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Enhancement and destruction of antibody function by somatic mutation: unequal occurrence is controlled by V gene combinatorial associations

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We examined the positive and negative effects of somatic mutation on antibody function using saturation mutagenesis *in vitro* to mimic the potential of the *in vivo* process to diversify antibodies. Identical mutations were introduced into the second complementarity determining region of two anti-phosphocholine antibodies, T15 and D16, which share the same germline VH gene sequence. T15 predominates in primary responses and does not undergo affinity maturation. D16 is representative of antibodies that co-dominate in memory responses and do undergo affinity maturation. We previously reported that >50% of T15 mutants had decreased antigen binding capacity. To test if this high frequency of binding loss was unique to T15 or a consequence of random point mutations applicable to other combining sites, we analyzed the same mutations in D16. We show that D16 suffers a similar loss of function, indicating an equally high potential for B-cell wastage. However, only D16 displayed the capacity for somatic mutation to improve antigen binding, which should enhance its persistence in memory responses. Mutation of residues contacting the haptenic group, as determined by molecular modeling, did not improve binding. Instead, productive mutations occurred in residues that either contacted carrier protein or were distant from the antigen binding site, possibly increasing binding site flexibility through long-range effects. Targeting such residues for mutation should aid in the rational design of improved antibodies.

Key words: affinity maturation/antibody/molecular modeling/saturation mutagenesis/somatic mutation

Introduction

Improved antigen binding resulting from somatic hypermutation is a central feature of antibody affinity maturation over the course of an immune response (Berek and Milstein, 1987; Rajewsky *et al.*, 1987; French *et al.*, 1989). Somatic mutation also has the potential to damage antibody function, but this aspect has been less well studied because such mutants generally escape detection. Thus, the contribution of harmful mutations to B-cell wastage has not been assessed thoroughly. It is generally assumed that mutations occur randomly over the length of the V region (Malipiero *et al.*, 1987), although recent studies have suggested that there may be mutational hot spots (Berek and Milstein, 1987; Levy *et al.*, 1988). The mechanism of somatic mutation is not known, and its impact on structure and function of antibodies is not well understood. To mimic effects of somatic mutation, we introduced random mutations *in vitro* by saturation mutagenesis of VH complementarity determining region 2 (CDR2) in murine anti-phosphocholine (PC) antibody, T15. The ability of T15 to bind antigen was dramatically decreased in >50% of the mutants (Chen *et al.*, 1992a); more recently, we reported that four of these mutants had secretion defects caused by certain CDR2 mutations (Chen *et al.*, 1994).

T15 is the prototype anti-PC antibody that predominates in primary responses and binds strongly to the free hapten (Claflin *et al.*, 1974; Lee *et al.*, 1974); we have called such antibodies Group I (Chang *et al.*, 1982a). Virtually all Group I antibodies use the VH1 gene of the S107 family (Perlmutter *et al.*, 1984). In contrast, Group II anti-PC antibodies are found mainly in the memory response to PC–protein and recognize an epitope that includes both PC and its phenyl linkage to carrier protein (Stenzel-Poore *et al.*, 1988). The phenyl ring is a dominant feature in the fine specificity of Group II antibodies, which bind free PC hapten weakly in the absence of the phenyl group (Bruderer *et al.*, 1989). Some Group II antibodies use the S107 VH1 gene, but in combination with other V gene segments that distinguish them from Group I antibodies (Stenzel-Poore and Rittenberg, 1989).

D16 is typical of Group II antibodies found in the memory response that use the S107 VH1 gene, and, like T15, it uses the germline VH1 sequence but with different DH, JH and Vκ segments. This allowed us to place the same mutant CDR2 sequences from T15 into D16 and to compare the effect of identical mutations on antigen binding function in these two closely related, but distinct, combining sites. We report that D16 antibody is equally susceptible to deleterious mutations in CDR2. Unlike T15, however, D16 also demonstrates a potential for mutation to improve antigen binding, which could aid its selection and expansion in the secondary response.

Computer modeling and anti-idiotypic analysis were used to derive a molecular basis for the ability of mutations to affect the combining sites of D16 and T15. Mutations affecting binding to VH1 by the anti-idiotope, TC-54, indicate that the epitope is encoded by a discontinuous set of residues in CDR2 that form a continuous solvent-

accessible surface structure. Comparison of the binding sites of T15 and D16 suggests that the position of D16-bound hapten is shifted relative to its position in T15. This shift provides a structural basis for fine specificity differences between the two antibodies. Such reorientation of the hapten in the D16 site would also alter CDR2 interaction with the carrier protein which may be responsible for the enhanced contribution of the phenyl linker to the epitope recognized by Group II antibodies.

Results

Experimental strategy

Previously we introduced point mutations into the H chain CDR2 of the T15 combining site by saturation mutagenesis (Chen *et al.*, 1992a). Forty six mutant antibodies containing 1–4 mutations each were analyzed. Statistical analysis showed the mutations were distributed randomly throughout CDR2 (Chen *et al.*, 1992a). Over 50% of the T15 mutants either lost or had reduced antigen binding activity (Chen *et al.*, 1992a). To test if this high frequency of binding loss was unique to T15 or a general consequence of random point mutations in the CDR2 of other combining sites, we have analyzed another anti-PC–protein antibody, D16. D16 uses genes typical of memory anti-PC phenyl antibodies that use the S107 VH1, DH SP2.2, JH2 and Vκ1c and Jκ1 gene segments. Wild-type (WT) D16, like T15, uses germline sequences for both VH and VL (Chen *et al.*, 1994). Moreover, use of the same VH gene segment in both the T15 and D16 active sites (although with different DH and JH) allowed the introduction of the same set of mutations to both antibodies expressed as IgG2b molecules, thus providing a valid comparison for the effects of these mutations on antibody function.

The D16 VH gene was isolated from the genomic library of the D16 hybridoma. Two restriction sites were generated flanking the CDR2 of T15 and D16 VH genes without changing the amino acid sequence. The CDR2 sequence of D16 was replaced by each of the mutated T15 CDR2 sequences. D16 VH genes with the same point mutations as the mutated T15 antibodies were transfected into D16 hybridoma cells lacking H chain but expressing L chain. Forty three mutant D16 transfectants were obtained; their CDR2 sequences are shown in Figure 1.

Binding to PC–protein

Binding activity of mutant D16 antibodies for PC–histone and PC–bovine serum albumin (BSA) was examined by direct binding enzyme-linked immunosorbent assay (ELISA) with increasing concentrations of antibody. The hapten is diazophenyl-linked to the protein, thus the epitope is viewed as $R\text{-}N{=}N\text{-}\Phi\text{-}PO_4\text{-}(CH_2)_2\text{-}N^+(CH_3)_3$, where R is either histone or BSA. Table I shows the results of one of three similar experiments with antigen coated on the plate at 1.0 μg/ml. The mutants fell into four antigen binding classes: improved binding (7/43); binding comparable to wild-type (9/43); decreased binding (5/43); no binding (22/43). The frequency of non-binding mutants is higher in D16 than in T15 (51% versus 37%), which conflicts with the original hypothesis that T15 would be more susceptible to loss mutations.

The most striking difference in the binding assays, however, is that seven mutant D16 antibodies (16%) acquired improved binding activity. In contrast, none of the T15 mutants had shown improved binding despite having identical mutations (Chen *et al.*, 1992a). An example of a binding assay comparing WT T15 with WT D16 and several D16 mutants is shown in Figure 2. Although the WT D16 bound PC–protein less well than WT T15, some of the D16 mutants had binding greater than that of WT T15.

Two other types of binding analysis were performed to verify that antigen binding had indeed been improved in the seven mutants. Antigen concentration on the ELISA plates was reduced 5-fold to reduce epitope density and favor binding by antibodies of greater functional affinity (Herzenberg *et al.*, 1980; Rothstein and Gefter, 1983; Pollack *et al.*, 1988). The results of one of two low antigen assays are also shown in Table I. All antibodies showed improved binding compared to wild-type, with mutants M106 and M159 improving 20- to 30-fold. In addition to these direct antigen binding assays, a competition ELISA was performed; an Fv fragment of the high affinity Group II antibody, M3C65 (Brown *et al.*, 1992), was used to compete with mutant or wild-type antibodies for binding to PC–protein. Based on the quantity of Fv required to achieve 50% inhibition, the seven mutants were again shown to have improved binding over WT, ranging from 3- to 12-fold better against PC–histone and 3- to 8-fold better against PC–BSA (data not shown).

Comparison of corresponding mutants revealed several T15/D16 pairs sharing CDR2 mutations with different binding phenotypes (Figure 3). Mutations in M161 and M183 caused loss of binding in the D16 antibody but did not affect binding in T15. The shared Glu H58→Gln substitution is probably responsible for this difference because it is the only mutation in M161. For mutants M41 and M244, the T15 binding activity was reduced ~5-fold, but binding ability of D16 antibody was completely lost. M41 has one mutation, Asn H52A→His, which causes the T15-decrease/D16-loss phenotypes. M244 has three mutations, one of which is the highly deleterious mutation at H52A, making it difficult to assign any additional contribution by the other two residues.

The seven D16 mutants with increased binding activity (M72, M106, M107, M142, M150, M159 and M296) have T15 counterparts with either unchanged or reduced binding activity. M296 is of particular interest because the single substitution Ser H51→Ile has contrasting effects, causing increased binding in D16 and decreased binding in T15. The same effects were also observed in mutant pair M106, which has three substitutions. Four of the D16 mutants with improved binding (M72, M107, M150 and M159) have their substitutions solely in the second loop of VH CDR2 (residues H60–H65). Together, these differences suggest that the combining sites of D16 and T15 are structurally distinct, even though both bind PC–protein and are encoded by the same VH gene. This conclusion is discussed further in the structural analysis described below.

Binding to non-PC antigens

To test the possibility that V region mutations would change the specificity of the antibody, we examined the reactivity of mutant D16 antibodies to the non-PC antigens, keyhole limpet hemocyanin (KLH), albumin, ubiquitin, actin and single-stranded DNA, and the haptens fluorescein

	50	51	52	a	b	c	53	54	55	56	57	58	59	60	61	62	63	64	65	binding to PC-protein	
WT	A	S	R	N	K	A	N	D	Y	T	T	E	Y	S	A	S	V	K	G	T15	D16
M20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	+	+
M22	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-
M28	-	-	-	-	-	S	-	-	-	-	-	-	-	R	-	T	-	T	-	-	-
M32	-	-	-	K	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	↓	-
M34	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	P	-	N	-	-	-
M41	-	-	-	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↓	-
M54	-	-	-	-	-	T	-	-	-	-	-	-	-	-	P	-	G	-	S	-	-
M66	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-
M67	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	-	-	+	↓
M72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	+	↑
M85	-	-	I	-	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-	-	-
M106	-	-	-	C	-	-	K	-	-	-	-	-	-	-	-	-	-	-	R	↓	↑
M107	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	+	↑
M111	-	-	S	-	-	-	-	-	H	-	-	-	-	-	-	-	-	N	-	-	-
M113	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D	+	+
M127	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	+	+
M135	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	↓	+
M142	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	+	↑
M145	-	-	-	-	-	-	-	H	-	-	-	Q	-	-	-	-	G	-	-	+	+
M148	-	-	-	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-	-	+	+
M150	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	+	↑
M152	-	-	S	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-
M153	-	-	-	-	-	-	-	-	-	-	-	-	-	T	V	-	M	T	-	-	↓
M154	-	-	-	K	-	-	-	-	-	I	-	-	-	-	-	-	-	-	-	-	-
M159	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	M	R	-	+	↑
M161	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	-	-	-	-	+	-
M164	-	-	I	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-
M166	-	-	-	K	-	-	-	-	-	-	-	G	-	-	-	Y	M	-	-	-	-
M171	-	-	G	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M173	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	+	+
M175	-	-	-	-	-	-	-	-	H	S	-	-	-	-	-	-	-	-	-	+	+
M183	-	-	-	-	E	-	-	Y	-	-	-	Q	S	-	-	-	-	-	-	+	-
M229	-	G	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	-
M240	-	-	-	-	-	-	-	-	-	-	-	-	D	-	-	-	-	N	-	+	-
M241	-	R	-	-	-	-	-	E	-	-	-	-	S	-	-	-	-	-	-	-	-
M242	-	-	S	-	-	V	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-
M244	-	-	-	T	-	-	-	-	-	-	-	-	-	R	-	-	-	-	A	↓	-
M255	-	-	-	-	Q	-	-	-	F	-	-	V	-	-	-	-	-	M	-	+	↓
M257	-	-	-	-	-	-	K	Y	-	-	-	K	-	R	-	-	-	-	-	+	+
M260	Q	C	-	-	L	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-
M276	-	G	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	+	↓
M289	-	-	-	-	-	V	S	E	-	-	-	-	-	-	-	-	-	-	-	↓	+
M296	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↓	↑

Fig. 1. Amino acid sequences of the wild-type (germline) and mutant S107 CDR2 regions and the PC–BSA binding of the corresponding T15 and D16 mutants. The wild-type (WT) sequence is shown on the top with the single-letter code. Mutant clone names are shown on the left. Substitutions are indicated by single-letter code and a dash indicates sequence identity. +: binding activity comparable to WT; –: no detectable binding; ↓: decreased binding; ↑: increased binding.

isothiocyanate (FITC), dinitrophenyl (DNP), oxazolone and soman. None of the mutant antibodies showed detectable binding to these antigens at concentrations up to 1 μg/ml of antibody; this was true also for the T15 mutants (Chen *et al.*, 1992a). Thus, random mutations in CDR2 of the VH1 gene do not readily give rise to altered antigenic specificities, in agreement with Malipiero *et al.* (1987), who suggested that such changes in specificity would be likely to be rare events.

Comparison of T15 and D16 combining sites by computer-assisted modeling

To ascertain the structural basis for the different effects observed for the same mutations in D16 and T15, a computer model of the D16 Fv was constructed (see Materials and methods). A model of the T15 Fv was made previously (Chien *et al.*, 1989) based on the structure of the closely related McPC603 antibody. In the T15 model, residues Phe L91, Tyr L94 and Leu L96 of VL CDR3,

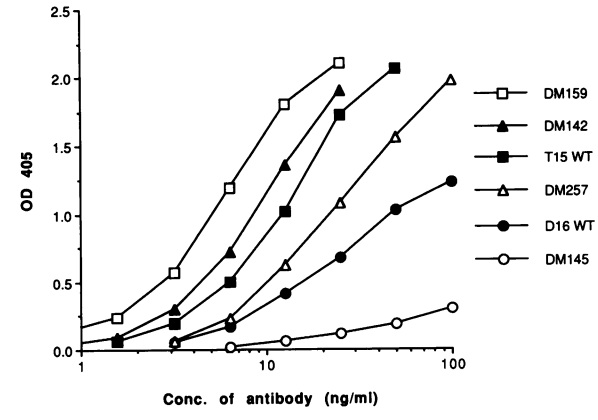

Fig. 2. Relative binding activity of wild-type T15 and wild-type and mutant D16 antibodies. Affinity-purified antibodies at various concentrations were tested by ELISA for binding to PC–histone. Each OD_{405} point represents the mean of triplicate determinations.

Table I. Binding features of the heavy chain CDR2 mutated D16 antibodies

Mutants[a]	Binding activity				
	Standard[b]		Low antigen[c]		Recognition by TC-54[d]
	PC–his	PC–BSA	PC–his	PC–BSA	
D16 WT	1.00	1.00	1.00	1.00	1.00
M72	***3.70***	***4.76***	***10.0***	***10.0***	1.06
M106	1.37	***3.85***	***24.0***	***33.0***	1.06
M107	2.80	***3.92***	***3.0***	***4.0***	1.09
M142	***3.30***	***5.67***	***6.0***	***12.0***	0
M150	***3.04***	***4.08***	***15.0***	***8.0***	1.07
M159	***8.33***	***10.00***	***20.0***	***15.0***	0.75
M296	2.80	2.63	***6.0***	***7.0***	0.62
M20	0.82	1.16			1.00
M113	1.00	0.76			0.97
M127	0.48	0.54			0.73
M135	0.65	0.58			1.03
M148	0.87	0.79			1.07
M173	1.82	1.67			1.13
M175	1.12	1.69			0
M257	1.52	2.08			1.03
M289	1.01	0.94			0.71
M67	0.56	**0.29**			1.02
M145	**0.15**	1.25			0
M153	0.68	**0.23**			0.59
M255	1.08	**0.25**			0
M276	**0.06**	**0.08**			1.16
M22	0[e]	0			0.87
M28	0	0			0.93
M32	0	0			0.92
M34	0	0			0.97
M41	0	0			1.00
M54	0	0			1.03
M66	0	0			0
M85	0	0			0
M111	0	0			0
M152	0	0			0
M154	0	0			0.46
M161	0	0			0.98
M164	0	0			**0.15**
M166	0	0			**0.08**
M171	0	0			0.36
M183	0	0			0
M229	0	0			0.81
M240	0	0			0
M241	0	0			0
M242	0	0			0.88
M244	0	0			0.81
M260	0	0			**0.09**

[a]The D16 wild-type and VH CDR2 mutant antibodies are all IgG2b and were expressed in the H chain-loss variant of D16 hybridoma cells. The corresponding CDR2 sequences for these antibodies are shown in Figure 2.

[b]In standard direct binding ELISA, plates were coated with 1 μg/ml PC–protein. Binding activity was calculated based on the antibody concentration (ng/ml) that gives an OD of 0.5 at 30 min in ELISA: Binding activity = WT concentration giving OD 0.5/mutant concentration giving OD 0.5. Binding activity values that are at least 3-fold lower than the wild-type D16 antibody are highlighted in bold face and those that are 3-fold higher than the WT are in bold face and italic.

[c]In low antigen ELISA, plates were coated with 0.2 μg/ml PC–protein.

[d]TC-54 is an anti-idiotypic antibody specific for the S107 V1 H chain. Binding to TC-54 was tested by direct-binding ELISA and the binding activity was calculated as above.

[e]These antibodies gave no detectable binding at concentrations of 2000 ng/ml.

and residues AspH95 and Trp H100B of VH CDR3 form the binding pocket containing the choline group of bound PC (Figure 4A). As in McPC603 (Satow *et al.*, 1986), there is an extensive hydrogen bonding network surrounding the trimethylammonium of bound PC, which cannot participate directly in hydrogen bond interactions. VH Glu H35, which is buried from solvent by the side chain of Tyr L94, does not contact antigen directly, but forms hydrogen bonds with residues Tyr L94 and Asp H95. Both Tyr L94 and Asp H95 are excluded from solvent upon antigen binding. Thus, it appears that T15 uses a buried, delocalized negatively charged region to complement the positively charged choline moiety, as has been deduced from sequence analysis of a number of PC binding antibodies (Padlan *et al.*, 1985).

Although D16 uses the same VH gene as T15, there are important differences between the two binding pockets. All of the residues in T15 that contact the choline group are replaced in D16 by side chains with very different functional groups: Gly L91, Leu L94 and Trp L96 in VL CDR3 and Gly H95 and Asp H98 in VH CDR3 (Figure 4B). Placing PC in the D16 combining site (Figure 4B) in the same orientation as in T15 causes the side chain of Trp L96 to interpenetrate the choline group, making this orientation unlikely. In addition, the electrostatic environment in the D16 binding pocket is very different from that of T15. D16 retains Glu H35, but its side chain cannot form hydrogen bonds with the surrounding residues (Gly H95 and Leu L94). The presence of two additional carboxylate side chains, Glu L34 and Asp H98, which are not present in McPC603 or T15, suggests that the choline moiety still binds near the center of the antigen binding pocket in D16. However, electrostatic complementarity of the Glu L34 and Asp H98 side chains with the choline group may be considerably weaker than the electrostatic interactions in T15. The side chain of Glu L34 (Ala in T15) is buried in the VL/VH interface and separated from the surface of the binding pocket by the side chain of Phe L89 and the VH CDR3 loop region. The side chain of Asp H98, which is at the top of the VH CDR3 loop, may stabilize the bound choline group through a solvent-exposed interaction. These shape and electrostatic differences in the T15 and Dl6 choline binding regions suggest that the D16-bound choline group is shifted away from VL CDR3 and towards VH CDR3 relative to its position in T15 (Figure 4B).

As in T15 (Chen *et al.*, 1992a) and McPC603 (Glockshuber *et al.*, 1991), mutation of Arg H52 caused loss of PC–protein binding in D16 (Figure 1), suggesting that the Arg H52 side chain has a similar role in all three antibodies: electrostatic complementarity with the phosphate group of PC through salt-bridge formation. However, the shift of the choline group in Dl6 relative to T15 would alter the position of the PC phosphate, affecting the strength of the salt bridge with Arg H52 and the orientation of the linker and carrier protein with respect to VH CDR2. Thus, carrier protein is likely to have very different interactions with the top of VH CDR2 in D16 than in T15. This is supported by the finding that D16 mutants with improved binding to PC–protein did not simultaneously acquire improved binding to haptenic PC or its analogs L-α-glycerophosphocholine and choline (data not shown).

mutant pairs	binding to PC-protein D16	T15	VH CDR2 sequence																	
			50	51	52	a	b	c	53	54	55	56	57	58	59	60	61	62	63	64	65
WT	+	+	A	S	R	N	K	A	N	D	Y	T	T	E	Y	S	A	S	V	K	G
M161	0	c	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	-	-	-	-
M183	0	c	-	-	-	-	E	-	-	Y	-	-	-	Q	S	-	-	-	-	-	-
M41	0	↓	-	-	-	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M244	0	↓	-	-	-	T	-	-	-	-	-	-	-	-	-	R	-	-	-	-	A
M72	↑	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-
M106	↑	↓	-	-	-	C	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-
M107	↑	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-
M142	↑	c	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-
M150	↑	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-
M159	↑	c	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	M	R	-
M296	↑	↓	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Fig. 3. CDR2 sequences of D16 and T15 mutants that share the same CDR2 mutation but displayed different binding features. Binding activities of D16 and T15 mutants for PC–protein are expressed as: –, no detectable binding; c, binding comparable to the wild-type antibody; ↓, binding activity is >3-fold lower than the wild-type; ↑, binding activity is >3-fold higher than the wild-type.

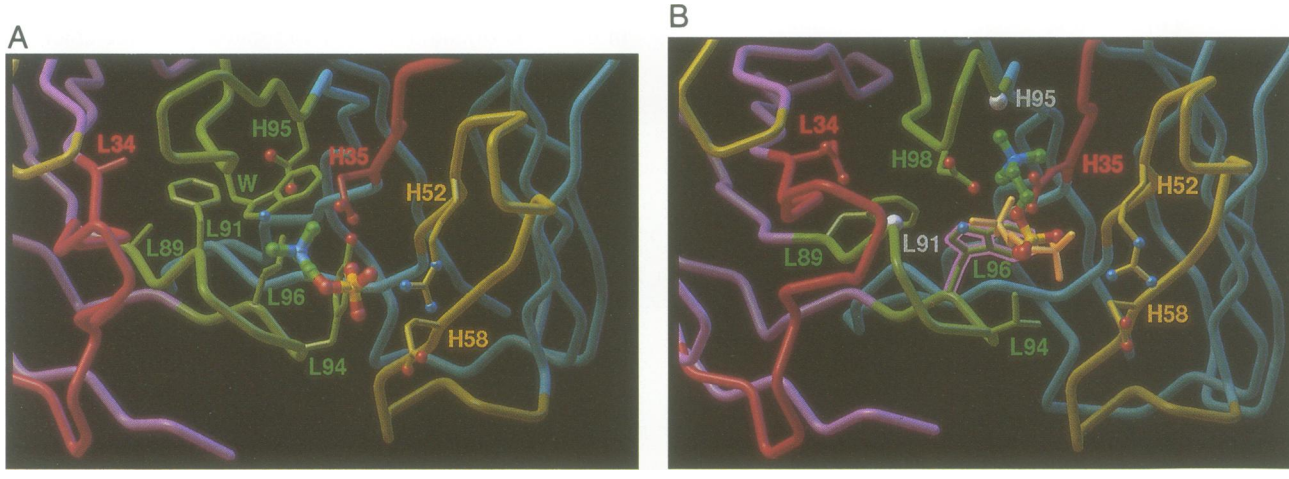

Fig. 4. Comparison of the antigen binding sites of the T15 and D16 antibodies. The Cα backbones for the framework residues (VL – purple, left; VH – blue, right) are shown with the CDRs of each chain color-coded: CDR1 – red; CDR2 – yellow; and CDR3 – green. Selected side chains are shown in the same colors as the CDR to which they belong, with polar side chain atoms shown as spheres (O – red; N – blue). VL residue numbers are preceded by 'L' and VH residue numbers are preceded by 'H'. View is looking into the antigen binding site from the solvent. (**A**) The T15 antigen binding site. The binding mode of PC (center, ball-and-stick model with atoms: C – green; N – blue; O – red; P – yellow) is similar to that observed in the McPC603 crystal structure. VL residues Phe L91, Tyr L94 and Leu L96 and VH residues Glu H35, Arg H52, Asp H95 and Trp H100B (labeled with a green 'W') form the binding pocket. (**B**) The D16 antigen binding site. VL residues Gly L91, Val L94 and Trp L96 and VH residues Glu H35, Arg H52, Gly H95 and Asp 98 (structurally equivalent to Trp H100B in T15) form the binding pocket. The replacement of Leu L96 in T15 by Trp L96 in D16 (emphasized by purple outline) causes steric clashes with PC as bound in T15 (shown as pale orange stick figure). In addition, the presence of Gly residues at positions L91 and H95 (indicated by white spheres at Cα atoms) changes the shape of the binding pocket. These alterations in the binding pocket suggest that the D16-bound choline group of PC (colored as in A) is shifted towards VH CDR3 (top center) and away from VL CDR3 (bottom center) relative to its position in T15.

Recognition by the anti-idiotope antibody, TC-54

As indicated above, the majority of the mutations in D16 affected antigen binding, but it was not clear whether this was due to local structural alterations or to more extensive conformational changes in the combining site. To pursue this question further, we tested mutant D16 antibodies with an S107 H chain-specific anti-idiotope mAb, TC-54. As shown in Table I, 11/43 mutants were no longer recognized by TC-54 and three more had greatly reduced binding to the anti-idiotope. Similar results were obtained for the corresponding T15 mutants (data not shown). No direct correlation between loss of binding to PC–protein and loss of TC-54 recognition was found; 54% of the mutants that no longer bound PC–protein could still be recognized by TC-54 and some antibodies (19%) that retained the ability to bind antigen, lost binding to TC-54 (Table I).

Binding of the anti-idiotope antibody TC-54 is sensitive to mutation of residues Lys H52B, Asp H54, Tyr H55 and Tyr H59 as shown here (Figure 5). Two mutants that lost binding to TC-54, M66 (Tyr H55→ Ser) and M142 (Asp H54→Val), have only single amino acid substitutions, suggesting that these residues provide a key feature of the D16 and T15 epitopes for TC-54. The D16 model shows that residues H52B, H54 and H55 form a continuous, solvent-exposed surface at the top of the first loop of VH CDR2 and that the solvent-exposed Tyr H59 side chain is on the following β-strand (Figure 6A). Others have

	50	51	52	a	b	c	53	54	55	56	57	58	59	60	61	62	63	64	65	TC-54 binding
WT	A	S	R	N	K	A	N	D	Y	T	T	E	Y	S	A	S	V	K	G	+
M66	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-
M85	-	-	I	-	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-	-
M111	-	-	S	-	-	-	-	-	H	-	-	-	-	-	-	-	-	N	-	-
M175	-	-	-	-	-	-	-	-	H	S	-	-	-	-	-	-	-	-	-	-
M255	-	-	-	-	Q	-	-	-	F	-	-	V	-	-	-	-	-	M	-	-
M142	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-
M145	-	-	-	-	-	-	-	H	-	-	-	Q	-	-	-	-	G	-	-	-
M152	-	-	S	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-
M183	-	-	-	-	E	-	-	Y	-	-	-	Q	S	-	-	-	-	-	-	-
M241	-	R	-	-	-	-	-	E	-	-	-	-	S	-	-	-	-	-	-	-
M240	-	-	-	-	-	-	-	-	-	-	-	-	D	-	-	-	-	N	-	-
M164	-	-	I	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	↓
M166	-	-	-	K	-	-	-	-	-	-	-	G	-	-	-	Y	M	-	-	↓
M260	Q	C	-	-	L	-	-	-	-	-	-	-	-	-	-	T	-	-	-	↓

Fig. 5. CDR2 sequences of the D16 mutants that are no longer recognized by TC-54 anti-idiotypic antibody.

implicated Ile H68 and Thr H57 in the binding of TC-54 (Chien *et al.*, 1988), which is consistent with their position in the epitope proposed here (Figure 6A). Thus, TC-54 binding provides a sensitive probe for structural changes in this localized region of VH CDR2, which was particularly useful for interpreting possible sources of improved binding in the D16 mutants, as discussed below.

Discussion

Random point mutations in VH CDR2 cause a high frequency of antigen binding loss antibodies

Somatic hypermutation in antibody V region genes must inevitably give rise to antigen binding-loss variants. The magnitude of this negative effect has not been clear. Such events have only rarely been detected in immune responses (Manser *et al.*, 1987; Siekevitz *et al.*, 1987; Apel and Berek, 1990), presumably because of the powerful selection by antigen of B cells whose antibody receptors had been improved by somatic mutation. Previously we introduced random point mutations in VH CDR2 of T15 anti-PC antibody in order to assess the impact of mutation in the absence of antigen selection pressure (Chen *et al.*, 1992a); 37% of the mutant antibodies had lost PC binding activity. Although this was the first evidence that a high proportion of B cells can lose antigen binding ability due to mutation in the hypervariable region, it was not clear if this result was unique to T15 which predominates in primary anti-PC responses but shows little affinity maturation as the response proceeds (Kluskens *et al.*, 1975; Rodwell *et al.*, 1983). Previous mutants of T15 usually had decreased binding (Claflin and Berry, 1988), suggesting that unmutated T15 has an optimal binding site for PC antigens fixed in the germline through evolutionary selection for this antibody which is highly protective against pneumococci and other PC-containing microorganisms. Mutations introduced into such a binding site could only be neutral or worsen the binding ability.

In this report, we tested the effects of the same VH CDR2 mutations in another antibody, D16. The heavy and light chain V region gene segments used by D16 are typical of a group of memory anti-PC–protein antibodies, which often undergo somatic mutation in the secondary response resulting in increased affinity (Stenzel-Poore and Rittenberg, 1989). Finding that 51% of D16 mutants did not bind PC–protein indicates that the high susceptibility to loss mutations is not unique to T15 and that other combining sites using the VH1 gene may be similarly susceptible. Such a high degree of loss implies that a large number of B cells within a mutating clone may cease to participate in the response. Thus, the generation of a high affinity antibody repertoire via somatic diversification is likely to be a costly process accompanied by extensive B-cell wastage.

Antibodies predominant in the memory response are those with a high potential to improve their binding activity

In many immune responses, the antibody population that dominates the primary phase differs from that of the memory response (Manser *et al.*, 1985). For example, the primary antibody repertoire responding to PC–protein is essentially comprised of a single population bearing the T15 idiotype (Claflin *et al.*, 1974), but, after a second or third challenge, the expressed repertoire is heterogeneous with a large proportion of non-T15 antibodies (Stenzel-Poore *et al.*, 1988). Manser *et al.* (1985) proposed an adaptability model to explain this phenomenon, defining adaptability as the ability to sustain somatic mutation without losing specificity for antigen. Accordingly, an antibody with low adaptability would only be expressed transiently, whereas antibodies with high adaptability would be expressed at increasing levels as the immune response progresses. Our comparative analysis of the effects of random mutations on T15 and D16 bears on this question. Both antibodies were equally susceptible to loss mutations, but only D16 was capable of improvement given the same restricted set of mutations in VH CDR2. Since both antibodies use the same VH gene segment but different DJ and L chain genes, it is the combination of gene segments that appears to govern the capacity for improvement. Further, the equal susceptibility to loss of function emphasizes that it is the ability to improve binding that is the critical factor determining whether a particular clone persists and expands in the response.

Differences between the T15 and D16 combining sites

Because D16 and T15 use the same germline VH gene sequence, their VH CDR2 and its surrounding framework residues are identical. This includes residues believed to control the conformation of VH CDR2 (Tramontano *et al.*,

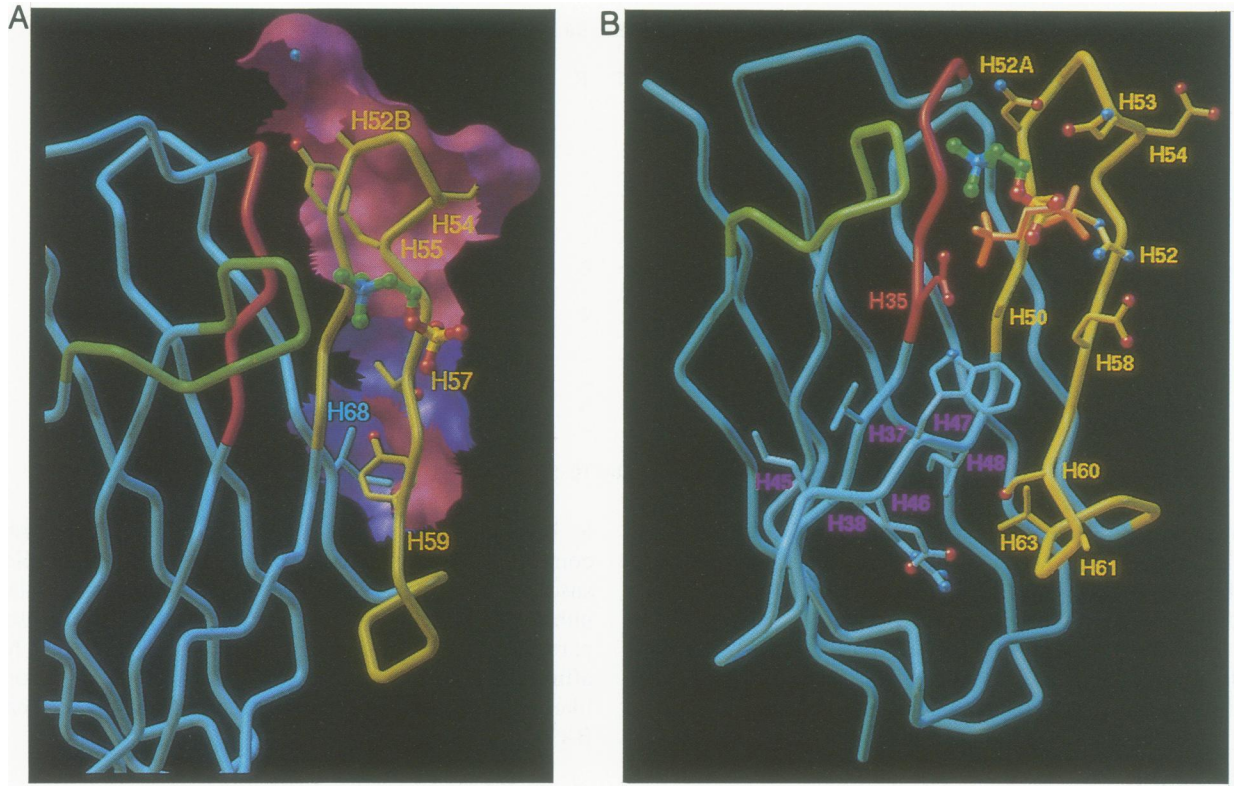

Fig. 6. Residues in VH CDR2 critical for TC-54 and antigen binding. The antibody and hapten are colored as in Figure 4. The region of the VH chain involved in the VL/VH interface is facing forward, with the antigen binding site at the top and VL not displayed. (**A**) Residues in the TC-54 epitope. The molecular surfaces (pink) of residues Lys H52B, Asp H54, Tyr H55 and Tyr H59, implicated in TC-54 binding by this study, together with the surfaces (purple) of residues Thr H57 and framework residue Ile H68, implicated in TC-54 binding in other studies (Chien *et al.*, 1988), form a continuous solvent-accessible surface that is part of the TC-54 epitope. (**B**) Residues critical for PC–protein binding. Although T15 and D16 are likely to have structurally similar VH CDR2 regions, the altered binding mode of PC in D16 results in different interactions of CDR2 with bound PC hapten. Residues in the first loop of CDR2 (Ala H50, Arg H52, Asn H52A, Asn H53, Asp H54 and Glu H58) are near the bound PC–protein and mutations here may affect antigen/antibody contacts directly. Residues Ser H60, Ala H61 and Val H63 lie in the second loop of CDR2, which is distant from the binding site. This loop interacts with side chains Arg H38, Glu H46 and Ile H48, which are on two adjacent β-strands that contain residues having large surface contacts with the VL chain (Chothia *et al.*, 1985), including Val H37, Leu H45 and Trp H47. Antigen binding in D16 may be improved indirectly by mutations that provide greater flexibility of the second CDR2 loop, allowing the VH interface residues to optimize the VL/VH geometry for antigen binding. This hypothesis is supported by recent crystallographic evidence of two humanized antibodies (Eigenbrot *et al.*, 1994). Replacing human residues H59–H65 with the murine sequence improved antigen binding >1000-fold and caused a 1.5 Å shift in the Trp H47 side chain and a 5° rotation of VH relative to VL.

1990), strongly suggesting that VH CDR2 is structurally similar in both antibodies. Consistent with this hypothesis, binding to TC-54 is similar in corresponding D16 and T15 mutants (data not shown). However, because of their different VL and VH CDR3 sequences, the D16-bound choline group is shifted towards VH CDR3, is more shallowly located and is no longer electrostatically stabilized by delocalized negative charge in the antibody (Figure 4). In addition, the D16-bound phosphate group lies deeper in the pocket, increasing contacts of D16 with the phenyl linker and the carrier protein. These changes in bound orientation would explain the low affinity of D16 for PC and its 36-fold stronger affinity for NPPC [based on inhibition ELISA (data not shown)]. The mutagenesis results show that D16 is more sensitive than T15 to mutations in VH CDR2 (Table II), further suggesting that CDR2 has critical interactions with the linker group and the carrier protein that do not occur in T15.

In most Group II antibodies, binding to free PC is virtually undetectable (Chang *et al.*, 1982a). Thus Group II antibodies including D16 require the presence of the phenyl group on the hapten in order to achieve functionally effective binding . In contrast, T15, with its higher affinity for free PC is functionally independent of the carrier. This would explain why T15 is able to bind both PC–protein and PC–polysaccharide, whereas D16 and other Group II antibodies cannot bind the polysaccharide form. The polysaccharide form of PC stimulates mouse B cells in a thymus-independent manner (Quintans and Cosenza, 1976) and is present on many microorganisms found in the environment in addition to the pneumococcus (Potter, 1970). Thus, even though mice may begin life with equal numbers of Group I and Group II precursors (Chen *et al.*, 1992b), environmental forms of PC would provide T15

Table II. Comparison of the binding properties of D16 and T15 mutant antibodies with identical CDR2 sequences

Phenotype	D16		T15	
	No. of mutants	%	No. of mutants	%
Increased	7	16%	0	0
Comparable	9	21%	22	48%
Decreased	5	12%	7	15%
Non-binding	22	51%	17	37%
Total	43		46	

with a selective advantage even in the absence of other forms of regulation.

Structural basis for improved binding to PC–protein

Seven D16 mutants showed improved binding for PC–protein, whereas the same mutations failed to improve binding in the T15 mutants (Chen *et al.*, 1992a). Four had mutations solely in the second H2 loop, residues H60–H65, which is distant from the antigen binding pocket (Figure 6B) and is unlikely to contact either the hapten or the carrier protein (Chothia and Lesk, 1987; Davies *et al.*, 1990). Three of the four are single-site mutants: M107, in which Ala H61 is replaced by Gly, the least conformationally restricted amino acid, and M72 and M150, in which the conformationally restricted, β-branched amino acid, Val H63, is replaced by the smaller Ala side chain and the more flexible Glu side chain, respectively. These results suggest that increasing the flexibility of residues H60–H65 can improve antigen binding, possibly by allowing greater conformational freedom of adjacent framework residues that contact VL (Figure 6B). This is exemplified by the greatly improved binding of the multi-site mutant M159 (Table I), which combines greater backbone flexibility (Ser H60→Gly) with a flexible side chain (Val H63→Met). The reduced binding by M67 (Val H63→Phe) may result from the limited rotational freedom of the Phe side chain. Together, these results suggest that somatic mutations in the second loop of VH CDR2 may provide an important mechanism for improving antigen binding in some antibodies, even though the residues in this loop do not contact antigen directly.

Two mutants with improved binding to PC–protein, M142 and M106, have substitutions of solvent-exposed residues Asn H53 and Asp H54, which lie at the top of the first VH CDR2 loop (Figure 6B). This loop contacts protein antigens (Davies *et al.*, 1990), suggesting that it may contact carrier protein or the linker group in D16. Both removal of the negative charge at H54 (Asp H54→Val in the single-site mutant M142) and possibly introduction of a positive charge at H53 (Asn H53→Lys, one of three mutations in M106) enhanced binding, suggesting that this part of the H2 loop may interact with a negatively charged region on the carrier. This is consistent with a previous suggestion that H53 binds to the carrier portion of the PC epitope in *Proteus morganii* polysaccharide (Claflin *et al.*, 1989).

The mutant M296 (Ser H51→Ile) shows improved binding under low antigen conditions (Table I). The H51 side chain extends into the VH framework (note the direction of the Cα atom preceding H52 in Figure 4B). Comparison of the McPC603 (Ser H51) with the 4-4-20, BV-04-01 and Yst9 crystallographic structures (Ile H51), revealed no significant differences in the side chain positions of framework residues surrounding H51. However, McPC603 (Satow *et al.*, 1986), T15 and D16 model structures all have a hydrogen bond between the side chains of Ser H51 and framework residue Arg H71, which influences the conformation of the first loop in CDR2 (Tramontano *et al.*, 1990). Mutation of Ser H51 to Ile changes the interaction between the side chains of H51 and H71 from a specific, directional hydrogen bonding interaction to a non-specific hydrophobic contact, which may allow the D16 antibody to accommodate antigen with less perturbation of the antibody structure.

Thus, none of the D16 mutants with improved PC–protein binding have substitutions of side chains likely to contact the PC haptenic group (Figure 6B). Instead, there may be two independent mechanisms for improved antigen binding: mutations that increase binding through direct interactions with carrier protein and linker and those that act through long-range effects. Mutants with substitutions solely in the second CDR2 loop (M72, M107, M150 and M159) must enhance PC–protein binding through long-range effects, possibly by increasing flexibility so that interactions between VL and VH can be optimized upon antigen binding. These mutants retained TC-54 binding, indicating little conformational change at the top of the first CDR2 loop. In contrast, M142 lost binding to TC-54, showing that a single functional group change in the first CDR2 loop can have opposite effects on antigen binding and the idiotope. The distinct effects of these two sets of mutations suggest that multisite mutants with both increased flexibility of the second CDR2 loop and improved electrostatic interactions of the first loop with carrier protein might have even greater affinity for PC–protein.

Structural basis for loss of PC–protein binding

Of the five residues that seem to be crucial for PC–protein binding by D16 (Figure 7), three (Ala H50, Arg H52 and Tyr H55) were previously identified to be important for binding to PC–protein by T15 (Chen *et al.*, 1992a), and thus are probably required for PC binding in general. Asn H52A and Gln H58 are also critical for antigen binding by D16. D16 mutants that replaced Asn H52A by Lys, His or Thr (Figure 7) showed no detectable antigen binding, but some of the corresponding T15 mutants retained partial binding (Figure 1). The shift of the positively charged choline group toward the Asn H52A side chain in D16 (Figure 6B), may make a positively charged residue at H52A more deleterious in D16. The side chain of Asn H52A also forms an extensive hydrogen bond network with the residues in the first loop of H2 at the top of the antigen binding site. Substitution by the more conformationally restricted Thr side chain may perturb this network, changing the conformation of the first H2 loop toward less favorable interactions with the carrier protein. Structural perturbation of this loop is also indicated by decreased binding of the anti-idiotope TC-54 for mutants M171 and M244 containing Thr H52A (Table I).

The single mutation of Glu H58→Gln (M161) has dramatically different effects in T15 and D16 with a loss of PC–protein binding in the D16 mutant but little change in the T15 mutant. Differences in the VL sequences may explain these results. The Arg H52 side chain lies between Glu H35 and Glu H58, and could, potentially, form a salt-bridge with either one. In McPC603 (Satow *et al.*, 1986) T15 and D16, the Arg H52 side chain forms a salt-bridge with Glu H58. In T15, the Glu H35 side chain is anchored by an extensive hydrogen bond network and is separated from the Arg H52 side chain by the Tyr L94 side chain (Figure 4A). In contrast, D16 has no hydrogen bonding network around Glu H35 and a smaller L94 (Val) side

	50	51	52	a	b	c	53	54	55	56	57	58	59	60	61	62	63	64	65	PC binding
WT	A	S	R	N	K	A	N	D	Y	T	T	E	Y	S	A	S	V	K	G	+
M22	**T**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-
M67	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	-	-	+
M72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	+
M150	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	+
M85	-	-	**I**	-	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-	-
M175	-	-	-	-	-	-	-	-	H	S	-	-	-	-	-	-	-	-	-	+
M152	-	-	**S**	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-
M142	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	+
M66	-	-	-	-	-	-	-	-	**S**	-	-	-	-	-	-	-	-	-	-	-
M41	-	-	-	**H**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M32	-	-	-	**K**	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	-
M171	-	-	**G**	**T**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M244	-	-	-	**T**	-	-	-	-	-	-	-	-	-	R	-	-	-	-	A	-
M161	-	-	-	-	-	-	-	-	-	-	-	**Q**	-	-	-	-	-	-	-	-

Fig. 7. CDR2 sequences of mutant D16 antibodies showing residues critical for antigen binding. The first three sets of binding-loss antibodies have more than one mutation and thus are compared with antibodies which have mutations in one of the same positions as the binding-loss mutant but retain binding activity for PC–protein. The amino acid substitutions which may be responsible for the altered binding phenotypes are indicated as bold face letters and underlined.

chain (Figure 4B), removing a steric constraint to the potential Arg H52–Glu H35 interaction. Mutation of the negatively charged Glu H58 to Gln in D16 may allow the side chain of Arg H52 to form a salt-bridge with Glu H35, which would have to be broken to allow antigen binding. The detectable antigen binding observed for triple-site mutant M145, which contains both Glu H58→Gln and Asp H54→His indicates that neutralizing the side chain of Asp H54 compensates for the deleterious effect of Gln H58, possibly by improving interactions with the carrier.

The results of this study demonstrate that random mutations in VH CDR2 of the S107 VH1 gene are equally harmful to antigen binding in two related but distinct antibody combining sites. Mutations in either site would thus have the potential to cause B-cell wastage resulting from loss of antigen binding function. Mutations that were identical to both sites improved antigen binding only in D16, emphasizing that the effects of mutation in VH may be modified by the other gene segments that distinguish D16 from T15. This is in keeping with the observation that D16 is representative of antibodies found in the memory response that display affinity maturation, a property lacking in T15. Furthermore, certain combinations of these CDR2 mutations prevent antibody secretion in T15 but not in D16 (Chen *et al.*, 1994). Thus, although some characteristics resulting from mutations in CDR2 may be shared, others are unique and not readily predicted from our current knowledge of antibody structure/function. Efforts to improve affinity by design through induced mutations can be expected to yield additional surprises. Fortunately, the immune system applies the power of antigen selection to achieve its ends and leaves the surprises for us.

Materials and methods

Generation of D16 hybridoma and its H chain loss variant

The production and characterization of the D16 hybridoma and its H chain loss variant have been described (Chen *et al.*, 1994). D16 produces an IgM, Vκ1c, Group II anti-PC–protein antibody. D16 is encoded by the S107 VH1 germline sequence and thus is identical to T15 in this respect; it differs from T15 in DJ and in Vκ.

Genomic cloning and mutation of D16 VH

Cloning of the productive V_H1-$D_HSP2.2$-J_H2 rearrangement of D16 and replacement of the germline CDR2 sequence of D16 with the same mutated VH1 CDR2 sequences from the T15 mutants generated previously (Chen *et al.*, 1992a) has been described (Chen *et al.*, 1994). The VH1 sequences and the CDR2 mutations were verified by sequencing in both pTZ18U and pSV2gpt vectors using primers for VH1 framework 3 and JH2 (Stenzel-Poore and Rittenberg, 1989; Chen *et al.*, 1992a).

Transfection

Transfection was performed by the lipofectin method as described previously (Brown *et al.*, 1992) except that the H chain loss variant of D16 hybridoma was used as recipient; 100 μl of lipofectin was used in each transfection. Stable transfectants that secreted IgG2b, κ antibody were saved for further analysis.

Antibody purification

Antibody secreted by transfectants was purified from tissue culture supernatants by affinity chromatography on either PC–Sepharose 4B or protein A–Sepharose CL-4B (Pharmacia, Uppsala, Sweden) as described previously (Chang *et al.*, 1982b).

ELISA

Direct binding ELISA and inhibition ELISA were described previously (Chen *et al.*, 1991). Various concentrations of purified antibody from D16 transfectants were examined for binding to PC–histone and PC–BSA by direct ELISA. Expression of an S107 H chain idiotope was tested with TC-54 monoclonal antibody, which is specific for the VH1 gene product independent of VL (Desaymard *et al.*, 1984). Purified TC-54 antibody was coated on the plates at 5 μg/ml and then overlaid with purified mutant or wild-type D16 or T15 antibodies at various concentrations. Binding was detected by further incubation with rabbit anti-mouse γ2b antibody coupled to alkaline phosphatase and developed with the substrate, nitrophenyl phosphate. Mutant antibodies were also tested for binding to various PC-unrelated antigens, including BSA, histone, KLH, albumin, ubiquitin, actin, FITC–BSA, DNP–BSA, oxazolone–BSA, soman–BSA and DNA, as described previously (Chen *et al.*, 1991).

Computer modeling of the D16 variable regions

Initial models of antibody variable regions were built with the Insight graphics program (Biosym Technologies, San Diego). The T15 variable region model was based on the closely related McPC603 antibody structure (Satow *et al.*, 1986), as previously reported (Chien *et al.*, 1989). During construction of the D16 antibody model, it was superimposed onto

an antibody structural database (ASD). The ASD contains coordinates of crystallographic antibody structures with VL, VH and Fv domains separately superimposed (Roberts *et al.*, 1994), allowing comparison of backbone conformations and analysis of conformational variability of specific side chains.

Structural templates for the D16 variable region model were selected based on high sequence identity of the framework regions. The selected template structures matched the canonical structures (Chothia *et al.*, 1989) for all the D16 CDRs except VH CDR3. Side chain replacements required for the D16 sequence were done with the Insight program, which follows the geometry of the starting side chain when possible. Identical side chains were left in the template conformation. The D16 VL was built from the coordinates of the B13I2 antibody [1IGF in the Protein Data Bank (PDB)] (Bernstein *et al.*, 1977), which has an identical sequence for the VL framework residues (as defined in Kabat *et al.*, 1991). The entire VL regions of D16 and B13I2 have just seven sequence differences, six in VL CDR1 and one in VL CDR3. The D16 VH was built from McPC603 antibody coordinates (kindly provided by Gerson Cohen at NIH) that are more highly refined, but similar to those in the PDB (1MCP). The VH sequences of the two antibodies are identical except for two differences in VH CDR2 (Gly H52C and Lys H54 in McPC603 are Ala H52C and Asp H54 in D16), two differences in framework region 4, and substantially different lengths and sequences for VH CDR3.

The structure of the D16 VH CDR3 (eight residues in length) was built from the coordinates of CDR3 of the D1.3 antibody (1FDL in the PDB), which has the same length. The first two and last four residues of the D1.3 VH CDR3 structure have the motif commonly found for these residues, including a main-chain hydrogen bond between the nitrogen atom of the second residue and the carbonyl oxygen atom of the fourth residue from the end of CDR3. The D1.3 VH CDR3 was grafted into the D16 structure by superposition of the backbone atoms (N, Cα, C, O) of the four residues preceding and following CDR3 onto the corresponding atoms of the D16 model, followed by appropriate residue replacements to provide the D16 sequence. The final CDR3 structure had a main-chain hydrogen bond between His H96 and Tyr H99 with a β-turn conformation for residues H96–H99 (His-Tyr-Asp-Tyr). The VL and VH domains were superposed onto the main-chain atoms of the structurally conserved β-sheet residues (Chothia and Lesk, 1987; Roberts *et al.*, 1990) of the corresponding McPC603 domains, giving the initial template-built model.

Energy minimization with the program Discover (Biosym Technologies, San Diego) was used to relieve steric constraints and relax bond lengths and angles for the transplanted VH CDR3 loop. Hydrogen atoms were added to all atoms. During minimization, all non-hydrogen atoms were strongly forced to the starting coordinates with harmonic constraints (forcing constant of 1000 kcal/Å). The model was subjected to 500 iterations of steepest descent minimization (final maximum derivative of 10 kcal/mol/Å) followed by conjugate gradient minimization until the final maximum derivative was <1.0 kcal/mol/Å (507 iterations). The initial, template-built model showed few steric interactions because of very high sequence identity. This was reflected in the small movement of the non-hydrogen atoms from their starting positions during minimization (root-mean-square deviation of 0.66 Å for VL, 0.53 Å for VH).

Simultaneous viewing of the D16 model with the structures of the templates (the B1312 VL and the McPC603 VH) and other sequentially related antibodies was done using the ASD to assist with model building and to check the effects of energy minimization. The 4-4-20 VL (4FAB in the PDB) has high sequence identity with D16, having just nine differences over the entire VL. These differences were at positions other than those of B13I2, providing a complementary check of the conservation of backbone and side chain conformations. Three antibodies in the ASD: 4-4-20; BV-04-01 (Herron *et al.*, 1991) and Yst9 (1MAM in the PDB), share a length of 19 residues for VH CDR2 with D16 and McPC603. Of the three, Yst9 has the highest sequence identity with the D16 VH, with just 13 differences in the framework residues and four differences in CDR2.

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References

Apel,M. and Berek,C. (1990) Somatic mutations in antibodies expressed by germinal center B cells early after primary immunization. *Int. Immunol.*, **2**, 813–819.

Berek,C. and Milstein,C. (1987) Mutation drift and repertoire shift in the maturation of the immune response. *Immunol. Rev.*, **96**, 23–41.

Bernstein,F.C., Koetzle,T.F., Williams,G.J.B., Meyer,J.E.F., Brice,M.D., Rodgers,J.R., Kennard,O., Shimanouchi,T. and Tasumi,M. (1977) The protein data bank: a computer-based archival file for macromolecular structures. *J. Mol. Biol.*, **112**, 535–542.

Brown,M., Stenzel-Poore,M.P., Stevens,S., Kondoleon,S.K., Ng,J., Bachinger,H.P. and Rittenberg,M.B. (1992) Immunological memory to PC-KLH: recurrent mutations in the λ1 light chain increase affinity for antigen. *J. Immunol.*, **148**, 339–346.

Bruderer,U., Stenzel-Poore,M.P., Bachinger,H.P., Fellman,J.H. and Rittenberg,M.B. (1989) Antibody combining site heterogeneity within the response to phosphocholine–keyhole limpet hemocyanin. *Mol. Immunol.*, **26**, 63–71.

Chang,S.P., Brown,M. and Rittenberg,M.B. (1982a) Immunologic memory to phosphorylcholine II. PC–KLH induces two antibody populations that dominate different isotypes. *J. Immunol.*, **128**, 702–706.

Chang,S.P., Brown,M. and Rittenberg,M.B. (1982b) Immunologic memory to phosphorylcholine III. IgM includes a fine specificity population distinct from TEPC 15. *J. Immunol.*, **129**, 339–346.

Chen,C., Stenzel-Poore,M.P. and Rittenberg,M.B. (1991) Natural auto and polyreactive antibodies differing from antigen-induced antibodies in the H chain CDR3. *J. Immunol.*, **147**, 2359–2376.

Chen,C., Roberts,V.A. and Rittenberg,M.B. (1992a) Generation and analysis of random point mutations in an antibody CDR2 sequence: many mutated antibodies lose their ability to bind antigen. *J. Exp. Med.*, **176**, 855–866.

Chen,C., Bruderer,U. and Rittenberg,M.B. (1992b) The developmental patterns of B cell precursors distinguishing between environmental and nonenvironmental forms of phosphocholine. *Cell. Immunol.*, **143**, 378–388.

Chen,C., Martin,T.M., Stevens,S. and Rittenberg,M.B. (1994) Defective secretion of an immunoglobulin caused by mutations in the heavy chain CDR2. *J. Exp. Med.*, **180**, 577–586.

Chien,N.C., Pollock,R.R., Desaymard,C. and Scharff,M.D. (1988) Point mutations cause the somatic diversification of IgM and IgG2a antiphosphorylcholine antibodies. *J. Exp. Med.*, **167**, 954–973.

Chien,N.C., Roberts,V.A., Giusti,A.M., Scharff,M.D. and Getzoff,E.D. (1989) Significant structural and functional change of an antigen binding site by a distant amino acid substitution: proposal of structural mechanism. *Proc. Natl Acad. Sci. USA*, **86**, 5532–5536.

Chothia,C. and Lesk,A.M. (1987) Canonical structures for the hypervariable regions of immunoglobulins. *J. Mol. Biol.*, **196**, 901–917.

Chothia,C. *et al.* (1989) Conformations of immunoglobulin hypervariable regions. *Nature*, **342**, 877–883.

Claflin,J.L. and Berry,J. (1988) Genetics of the phosphocholine-specific antibody response to *Streptococcus pneumoniae*. Germline but not mutated T15 antibodies are dominantly selected. *J. Immunol.*, **141**, 4012–4019.

Claflin,J.L., Lieberman,R. and Davie,J.M. (1974) Clonal nature of the immune response to phosphorylcholine. II. Idiotypic specificity and binding characteristics of anti-phosphorylcholine antibodies. *J. Immunol.*, **112**, 1747–1756.

Claflin,J.L., George,J., Dell,C. and Berry,J. (1989) Patterns of mutations and selection in antibodies to the phosphocholine-specific determinant in *Proteus morganii*. *J. Immunol.*, **143**, 3054–3063.

Davies,D.R., Padlan,E.A. and Sheriff,S. (1990) Antibody–antigen complexes. *Annu. Rev. Biochem.*, **59**, 439–473.

Desaymard,C., Giusti,A.M. and Scharff,M.D. (1984) Rat anti-T15 monoclonal antibodies with specificity for VH- and VH-VL epitopes. *Mol. Immunol.*, **21**, 961–967.

Eigenbrot,C., Gonzalez,T., Mayeda,J., Carter,P., Werther,W., Hotaling,T., Fox,J. and Kessler,J. (1994) X-ray structures of fragments from

binding and nonbinding versions of a humanized anti-CD18 antibody: structural indications of the key role of V_H residues 59 to 65. *Proteins*, **18**, 49–62.

French,D.L., Laskov,R. and Scharff,M.D. (1989) The role of somatic hypermutation in the generation of antibody diversity. *Science*, **244**, 1152–1157.

Glockshuber,R., Stadlmuller,J. and Pluckthun,A. (1991) Mapping and modification of an antibody hapten binding site: a site-directed mutagenesis study of McPC603. *Biochemistry*, **30**, 3049–3054.

Herron,J.N., He,X.M., Ballard,D.W., Blier,P.R., Pace,P.E., Bothwell, A.L.M., Voss,J.E.W. and Edmundson,A.B. (1991) An autoantibody to single-stranded DNA: comparison of the three dimensional structures of the unliganded Fab and a deoxynucleotide–Fab complex. *Proteins*, **11**, 159–175.

Herzenberg,L.A., Black,S.J., Tokuhisa,T. and Herzenberg,L.A. (1980) Memory B cells at successive stages of differentiation. Affinity maturation and the role of IgD receptors. *J. Exp. Med.*, **151**, 1071–1087.

Johnson,S. and Bird,R.E. (1991) Construction of single-chain Fv derivatives of monoclonal antibodies and their production in *Escherichia coli*. *Methods Enzymol.*, **203**, 88–98.

Kabat,E.A., Wu,T.T., Perry,H.M., Gottesman,K.S. and Foeller,C. (1991) *Sequences of Proteins of Immunological Interest*. 5th Edn, US Department of Health and Human Services, Washington, DC.

Kluskins,L., Lee,W. and Kohler,H. (1975) Immune response to phosphorylcholine 1. Characterization of the epitope-specific antibody. *Eur. J. Immunol.*, **5**, 489–496.

Lee,W., Cosenza,H. and Kohler,H. (1974) Clonal restriction of the immune response to phosphorylcholine. *Nature*, **247**, 55–57.

Levy,S., Mendel,E., Kon,S., Avnur,Z. and Levy,R. (1988) Mutational hot spots in Ig V region genes of human follicular lymphomas. *J. Exp. Med.*, **168**, 475–489.

Malipiero,U.P., Levy,N.S. and Gearhart,P.J. (1987) Somatic mutation in anti-phosphorylcholine antibodies. *Immunol. Rev.*, **96**, 59–74.

Manser,T., Wysocki,L.J., Gridley,T., Near,R.I. and Gefter,M.L. (1985) The molecular evolution of the immune response. *Immunol. Today*, **6**, 94–101.

Manser,T., Parhami-Seren,B., Margolies,M.N. and Gefter,M.L. (1987) Somatically mutated forms of a major anti-*p*-azophenylarsonate antibody variable region with drastically reduced affinity for *p*-azophenylarsonate. *J. Exp. Med.*, **166**, 1456–1463.

Padlan,E.A., Cohen,G.H. and Davies,D.R. (1985) On the specificity of antibody/antigen interactions: phosphocholine binding to McPC603 and the correlation of three dimensional structure and sequence data. *Ann. Immunol. (Inst. Pasteur)*, **136C**, 271–276.

Perlmutter,R.M., Crews,S.T., Douglas,R., Sorenson,G., Johnson,N., Nivera,N., Gearhart,P.J. and Hood,L. (1984) The generation of diversity in phosphorylcholine-binding antibodies. *Adv. Immunol.*, **35**, 1–37.

Pollack,R.R., French,D.L., Gefter,M.L. and Scharff,M.D. (1988) Identification of mutant monoclonal antibodies with increased antigen binding. *Proc. Natl Acad. Sci. USA*, **85**, 2298–2302.

Potter,M. (1970) Antigen-binding myeloma proteins in mice. *Ann. NY Acad. Sci.*, 306–321.

Quintans,J. and Cosenza,H. (1976) Antibody response to phosphorylcholine *in vitro*. Analysis of T-dependent and T-independent responses. *Eur. J. Immunol.*, **6**, 399–405.

Rajewsky,K., Forster,I. and Cumano,A. (1987) Evolutionary and somatic selection of the antibody repertoire in the mouse. *Science*, **238**, 1088–1094.

Roberts,V.A., Iverson,B.L., Iverson,S.A., Benkovic,S.J., Lerner,R.A., Getzoff,E.D. and Tainer,J.A. (1990) Antibody modeling: a general solution to the design of a metal-coordination site in an antibody binding pocket. *Proc. Natl Acad. Sci. USA*, **87**, 6654–6658.

Roberts,V.A., Stewart,J., Benkovic,S.J. and Getzoff,E.D. (1994) Catalytic antibody model and mutagenesis implicate arginine in transition-state stabilization. *J. Mol. Biol.*, **235**, 1098–1116.

Rodwell,J., Gearhart,P.J. and Karush,F. (1983) Restriction in IgM expression. IV. Affinity analysis of monoclonal anti-phosphocholine antibodies. *J. Immunol.*, **130**, 313–316.

Rothstein,T.L. and Gefter,M.I. (1983) Affinity analysis of idiotype-positive and idiotype-negative Ars-binding hybridoma proteins and Ars-immune sera. *Mol. Immunol.*, **20**, 161–168.

Satow,Y., Cohen,G.H., Padlan,E.A. and Davies,D.R. (1986) Phosphocholine-binding immunoglobulin Fab McPC603. An X-ray diffraction study at 2.7 Å. *J. Mol. Biol.*, **190**, 593–604.

Siekevitz,M., Kocks,C., Rajewsky,K. and Dildrop,R. (1987) Analysis of somatic mutation and class switching in naive and memory B cells generating adoptive primary and secondary responses. *Cell*, **48**, 757–770.

Stenzel-Poore,M.P. and Rittenberg,M.B. (1989) Clonal diversity, somatic mutation and immune memory to phosphocholine–KLH. *J. Immunol.*, ***143***, 4123–4133.

Stenzel-Poore,M.P., Bruderer,U. and Rittenberg,M.B. (1988) The adaptive potential of the memory response: clonal recruitment and epitope recognition. *Immunol. Rev.*, **105**, 113–136.

Tramontano,A., Chothia,C. and Lesk,A.M. (1990) Framework residue 71 is a major determinant of the position and conformation of the second hypervariable region in the VH domains of immunoglobulins. *J. Mol. Biol.*, **215**, 175–182.

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