expression vector pComb3, previously described by Barbas et al. (17), which allows expression of an antibody Fab on the surface of filamentous phage (antibodyphage) in the form of a fusion to coat protein III (cpIII). RNA from hybridoma BH-3H, which expresses a canonical IgG1, was used as a source of coding sequence for a canonical Fab (V_H-C_H1:$V_κ$-$C_κ$). The V region of BH-3H lacks somatic mutations and contains the most common single V_H-D (His) and D-J_H (Tyr) junctional amino acids found among canonical antibodies (24).

Restriction sites introduced at the ends of V_H coding sequence in the pCombfab10 vector allowed oligonucleotide-directed PCR mutagenesis of V_H codons 58 and 59. To control for possible artifacts due to bacterial expression of Fabs, we generated several defined mutations at 58 and 59. Subsequently, the cpIII sequences were deleted, allowing expression of free Fab molecules. Fabs were purified from periplasmic preparations by Ars affinity chromatography, and their intrinsic affinities for Ars-Tyr were determined. The values obtained for the Thr-Lys (wild type), Ile-Lys, and Ile-Thr (predominant in vivo) mutants were in good agreement with those obtained for site-directed mutants of a canonical V region expressed as complete antibodies in hybridomas (4, 21).

V_H codons 58 and 59 were "randomized" in pCombfab10 in two independent experiments (see Fig. 1 and Materials and Methods). A total of 480 colonies picked from the libraries were superinfected with helper phage, and supernatants were
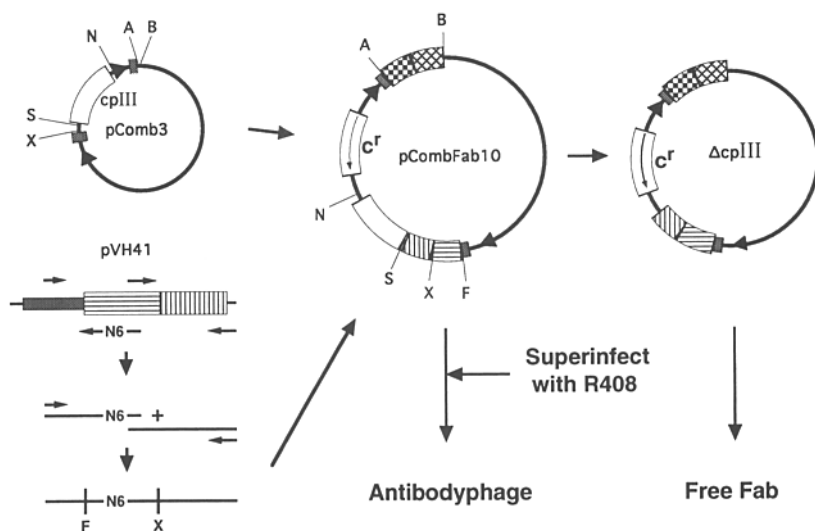
Figure 1. Construction of pCombfab10 and the V_H position 58 and 59 random libraries. A V_κ (*checkered boxes*)-C_κ (*cross-hatched boxes*) cDNA obtained by reverse transcription–PCR from hybridoma BH-3H RNA was cloned between the SacI (*A*) and XbaI (*B*) sites of pComb3, resulting in an in-frame fusion with one pel B leader region (*stippled boxes*). The germline sequence of V_κ-C_κ and in-frame fusion to pel B was confirmed by nucleotide sequencing. The resulting plasmid was modified by insertion of the pBR325 chloramphenicol resistance gene (*C'*) in the NheI (*N*) site, allowing selection against frequent deletions between the duplicated lac promotor (*filled triangles*)/pel B leader regions. A V_H (*horizontally striped boxes*)-C_H1 (*vertically striped boxes*) IgG1 cDNA obtained by reverse transcription–PCR from BH-3H RNA was then cloned between the XhoI (*X*) and SpeI (*S*) sites of pComb3, resulting in in-frame fusions with one pel B leader and the cpIII gene (*open boxes*). PCR-based site-directed mutagenesis procedures were used to insert unique SfiI (*F*) and XhoI (*X*) sites at the 5' and 3' ends of V_H, respectively, and to replace amino-terminal V_H codons present in pComb3 with those found in canonical V_H genes. An internal deletion of 74 bp in V_H was then made to ultimately allow distinction between wild-type and mutant clones. The BH-3H V_H-C_H1 cDNA/pel B leader region containing plasmid pVH41 was used as a template for oligonucleotide-directed PCR mutagenesis of V_H codons 58 and 59 with an oligonucleotide containing equimolar amounts of each base at all six positions of these two codons (*N6*). Pfu and Vent thermostabile DNA polymerases were used for this purpose in two separate experiments. The resulting populations of PCR products were used to replace the V_H sequences between the SfiI (*F*) and XhoI (*X*) sites of pCombfab10. Bacterial colonies containing the resulting members of the 58 and 59 random libraries were superinfected with R408 phage, resulting in the production of the mutant Fabs in the form of antibodyphage. Subsequently, individual members of the libraries were modified by deletion of the cpIII sequences between the SpeI (*S*) and NheI (*N*) sites, allowing expression of the mutant Fabs as free proteins.

collected for ELISA assay. To assess for dimerization of V_H-C_H1-cpIII with V_κ-C_κ, a monoclonal anti-C_κ capture antibody was used. To more specifically assess the structure of the V domain, the monoclonal antiidiotypic antibody E4 was used. Ars-binding activity of the antibodyphage was assessed using Ars-BSA. Repeated helper phage superinfection and ELISA screening of both libraries yielded reproducible results.

The vast majority of the antibodyphage clones gave rise to significant signals in all three ELISA assays (κ^+E4$^+$Ars$^+$, Fig. 2). Thus, most of the Fabs were expressed in a native state and retained specificity for Ars. Nevertheless, some variation in levels of expression was apparent, so the levels of anti-κ–binding activity were used to normalize the E4 and

Ars values. 33 antibodyphage that displayed high Ars/κ ratios (i.e., the highest apparent Ars affinities) were chosen for nucleotide sequencing analysis. Since only a few copies of the Fab-cpIII fusion protein are present at the tip of each phage particle (17), the values obtained from the ELISA assays are probably directly related to the affinity of the interaction between the Fabs and the plate-bound ligands. A number of the rare mutants that displayed either no anti-κ signal or anti-κ signals but no E4 or Ars reactivity were also sequenced. Most of the former were found to contain termination codons at positions 58 or 59 (data not shown).

The κ^+E4$^-$Ars$^-$ and κ^+E4$^+$Ars$^-$ clones contain a high frequency of Cys codons, or a high frequency of Pro and Gly
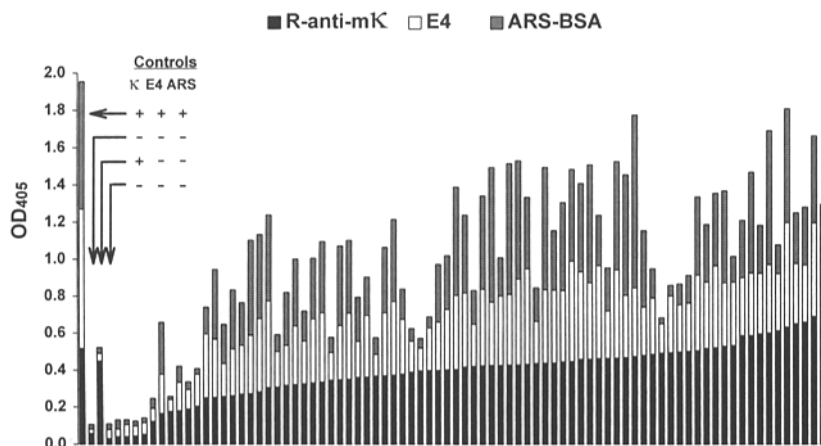
Figure 2. Representative ELISA screening data obtained from one of the V_H 58 and 59 antibodyphage random libraries. Culture supernatants were obtained and assayed by ELISA as described in Materials and Methods. Controls included were supernatants obtained from the wild-type clone that encodes a κ^+E4$^+$Ars$^+$ Fab; a mutant containing an amber termination codon in V_H (κ^-,E4$^-$Ars$^-$; *second from left*); a mutant containing six mutations distributed throughout V_H (κ^+E4$^-$Ars$^-$; *third from left*); and a mutant containing a frameshift mutation in V_H (κ^-E4$^-$Ars$^-$; *fourth from left*). Data obtained from individual members of the random library are plotted as single vertical bars in order of increasing anti-κ signal intensity from left to right, after the four controls.

Table 1. V_H 58 and 59 Antibodyphage Mutants with Different Idiotypic and Ars Binding Activity

58 + 59 Residues	58 + 59 Residues
κ+, E4-, Ars-	**κ+, E4+, Ars+ (high Ars/κ ratio)**
Val-Cys	Arg-Arg (5)
Ile-Cys	Arg-Thr (3)
Gly-Gly	Arg-Ser (3)
Glu-Pro	Thr-Lys* (wild type; 3)
Cys-Ser	Leu-Thr (3)
Arg-Cys	Arg-Gly (2)
κ+, E4+, Ars-	Pro-Arg (2)
	Lys-Arg
Pro-Cys (2)	Asp-Arg
Pro-Gly (2)	Ser-Arg
Gly-Gln	Arg-Leu
Leu-Asp	Arg-Ile
Ala-Asp	Arg-Lys
Asp-Gly	Leu-Asn
Ile-Pro	Tyr-Thr
Gly-Cys	Thr-Thr*
Tyr-Pro	Ala-Pro
Pro-Pro	Met-Val
Thr-Gln	Lys-Ser
κ+, E4+, Ars+/-	
Val-Pro	
Arg-Trp	

Most of the mutants were completely sequenced in the V_H region. 51 contained mutations only at positions 58 and 59. Three, the Arg-Leu and one each of the Arg-Arg and Arg-Ser mutants, contained a fortuitous Lys → Met change at V_H codon 67 and were not used for further analysis. The number of independent isolates of some of the mutants are indicated in parentheses.

* Amino acid combinations that have previously been observed among Ars-elicited in vivo antibodies.

codons, respectively (Table 1). The presence of Cys in CDR2 may often result in global alteration of V region folding, leading to loss of both Ars binding and the E4 idiotope. Proline or Gly may locally alter the conformation of CDR2, often leading to loss of Ars binding but not idiotypy. At position 58, 16 of the 33 κ+E4+Ars+ (high Ars/κ) mutants contained Arg codons (Table 1). At position 59, 2 predominant codons were observed; 8 Thr and 10 Arg codons. Five of these mutants contained Arg codons at both positions 58 and 59. These frequencies were all much greater than expected from Arg and Thr codon frequencies (Arg = 9.8%; Thr = 6.6% of all nontermination codons). Several other types of mutants were isolated multiple times from both libraries, many containing Arg at one position. The recurrent in vivo

58 Ile was not observed among the strong Ars-binding mutants but was seen in two of the Ars- mutants. Since control mutants with Ile at position 58 bound Ars with high affinity (Table 2), this may have resulted from biases in the random mutation procedure, or inadequate sample size.

The cpIII coding sequences of 14 representative strong Ars-binding mutants were removed (see Fig. 1), allowing expression and purification of free Fabs. The intrinsic affinities of these Fabs for Ars-Tyr were determined via fluorescence quenching and are shown in Table 2. The affinity of most of the mutants was equal to or greater than the wild-type (Thr-Lys) affinity. 4 of the 14 mutants displayed affinities equal to or greater than the Ile-Thr mutant. The rank order of these affinities was confirmed using a less sensitive ELISA assay in which various concentrations of soluble Ars-Tyr were used to compete Fab binding to plate-bound Ars (data not shown).

The abundance of Arg residues observed, an amino acid found frequently in CDRs of anti-DNA autoantibodies (10, 11, 25), taken together with previous data suggesting that unmutated canonical antibodies display weak binding to denatured DNA (23), led us to measure the binding of the purified

Table 2. Intrinsic Affinities of V_H 58 and 59 Fab Mutants for Ars-N-acetyl-L-tyrosine

58 + 59 Residues	K_a for Ars-Tyr (M-1)
Controls	
Thr-Lys* (wild type)	2.8 (2.7,2.8,2.9,3.0) × 10⁵
Ile-Lys*	6.0 × 10⁵
Ile-Thr*	1.1 × 10⁶ (8.9 × 10⁵)
Random mutants	
Asp-Arg	1.6 (2.0) × 10⁵
Ser-Arg	1.7 (1.8,1.8) × 10⁵
Arg-Gly	1.8 (1.9) × 10⁵
Arg-Ser	3.3 × 10⁵
Arg-Arg	3.5 (4.1) × 10⁵
Ala-Pro	3.9 (3.9) × 10⁵
Arg-Lys	4.1 × 10⁵
Arg-Thr	4.4 (4.8) × 10⁵
Thr-Thr*	4.6 × 10⁵
Tyr-Thr	8.0 × 10⁵
Met-Val	1.0 × 10⁶
Arg-Ile	1.3 (1.3) × 10⁶
Leu-Thr	1.6 (1.3,1.4,1.9) × 10⁶
Pro-Arg	1.7 (1.4) × 10⁶

When multiple affinities are shown for individual mutants (in parentheses), each value was obtained from an Fab preparation purified from an independent culture of that mutant. Many of the individual values shown were derived from two independent quenching analyses done on the sample.

* Amino acid combinations observed among Ars-elicited in vivo antibodies.

Fabs to this nucleic acid. Nine of the mutants displayed significant binding to denatured DNA (Table 3). Binding of eight of these mutants could be inhibited by low concentrations of Ars-Tyr, indicating that single or overlapping sites were responsible for both binding activities. DNA binding of seven of the mutants was weakly competed by RNA, and binding of one of the mutants could be somewhat inhibited by native DNA. Neither the Thr-Lys (wild-type) Fab nor the Ile-Lys or Ile-Thr (predominant in vivo) mutants displayed significant DNA binding in our assays. Because of their monovalence, it is likely that the Fabs that bound denatured DNA did so with significant affinity. Indeed, we found that Fabs with K_as for Ars-Tyr of $\sim 2 \times 10^5$ gave rise to Ars ELISA signals that were comparable to the signals obtained from the strongest DNA-binding Fabs on DNA plates.

Discussion

The bacteriophage Fab expression system relies on folding and secretion pathways that are distinct from those of mammals. Certain mutant antibodies that could not be efficiently expressed by B lymphocytes might be expressed more readily as Fabs via the bacteriophage pathway, and vice versa. In the case of canonical anti-Ars antibodies, however, this is unlikely, as numerous single and multiple amino acid substitution combinations have been introduced into canonical antibodies in all three V_H CDRs (including positions 58 and 59) with little or no effect on antibody production by B cell hybridomas (4, 21, 26, 27). Moreover, the only mutations we observed to ablate canonical Fab expression in the phage display format resulted in termination codons.

The frequency of antigen loss variants due to random mutagenesis of V_H CDR2 positions 58 and 59 is surprisingly low (Fig. 2). This indicates that loss of V region function due to mutations at 58 and 59 in vivo does not play a major role in the recurrent emergence of Ile and Thr as the predominant amino acid substitutions at these positions. A previous random mutagenesis study of the CDR2 region of a T15 idiotype expressing antiphosphorylcholine (PC) antibody revealed that a majority of mutants with two or more amino acid substitutions lacked affinity for PC (28). This study differed from ours in that mutations were randomly distributed throughout the length of CDR2. In addition, the T15 idiotype does not undergo affinity maturation (29), suggesting that germline T15 antibodies may contain a binding site for PC that is already optimal. These data support the idea that the influence of somatic mutation on affinity for antigen differs for different V regions, a parameter we have previously termed "adaptability" (30).

A variety of amino acid combinations at V_H CDR2 positions 58 and 59 result in increased affinity for Ars (Table 2). The spectrum of these combinations stands in marked contrast to the limited number that are products of the somatic mutation-affinity maturation process acting on canonical V regions in vivo. Only 1 (Thr-Thr) of the 11 ex vivo amino acid combinations that displayed increased affinity for Ars has been observed among in vivo mutants (combinations previously observed in vivo are indicated by asterisks in Tables 1-3). The recurrence of particular amino acid substitutions at 58 and 59 in vivo could be influenced by the type and frequency of mutations possible at these codons. The somatic mutation process displays a bias for introducing nucleotide transitions, may operate preferentially on small direct or inverted repeats and other small sequence motifs, and seems

Table 3. *Binding of V_H 58 and 59 Fab Mutants to Heat-denatured DNA*

58 + 59 Residues	ssDNA binding‡	Ars-Tyr§	RNA‖	dsDNA¶
Controls				
Thr-Lys*				
(wild type)	−			
Ile-Lys*	−			
Ile-Thr*	−			
Random mutants				
Met-Val	−			
Leu-Thr	−			
Asp-Arg	−			
Thr-Thr*	−			
Tyr-Thr	−			
Arg-Gly	+ / −	NI	NI	NI
Arg-Ser	+ / −	∼30	15%	NI
Ser-Arg	+	∼60	15%	NI
Arg-Thr	+	8.5	28%	NI
Arg-Ile	+	9.5	NI	NI
Arg-Lys	+ +	6.5	51%	NI
Ala-Pro	+ +	4.0	18%	11%
Pro-Arg	+ +	5.5	16%	NI
Arg-Arg	+ + +	16.0	25%	NI

* Amino acid combinations previously observed among Ars-elicited in vivo antibodies.

‡ Rankings were based on: (a) ELISA signals three to four times above background at + / − = 5 μg/ml; + = 2.5 μg/ml; + + = 1 μg/ml; + + + = 0.5 μg/ml; and (b) ability of binding to be competed 50% by 10–50 μg/ml soluble denatured DNA at an Fab concentration of 10 μg/ml. A minus sign indicates no reproducible ELISA signal obtained over plate coated without ssDNA at 10 μg/ml Fab. Binding of the conventional anti-ss and dsDNA 3H9 mouse mAb (10) under these conditions is inhibited 50% by 3 μg/ml denatured DNA.

§ The concentration of Ars-Tyr (μM) that gave 50% inhibition of binding to plate immobilized ssDNA at an Fab concentration of 10 μg/ml. A concentration of Ars-Tyr between 2 and 20 μM gives 50% inhibition to plate-bound Ars at this Fab concentration, depending on the intrinsic affinity of the particular Fab for Ars-Tyr.

‖ The percent inhibition of binding to plate-immobilized ssDNA obtained using 100 μg/ml soluble total yeast RNA or ¶40 μg/ml soluble native B cell hybridoma DNA, at an Fab concentration of 10 μg/ml. Binding of 3H9 was inhibited 50% by 0.7 μg/ml native DNA and ∼25% by 100 μg/ml RNA.

NI, no inhibition detected.

to act with a preference for one of the DNA strands (1, 5, 7). It was previously suggested that the recurrent ACT → ATT mutation to Ile at codon 58 in canonical V_H genes may be templated by a small direct repeat sequence upstream of codon 58 in CDR2 (7).

Another factor influencing amino acid recurrence at 58 and 59 is the number of mutations required to produce a given amino acid substitution. The somatic mutation process appears mainly to introduce single nucleotide replacements, although whether such replacements are usually introduced in clusters is a subject of debate (5, 6, 31). The recurrent in vivo Thr-58 → Ile and Lys-59 → Thr substitutions require one nucleotide replacement each. Isoleucine was not observed among the high affinity ex vivo mutants at position 58, but Leu was observed, and, in combination with Thr-59 gave rise to an affinity similar to the predominant in vivo Ile-Thr combination (Table 2). Mutation to Leu would require two nucleotide replacements in codon 58. The low frequency of Leu and the recurrence of Ile among in vivo mutants (1, 15) could therefore be due to restrictions on the mutation process at this codon. Many of the other high affinity ex vivo mutants also contain amino acid substitutions that would require two nucleotide replacements in a codon to take place in vivo.

Unmutated, canonical antibodies weakly bind denatured DNA, a property that is not displayed by canonical antibodies representative of the somatically mutated, Ars-selected memory repertoire (23). We previously proposed that autoreactivity intrinsic to preimmune V regions was lost upon their somatic mutational alteration in vivo (23). The data presented here suggest that this conclusion is probably an oversim-

plification. The affinity for both Ars and DNA of canonical V regions is frequently enhanced by the same combinations of amino acid changes at V_H positions 58 and 59. Among the 480 ex vivo mutants screened, 5–10% appeared, at the level of ELISA signal, to bind better to Ars than the wild-type Fab. Of these, nearly 80% had higher affinity for Ars, and >60% had significant affinity for DNA.

Fig. 3 presents a representation of the structure of the combining site of the canonical anti-Ars mAb 36-71, which contains the recurrent V_H 58Thr → Ile and Lys-59 → Thr substitutions (16). The side chains of the putative Ars contact residues in V_κ (Arg-96) and V_H (Asn-35, Trp-47, Tyr-50, Ser-99, and Tyr-106) are shown, as are the side chains of V_H Ile-58 and Thr-59. The Ars hapten is shown in green and is positioned based on the results of previous site-filling and site-directed mutagenesis studies (16, 26, 27, 32). It can be seen that Ile-58 is far from the binding pocket, and its side chain points away from this region. Threonine 59 is somewhat closer to the bound hapten, and its side chain points towards but not directly into the Ars-combining site. Given the orientation of the side chain of V_H amino acid 58, it is difficult to provide a simple structural explanation for the influence of this position on affinity for Ars and Ars conjugates (for a more detailed discussion of this issue, see reference 32). In contrast, the orientation of this side chain seems ideal to allow substitutions that increase affinity for DNA while not dramatically affecting affinity for Ars. Indeed, six of the nine DNA-binding mutants contain Arg at this position, and all but one of these mutants has slightly higher affinity for Ars than the wild-type Fab. Arginine is routinely found in the CDRs of anti-DNA antibodies derived from
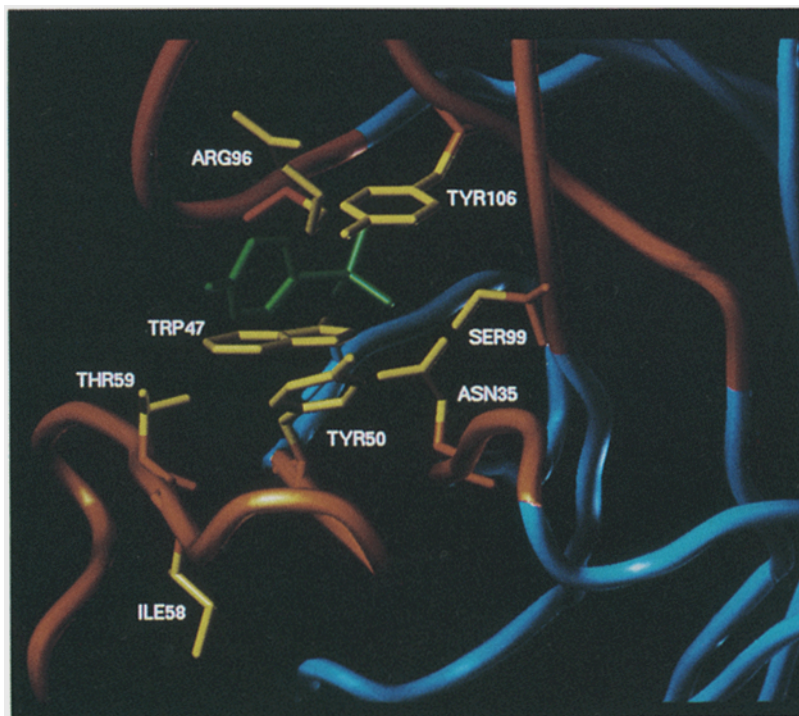
Figure 3. Graphic representation of the combining site of the canonical anti-Ars mAb 36-71. This image was generated as described in Materials and Methods. The main chain atoms of the framework regions of V_H and V_κ are depicted as blue tubes. The main chain atoms of the V_H and V_κ CDR regions are depicted as orange and red tubes, respectively. The side chains of the putative Ars contact residues Arg-96 in CDR3 of V_κ, Asn-35 in CDR1 of V_H, Trp-47 in FW2 of V_H, Tyr-50 in CDR2 of V_H, and Ser-99 and Tyr-106 in V_H CDR3 are shown explicitly in yellow. Also shown in yellow are the side chains of Ile-58 and Thr-59. The Ars hapten is shown in green, and the phenyl ring is depicted in the orientation presented in previous publications (16, 26, 27, 32), although docking procedures indicated that other orientations were no less favorable. The amino group that becomes linked to Tyr on carrier proteins is present at the para position of the phenyl ring relative to the arsonate group and points away from the binding pocket.

autoimmune mice (10, 11, 25) and in the binding sites of other DNA-binding proteins (33). It has been suggested that this amino acid is particularly well suited for interaction with DNA because of its long and flexible side chain and its positively charged guanidinium group, which contains many potential hydrogen bond donors and is planar with shared electrons, potentially allowing interaction with aromatic ring structures (34). The importance of Arg residues for the DNA-binding specificity of an anti-DNA mAb isolated from an autoimmune mouse has been formally demonstrated via site-directed mutagenesis (34).

The position 59 side chain, although apparently incapable of directly contacting the Ars hapten, might contact the Tyr side chain to which Ars is conjugated on carrier proteins. Replacements of Lys-59 may alter antigen binding through changes in this interaction. Although the data are limited, it appears that large, positively charged side chains (Lys and Arg) slightly reduce Ars-binding affinity, while smaller, uncharged side chains (Ser, Thr, Val, Ile) often enhance affinity.

The highly prevalent Arg-Arg mutant has an increased affinity for Ars-Tyr compared with wild type, perhaps due, in part, to an electrostatic effect of creating a more positively charged binding environment for the negatively charged hapten. The Pro-Arg mutant has the highest affinity for Ars-Tyr and the second highest apparent affinity for DNA. The presence of Pro-58 probably significantly alters the conformation of the amino-terminal half of V_H CDR2, which normally adopts a distorted β-sheet structure (16, 32), resulting in reorientation of the Arg-59 side chain. This reorientation apparently allows affinity enhancing interactions of Arg-59 with both Ars-Tyr and DNA. Alteration of CDR2 conformation may also explain the enhanced Ars affinity and DNA binding of the Ala-Pro mutant. In general, however, altering V_H CDR2 conformation destroys specificity for cognate antigen, as the majority of ex vivo mutants with Pro at either 58 or 59 (or both) lack affinity for Ars.

Others have suggested that autoreactivity is an expected and frequent outcome of V region hypermutation (10–13). For example, antibodies to foreign antigens display a dearth of Arg residues in their CDRs (10), while anti-DNA antibodies characteristic of systemic autoimmune diseases often contain multiple Arg residues in CDR regions (10, 11, 25). This difference has been interpreted to mean that, while Arg must arise frequently in CDRs during the maturation of the antibody response to foreign antigens, such mutants are either lost or selected against in normal individuals (10).

Many of the ex vivo mutants with high affinity for both Ars and DNA contain Arg at positions 58, 59, or both. Muta-

tional biases (see above) may limit substitutions to Arg at positions 58 and 59 in vivo. However, extensive amino acid diversity due to somatic mutation at codon 59 is observed among in vivo mutants (1, 15). In addition, our analysis shows that three (Cys, Gly, and Pro) of the seven amino acids (including Arg) that have not been observed at this position in vivo are often not compatible with specificity for Ars, although Pro in some contexts may increase both DNA and Ars binding (e.g., the Ala-Pro mutant). These data indicate that selective forces are largely responsible for the absence of Arg at position 59 among in vivo mutants.

Our data are compatible with the concept that there is a "second window" of tolerance induction before formation of the memory B cell compartment (14). If, during the mutation–selection process, antiself and increased antiforeign antigen affinity were always produced by independent combinations of somatic mutations, positive selection forces alone might effectively eliminate the autoreactive mutants. However, the "dual-specific" mutants, with increased affinity for both a foreign antigen (Ars) and a self antigen (DNA), our analysis indicates could be generated during the anti-Ars response could not be purged by positive selection alone, negative selection would have to operate as well. Evidence that active negative selection can act on B lymphocytes expressing antibodies specific for DNA has been obtained from transgenic mouse studies (35, 36). In the context of the germinal center, where most hypermutation and antigen selection is thought to take place, a relatively high concentration of extracellular nucleic acid may be available due to the cell death that takes place in this microenvironment (37). Direct support for this hypothesis requires the demonstration that somatic mutations that result in simultaneous increases in affinity for Ars and DNA take place during antigen-driven B cell differentiation in vivo.

A combination of positive and negative selection forces operating on rapidly mutating B cells in germinal centers would result in the specificity maturation of the immune response. The high specificity of the antibodies that resulted would maximize the sensitivity of the memory B cell compartment to low concentrations of foreign antigen while minimizing the chance of development of autoimmunity. Most ex vivo antibody mutation–selection strategies rely on increased affinity for cognate antigen as a measure of antibodies that will work better in therapeutic and diagnostic contexts (38). Our data suggest that this assumption is ill-founded. Under the influence of only antigen affinity–based positive selection forces, antibodies with increased affinity for both foreign and autoantigens might often arise.

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