

## Variable region framework differences result in decreased or increased affinity of variant anti-digoxin antibodies

(immunoglobulins/fluorescence-activated cell sorting/somatic mutation/complementarity/digoxin)

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**ABSTRACT** Rare spontaneous variants of the anti-digoxin antibody-producing hybridoma 40-150 ( $K_D = 5.4 \times 10^9 M^{-1}$ ) were selected for altered antigen binding by two-color fluorescence-activated cell sorting. The parent antibody binds digoxin 890-fold greater than digitoxin. The variant 40-150 A2.4 has reduced affinity for digoxin ( $K_D = 9.2 \times 10^6 M^{-1}$ ) and binds digoxin 33-fold greater than digitoxin. A second-order variant, derived from 40-150 A2.4 (designated 40-150 A2.4 P.10), demonstrated partial regain of digoxin binding ( $K_D = 4.4 \times 10^8 M^{-1}$ ). The altered binding of the variant 40-150 A2.4 was accounted for by a point mutation resulting in substitution of arginine for serine at position 94 in the heavy chain variable region. Antibody 40-150 A2.4 P.10 also contains this arginine but owes its enhanced antigen binding to deletion of two amino acids from the heavy chain amino terminus. This unusual sequence alteration in an immunoglobulin framework region confers increased affinity for antigen.

Although much is known about the genetic basis of antibody diversity (reviewed in refs. 1 and 2), knowledge of the precise molecular interactions between specific antibodies and antigens remains limited. The combining site of antibody molecules is composed of the amino-terminal domains [variable (V) regions] of two nonidentical polypeptides. The high degree of amino acid sequence variability found in three noncontiguous segments of the light (L) and heavy (H) polypeptide chains led to the prediction (3) that these "hypervariable" or "complementarity-determining regions" (CDR) would fold together in three dimensions to form the combining site. This prediction was verified by x-ray crystallographic analyses of hapten binding Fab fragments (4-7). [The Fab fragment ( $M_r$  50,000) obtained by limited proteolysis (8) is composed of one L and the amino-terminal half of the H chain and contains one of two identical antigen binding sites.] An examination of Fab crystal structures indicated that hypervariable region sequences are engrafted onto "frameworks" for which three-dimensional folding is remarkably uniform (9).

The availability of monoclonal antibodies, whether secreted by myeloma tumors or by hybridomas resulting from somatic cell fusion (10), makes possible further correlation of primary amino acid sequences with antigen binding specificity, through examination of structural variants obtained spontaneously or by design through site-directed mutagenesis. Spontaneous variant antibodies with reduced or absent antigen binding were reported for the phosphocholine (11, 12) and 4-hydroxy-3-nitro-5-iodophenylacetyl (13-15) haptens. In these cases the structural changes were due to point

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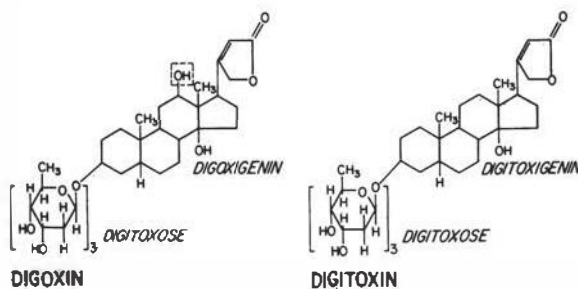


FIG. 1. Structures of digoxin (digoxigenin tridigitoxose) and digitoxin.

mutations in either hypervariable or framework regions, except for one instance where multiple amino acid differences, perhaps due to gene conversion, resulted in loss of an idiotope (15).

Digoxin (digoxigenin tridigitoxose, Fig. 1) is a useful hapten for studies of antibody complementarity as it approximates in size the antibody combining site, is uncharged, contains a rigid steroid nucleus, and has a known three-dimensional structure (16); multiple congeners of defined structure are known (17, 18). Anti-digoxin antibodies are characterized by high affinity and varying specificity for related cardiac glycosides. The anti-digoxin antibody 40-150 (18) was chosen for isolation of variants because of its sensitivity to the presence of a hydroxyl group at the 12 position on the steroid C ring of digoxin (Fig. 1) as it binds to digoxin more avidly than digitoxin, which lacks the hydroxyl group. We report the isolation and characterization from cell culture of a spontaneous antibody variant with reduced affinity due to a point mutation. A second-order variant demonstrated partial regain of binding due to a "compensatory" deletion of two amino acids from the framework region.

### MATERIAL AND METHODS

**Cell Lines and Antibody Purification.** The production of the murine A/J anti-digoxin hybridoma cell line 40-150 (IgG1,  $\kappa$ ) has been reported (18). Production of 40-150 and variant hybridoma proteins in ascites was performed as described (17). Antibodies were purified from ascites or

Abbreviations: CDR, complementarity-determining region(s); FACS, fluorescence-activated cell sorting; H, heavy; L, light; V, variable.

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Table 1. Binding of antibody 40-150\* and variants to cardiac glycosides

Antibody or fragment	$K_o \times 10^{-7} \ddagger$ (L/M)	Concentration of inhibitor <sup>†</sup> relative to digoxin giving 50% inhibition of binding to <sup>125</sup> I-labeled digoxin-bovine serum albumin					
		Digoxigenin	Digitoxin	Digitoxigenin	Gitoxin	Acetylstrophanthidin	Ouabain
40-150	540 ± 60	7.3	890	7300	400	8000	7300
40-150 A2.4	0.9 ± 0.1	19	33	36	39	44	46
40-150 A2.4 P.10	44 ± 9	6.9	1100	1200	260	1300	1300
Fab I	0.8 ± 0.2	21	38	39	43	44	36
Fab II	23 ± 4	7.5	830	1500	180	1300	1200

\*Minor differences in specificity determined here for antibody 40-150 as compared to reported values (18) are attributed to differences in the assay systems used and the <sup>125</sup>I-labeled digoxin-bovine serum albumin probe employed here (29).

<sup>†</sup>Binding of digoxin was set at 1.0 for all antibodies.

<sup>‡</sup>Affinity for digoxin.

culture medium by affinity chromatography on digoxin-bovine serum albumin-Sepharose or digitoxin-bovine serum albumin-Sepharose. Digoxin or digitoxin was coupled to bovine serum albumin through the terminal digitoxose moiety (17, 19). Antibodies were eluted by using 3 M KSCN; however, for separation of Fab fragments from variants, gradient affinity chromatography (0–3 M KSCN) was used.

**Fluorescence-Activated Cell Sorting (FACS).** Rare spontaneous variants of anti-digoxin hybridomas were isolated by two-color FACS as described (20). Parental cell populations were stained with digoxin-fluorescein to measure antigen binding by cell-surface antibody. The amount of cell-surface antibody was simultaneously estimated by using biotin-conjugated goat anti-mouse IgG and Texas red avidin. Upon measurement in a two-laser FACS system, cells whose antigen binding relative to surface IgG did not match that of the parental population were sorted as candidate variants. After a period of culture the sorted populations were restained and resorted. Typically on the third cycle, probable variant cells were cloned directly by using the FACS.

**Sequence Analysis.** For mRNA sequencing, total RNA and poly(A) mRNA were prepared as described (21, 22). Complementary DNA synthesis was performed simultaneously on H and L chains with poly(A)<sup>+</sup> RNA (100–200 µg) (23) by using complementary oligonucleotides to the respective constant regions (24). The remainder of the sequences through the N terminus were obtained by using oligonucleotides complementary to the third L chain framework region (see Fig. 3, positions 67–73) and the third H chain framework region (positions 68–73). The isolated full-length <sup>32</sup>P end-labeled cDNA transcripts were sequenced by base-specific chemical cleavage as described (24). Amino acid sequence analyses were performed on isolated chains and peptides obtained by cleavage with CNBr, *o*-iodosobenzoic acid, and tryptic digestion of completely reduced and alkylated citraconylated chains; the fragments were purified by gel filtration and high-pressure liquid chromatography (HPLC) (25, 26). Peptides were sequenced by automated Edman degradation on a Beckman 890C sequencer or an Applied Biosystems (Foster City, CA) 470A gas-phase sequencer. At cycles where proline was N-terminal, *o*-phthalaldehyde was used to reduce background or selectively sequence prolyl peptides (27). Phenylthiohydantoin-amino acids were identified by HPLC (28).

**Binding Assays.** A double-antibody precipitation assay using [<sup>3</sup>H]digoxin was employed to measure antibody affinity ( $K_o$ ) as described in detail elsewhere (29). A microtiter plate radioimmunoassay was used to assess relative antibody specificity for related cardiac glycosides (29). This method is applicable to lower affinity antibodies than that used previously (17, 18). The production of a rabbit polyclonal anti-40-150 idiotype serum and competition radioimmunoassay for the 40-150 idiotype were performed by

## RESULTS

In the initial FACS experiment a variant of 40-150 with reduced binding to digoxin, designated 40-150 A2.4, was isolated. This clone was subjected to a second round of sorting for cells with enhanced binding to digoxin, resulting in the selection of 40-150 A2.4 P.10. The binding characteristics of antibody 40-150 and the two variants are summarized in Table 1 and Fig. 2. Although antibody 40-150 binds digoxin ( $K_o = 5.4 \times 10^9 \text{ M}^{-1}$ ) 890 times more avidly than digitoxin, 40-150 A2.4 has reduced affinity for digoxin ( $K_o = 9.2 \times 10^6 \text{ M}^{-1}$ ) but now binds digoxin only 33 times more avidly than digitoxin. The second-order variant 40-150 A2.4 P.10 binds digoxin with an increased affinity ( $K_o = 4.4 \times 10^8 \text{ M}^{-1}$ ) compared to antibody 40-150 A2.4, from which it was derived, and distinguishes digoxin and digitoxin to an extent (1100-fold difference) similar to the parental antibody. This altered recognition of the steroid C ring 12-hydroxyl group among the three antibodies is also reflected in their relative binding to gitoxin (Table 1). Gitoxin, like digitoxin, lacks the 12-hydroxyl group but has an additional hydroxyl at position 16. The variant 40-150 A2.4 is relatively more sensitive to gitoxin inhibition of digoxin binding than the parent 40-150 and the second-order variant 40-150 A2.4 P.10. Unlike the results for digoxin binding, in a competition assay for the 40-150 idiotype, antibodies 40-150, 40-150 A2.4, and 40-150 A2.4 P.10 were indistinguishable (data not shown).

To determine the structural changes responsible for antigen binding differences among the three hybridoma proteins, the complete V region sequences were determined by nucle-

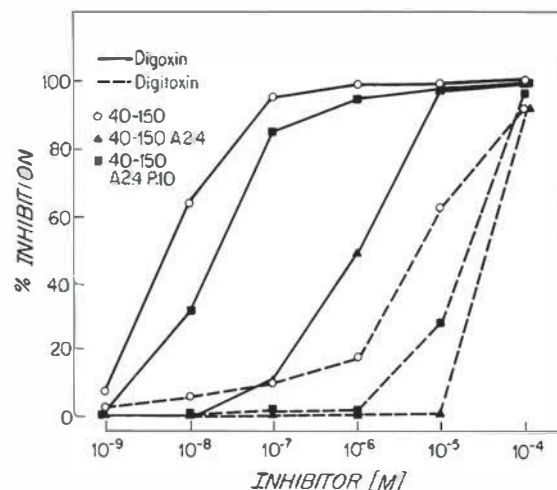


Fig. 2. Inhibition of binding of monoclonal anti-digoxin anti-



HEAVY CHAINS

40-150 H N P G L R L I P L V L T L R K G V Q C D V K L V E S G G G L V K P G G S L K L S C A A S G P T P R S Y T H A W V R O I P  
 40-150 A2.4  
 40-150 A2.4 P.10

40-150 ATGAACTTCGGCTCAGATTGATTTCTTGTCTTACTTTAAAAGGTCGACGTCGAGCTCAAGCTGGTCACCTCGGGGGAGCGTTACTGAACCTTCAAGCTCTCAAACTCTCCCTGCAAGCTCTCAATTCATTTCAGAAAGCTATTACATGCTTGGGTCGCGACATTCG  
 40-150 A2.4  
 40-150 A2.4 P.10

40-150 T I S I S D I T T T Y P D N V K G R P T T I S R O N A K N T L Y L O M S S L K S E D T A M Y Y G T S R R V G N T D I A H D Y W  
 40-150 A2.4  
 40-150 A2.4 P.10

40-150 ACCATTACTATTAGTGATATTTCACCTACTATCCAGACAATCTCAAGGCTCCATTCAACATCTCCAGAGACAATGCAAGAACCCCTCTACCTTCAAAATCAGCACTCGAAGCTCGAGGACACAGGCTATGATTTACTGTACAAGCTTGGGGAATTAAGCATATCTATGGACTACTG  
 40-150 A2.4  
 40-150 A2.4 P.10

LIGHT CHAINS

40-150 D V V H T O T P L T L S V Y T I G Q P A S I S C K S S O S L L N S D G K T Y L I V L L Q R P G Q S P K R L I  
 40-150 A2.4  
 40-150 A2.4 P.10

40-150 GATCTCTGATCAGCAGACTCGACTGACTCTCTCTGCTTACATTCACAAGCAGGCTCCATCTCTGCAAGTCAAGTCAGAGCCCTCTTAATAGTGTGAAAGACATATTGATTTGGTCTTACACAGGCCAAGCCAGTCTCAAAAGGCTCAATCT  
 40-150 A2.4  
 40-150 A2.4 P.10

40-150 K L D S G V P D R P T Y G S G S G Y D P T L K I S R V S A E D L G V Y Y C W O G T H P P Y T T P G G G T K L E  
 40-150 A2.4  
 40-150 A2.4 P.10

40-150 AAATGGACTCTGGAGTCCCTGACAGTTTCACTGGCAGTGGATCAGGACAGATTTCACACTGAAAATCAGCAGAGTGGAGGCTGAGGATTGGGAGTTTATTATTGTTGGCAAGGTACACATTTCCGTACACGTTCCGAGGGGGGACCAAGCTGAA  
 40-150 A2.4  
 40-150 A2.4 P.10

FIG. 3. Amino acid and nucleotide sequences of the V regions of 40-150, 40-150 A2.4, and 40-150 A2.4 P.10 H (Upper) and L (Lower) chains. Amino acid sequences are given at the top in a one-letter code (30). The numbering of amino acid residues and the designation of CDRs are as determined by Edman degradation results shown as well as from direct protein sequence analysis. The portions of the V region sequences determined by Edman degradation are indicated by brackets above the amino acid sequences: 40-150 L, completely sequenced; 40-150 H, all residues except 67-71 and 80-89; 40-150 A2.4 H, all residues except 82-89. See Fig. 4 for protein sequence results.

otide and amino acid sequence analyses. The L chain V region sequences of all three antibodies are identical (Fig. 3). The H chain V region sequences of 40-150 and 40-150 A2.4 are identical except for a single nucleotide difference (guanine/thymidine) resulting in the substitution of arginine for serine at position 94 (Fig. 3). This single amino acid difference in a "framework" residue (3) at the boundary with CDR 3, resulting in the substitution of a bulky polar side chain, must be responsible for the decreased affinity and altered specificity of 40-150 A2.4.

Since antibody 40-150 A2.4 P.10 was derived from 40-150 A2.4 we anticipated that the increased binding in the former antibody might be due to a compensatory mutation elsewhere in the V region. However, the V region nucleotide sequences of these two antibodies are identical for both H and L chains, including the mutation at position 94 (Fig. 3). All three antibody H and L chains were indistinguishable in size by PAGE in NaDodSO<sub>4</sub> under reducing conditions (not shown), precluding a major deletion. Fab fragments of all three antibodies had antigen binding properties identical to the intact antibodies from which they were derived, thus precluding structural alterations in Fc that affect antigen binding. We therefore analyzed the protein sequence of 40-150 A2.4 P.10. The sequence obtained by Edman degradation of the L chain (50 cycles) was identical to that predicted from the nucleotide sequence analysis shown in Fig. 3. However, sequence analysis (40 cycles) of the H chain revealed two amino acids at each position (Fig. 4). In addition to the full-length H chain corresponding to the nucleotide sequence (Fig. 3), a second species with an amino terminus at position 3 relative to the intact chain was also present. This result was obtained for antibody purified from ascites, from culture medium, and from fresh subclones of 40-150 A2.4 P.10, demonstrating that the mixed antibody is an inherent product of the clone.

To determine whether the truncated H chain species was indeed responsible for the observed enhancement of antigen binding by 40-150 A2.4 P.10, Fab fragments were prepared (8) and separated by gradient affinity chromatography (not shown). Two major peaks were identified. An early eluting peak (I) contained Fab fragments with affinity for digoxin and specificity indistinguishable from that of 40-150 A2.4 (Table 1). Sequence analysis of Fab I revealed a single sequence corresponding to the full-length H chain (Fig. 4). The later eluting peak (II) contained Fab fragments that bound digoxin with an affinity ( $K_D = 2.3 \times 10^8 \text{ M}^{-1}$ ) higher than peak I and close to that found for unfractionated 40-150

A2.4 P.10. The fine specificity of Fab II was identical to that of the intact antibody 40-150 A2.4 P.10 and its unfractionated Fab. Fab II contained a single sequence corresponding to the shortened H chain (Fig. 4). To confirm that the apparent enhanced affinity of 40-150 A2.4 P.10 was due to dominance of the higher affinity truncated species in the binding assay, Fabs I and II were mixed together in a molar ratio (40:60) corresponding to that observed in the protein sequence analysis of unfractionated 40-150 A2.4 P.10. The binding characteristics of this mixture duplicated the results for unfractionated 40-150 A2.4 P.10 (data not shown).

## DISCUSSION

The ability to isolate somatic mutants from antibody-producing cell lines in culture provides an opportunity to assess the effects upon antigen binding and idiotype of structural alterations limited to one or a few V region amino acids (11-15). Two-color FACS proved useful in isolating spontaneous variants of high-affinity anti-digoxin antibodies with frequencies in the  $10^{-6}$  range (20). The variant hybridoma protein 40-150 A2.4 binds digoxin with lower affinity than the parental antibody and is relatively less sensitive than the parent antibody to the absence of a 12-hydroxyl group on the steroid C ring of the hapten. The structural change responsible for the binding differences is due to a single amino acid substitution in the H chain framework region at position 94, at the edge of CDR 3 (Fig. 3). The finding that a framework mutation can alter binding to antigen is not unexpected. Previous studies of phosphocholine binding myeloma variants demonstrated that a mutation in the J<sub>H</sub> region reduces affinity for phosphocholine only when the hapten is bound to carrier protein. Although that J<sub>H</sub> mutation is formally in a hypervariable region based on sequence (3), it is not located in the binding site based on x-ray crystallography (32). In addition, crystallographic analysis of a lysozyme-antilysozyme complex (33) demonstrated that among 17 antibody residues that contact antigen, 2 were found in framework regions at the edge of CDR.

The results for a second-order variant of antibody 40-150 were, however, unexpected. Regardless of the mechanisms involved in H chain truncation of the digoxin antibody variant 40-150 A2.4 P.10 described here, the results demonstrate that a change in framework structure may significantly enhance antibody affinity. The marked effect of these structural changes on antigen binding was not accompanied by a

### ANTIBODY

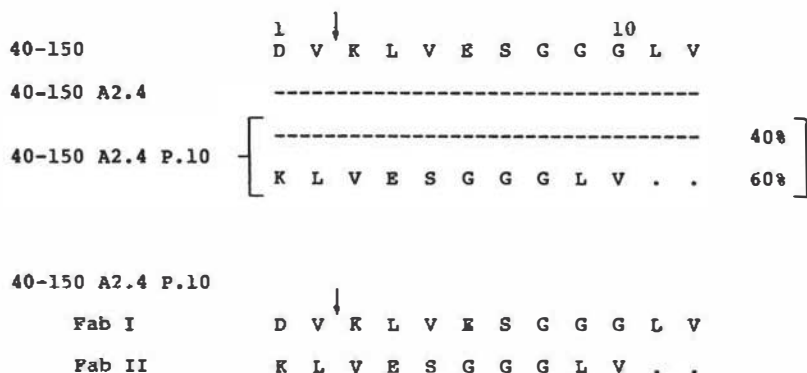


FIG. 4. Comparative amino-terminal sequences of H chains 40-150, 40-150 A2.4, and 40-150 A2.4 P.10. Antibody 40-150 A2.4 P.10 H chain contained two amino acids at each cycle for both the intact antibody and Fab. Amino acid sequences of 40-150 A2.4 P.10 Fab I and Fab II



change in idiotype, as detected by using a rabbit antiserum prepared against 40-150 (data not shown).

Inasmuch as (i) the nucleotide sequence of 40-150 A2.4 P.10, using an internal V region oligonucleotide primer (Fig. 3), revealed only a single sequence without ambiguity through the V<sub>H</sub> amino-terminal region and (ii) the H chain nucleotide leader sequences for 40-150 A2.4 and 40-150 A2.4 P.10 were identical, the appearance of a truncated H chain in 40-150 A2.4 P.10 was not due to a mutation encoded in V<sub>H</sub> or to a mutated leader signal sequence leading to an aberrant cleavage. It is probable that the (partial) H chain truncation in this cell line occurs from an as yet unidentified mutation affecting posttranslational modification of the H chain. The Val-Lys bond (positions 2 and 3, Fig. 3) is not a conventional cleavage site for signal peptidases (34, 35), although cleavage by an endopeptidase specific for the amino-terminal side of lysine (36) and aminopeptidase (37, 38) are possible explanations.

Models of the structures of 40-150 and its two mutants were constructed by computer (J. Novotny, R. E. Bruccoleri, and E.H., unpublished data) based on atomic coordinates of the anti-phosphocholine antibody McPC 603. The results indicate that when Arg(H)-94, is substituted for Ser(H)-94 (as in 40-150 A2.4), Arg(H)-94 can form a chain of hydrogen bonds to Asp(H)-101 and then to Arg(L)-46 and Asp(L)-55, all in the vicinity of the combining site. The resultant change in the antigen combining site surface may account for the affinity and specificity changes observed in 40-150 A2.4. Deletion of the two amino-terminal H chain residues (as in 40-150 A2.4 P.10) increases solvent accessibility to Arg(H)-94 by 100%. The solvation may result in the loss of a hydrogen bond between this residue and Asp(H)-101, restoring the structure to one similar to that of the parent 40-150 antibody.

Full molecular dissection of antigen-antibody complementarity requires production of new binding sites by site-directed mutagenesis, but the results presented here emphasize the risk of a narrow focus in designing such experiments. Information from an array of spontaneous mutants as well as from computer modeling studies and x-ray crystallographic analyses is needed to guide such work.

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