

# Antibodies that Are Specific for a Single Amino Acid Interchange in a Protein Epitope Use Structurally Distinct Variable Regions

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## Summary

We have analyzed how the immune system generates antibodies that are specific for analogues of an epitope on the influenza virus hemagglutinin (HA) that differ solely by the presence of Asp or Gly at amino acid 225. Most antibodies induced in response to HA(Asp225) use one of a few closely related variable (V) region structures that are encoded by characteristic VH/Vk gene segment combinations. Remarkably, none of these VH/Vk combinations was induced in response to HA(Gly225). Instead of modifying the HA(Asp225)-specific V regions by junctional variation or somatic mutation to recognize the altered epitope, new VH/Vk combinations were used. The expression of unique VH/Vk combinations appears to confer exquisite specificity to the selection of HA-specific B cells from the pre-immune repertoire.

In both B and T lymphocytes, specific recognition is mediated through clonally distributed heterodimeric receptors. For each type of receptor, two polypeptide chains are generated by somatic rearrangement of separate V, J, and in the case of VH and V $\beta$ , D gene segments (reviewed in reference 1). Crystallographic analyses of antibody molecules have demonstrated that the regions of greatest sequence variability, called complementarity-determining regions (CDR), form loops (2, 3); for each polypeptide, CDR1 and CDR2 are encoded by the V gene segment, whereas the junctionally encoded CDR3 is somatically generated during V(D)-J gene assembly (1, 4, 5). The spatial arrangement of the amino acid residues in these loops establishes the specificity of antibody molecules for protein molecules.

Previous studies have clearly demonstrated that somatic alterations of V region sequences by junctional variation and somatic mutation can play an important role in determining the specificity of antibody molecules (6-8). For example, somatic alterations of antibodies that recognize the influenza virus A/PR/8/34 hemagglutinin (PR8 HA)<sup>1</sup> can influence their interaction with the HA, as measured by their ability to crossreact with mutant viruses that possess different amino acid substitutions in the antibody combining site (9-14). Somatic alterations of V region sequences can also change the specificity of antibody molecules such that they recognize different antigens. An example of this is the somatic mutation of a phosphocholine-specific antibody to acquire specificity for DNA (15). Similarly, somatic mutation of V region se-

quences can alter antibody specificity for analogues of the hapten arsonate (16). However, the extent to which somatic alterations of V region sequences can give rise to antibodies that are specific for different protein antigens has been unclear. This question is of particular interest in view of the extensive interactions that take place in the combining site between antibodies and protein epitopes. Analyses of complexes formed between antibodies and protein antigens have demonstrated interactions between 15 and 22 amino acids on both the antigen and the antibody molecule (17-23). Nevertheless, single amino acid substitutions that induce only minor local alterations in the epitope can abolish antibody binding, and can lead to the induction of noncrossreactive antibodies (22, 24-26). How the immune system distinguishes between distinct analogues of epitopes that display such extensive structural similarity has not yet been explained.

We have addressed this issue by comparing hybridoma antibodies that were induced in response to two HA molecules that differ from each other solely by the presence of Asp or Gly at amino acid 225. Based on this comparison, we have assessed how antibodies that are specific for similar but not identical overall shapes of determinant are generated. Even though certain characteristic VH/Vk combinations were used to recognize Asp225, none of these combinations was used to recognize Gly225. The results indicate that somatic alterations of these characteristic VH/Vk combinations did not provide the basis for distinguishing these epitope analogues, and further suggest that the expression of specific VH/Vk combinations can be critical in establishing the specificity of antibodies for the HA. These findings also demonstrate the capacity for single amino acid interchanges to influence the immune recognition of viruses, such as influenza virus and

<sup>1</sup> Abbreviations used in this paper: HA, hemagglutinin; HAU, hemagglutinating units.

HIV, which can accumulate numerous amino acid substitutions during their evolution in man.

## Materials and Methods

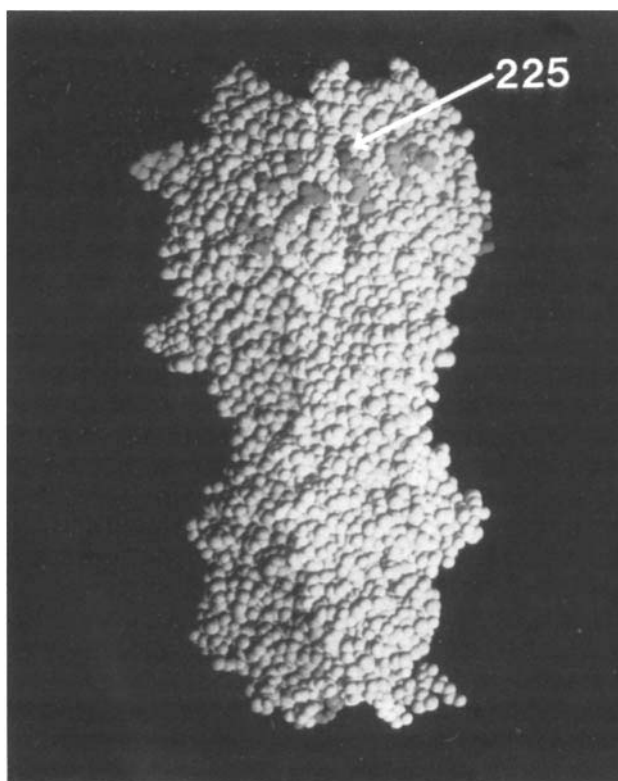
**Hybridoma Antibodies.** HA-specific antibodies were generated as described previously by fusion of SP2/0-Ag14 myeloma cells with splenocytes from BALB/c mice (Jackson Laboratories, Bar Harbor, ME) (27). In the hybridoma designation, the first number indicates the fusion from which the hybridoma was derived; for example, H36-6 was derived from fusion H36 after immunization with PR8 virus, whereas T1-107 was derived from fusion T1 after immunization with mutant virus T3. The PR8-specific hybridoma antibodies were generated by various primary and secondary response immunization protocols, as have been described elsewhere (11, 27, 28). The T3-specific hybridomas were generated after either primary or secondary immunization of adult mice as follows. For fusions T1, T2, T4, T7, T8, T9, and T10, mice were immunized intravenously with 1,000 hemagglutinating units (HAU) of virus T3, followed 5 d later by splenectomy and fusion. For fusions T5 and T6, mice were primed for 24 d and given a booster injection 3 d before death with 1,000 HAU by the intraperitoneal and intravenous routes, respectively. T3 HA-specific antibodies were identified as those that were able to bind the immunizing virus, but that could not bind the reassortant virus J1 (which contains a serologically noncrossreactive HA) (27). Culture supernatants were analyzed for their binding to purified T3 and J1 viruses in the ELISA by detection first with a murine Ck-specific rat mAb/biotin reagent, then with extravidin-alkaline phosphatase (Sigma Chemical Co., St. Louis, MO), and finally developed with *p*-nitrophenyl phosphate, as described (29).

**Specificity Analysis of Hybridoma Antibodies.** Specificity analysis of the PR8-specific antibodies has been described previously (11, 27, 30). HA-specific hybridoma culture supernatants from the T3 fusions were first screened at two dilutions (1:5 and 1:25) for their ability to bind 20 HAU of purified viruses T3 and PR8 in the ELISA. Antibodies that displayed more than twofold higher binding to T3 than to PR8 in this primary screen were then cloned by limit dilution. The ability of culture supernatants to bind T3 and PR8 virus was then compared in serial dilution in the ELISA, and those that displayed at least fourfold higher binding to T3 than to PR8 in the linear titration range were selected for sequence analysis.

**Sequence Analysis of Hybridoma V Regions.** Total cellular RNA was prepared from hybridoma cell lysates and used to prepare cDNA copies of Ig V regions using <sup>32</sup>P-labeled constant region-specific primers as described (11). Full-length cDNA products were isolated by PAGE, and VH and Vk region sequences were amplified in the PCR using either degenerate V region primers that hybridize to the 5' ends of many VH and Vk regions, or using one-sided PCR, and were subjected to direct sequence analysis as described (28).

## Results

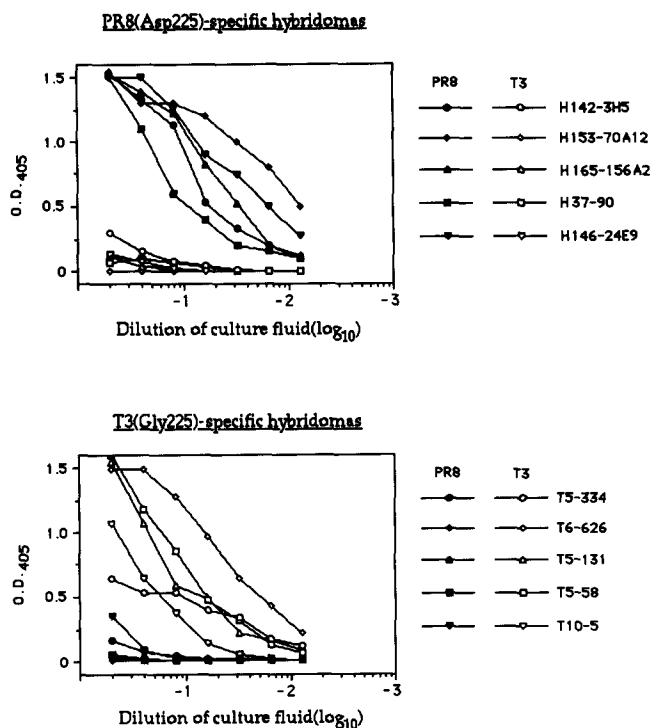
**Generation and Specificity Analysis of PR8- and T3-specific Hybridoma Antibodies.** Virus T3 is a neutralization escape mutant that differs from influenza virus PR8 by an Asp to Gly interchange at amino acid 225 in the HA (10). T3 is one of 12 mutant viruses that define the antigenic region Ca, containing amino acid substitutions that are closely located on the surface of the H3 subtype HA (Fig. 1). 16% of PR8



**Figure 1.** Location on the HA of amino acid 225 and of other amino acids that define the Ca antigenic site. A side view of the crystallographic structure of the H3 subtype HA trimer is shown (36); two of three monomers from the trimer can be seen in this view. The Ca site spans the interface between adjacent monomers (10). The amino acid residues that define the Ca antigenic site are shaded, and the location of amino acid 225 is indicated.

HA-specific hybridomas display reduced or undetectable binding to T3 (W. Gerhard, personal communication). We analyzed 27 hybridomas that bind to antigenic region Ca on the PR8 HA for their VH and Vk region sequences: 19 of these hybridomas fail to bind to mutant virus T3 (11, 30). The remaining Ca-specific hybridomas can bind to T3, but fail to bind to one or more of the other mutant viruses that have substitutions in this region (30). The 19 hybridomas that fail to bind to T3 were obtained from 12 individual donor mice following a variety of primary and secondary response immunization protocols. Since these antibodies can bind to the PR8 HA (which contains Asp at residue 225) but display reduced, and in most cases, undetectable binding to the T3 HA (which contains Gly at residue 225), we designate these hybridomas as being PR8(Asp225) specific. The reaction of several PR8(Asp225)-specific hybridomas with the PR8 and T3 viruses in ELISA is shown in Fig. 2.

We have now generated a corresponding panel of T3(Gly-225)-specific hybridoma antibodies. Nine individual BALB/c mice were immunized with mutant virus T3; spleens were removed 5 d after primary or 3 d after secondary immunization and used to produce T3 HA-specific hybridomas. The



**Figure 2.** Specificity analysis of PR8(Asp225)-specific and T3(Gly225)-specific hybridoma antibodies. Serial dilutions of culture supernatants from representative hybridomas were incubated with 20 HAU each of purified PR8 and T3 viruses adsorbed to polyvinyl plates, and antibody binding was measured in ELISA.

hybridomas were screened first to identify those that were T3 HA specific, and then for their ability to bind to the T3 and PR8 viruses; 15% of the T3 HA-specific hybridomas failed to bind to PR8. 20 T3-specific hybridoma antibodies were isolated that displayed reduced, and in most cases, undetectable binding to PR8 (Fig. 2). Accordingly, the T3 (Gly225)-specific hybridomas recognize a region of the HA that is related to the one recognized by the PR8(Asp225)-specific hybridomas in that amino acid 225 is a crucial part of the epitope, but Gly instead of Asp is required at amino acid 225.

**Variable Region Expression among PR8- and T3-specific Hybridoma Antibodies.** The VH and Vk region nucleotide sequences of some of the PR8(Asp225)-specific hybridoma antibodies have been described (11). The VH and Vk region nucleotide sequences of the remaining PR8(Asp225)-specific, and of the T3(Gly225)-specific hybridomas are shown in Figs. 3 and 4. The deduced amino acid sequences of the PR8 (Asp225)- and T3(Gly225)-specific hybridoma VH and Vk regions are shown together in Figs. 5 and 6. The sequences have been grouped according to previously defined VH and Vk gene families (31–34); the gene family from which each hybridoma VH and Vk region was derived is listed in Table 1. The VH and Vk gene families represent groups of similar gene segments that share >80% sequence similarity. It should be noted that even though a set of hybridomas might express members of the same VH or Vk gene family, they neverthe-

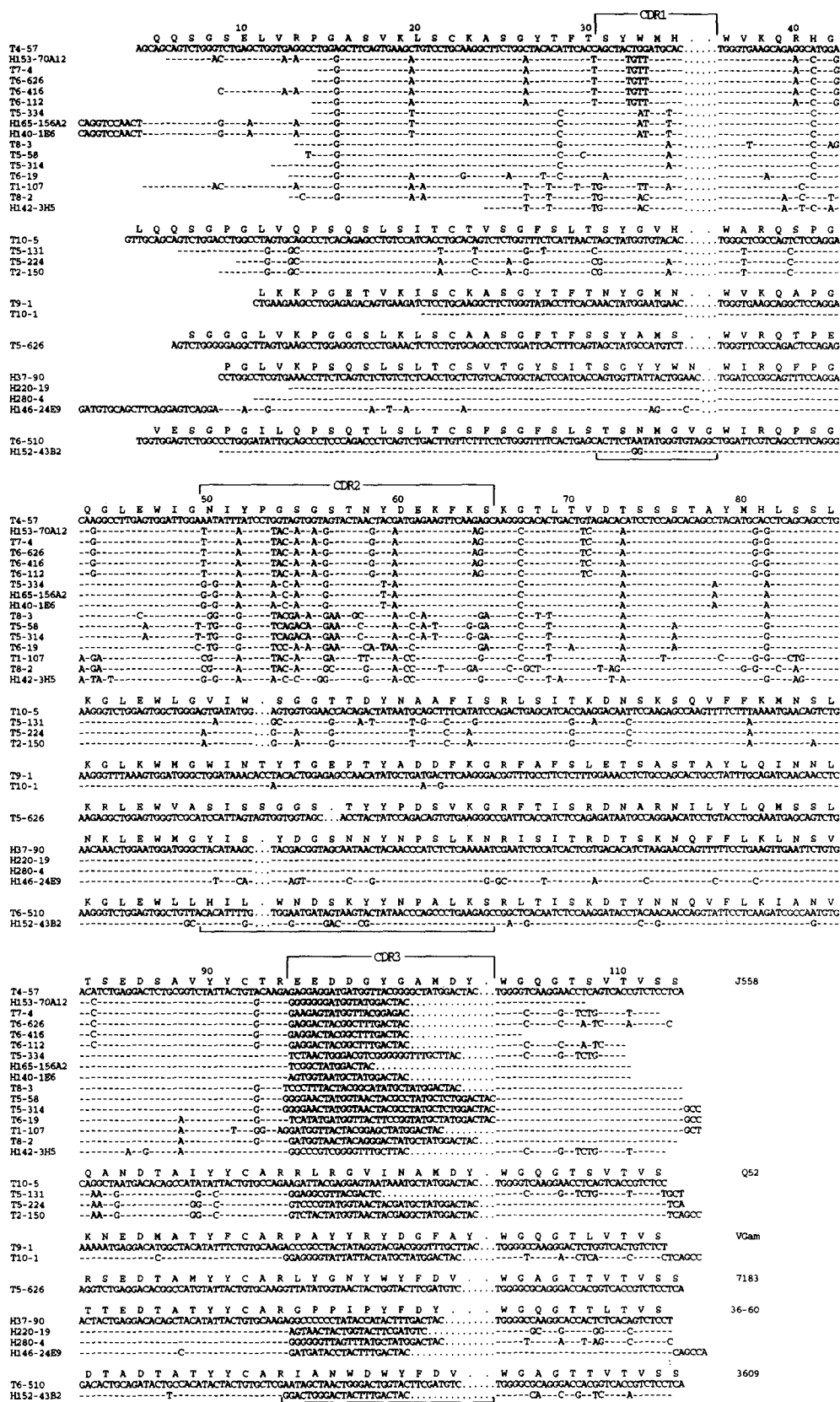
less can display many differences in their V region sequences. For example, several VH genes derived from the J558 gene family displayed quite different sequences. In some cases, however, sets of hybridomas expressed VH or Vk sequences that were identical or that differed by only one or two amino acids. These hybridomas appear to utilize common germ-line gene segments, as indicated by shared symbols in Table 1.

The PR8(Asp225)-specific panel contains gene segments drawn from five different VH and five different Vk gene families. As shown in Table 1, these VH and Vk gene segments did not associate in random combinations to generate the PR8(Asp225) specificity. Indeed, the majority of the PR8 (Asp225)-specific hybridomas can be placed into one of four groups based on the VH/Vk gene segment combination. Two large groups utilized VH gene segments drawn from the 7183 gene family in conjunction with a unique gene segment from the Vk1 or Vk24 gene families. A third group of three hybridomas utilized a specific 36-60/VkRF gene segment combination, and a pair of hybridomas utilized a specific J558/Vk1 gene segment combination. The Vk1 gene segment utilized by this latter group is quite different (20 amino acid differences) from the Vk1 gene segment utilized in conjunction with members of the 7183 family. These four VH/Vk gene segment combinations make up 80% of the hybridoma panel and are thus quite characteristic of the PR8(Asp225) response.

The overall number of VH and Vk gene families utilized by the T3(Gly225)-specific hybridoma panel is similar to that used in the PR8(Asp225) response. Members of five different VH and seven different Vk gene families are expressed by the T3(Gly225)-specific hybridomas. However, none of the VH/Vk combinations characteristic of the PR8(Asp225)-specific hybridomas was used in response to T3(Gly225). Interestingly, some of the individual VH and Vk gene segments from the PR8(Asp225) combinations were used by T3(Gly225)-specific hybridomas. With only one exception, these gene segments were expressed in association with a member of a different VH or Vk gene family to recognize T3(Gly225). The notable exception is hybridoma T5-334, which expresses the same J558 gene segment as is utilized as part of the characteristic J558/Vk1 combination in the PR8(Asp225) response. T5-334 utilizes this J558 gene segment in conjunction with the Vk1 gene segment that associates with members of the 7183 gene family in the PR8(Asp225) response. In this case, then, a novel reassortment of gene segments from the PR8 (Asp225) response appears to have provided the basis for establishing specificity for T3(Gly225).

A small group of T3(Gly225)-specific hybridomas from two different mice express an identical J558/Vk4/5 gene segment combination. As was observed with the characteristic PR8(Asp225)-specific VH/Vk combinations, the individual VH and Vk gene segments from this T3(Gly225)-specific VH/Vk combination were utilized in the PR8(Asp225) response. Once again, however, they were expressed in association with gene segments from different VH and Vk gene families to generate the alternate specificity.

The general lack of overlap in VH/Vk expression between the T3(Gly225) and PR8(Asp225) hybridoma panels extends



**Figure 3.** Nucleotide sequences of VH regions from PR8(Asp225)- and T3(Gly225)-specific hybridomas. Sequences are grouped according to VH gene family and are displayed relative to a representative from each group (e.g., T4-57 for the J558 group; the deduced amino acid sequence for each group representative is also shown). Dashes indicate identity with the representative sequence. The deduced amino acid sequence of each family representative is numbered according to Kabat et al. (41). The locations of CDR are also shown. Dots have been introduced to facilitate alignment of the sequences. The nucleotide sequences of some of the PR8 (Asp225)-specific hybridomas have previously been described (11). These sequence data and the data in Fig. 4 are available from EMBL/GenBank/DBJ under accession numbers X59172-X59211, M64134, M64135, M64137, M64140-M64142, M64147, M64148, M64150, M64152, M64153, M64155, M64159-M64161, M64166, M64167, and M64169.





to those VH/Vk combinations that are present only once in each panel. Even though three hybridomas from the T3(Gly225) response express the same VH/Vk gene family combinations as are expressed by three hybridomas from the PR8(Asp225)-specific panel, the individual VH and Vk gene segments that are used in each response display many differences that indicate their derivation from different germ-line gene segments. The possibility that these represent somatic variants of single germ-line gene segments can be excluded because in most cases they are primary response hybridomas that display little or no somatic mutation (6, 8). The only secondary response hybridoma among this group that could potentially represent a somatic variant is T6-510. However, it displays a different length of L chain CDR1 region that indicates a distinct germ-line gene origin.

## Discussion

Corresponding panels of hybridoma antibodies were generated that recognize related structures on the HA in which amino acid 225 forms a crucial part of the epitope, but that differ in their requirement for Asp or Gly at this residue. Many different V gene segments appeared to be used preferentially in the PR8(Asp225)- and T3G(Gly225)-specific hybridoma panels. Even though seven individual V genes were used in both panels, they were always utilized as part of different VH/Vk combinations in the two panels. Moreover, a large proportion of the PR8(Asp225)-specific hybridomas utilized one of four characteristic VH/Vk gene segment combinations. Remarkably, none of these combinations was utilized among T3(Gly225)-specific antibodies. Despite the large sequence variability that could potentially have been introduced, particularly in the H chain CDR3, to alter the antibody paratopes, this mechanism was not used to generate antibodies that distinguish this single amino acid interchange. Instead, unique VH/Vk gene segment combinations were used in each response.

The failure to observe somatically altered versions of the characteristic PR8(Asp225) VH/Vk combinations among the T3(Gly225)-specific hybridomas was unexpected. Both somatic mutation and junctional variation can influence how antibodies interact with the HA, as measured by their ability to recognize mutant viruses (such as T3) that contain single amino acid substitutions (11–14). In addition, the paratopes

of the PR8(Asp225)-specific antibodies described here can all be distinguished based on their reactivities with mutant viruses, and some of these differences in specificity for the HA are likely to be the result of junctional variation or somatic mutation. For example, the sets of PR8(Asp225) hybridomas that utilize the characteristic J558/Vk1 and 36-60/VkRF combinations each express identical VH and Vk gene sequences, differing only in their junctional sequences (Figs. 5 and 6). Since these antibodies also display unique reactivities with other PR8 mutant viruses (30), the junctional sequences of these antibodies influence how they interact with the HA. Why then was junctional variation not utilized to modify the paratopes and allow recognition of the structurally related region of T3(Gly225)? One possibility is that the characteristic PR8(Asp225)-specific antibodies bind to the HA with orientations that do not involve interactions between the junctional regions and residue 225. Thus, modifications of the junctional regions would not affect recognition of the amino acid at residue 225. Although this may have provided a plausible mechanism to account for a single VH/Vk combination, it seems less likely to explain the failure of junctional variation to adapt any of four different PR8(Asp225)-specific VH/Vk combinations to recognize T3(Gly225).

A second possibility is that although junctional variation could potentially generate the alternate specificities, the appropriate types of modification are rare. It was anticipated that junctional variation would modify the specificity of some of these VH/Vk combinations because it has the capacity to introduce an enormous amount of sequence variation. Estimates of the size of the antibody repertoire generally introduce factors of several orders of magnitude to account for the number of different junctional sequences that can be expressed by any individual VH/Vk combination (see for example references 1 and 8). It was anticipated that at least some of the different junctional sequences from this vast potential pool would modify the specificity of these antibodies. It is significant, however, that these estimates predict total repertoire sizes that greatly exceed the number of lymphocytes in a mouse. Thus, even if junctional variation has the capacity to modify the PR8(Asp225) VH/Vk sequences and generate specificity for T3(Gly225), these sequences may be so rare that there is a low probability that any individual mouse would contain a B cell that expresses the appropriate modification.

**Figure 5.** Deduced amino acid sequences of PR8(Asp225)- and T3(Gly225)-specific H chain V regions. Hybridomas whose designation contains the H prefix are PR8(Asp225) specific; those whose designation contains the T prefix are T3(Gly225) specific. Sequences are grouped according to the VH gene family from which they were derived (which is indicated), and are displayed relative to a representative sequence from that family (e.g., T4-57 for the J558 group). Dashes indicate identity with the representative sequence. Within each family, V regions that display near identical amino acid sequences and are likely to have been derived from the same VH gene segment are grouped together. Members of the 7183 gene family have been grouped according to a previous characterization that included Southern hybridization analysis and the presence of shared silent substitutions (11). The H chain isotype, and the JH gene segment that is utilized are indicated. Also shown is the Vk gene family that each hybridoma expresses, and the protocol used for immunization; 1° indicates primary response, 2° indicates secondary response, and 1°n indicates hybridomas derived from neonatal mice primed intravenously on day of birth followed by immunization and fusion at various times during the next few weeks. Although these mice received two immunizations, they are referred to as primary response antibodies because there is no evidence that injection at birth primed HA-responsive B cells (28). The sequence of T4-57 is numbered according to Kabat et al. (41), and the location of CDR is indicated. Dots have been introduced to facilitate alignment of the sequences. Two groups of T3-specific hybridomas (T5-58 and T5-334; and T6-626, T6-416, and T6-112) express identical CDR3 sequences, and therefore represent clonally related B cells (42). This latter group utilizes identical H and L chain nucleotide sequences to T7-4, which was derived from an independent donor and displays a unique CDR3 sequence.

			10	20	30	40	50	60	70	80	90	100	
			CDRI1	CDRI2	CDRI3								
			10	20	30	40	50	60	70	80	90	100	
			DIQITQSPSSLSASLGGKVTITC	DIQKVIAR	LVVQKPKGPKRLLIH	VTSTLQP	GIPRFSGSGSGTDFTLTISSVQREDIADYFC	LVVQKPKGPKRLLIH	VTSTLQP	GIPRFSGSGSGTDFTLTISSVQREDIADYFC	LVVQKPKGPKRLLIH	VTSTLQP	GIPRFSGSGSGTDFTLTISSVQREDIADYFC
T1-107	JK2	JK1	---	---	---	---	---	---	---	---	---	---	---
T5-224	JK2	JK1	---	---	---	---	---	---	---	---	---	---	---
T5-58	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
T5-314	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
T4-57	JK1	JK1	---	---	---	---	---	---	---	---	---	---	---
H146-2483	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
H37-60	JK1	JK1	---	---	---	---	---	---	---	---	---	---	---
H37-62	JK1	JK1	---	---	---	---	---	---	---	---	---	---	---
H37-82	JK1	JK1	---	---	---	---	---	---	---	---	---	---	---
H38-2	JK1	JK1	---	---	---	---	---	---	---	---	---	---	---
H146-24E9	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
T5-334	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
H165-156A2	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
H140-1E5	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
T5-131	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
T6-19	JK1	JK1	---	---	---	---	---	---	---	---	---	---	---
T5-626	JK4	JK4	---	---	---	---	---	---	---	---	---	---	---
T6-415	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
T6-626	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
T6-112	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
T7-4	JK1	JK1	---	---	---	---	---	---	---	---	---	---	---
H152-4382	JK1	JK1	---	---	---	---	---	---	---	---	---	---	---
H142-3HS	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
T2-150	JK4	JK4	---	---	---	---	---	---	---	---	---	---	---
T6-510	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
T8-2	JK4	JK4	---	---	---	---	---	---	---	---	---	---	---
T8-3	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
T10-1	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
H153-70A12	JK1	JK1	---	---	---	---	---	---	---	---	---	---	---
T10-5	JK5	JK5	---	---	---	---	---	---	---	---	---	---	---
H220-19	JK1	JK1	---	---	---	---	---	---	---	---	---	---	---
H280-4	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
H37-90	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
T9-1	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
H158-98H4	JK5	JK5	---	---	---	---	---	---	---	---	---	---	---
H220-25	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
H35-06	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
H37-90	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
H37-80	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---
H37-40	JK2	JK2	---	---	---	---	---	---	---	---	---	---	---

Figure 6. Deduced amino acid sequences of PR8(Asp225)- and T3(Gly225)-specific L chain V regions. The hybridoma designations are as described in Fig. 4. Sequences are grouped according to the Vk gene family from which they are derived (which is indicated), and displayed relative to a representative sequence from that family (e.g., T1-107 for V<sub>k</sub>9); within each family, V regions that display near identical amino acid sequences or that share common, systematic substitutions relative to other family members have been grouped together. Dashes indicate identity with the representative sequence, and ambiguities are indicated by X. The Jk gene segment that is utilized is indicated. Also shown for each hybridoma is the VH gene family that each hybridoma expresses, and the protocol used for immunization (see legend to Fig. 4 for details). The sequence of T1-107 is numbered according to Kabat et al. (41), and the locations of CDR are indicated. Dots have been introduced to facilitate alignment of the sequences.



**Table 1.** Variable Region Gene Expression by the PR8(Asp225)- and T3(Gly225)-specific Hybridomas

Hybridoma	PR8(Asp225)		T3(Gly225)		
	VH	Vk	Hybridoma	VH	Vk
H37-40	7183*	Vk24‡			
H220-25	7183§	Vk24‡			
H37-80	7183	Vk24‡			
H35-C6	7183	Vk24‡			
H158-89H4	7183	Vk24‡			
H146-24B3	7183§	Vk1¶			
H36-2	7183§	Vk1¶			
H37-62	7183**	Vk1¶			
H37-60	7183**	Vk1¶			
H37-82	7183*	Vk1¶			
H140-1E6	J558##	Vk1	T5-334	J558##	Vk1¶
H165-156A2	J558##	Vk1			
H37-90	36-60¶¶	VkRF***			
H280-4	36-60¶¶	VkRF***	T10-5	Q52	VkRF***
H220-19	36-60¶¶	VkRF***			
H152-43B2	3609	Vk4/5*			
			T6-626	J558###	Vk4/5*
			T7-4	J558###	Vk4/5*
H153-70A12	J558##	Vk21§§§			
			T10-1	VGam	Vk21§§§
H142-3H5	J558	Vk4/5			
H146-24E9	36-60	Vk1§§			
			T8-2	J558	Vk21
			T8-3	J558	Vk21
			T1-107	J558	Vk9¶¶¶
			T5-224	Q52****	Vk9¶¶¶
			T2-150	Q52****	Vk4/5
			T4-57	J558	Vk9
			T5-58	J558	Vk9
			T6-510	3609	Vk4/5
			T9-1	VGam	Vk23
			T6-19	J558	Vk8
			T5-626	7183	Vk8
			T5-131	Q52	Vk1

For each hybridoma, the VH and Vk gene family that was utilized is shown. Closely related VH and Vk gene segments that are likely to have been derived from the same germ-line gene segment are indicated by the same symbols. The hybridomas have been grouped together according to the VH/Vk combinations they express. Where the same gene segment was utilized by both a PR8(Asp225)- and a T3(Gly225)-specific hybridoma, they have been placed opposite each other in the table. Only one representative from groups of clonally related hybridomas is included.

These findings may then illustrate the difficulty in defining how distinct mechanisms contribute to the generation of the immune repertoire based on estimates of potential diversity that exceed the total lymphocyte pool of an individual.

In view of the failure of junctional variation to modify the PR8(Asp225 VH/Vk combinations, it is intriguing that there are several examples in which specific VH/Vk pairing appears to have provided the basis for generating these alternate specificities. For example, the T3(Gly225)-specific hybridoma T5-334 uses VH and Vk gene segments that are drawn from characteristic PR8(Asp225)-specific hybridomas, but does so in a novel pairwise combination that has not been observed in the PR8(Asp225) response (see Table 1). Hybridoma T5-334 demonstrates that the absence of this combination from the PR8(Asp225)-specific hybridoma panel is not a consequence of the inability of this VH and Vk gene to associate and produce an HA-specific antibody. Rather, the pairing of this VH with this Vk gene segment appears to influence the specificity of the resulting antibody, leading to its utilization in the T3(Gly225) response. This model suggests that the specificity of antibodies for the HA may be exquisitely dependent on specific VH/Vk pairing, even to the extent that junctional variation cannot modify these antibody paratopes and create specificity for T3(Gly225). Future experiments in which these VH and Vk gene segments are reassorted in bacterial expression systems (35, 36) should allow this model to be directly tested.

It remains possible that certain VH/Vk combinations have the potential to acquire appropriate somatic mutations that would allow them to interact with either T3(Gly225) or PR8(Asp225). The failure to identify somatic variants of the characteristic PR8(Asp225) VH/Vk combinations among secondary response T3(Gly225)-specific hybridomas may in fact reflect the specificity with which primary response B cells were stimulated. Since none of the T3(Gly225)-specific primary response hybridomas utilized the characteristic PR8(Asp225) VH/Vk combinations, these combinations were apparently not stimulated during primary immunization with T3. Hence, somatic variants of these combinations were not obtained among the secondary response hybridomas. It may therefore be possible to obtain T3(Gly225)-specific somatic variants of the characteristic PR8(Asp225) VH/Vk combinations by priming with PR8 followed by a secondary boost with T3. Experiments to address this possibility are in progress.

Central to this analysis is the postulate that the Asp to Gly interchange at amino acid 225 does not induce major structural alterations in the HA. Several lines of evidence suggest that this interchange is unlikely to impart a large effect on the structure of the HA. Most importantly, a significant number of HA-specific antibodies recognize the antigenic region Ca (in which residue 225 is located), and crossreact with

PR8(Asp225) and T3(Gly225) (10, 30). These antibodies recognize the same region of the HA as do the antibodies described here, but the Asp to Gly interchange at residue 225 does not affect their binding, presumably because they do not make important contacts with this residue. In addition, the structures of seven HA and neuraminidase neutralization escape mutants have been determined (22, 24–26). In all cases, escape mutants that differ by single amino acid interchanges display only local structural alterations on the surface of the molecule. In one of these comparisons, the effect of an Asp to Gly interchange at residue 146 of the X-31(H3 subtype) HA was examined (24). This Asp to Gly substitution resulted in a repositioning of the amino acid side chain over a range of <1 Å, and led to a >1,000-fold reduction in the binding of the antibody used for selection. Residue 146 is located in a loop on the HA (37); comparison of the antigenic structure of PR8 with the crystallographic structure of the H3 subtype HA suggests that PR8 amino acid 225 is similarly located on a loop and is exposed to solvent (10). It seems likely that a similar local conformational change in the HA monomer was induced by the Asp to Gly interchange in virus T3. It may, however, be significant that the antigenic region Ca spans the interface between adjacent HA monomers (Fig. 1). It is therefore possible that subtle conformational changes in this region could nevertheless have a complex effect on the antigenic structure of this site on the HA trimer. If this indeed is the case, it remains intriguing that different pairings of the same VH and Vk gene segments were in some cases utilized to generate the alternate specificities. A similar comparison of antibodies induced in response to PR8 and an escape mutant from an antigenic region that does not span the monomer interface (such as the site Cb) should allow this possibility to be addressed.

Finally, it is significant to note that antigenic variability is the prominent feature of the immune recognition of the HA (as well as some other viruses, such as HIV) (38, 39). Influenza A viruses can escape the existing antiviral immunity of the host population gradually (antigenic drift) by accumulating individual, serologically significant amino acid differences (38). For example, the mutation of Asp to Gly at residue 225 has occurred during the evolution of the H1 subtype influenza virus in humans (40), and residue 225 is one of at least 30 positions at which serologically significant amino acid substitutions can occur on the H1 subtype HA (10). The dissimilarity between these two hybridoma panels serves to demonstrate how relatively minor structural variation of the type that occurs during antigenic drift of the HA can nevertheless exert a considerable impact on the structural and genetic basis by which a protein, or indeed, a virus, is recognized by the immune system.

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