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THE SPECIFICITY PROPERTIES THAT DISTINGUISH MEMBERS OF A SET OF HOMOLOGOUS ANTI-DIGOXIN ANTIBODIES ARE CONTROLLED BY H CHAIN MUTATIONS[1]

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Five murine A/J strain anti-digoxin mAb (35-20, 40-40, 40-120, 40-140, and 40-160) have highly homologous H and L chain V regions, only differing by somatic mutation, yet differ in affinity and specificity. The availability of the VH and VL genomic clones from one hybridoma, 40-140, has now allowed studies involving in vitro mutagenesis and chain recombination among these five hybridomas. To determine the relative contributions of the mutations found in either VH or VL to the overall binding properties of these antibodies, we recombined the 40-140VH with the VL of each hybridoma. The 40-140VH gene was transfected into hybridoma variants that produce only VL. The recombinant antibodies show that the mutations present in VH, rather than in VL, affect the fine specificity properties of these antibodies, whereas, the mutations among both VH and VL chains are important in determining antigen affinity. From mutations present in VH that affect fine specificity properties, the comparison of the antibody sequences, and from the previously measured binding properties, we predicted and tested selected VH mutations for their ability to alter specificity or affinity by doing site-directed in vitro mutagenesis. The results for the somatic mutations found in this group of antibodies show: 1) VH mutations control the fine specificity properties that distinguish different members of this group; 2) in particular, VH residues 54 and 55 in CDR2 control the distinguishing characteristics of specificities between these antibodies; and 3) by mutagenesis, we had the unusual result of being able to alter Ag specificity without affecting affinity. A computer model of the 40-140 antibody binding site was generated which indicates that VH residues 54 and 55 are highly accessible.

The hapten digoxin, a cardiac glycoside, offers several advantages as a model for antibody-hapten interaction: 1) Digoxin is unusually large for a hapten, and probably occupies most of the antibody combining site (1, 2); 2) x-ray crystallographic studies of the structure of digoxin and several analogs are known and show that the cardiac glycoside steroid ring is conformationally rigid, regardless of functional group substitutions (3); 3) the availability of more than 60 structural analogs of digoxin is useful for the investigation of fine specificity of Ag binding (4); and 4) monoclonal anti-digoxin antibodies generated from immunized A/J strain mice have unusually high affinities (more than 10^9 M^{-1}), which suggests that multiple antibody-Ag contacts exist within the antibody combining site (5).

Sets of mAb that have homologous H and L chains are useful in structure-function studies because the small mutational sequence differences are responsible for binding alterations. This allows the assignment of certain residues to certain contributions to Ag binding. Such sets of mAb often are a large part of certain immune responses, comprising a major idiotypic family whose members are derived from one set of germline V region gene segments (6–8). Among anti-digoxin mAb, we had previously found that one homologous group of five antibodies, the 35-20 group, each use the same H and L chain V region germline gene segments in the assembly of their expressed antibody genes (9). The expressed H and L chain genes from one of these hybridomas, 40-140, have been cloned and sequenced (9).

Although the 35-20 group probably does not represent a major idiotypic family (M. Mudgett-Hunter, unpublished observation), it is useful for structure-function studies. The antibodies in the 35-20 group share sequence homology, varying by somatic mutation, but have significant differences in their binding properties (5). In the present studies, the H chain 40-140 clone was expressed in genetic constructs to form chain recombinants among this group of antibodies or to mutate it to test predictions of which residues contribute to binding. We have found that the H chain mutations control the specificity properties that distinguish different members of the 35-20 group. Specifically, two residues in H chain CDR2 are major contributors for affecting fine specificity. We also showed that the somatic mutations present in both the H and L chains contribute to affinity. It is noteworthy that by mutating H chain residues 54 and 55 we were able to alter fine specificity properties without affecting affinity for digoxin. A computer model of the 40-140

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binding site indicates that these two residues are among the most accessible.

MATERIALS AND METHODS

Cell lines. The 35-20 group of homologous anti-digoxin mAb examined in these studies are 40-40 ($\gamma1,\kappa$), 40-120 ($\gamma2a,\kappa$), 40-140 ($\gamma1,\kappa$), 40-160 ($\gamma1,\kappa$), and 35-20 ($\gamma1,\kappa$). The 35-20 group hybridomas were generated from spleen cells from A/J mice that had been injected with digoxin-human serum albumin (5). Hybridomas that produce only L chains were selected in soft agar as described (10). All cells were maintained in DMEM supplemented with 15% FCS (GIBCO, Grand Island, NY), 50 μg/ml of gentamycin sulfate (GIBCO), and 0.6 mg/ml L-glutamine.

Expression vector construction. For chain recombination, the 4.8-kb 40-140VH *Eco*RI fragment (9) was cloned in the correct orientation into the vector pSV2gpt (11) that had been modified by the insertion of a polylinker at the *Eco*RI site (R. I. Near, unpublished observations) and by the insertion into the *Xba*I site of the polylinker of a 5.5-kb *Xba*I fragment containing the germline murine $\gamma2b$ C region gene (12). For expression of the mutated 04-140VH gene, the mutated 1.8-kb *Xba*I piece was cloned in the correct orientation into pSV2gpt that contains a polylinker between the *Eco*RI and *Bam*HI sites, a *Xba*I-*Bgl*II $\gamma2b$ 3.6-kb piece, and an *Eco*RI-*Xba*I H chain enhancer fragment (from the 40-140VH *Eco*RI clone).

DNA transfection. Construct DNA was introduced into myeloma and hybridoma cells using the Gene-Pulsar electroporation apparatus (Bio-Rad Laboratories, Richmond CA). Briefly, 1×10^7 cells in 1.0 ml PBS at 4°C in the presence of 20 μg construct DNA (linearized with *Sal*I for the chain recombination construct and with *Eco*RI for the mutagenesis construct) were given a 450 V/25 uFD pulse. After 10 min on ice, the cells were diluted in 12 ml normal medium, distributed into 96-well plates at 3×10^4 cells/well, and incubated at 37°C for 48 h, after which selection medium was added. For Ecogpt selection (11), 0.4 μg/ml mycophenolic acid, 250 μg/ml xanthine, and 15 μg/ml hypoxanthine was added to the normal medium. Transfected cell colonies were usually visible within 2 to 3 wk of selection.

In vitro mutagenesis. Mutagenesis was done according to the technique of Kunkel (13) with a Muta-Gene in vitro mutagenesis kit (Bio-Rad). The 1.8-kb *Xba*I 40-140VH fragment (9) was cloned into M13 and phage were grown in CJ236 to obtain uridinylated template. Oligonucleotides were annealed to the uridinylated template strand, second strand was synthesized, the products transfected into MV1190, and mutants were screened for according to the manufacturers' instructions. The mutated 40-140VH region was sequenced to confirm correct mutagenesis.

DNA and RNA sequencing. The V regions were sequenced by the dideoxy chain-termination method (14), using Sequenase (United States Biochemicals, Cleveland, OH) and adenosine 5′-[α-^{35}(S)thio] triphosphate (Amersham Corp., Arlington Heights, IL). Oligonucleotide primers were synthesized on the Applied Biosystems (Foster City, CA) 380B DNA Synthesizer. Transfectomas were checked for proper transcription by synthesizing ^{32}P-cDNA from the H chain mRNA (15) and sequencing by the Maxam and Gilbert (16) method.

Competition RIA for fine specificity. The fine specificities of anti-digoxin antibodies from the hybridoma and transfectoma supernatants were determined by measuring the competition between digoxin and its analogs in an RIA (5, 10). Briefly, cell supernatants were incubated in microtiter plates coated with affinity-purified goat anti-mouse $F(ab')_2$ (ICN Biomedicals, Lisle, IL) at 4°C overnight. The next day, the plates were washed with distilled water and the wells were filled with 1% horse serum in PBSA[3] to block any remaining nonspecific protein binding sites. After the plates had been washed, cardiac glycosides (10^{-10} to 10^{-4} M) in PBSA containing 1% horse serum and 5% ethanol were added to the wells. Immediately after addition of each analog, 50,000 cpm of [^{125}I]-digoxin (Cambridge Medical Diagnostics, Burlington, MA) was added to the same wells and allowed to incubate overnight at 4°C. The next day, radioactive ligand was removed and the plates were extensively washed with distilled water before each well was cut from the plate and counted (Micromedic Systems Automatic Gamma Counter, model 4/600, INC Biomedicals Inc., Costa Mesa, CA). Antibodies were compared by determining the amount of unlabeled analog required to achieve 50% inhibition relative to the amount of unlabeled digoxin required for 50% inhibition. Antibody isotypes were determined with the Mouse Ig Subtype Identification Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Affinity measurement. A double antibody precipitation assay was used to determine the affinity constant, K_o, as previously described (10). Briefly, supernatant from each hybridoma or transfectoma was diluted with 1% horse serum in PBSA to a concentration estimated to be approximately the K_d or lower and was incubated with varying concentrations of [^{3}H]digoxin (25 μCi/mmol, New England Nuclear, Boston, MA) overnight at 4°C. The Ag-antibody complex was precipitated with rabbit anti-mouse IgG (ICN Immunobiologicals, Inc., Lisle, IL) overnight followed by goat anti-rabbit IgG (a gift of Dr. Charles Homcy, Massachusetts General Hospital, Boston, MA) for 4 h at 4°C. The precipitated complex was filtered onto glass fiber filters (24 mm, no. 32, Schleicher & Schuell, Keene, NH) on a Millipore manifold (Millipore, New Bedford, MA). The filters were placed in 5 ml scintillation fluid (Ultima Gold, Packard, Downers Grove, IL) and were counted in a Packard 1500 Tri-Carb Liquid Scintillation Analyzer (Packard Instrument Co., Sterling, VA). The data were analyzed with the curve fitting program LIGAND (17) which calculates K_o and the antibody concentration.

Computer-generated model of 40-140 Fv fragment. To construct an approximate model of the 40-140 Ag combining site, the amino acid sequences of the 40-140VL and VH regions were aligned with those of the mouse myeloma protein McPC603 whose atomic coordinates are known (18). The alignment with 40-140VL and VH regions resulted in 29% identical residues in V regions (71% chemically similar side chains, such as Glu-Asp or Lys-Arg), indicating that both domains share a common fold. Based on the alignment, the three CDR in each V domain were delineated. The H chain CDR loops H1, H2, and H3 comprised residues 28-36, 49-56, and 100-106, respectively; The L chain loops L1, L2, and L3 comprised residues 28-32, 51-57, and 90-96, respectively (consecutive numbering). In generating the three-dimensional 40-140 model, coordinates for the framework were directly copied from those of the McPC603 structure, and framework side chains were replaced using the conformational search program CONGEN (19). The backbone and the side chains of the CDR loops were constructed with CONGEN using the previously described protocol (20). The loops were generated successively in the order L2-H1-L3-H2-L1-H3. In the case of the H1, H2, H3, and L2 loops, the backbone conformational space was sampled with the EMAX search parameter of 0.6 kcal (19), so that the lowest-energy conformations of the principal minima of the ϕ, ψ conformational space were taken into account. This protocol allowed the model to be constructed in an acceptably short time (about 1 day of CPU time on a MicroVax II), but for the price of an incomplete sampling of conformations. Thus, the model should be viewed as an approximate one.

RESULTS

Sequences and binding properties of 35-20 antibodies. The 35-20 group of homologous anti-digoxin antibodies consists of five mAb (35-20, 40-40, 40-120, 40-140, and 40-160) that differ mostly by point mutations. Figure 1 shows the H and L chain V region amino acid sequences from these antibodies (9, 15). The four antibodies from fusion 40 (40-40, 40-120, 40-140, and 40-160) use identical D, JH2, and Jk5 gene segments and have VH and VL segments that share more than 95% homology. The fusion 40 antibodies are derived from one fusion (5). Hybridoma 35-20 is from a separate fusion and uses a different D region and JH1 instead of JH2. Excluding the D and JH regions, the 35-20 VH and Vk segments are about 90% homologous with their fusion 40 antibody counterparts.

Although these antibodies are very similar, they do have some distinguishing binding properties (5). Antibody 35-20 and 40-160 have unique fine specificity profiles, whereas 40-40, 40-120, and 40-140 have very similar fine specificity properties. The preliminary affinity data had indicated that, of the fusion 40 antibodies, 40-140 had roughly a fivefold lower affinity for digoxin than the other three antibodies. The cloned VH gene of 40-140 (9) was inserted into constructs and expressed to examine the contributions of the somatic mutations present on the 35-20 group H and L chains to these different binding properties.

Ig chain recombination by transfection. To determine

[3] Abbreviations: used in this paper: PBSA, PBS containing 0.10% sodium azide.

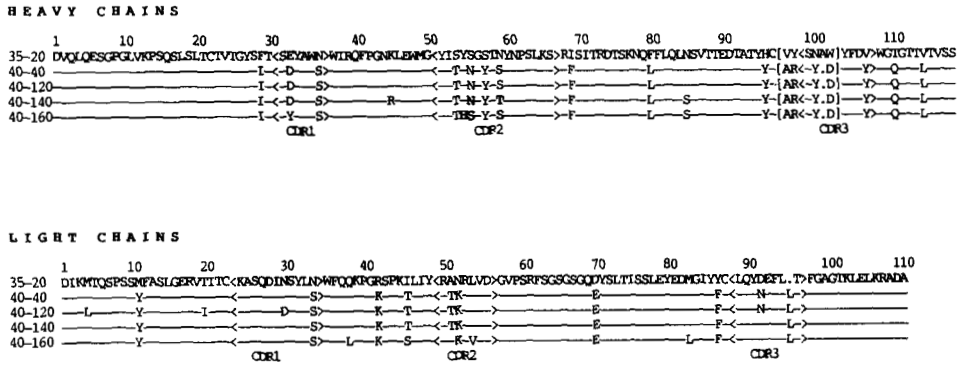

Figure 1. Amino acid sequences of H and L chain V regions of the 35-20 group of antibodies. Sequences are shown in the one-letter code with sequential numbering. H chains are shown at the *top*. L chains at the bottom. The sequences have been previously been reported (5, 9, 15). The CDR segments are denoted by *angle brackets* and the approximate D region is denoted by *square brackets*. A *line* indicates identity and a *dot* indicates a deletion as compared with the first sequence (antibody 35-20).

the relative contributions from the mutations found on the H and L chains to binding properties, we needed to pair one H chain with the different L chains among the 35-20 hybridomas. To do this, a variant cell line that has lost H chain production was generated from each hybridoma from the 35-20 group. A genetic construct that expresses VH40-140 attached to a γ2b C region was transfected into each of these L chain-producing cell lines and stable transfectomas were subsequently selected with mycophenolic acid. This strategy is depicted in Figure 2. The 4.8-kb *Eco*RI piece containing VH40-140 was inserted in pSV2gpt with a polylinker and a genomic γ2b gene. Transfectomas that express this construct were first detected by ELISA screening for the γ2b isotype (no hybridoma in the 35-20 group originally used this isotype). Transfectomas were further checked by ELISA for the ability to bind digoxin-keyhole limpet hemocyanin. To check for correct expression (not shown), mRNA from several transfectoma lines were sequenced, protein A purified transfectoma antibody was run on PAGE gels and showed H chain of the appropriate size (about 55 kDa), and Northern blots showed mRNA of the correct size (1.8 kb). Inasmuch as all the data indicated that the construct was functioning correctly, supernatant from each recombinant transfectoma line was assayed for binding properties.

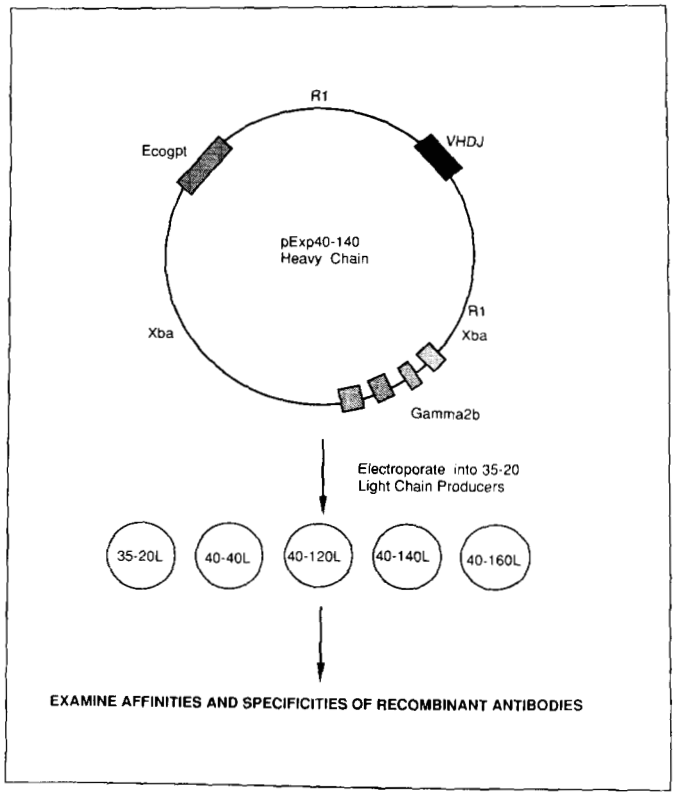

Figure 2. Vector used for Ig chain recombination by transfection of the 40-140 H chain. The expression vector pExp40-140H containing a polylinker, an *Eco*RI genomic 4.8-kb fragment with the rearranged 40-140 H chain gene, an *Xba*I 5.5-kb piece with a γ2b gene, and the pSV2gpt plasmid with the Ecogpt gene (11), was transfected into variant hybridomas from the 35-20 group that have lost H chain production. Recombinant antibodies secreted into the transfectoma supernatants were assayed for binding properties. Recombinant transfectomas were referred to as "(L donor)"t (i.e., VH140 into 40-160L is called 40-160t). *R1*, *Eco*RI; *Xba*, *Xba*I. *Boxes* in the γ2b piece indicate exons.

Binding properties of recombinant antibodies. Tables I and II show the fine specificity properties and digoxin affinities, respectively, of the parental and recombinant antibodies. As described previously (5) 40-140, 40-40, and 40-120 have similar specificity profiles whereas 40-160 and 35-20 are unique (Table I). However, the recombinant antibodies in which 40-140VH is expressed with different L chains, have specificity profiles (with the exception of 35-20 binding to ouabain) that are all similar to that of 40-140. This indicates that the mutations (somatic differences as compared with other members of the 35-20 group of H chains) present in the H chain of 40-140, rather than the mutations present in the recombinant L chain partners, are the more significant contributors to the fine specificity properties of the recombinant antibodies.

The affinity for digoxin shows a different relative dependence on H and L chain origin than does the fine specificity. The recombinant antibodies show that their affinity is affected by both the choice of H chain and of L chain (Table II). The recombinant antibodies 40-40t, 35-20t, and probably 40-120t (although this is only a two- to threefold difference) have affinities significantly different than either donor parental antibody (Table II). However, antibody 40-160t has the same affinity as its L chain donor parental antibody, 40-160. Therefore, in this case, the mutational differences between 40-140 and 40-160 present in the 40-160 L chain are more important than those in the H chain for affecting affinity. The control transfectant 40-140t (VH140 into the 40-140 L chain producing line) shows the same affinity as the parental line 40-140.

Mutagenesis of 40-140VH. Inasmuch as the somatic mutations in 40-140 H chain have a dominant role over those in L chain in determining the distinguishing overall fine specificity characteristics among the 35-20 antibodies, we chose to mutate VH40-140 (Fig. 3). The sequence of 35-20VH is too disparate from that of 40-140VH to

TABLE I

Fine specificity of 40-140VH recombinant antibodies[a]

Parent Antibody	Glycoside Inhibitor of Digoxin Binding					
	Digoxigenin	Digitoxin	Digitoxigenin	Gitoxin	Acetylstrophanthidin	Ouabain
40-140	14	1.9	19	35	109	393
40-40	13	1.8	25	32	116	420
40-120	16	1.4	16	54	111	624
40-160	4.0[b]	1.6	8.5	79	46	140
35-20	0.8	2.2	1.5	34	33	93
Recombinant Antibodies						
L Chain Donor	Digoxigenin	Digitoxin	Digitoxigenin	Gitoxin	Acetylstrophanthidin	Ouabain
40-140	15	2.1	13	34	97	463
40-40	12	1.9	15	37	110	520
40-120	11	2.1	12	34	140	610
40-160	13	1.5	14	26	65	480
35-20	11	2.7	15	53	110	190

[a] Concentration of inhibitor relative to digoxin yielding 50% inhibition of [^{125}I]digoxin binding. The concentration of cold digoxin that inhibits at 50% was set at 1.0. All experiments are in duplicate.

[b] Underscored numbers are specificity measurements more than two-fold different than those of normal antibody 40-140.

TABLE II

Affinity of 40-140VH recombinant antibodies[a]

Parent Antibodies	Affinity	
	(10^9 M^{-1})	±(10^9 M^{-1})
40-140	1.3	0.2
40-40	5.3	1.0
40-120	1.1	0.4
40-160	12	2.0
35-20	4.2	0.9
Recombinant Antibodies		
L Chain Donor	Affinity	
	(10^9 M^{-1})	±(10^9 M^{-1})
40-140	1.8	0.5
40-40	0.35	0.06
40-120	3.0	0.2
40-160	12	0.2
35-20	0.39	0.07

[a] The average intrinsic affinity constant (K_0) and error were calculated according to the algorithm LIGAND (see *Materials and Methods*).

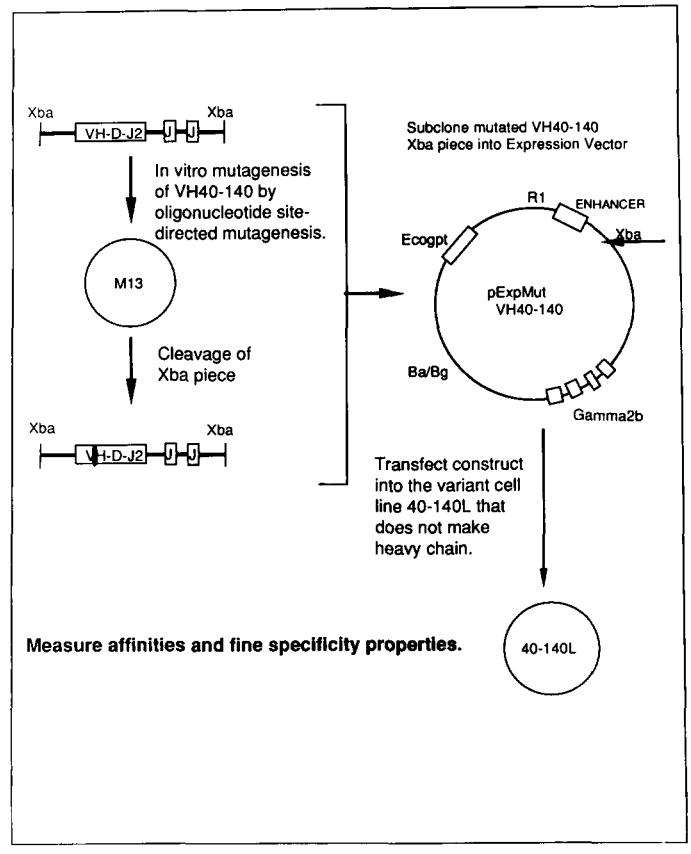

Figure 3. Vectors for mutagenesis and expresion of 40-140 H chain mutants. A 1.8-kb *Xba*I fragment containing the rearranged 40-140VH gene was cloned into M13mp18 and mutated (see *Materials and Methods*). The *Xba*I fragment was then recleaved and ligated (at the *Xba*I site denoted by the *arrow*) into the expression vector pExpMutVH40-140 that contains the H chain enhancer, the *Eco*RI-*Bam*HI fragment from pSVgpt2 (11), and a 3.6-kb *Xba*I-*Bgl*II γ2b piece. The construct was transfeced into the variant line 40-140L that only produces L chain. *RI*, *Eco*RI; *Ba*, *Bam*HI; *Xba*, *Xba*I; *Bg*, *Bgl*II. *Boxes* in the γ2b piece indicate exons (as do J region *boxes* in the *Xba*I piece).

indicate particular positions to mutate (Fig. 1). The antibodies 40-40 and 40-120 have fine specificity properties very similar to those of 40-140; therefore, the sequence differences among these H chains are not of interest here. However, 40-160VH only has somatic mutational differences from 40-140VH at positions 32, 54 and 55, and 59, yet has significantly different binding properties (Fig. 1; Table I). In our computer model, Figure 4, position 32 is buried. Preliminary affinity data had indicated that 40-140 had the lowest affinity among the 40-fusion antibodies. Because the only site unique to 40-140 is H chain Thr59 (the other antibodies have Ser), we first predicted that mutating Thr59 to Ser would increase affinity. Second, because among the 40-fusion antibodies, only 40-160 has a unique fine specificity and is unique at positions 54 and 55, we predicted that mutating TyrAsn to HisSer would alter the fine specificity of 40-140 to that of 40-160. Figure 3 shows the method of mutagenesis followed by transfection of the mutated construct into the 40-140 L chain producing line. Tables III and IV show the resultant mutant antibody binding properties. The double mutation at positions 54 and 55 does indeed alter the fine specificity of 40-140 so that it is not significantly different from that of 40-160 (Table III). In addition, the double mutant has no effect on digoxin affinity (Table IV). The single mutation at Thr59 has little effect on either affinity or fine specificity and, when combined with the double mutant to make a tipple mutant, there is no additional change from that of the double

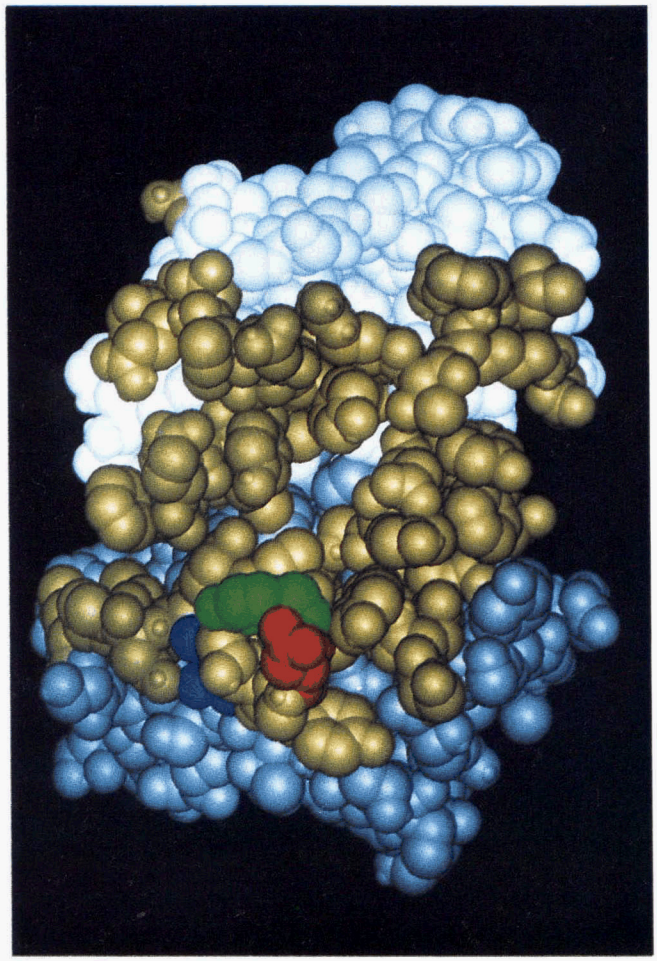

Figure 4. Computer generated model of the 40-140 combining site. The model was generated as described (see *Materials and Methods*) and is shown here as a *space-filling figure*. The view is looking directly into the antibody binding site. The different segments of the antibody chain are color coded: gray, H chain framework; white, L chain framework; brown, CDR loops; green, Tyr54; red, Asn55; and blue, Thr59.

mutant alone. This is not surprising because the final affinity measurements from the chain recombination studies (Table II) shows that 40-140 does not have affinity lower than the other 40-fusion antibodies. The recombination studies also show that the choice of 40-160L chain vs 40-140 L chain is more important in determining the overall affinity than the choice of the 40-140 H chain. It is noteworthy that we are able to separately alter specificity without changing affinity.

Computer model. A computer model of the 40-140 antibody combining site (Fig. 4) was generated as described in *Materials and Methods* using the protein McPC603 atomic coordinates (18) to help align 40-140VL and VH framework amino acids. Because this model used an incomplete sampling of conformations, it is an approximate one. The model depicts residues 54-55 (TyrAsn 40-140 H chain) to be highly accessible to solvent, whereas, residue Thr59 is relatively inaccessible. This is consistent with the mutagenesis data that shows TyrAsn mutated to HisSer results in altering fine specificity of 40-140 to that of 40-160 although mutation at Thr59 has no effect.

DISCUSSION

There are several approaches currently pursued for the study of structure-function of Ag-antibody complexes. Most of the understanding of Ag-antibody complexes derives from x-ray crystallographic structures (21). Alternative systems are based on computer modeling (22–24), the study of effects of different but related Ag on binding properties (25, 26), and the effect of different but related antibody sequences on binding properties (8, 27, 28). Predictions based on these models may be tested by assaying the binding to different Ag (5, 25, 29), by showing the effects of specific antibody amino acids by in vitro mutagenesis (30, 31) or by altering sections of or entire H and L chains (chain recombination) (24, 32, 33).

The antibodies in the 35-20 group provide a model for the study of structure-function in Ag antibody interactions. The 35-20 group of homologous anti-digoxin antibodies consists of five mAb (35-20, 40-40, 40-120, 40-140, and 40-160) that differ mostly by point mutations and in their binding properties (5). Antibodies 40-140, 40-40, and 40-120 have similar specificity profiles whereas 40-160 and 35-20 are unique (Table I). Antibodies 40-140, 40-40, and 40-120 are sensitive to the presence of the digitoxose groups of digoxin and digitoxin, binding 10–15 times more poorly to the aglycones digoxigenen and digitoxigenin (Table I). Antibody 40-160 shows modest sensitivity to the sugar group and 35-20 is indifferent to the sugar moieties. Substitutions on the β surface of steroid rings A and B characteristic of both acetylstrophanthidin and ouabain greatly inhibit antibodies 40-140, 40-40, and 40-120, whereas, these substitutions result in more modest inhibitions in 40-160 and 35-20 and may be less important for binding recognition.

The chain recombination study in which the 40-140 H chain is recombined with the different 35-20 group L

TABLE III

Fine specificity of 40-140VH mutant antibodies[a]

Antibody	Glycoside Inhibitor of Digoxin Binding					
	Digoxigenin	Digitoxin	Digitoxigenin	Gitoxin	Acetylstroph[b]	Ouabain
40-140	13	2.6	20	42	143	300
4 H59(S:T)[c]	21	3.0	29	57	154	475
17 H54(Y:H) + H55(N:S)	3.0	2.1	4.2	43	39	130
4 + 17	4.2	1.6	6.4	35	23	114
40-160	2.6	2.1	6.3	57	37	93

[a] Concentration of inhibitor relative to digoxin yielding 50% inhibition of [^{125}I]digoxin binding. The concentration of cold digoxin that inhibits at 50% was set at 1.0. All experiments are in duplicate.

[b] Acetylstroph, acetylstrophanthidin.

[c] Mutations refer to H chain-position (from first to second amino acid). Mutant 17 is a double mutant and mutant 4 + 17 contains all three mutations.

TABLE IV
Affinities of 40-140VH mutant antibodies[a]

Antibody	Affinity	
	(10^9 M^{-1})	±(10^9 M^{-1})
40-140	1.3	0.2
Mutant 4 H59(S:T)[b]	2.0	0.4
Mutant 17 H54(Y:H) + H55(N:S)	1.7	0.2
Mutant 4 + 17	1.5	0.1
40-160	12	2.0

[a] The average intrinsic affinity constants (K_0) and error were calculated according to the algorithm LIGAND (see *Materials and Methods*).

[b] Mutations refer to H chain-position (mutation from first to second amino acid). Mutant 17 is a double mutant and mutant 4 + 17 contains all three mutations.

chains shows that the mutations (somatic differences as compared with other members of the 35-20 H chains) present in the H chain of 40-140, rather than the mutations present in the recombinant L chain partners, are the more significant contributors to the fine specificity properties of the recombinant antibodies. Inasmuch as the use of the 40-140 H chain in the chain recombinants imparts sensitivity to the digitoxose groups of digoxin and digitoxin and relatively weak binding to acetylstrophanthidin and ouabain, these H chain mutations contribute to the recognition of the sugar moieties and somewhat to the β surface of steroid rings A and B, respectively. In addition, the similarity between the specificity properties of antibodies 40-140, 40-40, and 40-120 is, therefore, understandable because these antibodies have identical VH regions (with the two exceptions of 40-140 Arg45 and Ser85 in framework 2 and 3) (Fig. 1).

Examples are known in which H chain is a major component in Ag binding. It has been reported, in some cases, that isolated H chain can still bind Ag, with loss of affinity (34, 35), and that it can also bind Ag when combined with nonspecific L chains (36). A VH expression library has also been described from which clones have been selected for Ag binding (37). A different digoxin antibody chain recombinant has been reported, generated by fusion of hybridomas, in which the evidence also supports significant H chain contribution to fine specificity (10). The 40-140H chain, when transfected into the L chain producing myeloma J558L (λ1), secretes 40-140H + λ1 that does not bind digoxin (R. I. Near, unpublished results). However, when 40-140VH was placed next to the constant portion of a TCR, and then when this hybrid construct was expressed in transgenic mice, digoxin was able to activate splenic T cells, although the affinity at which digoxin binds could be very low (38). The evidence indicates that if VH40-140, itself, is capable of binding digoxin, it probably binds with a very low affinity. Therefore, the L chain must be contributing significantly to the binding to digoxin. This is borne out in the recombinant antibodies, because the resultant affinity constants reflect a combination of both H and L chain donors with the exception of the 40-160 L chain donor, in which the affinity constant of the recombinant antibody matches that of the L chain donor and not of the H chain donor (Table II). Inasmuch as 40-140VL and 40-160VL differ in CDR2, and in no other CDR, it is likely that L chain differences Thr51 vs Ala and/or Leu 54 vs Val would affect digoxin affinity.

Because the mutations in 40-140 H chain rather than those in L chain are responsible the fine specificity characteristics that distinguish 40-140 from the other antibodies, and because among the 40-fusion antibodies, only 40-160 has a unique fine specificity and is unique at positions 54 and 55, we predicted that mutating 40-140VH Tyr54Asn55 to HisSer would alter the fine specificity of 40-140 to resemble that of 40-160 (Fig. 1; Table I). Indeed, mutating TyrAsn to HisSer did alter the fine specificity of 40-140 to resemble that of 40-160 without significantly changing the affinity (Tables III and IV). Mutating Tyr54Asn55 to HisSer results in the loss of recognition of the sugar moieties and (to a lesser extent) recognition of the β surface of steroid rings A and B. Because preliminary affinity data had indicated that 40-140 had the lowest affinity among the 40-fusion antibodies and because the only site unique to 40-140 is H chain Thr59, we had predicted that mutating Thr59 to Ser would increase affinity. However, the Thr59-Ser mutation had no significant effect on affinity. This is consistent with the final affinity measurements (Table II) that indicate the differences between 40-140 and 40-160 L chain affect the affinity.

It is noteworthy that we were able to mutate to alter fine specificity without changing affinity. This may be related to the large size of digoxin that probably has multiple areas of interaction with the combining site. Mutations in antibodies that bind to small haptens usually affect both specificity and affinity (39, 40). This is probably related to the fewer number of residues in contact with a small hapten (18, 41) as opposed to Ag that fill the combining site (42); a change in one of many residues in contact with a large Ag may affect a relatively smaller area of the contact surface. Alternatively, positions 40-140VH 54 and 55 may not directly bind digoxin, but may affect the shape of the combining site or the access of digoxin to sterically fit into the combining site. The mutations themselves are relatively nonconservative and alterations in hydrogen bonds (Tyr to His) or changing an amide to an hydroxyl group (Asn to Ser) could have large effects. Inasmuch as there is no accurate structure, we can only speculate on this point. However, according to the computer model (Fig. 4), positions 54 and 55 are among the most accessible to solvent and could be an area that digoxin must contact. This area of the combining site may need to undergo steric adjustments to optimize binding. Inasmuch as digoxin is a relatively rigid molecule (3), the antibody and not the Ag would make such steric adjustments.

We have shown that, in this system, the mutations present among the 35-20 group H chains control the distinguishing characteristics of fine specificity, whereas the mutations in the combination of L chain and H chain control the distinguishing characteristics of the affinities among this set of antibodies. Specifically, H chain CDR2 residues 54 and 55 are sufficient to account for the fine specificity differences between antibodies 40-140 and 40-160. It should be carefully stated that, although H chain in the 35-20 group seems to control the overall specificity properties, this does not mean that mutations in L chain or mutations at alternate positions in H chain could not be found that had significant effects on specificity. Rather it means that, of the mutations present in the 35-20 group, those in H chain are more significant contributors to the specificity properties that distinguish these antibodies than are those in the L chain.

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