Different Epitope Structures Select Distinct Mutant Forms of an Antibody Variable Region for Expression During the Immune Response

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Summary

Antibody variable (V) regions that initially differ from one another by only single amino acid residues at V\textsubscript{D} and D-J\textsubscript{\text{a}} segment junctions (termed canonical V regions) can be elicited in strain A/J mice by three different haptens. Among such V regions an amino acid substitution due to somatic mutation is recurrently observed at V\textsubscript{H} CDR2 position 58, regardless of which of these haptens is used for immunization. This substitution confers upon a canonical V region a generic increase in affinity for all the haptens. Conversely, the type of amino acid substitution at V\textsubscript{H} position 59 resulting from somatic mutation that is recurrently observed among such V regions changes with the eliciting hapten, in a manner that correlates directly with the cognate affinity increases (or decreases) for hapten conferred by the observed substitutions. This small subregion of V\textsubscript{H} CDR2 therefore plays a major role in determining both affinity and specificity for antigen. The data confirm that affinity for antigen is of pivotal importance in determining the degree of selection of different mutant forms of a V region. Moreover, during an immune response a sufficiently diverse mutant repertoire can be generated from a single canonical V region to allow adaptation to increased affinity for three different epitopes.

Extended changes in the structure and function of expressed antibody V regions occur during the course of most murine immune responses due to somatic hypermutation of the genes encoding them (1). Hybridomas isolated at late stages of immune responses express V region genes that display a nonrandom distribution of somatic mutations. The ratio of mutations causing amino acid replacements (R) to those that do not, silent (S), is often very high in CDR regions and low in framework regions (2, 3). Further, many V regions encoded by the same germline V\textsubscript{H} or V\textsubscript{L} gene segments and expressed by hybridomas derived from different mice immunized with the same antigen contain identical amino acid substitutions resulting from mutation (4–9). These observations are often cited in support of the notion that antigen selective forces cause a dramatic skewing of the somatically mutated V region repertoire generated during an immune response (2–7). However, it has recently become apparent that the somatic mutation process is nonrandom, with respect to both the location and outcome of its action. Mutational hot-spots and perhaps hot areas exist in many V genes (4, 8, 10, 11), and a given base does not mutate to the other three available bases with equal frequency (9, 12, 13). How the nonrandom nature of somatic mutation contributes to the differences in R to S ratios in subregions of V genes and the recurrence of particular R mutations is not understood.

Affinity for antigen also plays a role in shaping the composition of the elicited V region repertoire. The intrinsic affinity for antigen of both serum (14, 15) and mAbs tends to increase with time after immunization (1, 4–7). Somatic mutation clearly contributes to this affinity maturation since several recurrently observed somatic mutations have been shown to confer increased affinity for antigen (4, 5, 6, 16). The influence on affinity of such mutations is, however, sometimes very small and it is uncertain how small differences in intrinsic affinity could be translated into large differences in the degree of clonal selection of B cells.

Thus, our mechanistic understanding of antigen selection and somatic mutation is very limited. It is, therefore, currently difficult to evaluate the relative importance of these two processes, as well as immunoregulatory processes (17) that are not directly perceptive of antigen structure, in determining the structure of V regions expressed in the elicited antibody repertoire. If antigen plays a pivotal role in the se-

1 Abbreviations used in this paper: Ars, p-azophenylarsonate or p-aminophenylarsonate; IArs, p-azo-meta-iodo-phenylarsonate or p-amino-meta-iodo-phenylarsonate; Phos, p-azophenylphosphonate or p-aminophenylphosphonate; R, replacements; S, silent; Tyr, N-acetyl-L-tyrosine.
lection of V regions during an ongoing immune response, and the size of the mutant repertoire generated from a single V region is very large, then different epitope structures should select different mutant forms of a given V region for predominant expression. Moreover, if affinity differences among mutant V regions for the eliciting epitope are directly translated into differences in the degree of expression of those V regions, then a mutation that confers an affinity increase specific for one epitope should be predominantly observed only among the V regions elicited by that epitope. Conversely, mutations that confer generic increases in affinity for a variety of epitopes would be predicted to be observed among V regions elicited with all of these epitopes.

The immune response of strain A/J mice to p-azophenylarsonate (Ars) provides an ideal model system to experimentally address these issues. Previous molecular and serological analyses of this response have revealed that antibody V regions encoded by a single combination of gene segments, composed of a V\(_\gamma\) gene segment called V\(_\gamma\)Id\(_{CR}\) (18), a D segment encoded by the A/J DFL16.1 locus (19), J\(_\gamma\)2, a V\(_\gamma\)10 gene segment termed V\(_\gamma\)Id\(_{CR}\) (20), and J\(_\gamma\)1, are expressed by a major fraction of the stimulated B cell population at late times in the primary and in the secondary anti-Ars response (21, 22). Unmutated V regions of this canonical type differ from one another by at most two amino acids whose codons are generated by V\(_\gamma\)-D and D-J\(_\gamma\) joining.

Materials and Methods

Synthesis, Purification, and Conjugation of Hapten.

Ars and p-aminoarylphosphonate (Phos) were obtained from Aldrich Chemical Co. (Milwaukee, WI), p-amino-meta-iodophenylarsonate (IArs) was prepared as described (23). N-acetyl-t-tyrosine (Tyr) derivatives of Ars, IArs, and Phos were prepared and purified as previously described (24). Proton NMR spectra of the conjugates were taken on a 300-MHz instrument (General Electric Co., Wilmington, MA) in the Department of Chemistry, Princeton University (Princeton, NJ) and revealed that each was free of detectable contamination. Hapten conjugates of KLH (Calbiochem Corp., La Jolla, CA) were prepared as described (25) using identical weight ratios of hapten to protein. Hapten to protein conjugation ratios were determined by absorption at 280 nM and by As or P analysis forArs, IArs, and Phos-KLH conjugates (Schwarzkopf Microanalytical Laboratory, Woodside, NY) and found to be within twofold of one another. We observed that Phos-KLH preparations that had been stored at 4°C in PBS for >1 mo failed to elicit E4\(^*\) antibodies. Freshly conjugated preparations of Phos-KLH were therefore used in all experiments.

Immunizations and Hybridoma Formation, Screening and Analysis.

Female A/J mice of 8–12-wk of age that had been originally obtained from The Jackson Laboratory (Bar Harbor, ME) were used in all experiments. Primary immunizations were of 100 \(\mu\)g antigen emulsified in CFA and secondary immunizations were of 100 \(\mu\)g antigen in saline. Both injections were intraperitoneal. Hybridomas were constructed and screened for expression of the V\(_\gamma\)Id\(_{CR}\) gene segment and E4 idiotope as described (8). V region sequencing was performed using either the primer extension-dideoxynucleotide direct mRNA sequencing procedure (26), or by converting mRNA to cDNA using reverse transcriptase followed by the PCR. Direct sequencing of the total PCR cDNA product was done using the primer extension-dideoxynucleotide-Sequence protocol (27).

Southern blotting analysis of hybridoma DNA was performed as described (8) using nylon membranes. All probes were labeled with \(^{32}\)P via nick translation.

Generation of Engineered Mutant Antibodies.

The lle 58 and Thr 59 single mutants and the lle 58 Thr 59 double mutant of mAb 36–65 were previously described (16). The Tyr 59 V\(_\gamma\) mutation was introduced into the rearranged 36–65 VDJ gene in M13MP19 by the Eckstein method of oligonucleotide-directed mutagenesis (28) using a mutagenesis kit from Amersham Corp. (Arlington Heights, IL) and the oligonucleotide 5’ GT TAT ACT TAT TAC AA T G’3’ (substituting nucleotides are underlined). All other procedures were as previously described (16).

Serology, Antibody Purification, and Affinity Analysis.

Determination of quantities of idioype-bearing and hapten-binding antibody in immune sera and hybridoma culture supernatants were performed by solid phase competition RIA as described (8) using a prototypical Ars-binding, idioype-positive mAb (36–65) as a standard. Ars binding mAbs were purified from culture supernatants by both affinity chromatography and gel filtration. Affinities of mAbs for hapten-tyrosine conjugates were determined by fluorescence quenching at 23–24°C as described previously (29) using a Perkin-Elmer LS-3 fluorescence spectrophotometer and a curve fitting program written by A. Sharon (Massachusetts Institute of Technology, Cambridge, MA) and J. Sharon. Two binding sites per molecule were assumed for all antibodies.

Results

We previously reported that canonical antibody V regions can be elicited in strain A/J mice by the Ars structural ana-

| Table 1. Frequency and Level of Expression of Anti-hapten Antibody and the E4 Idiotype among A/J Immune Sera Elicited with Ars, Phos, and IArs Conjugates of KLH |
|-----------------------------------------------|
| Antigen | Response | Anti-hapten antibody | E4 idiotype expression | Average level |
|---------|----------|----------------------|-----------------------|--------------|
|         |          |                      | µg/ml                 |              |
| Ars-KLH | 1'       | 12/12                | 11/12                 | 146          |
|         | 2'       | 12/12                | 12/12                 | 1,325        |
| Phos-KLH| 1'       | 21/21                | 7/21                  | 19           |
|         | 2'       | 21/21                | 13/21                 | 184          |
| IArs-KLH| 1'       | 18/18                | 11/18                 | 26           |
|         | 2'       | 18/18                | 17/18                 | 323          |

The number of mice that responded with detectable levels of anti-hapten or E4 expressing serum antibody (equivalent to >1 µg/ml 36-65 as measured in either a direct binding assay to hapten-BSA [anti-hapten antibody] or an E4 binding competition assay [E4 expressing antibody]) over the total number of mice immunized is shown. In the case of anti-hapten antibody, all of the mice responded with high levels: serum dilutions of >1/500 were required for primary antisera, and >1/5,000 of secondary antisera, before binding was reduced to half-maximum. The average levels of E4\(^*\) antibodies in sera from responding mice, expressed as µg/ml equivalents of 36-65, are shown. Ars- and Phos-elicited mAbs were found to bind equally well to Ars-BSA on plates, and so Phos-KLH immune sera were assayed on Ars-BSA plates; IArs-KLH immune sera were assayed on IArs-BSA plates. Mice were bled and sera collected 21–23 d after primary immunization. 30 d after primary immunization mice received 100 µg of antigen intraperitoneally in saline and secondary bleeds were performed 10 d later.
Figure 1. Southern blotting analysis of rearranged V genes in representative Phos- and IArs-induced hybridomas. The upper two panels show autoradiograms obtained from a single EcoRI blot hybridized with either a probe specific for the Jκ region (Jκ, encompassing a region just 3' of Jκ4 to just 3' if the IgH enhancer) or a VκIdCR coding region probe (Vκ, see reference 18). The VκIdCR blot was hybridized at a moderate stringency to avoid crosshybridization of the probe to a large number of J558 Vκ gene segment family members. Lanes corresponding to A/J kidney (A) and the Sp2/0 fusion partner (S) are indicated. The other lanes correspond to (from left to right) anti-Phos hybridomas 2F3A and 2F3M, and anti-IArs hybridomas 212A, 214G, and 1303. The arrows indicate the 5.5-kb band characteristic of the VκIdCR gene segment rearranged to Jκ2 (18). Molecular weight standards were run in adjacent lanes but are not shown. The lower two panels show the results of a single BamHI blot probed with either a Jκ region probe (Jκ, containing all of the Jκ locus) or a probe specific for a region just 5' of the VκIdCR gene segment (Vκ, see reference 20). Hybridization at high stringency of the VκIdCR blot was not necessary since there are apparently only three members of the Vκ0 gene family to which VκIdCR belongs (20). The arrows indicate the position of the 9.8-kb band characteristic of the VκIdCR gene segment rearranged to Jκ1 (20). Once again, the lanes corresponding to A/J kidney DNA (A) and Sp2/0 DNA (S) are indicated. The hybridoma lanes correspond to (from left to right) 2F3A, 2F3M, 212A, 214G, and 1303.

An analysis of the V genes encoding E4+ Phos-KLH-induced mAbs and several previously uncharacterized IArs-KLH-induced E4+ mAbs confirmed that they were encoded by the same gene segments that encode canonical anti-Ars V regions. This analysis involved characterization of restriction enzyme sites flanking rearranged V genes by Southern blotting, and direct analysis of Vκ and Vκ sequences in mRNA. Fig. 1 shows the results of Southern blotting anal-
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Figure 2. Sequences of the Vκ and Vγ genes expressed by Phos- and IArs-induced hybridomas. Sequences were determined as described in Materials and Methods and are presented compared to the sequences of prototype canonical Vκ and Vγ genes encoded by germ-line forms of the VκIdCR (18), DF116.1 (19), J2 (40), VγIdCR (20), and J1 (41) gene segments, and the consensus sequence of the J558 Vκ gene subfamily most homologous to VκIdCR (42). In this consensus sequence (Rel.Con.) nucleotide differences from the VκIdCR sequence found in at least 25% of the related genes are indicated in plain type, and differences found in at least 70% of these genes shown in bold type. The name of the hybridomas from which each sequence was derived is indicated at the beginning of each sequence. The regions encoded by different gene segments are separated by gaps. The V-D and D-J junctional nucleotides that vary among canonical Vκ genes are indicated (N). Nucleotide identity is indicated by a dash. Differences are shown explicitly. Positions that could not be unambiguously determined are indicated by a gap. Hybridomas 2F3A, 2F3M, and 2F3K were derived from an anti-Phos-KLH secondary response. Hybridomas 2I2A and 2I4G were derived from anti-IArs-KLH secondary responses and hybridoma 1303 was derived from an anti-IArs-KLH tertiary response. For hybridoma formation, mice were either: (a) immunized intraperitoneally with 100 μg antigen in CFA, rested 30 d (120 d for IArs-immunized mice), boosted with 100 μg antigen in PBS intraperitoneally, and spleens taken 3 d later (secondary response) or; (b) primed, rested, and boosted as in a, rested an additional 30 d, boosted intraperitoneally with 100 μg antigen in PBS intraperitoneally, and spleens taken 3 d later (tertiary). All of the IArs hybridomas were derived from different mice, and so represent distinct events. While all of the Phos hybridomas were derived from a single mouse, the sequences of their expressed Vκ genes reveal differences at Vκ-D and D-J junctions, a strong indication that these hybridomas were derived from distinct clonal precursors and so are representative of independent events.
yses of DNAs isolated from two Phos-induced hybridomas (2F3A and 2F3M) and three IArs-induced hybridomas (2I2A, 2I4G, and 1303) using Jα, VαdCR, Jκ, and VκdCR probes. In this figure, lanes corresponding to A/J kidney (germline) DNA and DNA from the Sp2/0 fusion partner are indicated by A and S, respectively. These analyses reveal that the novel restriction fragments detected with the Jα and VαdCR probes, and the Jκ and VκdCR probes (both indicated with arrows in Fig. 1) are of the sizes expected for rearrangement of the VαdCR gene segment to the Jα gene segment (18) and rearrangement of the VκdCR gene segment to the Jκ gene segment (20), respectively. Fig. 2 shows the nucleotide sequence of the Vα and Vκ genes expressed by these Phos- and IArs-induced hybridomas as compared with the consensus sequences that encode canonical anti-Ars V regions. The sequence analyses provide further support for the conclusion

Figure 3. Amino acid substitutions observed in the Vκ CDR2 subregion of canonical V regions elicited with Ars, IArs, and Phos-KLH. Amino acid sequences in the CDR2 subregion of the canonical Vκ regions expressed by hybridomas elicited with the various hapten are shown above the germline sequence. In each case, the germline-encoded amino acid sequence is shown using the three letter code, and amino acid positions, numbered sequentially from the mature amino terminus, are indicated. Only the amino acid substitutions due to somatic mutations are indicated; blanks denote identity with the germline sequence. The hybridoma names are listed next to each sequence, and in parentheses next to this name is a reference number indicating where the sequence was previously published. If the sequence of the Vκ gene encoding one of the CDR2 regions has not been previously published, this is also indicated in parentheses next to the hybridoma name. In compiling the data, we strived to consider only independent mutational events. To this end, we considered only the sequences of canonical V regions expressed by hybridomas that were derived from different mice, canonical V regions encoded by Vκ genes that differed at Vκ-D or D-Jκ junctions (and hence were probably derived from different clones), or Vκ genes that were derived from clonally related hybridomas but differed in all mutations in their Vκ CDR2 subregions.
eral differences in mutation pattern. The most dramatic differences are observed at VH position 59.

Phos-induced V regions, IArs-induced V regions reveal several V genes encoding canonical V regions (Fig. 2; references 32, 33). We have observed at the VH position 58 Ile substitution, seen among other V regions encoding E4+ V regions elicited with Phos and IArs, must be due to somatic mutation. The amino acids we have observed at the VH/D, DJ, and VJ, junctions in such V regions are identical to those that have previously been observed among Ars-elicited canonical V regions (32, 33).

A survey of the sequences of a large number of VH and VK genes encoding canonical V regions (Fig. 2; references 8, 9, 34–38; and S. Fish and T. Manser, unpublished results) expressed by hybridomas derived from secondary and hyperimmune responses of A/J mice to Ars, IArs, and Phos showed that recurrently observed somatic mutations causing amino acid substitutions (observed in >25% of the genes encoding antibodies elicited with a given hapten) were located only in the VH CDR2 region. Fig. 3 presents a compilation of the amino acid sequences in this region. While similar types of substitutions are observed in VH CDR2 among Ars- and Phos-induced V regions, IArs-induced V regions reveal several differences in mutation pattern. The most dramatic differences are observed at VH position 59.

All of the antibodies that contain these recurrent VH CDR2 amino acid substitutions also contain unique substitutions in their VH and VK regions. To dissect the effect of certain recurrent mutations away from all other changes, we used variants of canonical, unmutated mAb 36–65 that were generated by site-directed mutagenesis. The affinities of these mutant antibodies for N-acetyl-L-tyrosine (Tyr) conjugates of Ars, IArs, and Phos were measured, and are shown in Table 2. The unmutated antibody has a Kd for IArs-Tyr that is 5.1-fold higher than for the eliciting epitope (Ars-Tyr), that is, this binding is heteroclitic. Its affinity for Phos-Tyr is 2.2-fold lower than for Ars-Tyr.

The recurrent VH position 58 Ile substitution, seen among mAbs elicited with all three haptens (Fig. 3), results in similar increases in affinity for all the tyrosine conjugates. The recurrent position 59 Thr substitution, seen only among Ars-KLH and Phos-KLH elicited mAbs, results in 2.5- and 2.3-fold increases in affinity for the respective haptens, but a 2.4-fold decrease in affinity for IArs-Tyr. Both these changes result in 5.9-, 4.3-, and 1.9-fold increases in affinity for Ars-Tyr, Phos-Tyr, and IArs-Tyr, respectively. The VH Thr 59 substitution, observed recurrently only among IArs-KLH-elicited mAbs, confers a 6.3-fold increase in affinity for IArs-Tyr, while it does not significantly affect affinity for Ars-Tyr and Phos-Tyr.

**Table 2. Affinities for N-Acetyl-L-Tyrosine Conjugates of Ars, Phos, and IArs (Expressed as Kd in M⁻¹ × 10⁻⁷) of 36–65 and Site-directed Mutants in the 36–65 VH Region**

| Antibody          | Ary-Tyr | Phos-Tyr | IArs-Tyr |
|-------------------|---------|----------|----------|
| 36–65 (wild-type Th 58 Lys 59) | 3.9; 0.43* | 1.8 | 20; 1.2* |
| Ile 58            | 11 (2.8) | 4.4 (2.4) | 66 (3.3) |
| Thr 59            | 9.7 (2.5); 0.21* | 4.1 (2.3) | 8.4¹ (0.42) |
| Ile 58 Thr 59     | 23 (5.9) | 7.7 (4.3) | 38 (1.9) |
| Tyr 59            | 3.8 (0.97); 0.25* | 1.6 (0.89) | 125¹ (6.3) |

The affinity values are shown in bold type. These values were obtained from fluorescence quenching measurement using single preparations of Ars-Tyr, Phos-Tyr, and IArs-Tyr. Tyr conjugates of the haptens were used since the predominant attachment site on carrier proteins of these haptens is tyrosine side chains. The relative increase (or decrease) in affinity conferred by the mutation(s), obtained by normalizing the affinity of the mutant to the affinity obtained for 36–65 for each hapten is shown in parentheses after each affinity value. The maximum quench value, used to calculate the affinities, ranged between 53.3 and 65.1 for Ars-Tyr affinities, between 51.6 and 59.9 for Phos-Tyr, and between 53.4 and 60.5 for IArs-Tyr.

The values obtained for several of the affinities differ slightly from values previously reported. This is most probably due to variations in the hapten-tyrosine preparations, as we observe that affinity measurements conducted with the same preparations of hapten-tyrosine are highly reproducible (see SDs in this table) but those conducted with different preparations of the same hapten-tyrosine conjugate vary somewhat.

* The SD is given for values that are averages of three or four determinations.
  t Values that are averages of two determinations. The individual values were within 8% of each other.

Discussion

The frequency of particular amino acid substitutions resulting from somatic mutations at codons 58 and 59 in the CDR2 region of the VH genes encoding canonical mAbs elicited with Ars, Phos, and IArs correlates directly with the cognate affinity increases (or decreases) conferred by those substitutions. Most significant are the substitutions at position 59, where a striking difference in the type of recurrent amino acid change obtained with IArs vs. Phos and Ars is
observed. The type and location of somatic mutations introduced into a V gene during an immune response should not be influenced by the antigen. Therefore, the amino acid substitution frequency variations we observe at V_{H} CDR2 position 59 must be due entirely to biases imposed by antigenic selection. Since these variations directly correlate with affinity differences, our findings confirm that affinity for antigen must dramatically affect the clonal selection process. Moreover, the data show that while the size of the mutant repertoire generated from a single canonical V region may be somewhat restricted by the presence of mutational hotspots, this size is sufficient to allow efficient mutational adaptation (22) to increased affinity during immune responses to different epitopes.

It is noteworthy that the intrinsic affinities measured for the site directed mutants (Table 2) are so simply related to the frequency of observation of the corresponding in vivo mutations (Fig. 3), in a variety of different V_{H} and V_{L} mutant backgrounds. Similar results have been obtained by Berek and Milstein (4) and Rajewsky et al. (5) studying other antigen-antibody systems. Our analysis illustrates that this can be the case for both mutations that confer a generic increase in affinity for different epitopes (the position 58 Thr to Ile) and for mutations that confer more specific affinity increases (the position 59 Lys to Thr and Lys to Tyr). These data attest not only to the efficiency of the affinity-based antigen selection process, but also suggest that the magnitude of the influence on affinity of recurrently observed amino acid substitutions is relatively unaffected by substitutions at other positions. Thus, changes even at the adjacent V_{H} ID positions 58 and 59 can additively influence the affinity of a canonical V region for three different haptenes, and canonical V regions elicited in vivo contain recurrent substitutions either alone, or together at these positions. These observations provide additional support for the idea that amino acid substitutions in a V region can be selected in a stepwise, unordered fashion during the immune response (16, 39).

Finally, the data provide an explanation for the lower frequency and average level of expression of E4^{+} (canonical) V regions observed in response to iArs as compared with Ars (Table 1). Since the affinity of an unmutated, canonical V region for iArs-Tyr is 5.1-fold higher than for Ars-Tyr (Table 2), an affinity-based clonal selection hypothesis would predict that the frequency and level of expression of E4^{+} antibodies in the anti-iArs response should be higher than in the anti-Ars response (this correlation of frequency and level of expression with affinity is indeed apparent in the case of the Phos response). However, the recurrent amino acid substitutions observed at V_{H} CDR2 position 59 in Ars- and iArs-elicited antibodies require fundamentally different mutational alterations of codon 59. The Ars (and Phos)-specific Lys to Thr substitution requires a single nucleotide change (AAG to TAG), whereas the iArs-specific Lys to Tyr substitution requires that two nucleotide changes occur (AAG to TAC, or TAT), one of which alone will generate a termination codon (AAG to TAG). Therefore, the affinity maturation of canonical V regions during the immune response to iArs may take place more slowly than during the response to Ars, due to the lower probability of occurrence of amino acid substitutions at V_{H} codon 59 that confer an increased affinity for antigen. This may allow B cell clones expressing other types of iArs-specific V regions to gain predominance over clones expressing canonical V regions.

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