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Altered Hapten Recognition by Two Anti-digoxin Hybridoma Variants Due to Variable Region Point Mutations*

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Two spontaneous variants of the murine anti-digoxin antibody-producing hybridoma cell line 26-10 were isolated by two-color fluorescence-activated cell sorting on the basis of altered hapten binding. The variable region sequences of the antibodies produced by the mutant lines revealed that each contains a single amino acid change in the heavy chain second complementarity determining region. A Tyr to His change at position 50 leads to a 40-fold reduction in affinity for digoxin. A Ser to Phe mutation at position 52 results in a 300-fold reduction in affinity for digoxin.

A competition assay involving 33 digoxin analogues was used to examine the specificity of hapten binding of 26-10 and the two mutant antibodies. The position 50 mutant has a distinct specificity change; it exhibits a preference for digoxin congeners containing a hydroxyl group at the steroid 12 position, whereas the 26-10 parent does not. The affinities of all three antibodies for hapten are progressively lowered by substitutions of increasing size at the digoxin steroid D ring 16 position. Although 26-10 binds digoxin and its genin form equally, 12 and 16 steroid position substitutions which lower affinity also confer a preference for a sugar at the steroid 3 position. These results suggest that position 50 contributes to specificity of the antibody and that alterations of the hapten can lead to differences in recognition, possibly through a shift in hapten orientation within the binding site.

Defining the structural requirements for antibody and antigen interactions has been elusive. Correlation of amino acid sequence to antibody specificity and tertiary structure is as yet imperfect. Compounding the problem is the diversity of the humoral immune response which can result in the production of antibodies with similar affinities for a given antigen but divergent primary structures.

A useful approach to correlation of structure and function of antibodies has been to select and analyze antibody mutants with altered antigen binding due to single V[1] region mutations. Scharff and his colleagues (1–3) isolated myeloma variants having altered hapten specificity, and Rajewsky and his coworkers (4–7) selected several idiotypic mutants of a hybridoma line, two of which had altered hapten recognition. Although analyses of these mutants implicated certain amino acid side chains in hapten binding, limitations of these systems, including a low V region mutational rate compared with that *in vivo* (reviewed in Ref. 8), and relatively low affinities of the parental antibodies and consequently often unmeasurable affinities of the mutants hampered extensive correlation of structure with binding.

Immunization with protein conjugates of the cardiac glycoside digoxin, however, elicits high affinity polyclonal (9) and monoclonal antibodies (10, 11). A large variety of natural and synthetic digoxin analogues, which differ by substitutions on the steroid body as well as different sugars linked through the 3 position oxygen (Fig. 1), are available for determination of binding specificity. We previously used a two-color fluorescence-activated cell sorting procedure to select binding variants of the anti-digoxin hybridoma cell line 40-150 (12). Fluorescence-activated cell sorting permits selection of hybridoma variants despite a relatively low mutation rate (13). We have used a similar procedure to isolate multiple variant lines from the anti-digoxin hybridoma 26-10. Nucleotide and protein sequence analyses of two of these variant lines reveal unique mutations in the H chain CDR2 which encode single amino acid changes. Both mutations result in lowered affinity for digoxin. To define the pattern of recognition for hapten, 33 analogues of digoxin were used in a competition assay to determine the affinity for each analogue as compared to digoxin. One of the mutants can distinguish between the presence and absence of a single hydroxyl on the hapten, whereas the 26-10 parent antibody does not.

MATERIALS AND METHODS AND RESULTS[2]

Selection of Mutant Lines—Because initial experiments indicated that Ig surface expression of 26-10 cells was too low

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[1] The abbreviations used are: V, variable, FACS, fluorescence-activated cell sorting; H, heavy; L, light; CDR2, second complementarity determining region; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PE, phycoerythrin; HSA, human serum albumin; H50, heavy chain position 50; H52, heavy chain position 52; PCR, polymerase chain reaction; Fab, antigen-binding fragment of antibody; BSA, bovine serum albumin; Ig, immunoglobin.

[2] Portions of this paper (including "Materials and Methods," part of "Results," part of "Discussion," Figs. 3 and 4, and Footnotes 3 and 4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

for sorting, subclones with increased expression were isolated (see "Materials and Methods"). The 26-10 variant lines LL2 and LB4 were isolated from these 26-10 subclones by selection of cells exhibiting high levels of staining with a conjugate of digoxin, human serum albumin and phycoerythrin, relative to labeling for surface Ig, following preincubation with digitoxin (12-deshydroxydigoxin; see Fig. 1). The H and L chains of purified LL2 and LB4 antibody migrated with those of the parent 26-10 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). Both variants expressed γ2a and κ isotypes.

V Region Sequence of 26-10 Variants—The V region sequences of the antibodies are shown in Fig. 2. The L chain V region nucleotide sequences of all three lines are identical. However, the LB4 H chain contains a single base change (C to T) causing a Ser to Phe substitution at position 52 (H52). In variant LL2, a point mutation (T to C) results in a Tyr to His change at H chain position 50 (H50). These mutations were detected both by sequencing V region cDNA by chemical cleavage and by chain termination sequencing of cloned polymerase chain reaction-amplified copies of V region cDNA. The mutations were confirmed by Edman degradation of the purified H chains (60 cycles for LL2, cycle 52 not identified; 59 cycles for LB4, cycles 43 and 46 not identified). No differences between translated cDNA and amino acid sequences were detected, nor were any detected by amino acid sequencing of L chains (32 cycles for LL2; 54 cycles for LB4).

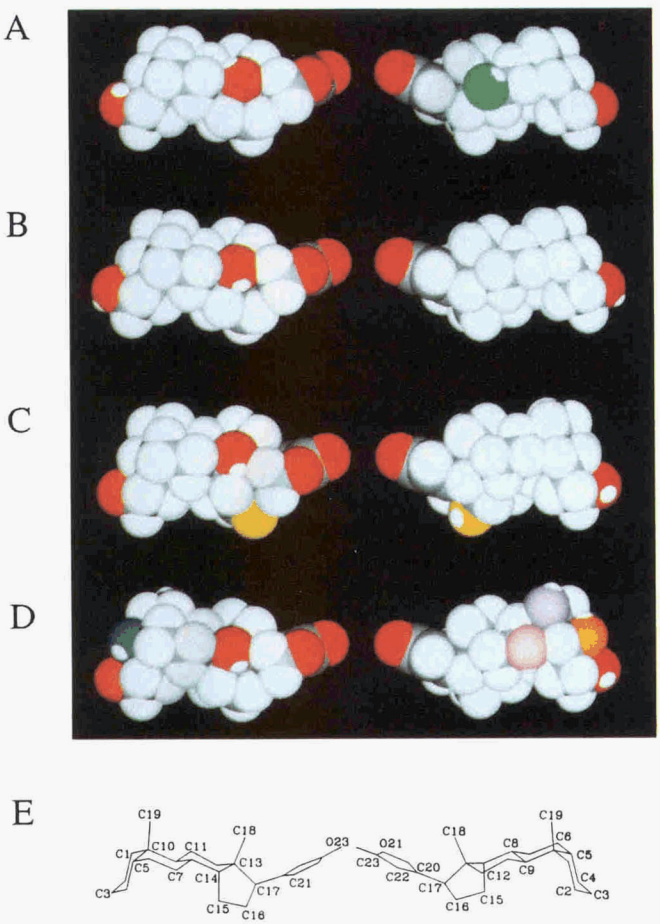

FIG. 1. **Space-filling models displaying "front" and "rear" of digoxigenin (*A*), digitoxigenin (*B*), gitoxigenin (*C*), and ouabagenin (*D*) and a corresponding vector diagram showing the cardenolide numbering system (*E*).** Carbons and hydrogens are shown in *gray* and *white*, respectively. Oxygens at the 3, 14, 21, and 23 positions are shown in *red*. The 12-oxygen of digoxigenin is shown in *green*, and the 16-oxygen of gitoxigenin is shown in *yellow*. For ouabagenin, the 1-oxygen is *orange*, the 5-oxygen is *blue*, the 11-oxygen is *coral*, and the 19-oxygen is *purple*.

Affinity Measurements—Affinities were determined with an equilibrium saturation method which exploits the adherence of these antibodies to glass fiber filters to separate bound and free tritiated ligand. The affinity of antibody 26-10 for digoxin is 2.4×10^{10} M^{-1} by this method (Table I). Because this is higher than previously published values using double antibody precipitation (2.6×10^{9} M^{-1}; Ref. 14) and dextran-coated charcoal (6.9×10^{9} M^{-1}; Ref. 10), kinetic experiments using filtration separation were undertaken to establish the consistency of the method reported here. From a representative kinetic measurement, the resulting affinity constant is 2.2×10^{10} M^{-1} (Fig. 3). The discrepancy between the affinities reported here and those reported previously is due in part to the different antibody concentrations used (0.05–0.1 of the apparent K_D *versus* $\geq$ the apparent K_D for previous measurements). Apparent affinities tend to decrease when measured at increasing receptor concentrations (data not shown; Refs. 15 and 16).

The affinity of variant antibody LL2 for digoxin is reduced 40-fold in comparison to 26-10 (6.4×10^{8} M^{-1}, Table I). Variant antibody LB4 demonstrates a 300-fold reduction (8.6×10^{7} M^{-1}). The affinities of the antibodies for ouabain were also measured. Ouabain lacks the steroid C ring 12-OH of digoxin, but has additional hydroxyls at the 1β, 5β, 11α, and 19 positions (Fig. 1). In contrast to digoxin, which has a tridigitoxose moiety, ouabain has a rhamnose at position 3. Using saturation equilibrium methods, 26-10 has an affinity for ouabain of 6.0×10^{8} M^{-1} (Table I), a reduction of approximately 40-fold relative to digoxin, consistent with previous competition binding studies (10, 11). LL2 and LB4 also show reduced affinity for ouabain compared to digoxin.

Idiotype of Variant Antibodies—Inhibition curves of antibodies 26-10, LL2 and LB4 using a competitive radioimmunoassay for idiotype (14) were indistinguishable (data not shown).

Specificity of Hapten-Antibody Interaction—In order to examine changes in recognition due to alteration of hapten structure and to detect differences in specificity of variant antibodies, a competition assay using 33 digoxin analogues was used (Tables II–IV). The tables list structural features which distinguish each hapten from digoxin (Tables II and III) or ouabain (Table IV).

As shown in Table II, and as noted previously (10, 11), antibody 26-10 has no apparent recognition of the digoxin sugar moiety. Digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside, and digoxin are bound equally. The absence of sugars (digoxigenin) also does not substantially affect binding. Conversion of the 3β-OH to 3α-OH (3-epidigoxigenin) results in only a slight decrease in binding relative to digoxigenin. Mutants LL2 and LB4 exhibit a similar pattern.

As shown previously (10, 11), 26-10 does not distinguish between the presence (digoxin) or absence (digitoxin) of the 12-OH. Binding is also not significantly affected by removal of the sugar (digitoxigenin) or by the replacement of tridigitoxose with rhamnose (evomonoside) or thevetose (neriifolin). The presence of the 12-OH is more important for binding of LL2, however, as the relative affinity for digitoxin, digitoxigenin, evomonoside, and neriifolin are lower than for digoxin. LB4 is less reliant upon the 12-OH for optimal binding compared with LL2. The importance of the 12-OH in binding by LL2 and LB4 is further illustrated by experiments using gitoxin and diginatin (Table II). Gitoxin and digitoxin both lack a 12-OH, but gitoxin possesses a 16-OH. The presence

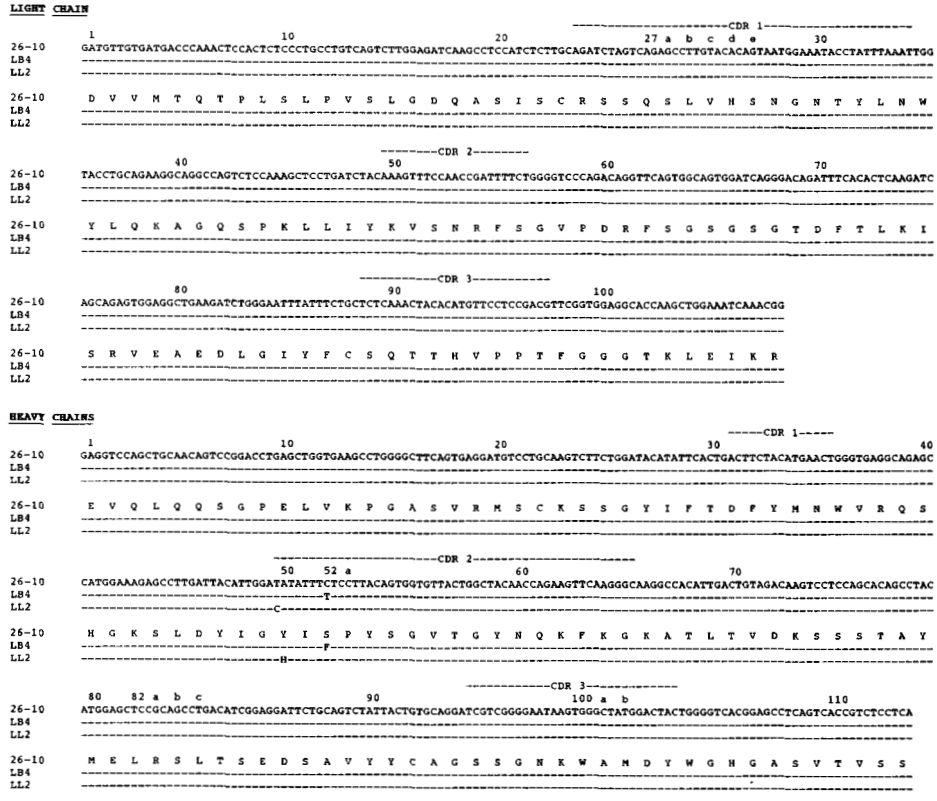

FIG. 2. **Nucleotide and amino acid sequences of 26-10, LL2, and LB4 L (*top*) and H (*bottom*) chains.** Amino acid sequences were translated from the nucleotide sequences and confirmed in part by protein sequence analysis (see "Results"). Amino acid sequences are given in one-letter code. Amino acid residue numbering and designation of complementary determining regions are as defined by Kabat (35). A dash indicates identity to above sequence. The complete genomic nucleotide and partial protein sequences of 26-10 have been reported previously (11, 14, 36).

TABLE I

Affinity of 26-10 and mutant antibodies

Affinities of antibody 26-10 and its mutants for digoxin and ouabain were measured using an equilibrium saturation method using filtration through glass fiber filters for separation of bound and free ligand (see "Materials and Methods").

Antibody	Affinity	
	Digoxin	Ouabain
	M^{-1}	
26-10	$2.4 \pm 0.5 \times 10^{10}$	$6.0 \pm 0.4 \times 10^{8}$
LL2 (Y50H)	$6.4 \pm 0.9 \times 10^{8}$	$8.3 \pm 0.7 \times 10^{6}$
LB4 (S52F)	$8.6 \pm 0.4 \times 10^{7}$	$9.9 \pm 0.8 \times 10^{5}$

of the 16-OH group lowers the affinity of each antibody for gitoxin, relative to both digoxin and digitoxin. Each antibody, but in particular LL2 and LB4, has greater affinity for diginatin, which has both 12-OH and 16-OH, than gitoxin, suggesting that the presence of the 12-OH compensates for impaired binding due to the 16-OH. Given the relatively higher affinities for digoxin *versus* digitoxin displayed by LL2 and LB4, a greater affinity for diginatin than gitoxin is expected for LL2 and LB4, but the slight improvement seen with 26-10 is unexpected.

The affinity of all three antibodies is substantially lower for 12-acetyldigoxin (Table II). In addition, replacing the position 3 sugars with an acetyl group (digoxigenin-3,12-diacetate) further impairs binding. Thus, unlike congeners with a 12-OH, which are indifferent to the substituent at the 3 position, the presence of a 12-acetyl group confers recognition of the 3 position substituent upon the antibodies. The three antibodies are also sensitive to saturation of the C-20–C-22 bond of the lactone ring, as shown by greatly reduced affinity for dihydrodigoxigenin.

The effects of substitutions at the steroid D ring 16 position are summarized in Table III. All antibodies bind gitoxin congeners containing a 16-OH but lacking a 12-OH with slightly higher affinity when the congeners have a sugar at the 3 position (gitoxin, gitoxigenin monodigitoxoside, strospeside) than when they possess a 3-OH (gitoxigenin) or 3-acetyl group (gitoxigenin-3-acetate). Addition of a formyl group at the 16 position (gitaloxin) further decreases affinity and, as is the case with gitoxin, the genin form of the 16-formyl analogue (gitaloxigenin) has slightly lower affinity than the glycoside. A 16-acetyl group causes an even more marked decrease in binding. Glycoside forms of 16-acetylated congeners are again favored over genin and 3-acetylated forms, but 16-acetylgitoxin, which has a tridigitoxose, is bound with higher affinity than those congeners with single sugars (oleandrin, oleandrigenin monodigitoxoside). The same trends were observed for the two mutants, although LB4 exhibits a smaller difference in relative affinity between 16-acetylgitoxin and the 3-acetylated and genin compounds than does 26-10.

The pattern of specificity for ouabain and related compounds is shown in Table IV. Antibody 26-10 binds ouabain and ouabagenin with affinities reduced by one to two orders of magnitude (10, 11; Tables I and IV), relative to digoxin. Both acovenoside A, which has a 1-OH but differs from ouabain in its sugar, and strophanthidol, which has the 5- and 19-hydroxyls of ouabain but no sugar, bind 26-10 nearly as well as digoxin. This suggests that the 11α-OH of ouabain may account for the reduced binding to 26-10. Strophanthidin, with a 19-oxo group in place of the 19-OH of strophanthidol, and several compounds differing from strophanthidin at the 3 position (acetylstrophanthidin, erysimoside, helveticoside, and cymarin) did not differ in binding from strophanthidol or digoxin, indicating an insensitivity to the difference between a 19-OH and 19-oxo group and the groups at the 3 position.

The specificity of LB4 for these compounds varied only slightly from that of 26-10, showing a lowered affinity for acovenoside A, suggesting that the 1-OH may have a minor role in the decreased affinity of LB4 for ouabain. Antibody

TABLE II

Specificity of binding of digoxin analogues

Digoxin analogues were used to compete with ^{125}I-digoxin for binding to antibody 26-10 and its mutants (see "Materials and Methods"). The results are presented as the ratio of concentrations of analogue to digoxin which inhibit 50% of binding of ^{125}I-digoxin to antibody.

Analogue	Substitutions at steroid positions[a]			Ratio of inhibitory concentrations		
	3	12β	16β	26-10	LL2	LB4
Digoxin (digoxigenin tridigitoxoside)	Tridigitoxose	-OH		1	1	1
Digoxigenin bisdigitoxoside	Bisdigitoxose	-OH		1	2	1
Digoxigenin monodigitoxoside	Monodigitoxose	-OH		1	2	1
Digoxigenin	-OH	-OH		2	4	1
3-Epidigoxigenin	α-OH	-OH		4	3	2
Digitoxin	Tridigitoxose			1	8	4
Digitoxigenin	-OH			2	13	5
Evomonoside (digitoxigenin-L-rhamnoside)	L-Rhamnose			3	14	5
Neriifolin (digitoxigenin thevetoside)	Thevetose			2	12	4
Gitoxin	Tridigitoxose		-OH	5	46	15
Diginatin (16-hydroxydigoxin)	Tridigitoxose	-OH	-OH	2	4	2
12-Acetyldigoxin	Tridigitoxose	$-OCOCH_3$		160	150	130
Digoxigenin-3,12-diacetate	$-OCOCH_3$	$-OCOCH_3$		750	930	480
Dihydrodigoxigenin (C-20–C-22 bond saturated)	-OH	-OH		1700	1100	590

[a] Refer to Fig. 1 for steroid numbering system. Orientation of substitutions at the 3 position is β, except as noted.

TABLE III

Specificity of binding of gitoxin analogues

Gitoxin analogues were used to compete with ^{125}I-digoxin for binding to antibody 26-10 and its mutants (see "Materials and Methods"). The results are presented as the ratio of concentrations of analogue to digoxin which inhibit 50% of binding of ^{125}I-digoxin to antibody.

Analogue	Substitutions at steroid positions[a]			Ratio of inhibitory concentrations		
	3β	12β	16β	26-10	LL2	LB4
Digoxin	Tridigitoxose	-OH		1	1	1
Gitoxin	Tridigitoxose		-OH	5	46	15
Gitoxigenin monodigitoxoside	Monodigitoxose		-OH	4	40	14
Strospeside (gitoxigenin monodigitaloside)	Monodigitalose		-OH	5	40	12
Gitoxigenin	-OH		-OH	13	66	22
Gitoxigenin-3-acetate	$-OCOCH_3$		-OH	12	74	29
Gitaloxin (16-formylgitoxin)	Tridigitoxose		-OCHO	30	160	76
Gitaloxigenin	-OH		-OCHO	150	540	170
16-Acetylgitoxin	Tridigitoxose		$-OCHOCH_3$	150	430	220
Oleandrin	Oleandrose		$-OCHOCH_3$	740	3,900	580
Oleandrigenin monodigitoxoside	Monodigitoxose		$-OCHOCH_3$	770	3,300	630
Oleandrigenin	-OH		$-OCHOCH_3$	17,000	>16,000	2,100
Gitoxigenin-3,16-diacetate	$-OCOCH_3$		$-OCHOCH_3$	36,000	>13,000	2,200

[a] Refer to Fig. 1 for steroid numbering system.

LL2 likewise displayed a lowered affinity for ouabain, relative to digoxin, but the reduction cannot be readily attributed to a single substitution. Binding of acovenoside A differs little from ouabain, whereas the other congeners have a higher affinity. In addition there is an overall reduction in the affinity of LL2 for all the ouabain analogues compared to digoxin. This may be due to substitutions at positions 5 and 19 or, more likely, the absence of a 12-OH (see Table II) as binding of LL2 is more dependent than the other antibodies on the 12-OH.

DISCUSSION

Examination of the mammalian humoral response, which attains high specificity by selection of B cell mutants with altered binding (17), can assist in the correlation of antibody structure to function. The increased affinity of antibody response over time (18–20) has been attributed to accumulation of mutations within germline-encoded V regions; experiments employing *in vitro* mutagenesis confirm this (21, 22), identifying binding site residues responsible for improved affinity. *In vitro* selection techniques, whereas most frequently isolating mutants with decreased or absent affinity or idiotype (1–7), also allow identification of residues important in binding antigen.

We used two-color fluorescence-activated cell sorting to isolate multiple spontaneous variants of the anti-digoxin hybridoma line 26-10. Evaluating antigen binding in relation to surface Ig expression avoids selection of lines with altered levels of antigen binding due simply to changes in surface Ig expression. The 26-10 variant lines LL2 and LB4 were selected for high staining with digoxin-human serum albumin-phycoerythrin relative to surface Ig following preincubation with digitoxin. Such a decrease in effectiveness of the digitoxin inhibition might occur by two mechanisms: lowered affinity for digitoxin relative to digoxin or lowered affinity for free hapten relative to the multivalent digoxin-human serum albumin-phycoerythrin conjugate. Both variant lines produce antibody with moderate reductions in relative affinity for

TABLE IV

Specificity of binding of ouabain analogues

Ouabain analogues were used to compete with ^{125}I-digoxin for binding to antibody 26-10 and its mutants (see "Materials and Methods"). The results are presented as the ratio of concentrations of analogue to digoxin which inhibit 50% of binding of ^{125}I-digoxin to antibody.

Analogue	Substitutions at steroid positions[a]					Ratio of inhibitory concentrations		
	1β	3β	5β	11α	19	26-10	LL2	LB4
Ouabain	-OH	L-Rhamnose	-OH	-OH	-OH	35	27	39
Ouabagenin	-OH	-OH	-OH	-OH	-OH	50	24	74
Acovenoside A	-OH	6-Deoxy-3-*O*-methyl-L-talose				2	18	6
Strophanthidol		-OH	-OH		-OH	2	8	3
Strophanthidin		-OH	-OH		=O	2	7	2
Acetylstrophanthidin		-$OCHOCH_3$	-OH		=O	2	7	2
Erysimoside (strophanthidine digilanobioside)		Digilanobiose	-OH		=O	1	4	2
Helveticoside (strophanthidine digitoxoside)		Digitoxose	-OH		=O	1	4	1
Cymarin (strophanthidin-D-cymaroside)		Cymarose	-OH		=O	1	4	1

[a] Refer to Fig. 1 for steroid numbering system.

digitoxin as compared with digoxin (Table II). Homogeneous populations of both LL2 and LB4, however, show lesser inhibition of digoxin-HSA-PE binding by digoxin, ouabain, and digitoxin than does the 26-10 subclone P2.3 (see "Materials and Methods") from which LL2 was derived (data not shown), suggesting that the variants retain affinity for the conjugate while losing affinity for free hapten. Thus both mechanisms may have contributed to the selection of these variants.

The structural bases for the alteration in binding were single amino acid replacements in the antibody H chain CDR2 in each variant (Fig. 2). Antibody LL2 has an H50 Tyr to His mutation; antibody LB4 contains an H52 Ser to Phe mutation. The effects of mutations on affinity were measured in a saturation equilibrium assay using filtration through glass fiber filters to separate bound from free ligand (23). The mutant antibodies have reduced affinities relative to the parent 26-10 (Table I).

Competition studies using a panel of digoxin analogues have provided added insight into 26-10 binding. Although, as noted previously (10, 11, 14), a 12-OH does not contribute to binding, 12-acetylation significantly inhibits hapten-antibody interaction (Table II). Similarly, reductions in affinity were also observed for congeners with substitutions of increasing size at position 16 (Table III). The reduced affinity may be due to steric hindrance caused directly by bulky substitutions at the 12 and 16 positions. Additionally, the 16 substitutions may restrict the rotation of the lactone ring about the C-17–C-20 bond, preventing the orientation required for optimal binding, analogous to the lowered inhibition of Na^+,K^+-ATPase by certain digoxin analogues which was correlated with altered position of the lactone carbonyl oxygen (24, 25).

Saturation of the lactone ring (dihydrodigoxigenin) also reduces affinity (Table II). Because this modification results in a multiplicity of structural alterations (Fig. 4), it is difficult to assign the cause of the lowered affinity.

Based upon the lack of difference in binding of digoxin and digoxigenin and digitoxin and digitoxigenin, it was concluded that the sugars had no role in the interaction between 26-10 and hapten (10, 11). Although the results with digoxigenin, 3-epidigoxigenin, and digoxigenin bis- and monodigitoxoside support this conclusion (Table II), other analogues with different steroid ring substitutions reveal an interaction between antibody and 3-position substituents. The relative affinities for the glycosylated and the aglycone or 3-acetylated forms of the 16-substituted analogues differ. This difference increases with increase in size of the 16 substituent (Table III). Antibody 26-10 also demonstrates a higher affinity for 12-acetyldigoxin than for digoxigenin-3,12-diacetate (Table II). Thus the presence of the tridigitoxose, either by direct binding to antibody or by removal of an unfavorable interaction between antibody and a 3-acetyl or 3-OH group, increases the affinity for analogues substantially modified at the 16 or 12 positions. In addition, the greater relative affinity of 26-10 for 16-acetylgitoxin than for oleandrin or oleandrigenin monodigitoxoside indicates a contribution by the second and/or third digitoxose to binding.

The observation that 3-position substitutions affect binding only in the context of 12- and 16-position substitutions may be due to a relatively minor contribution of the sugar moiety to the overall high affinity interaction between 26-10 and digoxin. When lower affinity interactions are examined, the difference between glycosylated and aglycone analogues may become more prominent. Another possible explanation is that bulky 12- or 16-position groups force a shift in the position of hapten in the binding site, resulting in contact with 3-position substituents.

Consistent with the latter model are the effects on 12-acetyldigoxin and 16-acetylgitoxin, which have similar affinities for 26-10, of 3-acetylation. Although digoxigenin-3,12-diacetate and 12-acetyldigoxin differ moderately in relative affinities, 16-acetylgitoxin and gitoxigenin-3,16-diacetate differ by a much greater margin (compare Tables II and III). The position of 12-acetyldigoxin within the binding site may differ from that of 16-acetylgitoxin, allowing similar affinity but disparate recognition of 3-position substituents.

The high relative affinity of 26-10 for strophanthidol (Table IV) suggests the 5- and 19-hydroxyls of ouabain and ouabagenin are not responsible for their lowered binding. The 1β-OH, at least in the context of the sugar of acovenoside A, also does not inhibit binding. The reduced binding of ouabain could be accounted for by presence of an 11α-OH; however, it is also possible that a combination of the 1, 5, 11, and 19-OH groups or the absence of the sugar of acovenoside A diminishes binding to 26-10. A possible role for the 11α-OH cannot be assigned without testing sarmentogenin (11α-hydroxydigitoxigenin), which was not available.

Antibodies LL2 and LB4 were also assayed to determine if

the reduction in affinity was accompanied by a change in specificity. The specificity differences between 26-10 and LB4 are minor (Tables II–IV). It is possible that the loss of affinity of LB4 is due to steric hindrance caused by substitution of Phe for Ser at H52. Although the Phe could also disturb local conformation of the binding site, it is unlikely there is a global deformation because LB4 still retains relatively high affinity. Alternatively, the loss of affinity could be due to the loss of a hydrogen bond to the Ser hydroxyl.

The Tyr to His mutation at H50 of mutant LL2 confers distinct specificity differences. Although the affinity of LL2 for digoxin was reduced, the specificity for digoxin was enhanced, as shown by a preferential binding to digoxin and diginatin which have a 12-OH (Table II). In addition, LL2 better accommodates the combination of the 1β and 11α hydroxyls of ouabagenin than 26-10 or LB4 (Table IV). Relative to strophanthidol, which lacks these groups, the apparent affinity of 26-10 and LB4 for ouabagenin is reduced by 25-fold, whereas for LL2 the difference is only 3-fold.

The altered specificity and lowered affinity of LL2 may result from a direct interaction between H50 His and the hapten 12 position. The hydrophilic imidazole may preferentially bind to analogues with a 12-OH, but it would otherwise hinder binding of the generally hydrophobic hapten. Alternatively, the His may be oriented differently than the Tyr within the binding site, forcing hapten to bind with lower affinity but permitting a direct interaction between the 12-OH and a hydrophilic side chain elsewhere in the binding site.

Despite the suggestion, based on V region sequence comparisons, that H50 and H52 are residues integral to antibody specificity (26), and the isolation of an H50 mutant with altered recognition for a different hapten (7), it does not necessarily follow that specific complementarity determining region positions are critical in all binding sites. Examination of available Fab x-ray crystal structures also indicate a varying role for H50 and H52 in antigen recognition. Contact between H50 and hapten or antigen occurs in the anti-azophenylarsonate Fab 36-71 (27) and the anti-lysozyme Fabs HyHEL-5 (28) and HyHEL-10 (29). This position, however, is not involved in the binding of the anti-lysozyme Fab D1.3 (30), nor does the H50 Ala in McPC603 Fab (31) contact hapten, despite conservation of this residue in anti-phosphorylcholine antibodies (32). The H50 Gln of anti-fluorescein Fab 4-4-20 is also not directly involved in binding, but it may stabilize the binding site through a hydrogen bond to an H chain Trp residue which does contact hapten (33). The H52 Arg of McPC603 directly contacts phosphorylcholine (31), and H52 is in contact with antigen for anti-lysozyme antibodies HyHEL-5 (28), HyHEL-10 (29), and D1.3 (30). However this residue does not interact with hapten in either Fab 4-4-20 (33) or 36-71 (27).

The role of H50 and H52 in high affinity binding of digoxin by antibody 26-10 may be further examined by site-directed mutagenesis. The information from these experiments, in combination with the results of chain recombination (14, 34) and studies of other 26-10 variants, will provide the means to test and refine combining site models arising from molecular modeling and crystallographic studies.

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SUPPLEMENTAL MATERIAL TO
ALTERED HAPTEN RECOGNITION BY TWO ANTI-DIGOXIN HYBRIDOMA
VARIANTS DUE TO VARIABLE REGION POINT MUTATIONS

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MATERIALS AND METHODS

Haptens [^{3}H]digoxin and [^{3}H]ouabain were obtained from New England Nuclear (Boston, MA). ^{125}I-digoxin was obtained from Cambridge Medical Diagnostics (Billerica, MA). Digoxin, digoxigenin, digoxigenin-3,12-diacetate, digitoxin, digitoxigenin, ouabain, ouabagenin, acovenoside A, oleandrin, oleandrigenin, acetylstrophanthidin, strophanthidol, gitoxigenin-3-acetate, and gitoxigenin-3,16-diacetate were obtained from Sigma (St. Louis, MO). Other analogues (Tables II–IV) were obtained from Serva (Westbury, NY).

Selection of variant cell lines The murine antidigoxin hybridoma 26-10 (IgG2a, K) was isolated following fusion of Sp2/0-Ag14 myeloma cells with A/J immune splenocytes (10). Initial FACS indicated that the surface antibody expression of 26-10 cells was inadequate for efficient sorting of variants. Therefore derivative clones with stable high level expression were selected. Cells were labeled by incubation with digoxin-fluorescein as described (13), or with a conjugate of digoxin, HSA and PE (37,38). Initially the brightest 0.15% of 5×10^6 cells were sorted and grown for 3 weeks. These cells were stained and the brightest 0.2% of viable cells resorted. After another growth period, a subpopulation demonstrated bright staining with digoxin-HSA-PE. These cells, designated 26-10P2, were cloned directly by FACS. The resulting clones expressed high levels of surface antibody; their surface and secreted antibody did not differ from the original as judged by 2-dimensional SDS-PAGE (data not shown). The mutant lines described here were selected from two different subclones, designated 26-10P2.26 and 26-10P2.3.

Mutants were selected by incubating the subclones (4×10^6 cells/ml in RPMI-1640 without dye, with 3% fetal calf serum) with digitoxin (4 nM) for 10 minutes on ice, followed by addition of digoxin-HSA-PE and fluorescein-conjugated goat anti-mouse IgG2a (Southern Biotechnology Associates, Birmingham, AL). The digitoxin concentration was chosen to reduce digoxin-HSA-PE binding by 26-10P2 cells to 1/3 of the level in the absence of inhibitor. Following a 20 minute incubation on ice, cells were washed 3 times and sorted to isolate cells with staining different from that of typical 26-10P2 cells. Between rounds of sorting, selected cells were grown for 8 to 11 days. Cell line 26-10P2.26LB4 (referred to herein as LB4) was cloned following three cycles of enrichment for a population demonstrating lowered sensitivity to digitoxin inhibition. This cell line thus stained brighter with digoxin-HSA-PE relative to staining with fluorescein-conjugated anti-IgG2a as compared to the parent. Cell line 26-10P2.3LL2 (referred to herein as LL2) was cloned in a similar fashion.

Antibody purification Production of antibody in ascites and purification by affinity chromatography on ouabain-amine-sepharose were performed as described (11) except 20 mM ouabain was used for elution. Isotype was determined with a mouse monoclonal antibody isotyping kit (Amersham, Arlington Heights, IL).

Nucleotide sequence analysis Sequencing of V region cDNA by chemical cleavage was initially done as described (39). The sequences were verified and completed by dideoxy chain termination sequencing of cloned copies of PCR amplified V region cDNA. Oligonucleotide primers used for amplification encoded restriction enzyme sites, facilitating cloning into M13 (40).

Protein sequencing Automated Edman degradation of partially reduced and alkylated H and L chains was performed in a Beckman 890C sequencer using a 0.1 M Quadrol program (41,42). At cycles at which proline was N-terminal, *o*-phthalaldehyde treatment was used to reduce background (43). Phenylthiohydantoin-amino acids were identified by high pressure liquid chromatography (44).

Determination of affinity An affinity assay using filtration through glass fiber to separate free from bound ligand was designed. The adherence of antibodies to #32 glass fiber filters (Schleicher and Schuell, Keene, NH) was measured by retention of trichloroacetic acid precipitable counts of radioiodinated antibody (45) under assay conditions (see below). Approximately 90% of 26-10 antibody is retained, which is comparable to a double antibody precipitation method previously used (14). The adherence of radiolabeled 26-10 antibody is not inhibited by up to 50 μg/ml of 26-10 antibody, although undiluted culture supernatants with 16% fetal calf serum reduce adherence to 60%. Washes of up to 20 ml of cold phosphate buffered saline with azide (PBSA; 0.1 M sodium chloride, 0.01 M potassium phosphate, 0.02% sodium azide, pH 7.4) do not affect antibody adherence.

Equilibrium affinity studies were performed using affinity purified antibody or culture supernatant at antibody concentrations of 0.05 to 0.1 K_a. Antibody was diluted into PBSA containing 1% gamma globulin-free horse serum (Gibco, Grand Island, NY) (1%HS-PBSA) or 0.2% BSA (0.2%BSA-PBSA) (Sigma, St. Louis, MO). Solutions of ^{3}H-ligands (0.1 to 10 K_a), were diluted into PBSA. For affinity measurements of antibodies LL2 and LB4 for ouabain, [^{3}H]ouabain was diluted 1:10 and 1:50, respectively, with unlabeled ouabain in order to lower specific activity. Ligand and antibody solutions were combined in duplicate or triplicate in 5 ml polystyrene tubes (No. 55.476, Sarstedt, Princeton, NJ). Nonspecific binding was estimated by substituting 1%HS-PBSA or 0.2%BSA-PBSA for the antibody solution. Incubation volumes varied from 2 ml for the highest affinity interactions to 0.35 ml for lower affinity interactions to maintain adequate signal to noise ratio. Solutions were incubated at 20°C for one to two hours, sufficient to achieve equilibrium as indicated by association kinetics data (see below). Bound ligand was separated from free by filtration, under vacuum, through glass fiber filters using a Millipore 1225 sampling manifold (Millipore, Bedford, MA). Filters were immediately washed with PBSA (1°C), the tubes rinsed once with PBSA, the rinse filtered, and the filters washed again with PBSA. Total volume of the washes was 10 ml. Filters were vials containing scintillation fluid and counted. Affinities were calculated using the LIGAND program (16,46,47) as modified by G. A. McPherson for IBM PC (Elsevier-BIOSOFT, Cambridge, UK). Reported values were obtained by combining a minimum of three separate assays in one fit.

Specificity of antibody for cardiac glycoside congeners Antibody specificity was determined using a competition radioimmunoassay modified from that previously described (14). Briefly, wells of polyvinyl chloride 96-well assay plates were coated with affinity-purified goat anti-mouse Fab (ICN ImmunoBiologicals, Lisle, IL) in PBSA and blocked with 10% HS-PBSA. Culture supernatants (LL2, undiluted; 26-10, 1:100 in 1% HS-PBSA) or purified antibody (LB4, 10 μg/ml in 1% HS-PBSA) were added and incubated for 3 hr at room temperature. These conditions gave the lowest antibody concentration providing maximal binding of ligand. Thereafter, plates were washed, and 25 μl of a solution of digoxin analogue was added in duplicate. Stock solutions (10 mM) of digoxin and its analogues were made in pyridine and diluted (0.1 mM to 0.1 nM) in 15% t-butanol in PBSA. This solvent, without substantially

reducing ligand binding, improved solubility of more hydrophobic digoxin analogues, notably gitoxigenin-3,16-diacetate, as shown by the ability of a 0.1 mM solution to withstand centrifugation (16,000xg, 30 minutes) without pelleting. Relative analogue concentrations of supernatant and pellet fractions were measured by a modified Raymond test (48,49).

Each assay plate contained a series of digoxin dilutions as internal controls. Twenty five μl of ^{125}I-digoxin (50,000 cpm/25 μl, in 1% HS-PBSA, approximately 0.3 nM final concentration) was added to each well. Plates were incubated, washed, and counted as before. The averages of at least three assays are reported as the concentration of analogue required for 50% inhibition of ^{125}I-digoxin binding relative to the concentration of unlabeled digoxin required for comparable inhibition.

Graphic display of digoxin analogues Displays of digoxin analogues were made using coordinates for digoxin (50), dihydrodigoxigenin (51), digitoxigenin (52), gitoxin (53), and ouabain (54). Any hydrogen atoms not mapped in the crystal were added using established parameters (55) and the structures were energy minimized. To ease comparison between structures, the lactone rings were rotated about the C17-C20 bond to an equivalent position. Manipulations were performed using CONGEN (56), and displayed using PLT2[3] for vector drawings and PEER[4] for space-filling display on a Silicon Graphics Personal Iris (Mountain View, CA).

RESULTS AND DISCUSSION

The slope of the inset plot, $\ln(B_d(t)/B_{eq})$ where $B_d(t)$ is bound digoxin at time t and B_{eq} is digoxin bound at equilibrium, is k_{-1}. The true association constant, k_1, is $(k_{ob}-k_{-1})/[[^3H]digoxin]$, and the affinity constant, K_a, equals k_1/k_{-1}. The k_{ob} is 0.32 min^{-1}, the k_{-1} is 0.050 min^{-1}, the k_1 is 1.08 x 10^9 min^{-1} M^{-1}, and the K_a 2.2 x 10^{10} M^{-1}.

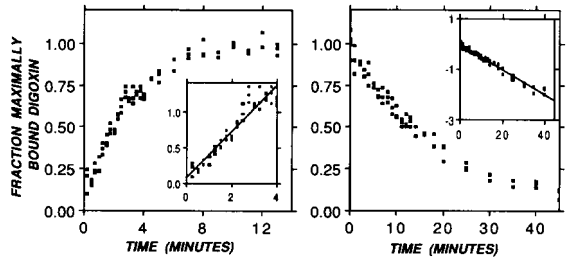

Figure 3 Kinetics of association (left) and dissociation (right) of digoxin and 26-10. Apparent association rate, k_{ob}, was determined by adding [^{3}H]digoxin (0.25nM) to 26-10 (0.012 nM) and stopping the reaction at various time points by filtration (as for affinity assay; see Materials and Methods). The slope of the inset plot, $\ln[B_{eq}/(B_{eq}-B_a(t))]$ versus time where B_{eq} is [^{3}H]digoxin bound at equilibrium and $B_a(t)$ is [^{3}H]digoxin bound at time t, is the k_{ob}. Dissociation rate, k_{-1} (right) was determined by adding a 1000-fold excess of digoxin, relative to [^{3}H]digoxin, to tubes in which association had been shown to reach equilibrium, and stopping the reaction by filtration.

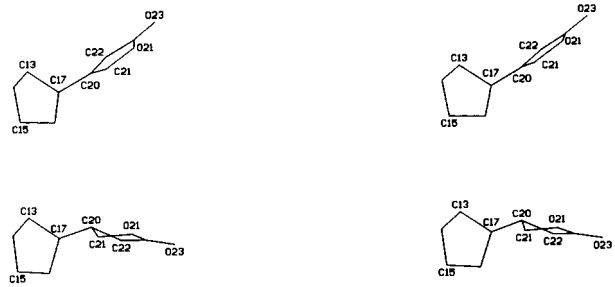

Figure 4 Stereo views of steroid D ring and attached lactone ring of digoxin (top) and (20S)-dihydrodigoxigenin (bottom). Saturation of the C20-C22 bond allows the lactone ring to change from planarity to a half-chair or envelope shape (24,51). In addition, the bonds about C20 change from a planar to a tetrahedral arrangement. This shifts the lactone ring in relation to the steroid body, which is most marked for the lactone carbonyl oxygen. Any binding reliant upon the planarity or resonance of the ring, or the location of the ring in relation to the steroid would therefore be compromised by saturation of the ring. Furthermore the introduction of a chiral center about C20 leads to S and R isomers, in an approximately equimolar ratio (57); it is possible one isomer has a lower affinity for the antibody than the other. Even complete loss of affinity for one isomer, however, would not account for more than a two-fold drop in the affinity, providing affinity for the other isomer is unaltered. A three log drop in affinity is in fact observed (Table II), thus the difference in affinity of 26-10 for digoxigenin and dihydrodigoxigenin must reflect lowered affinity for both isomers.

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[3] R.E.B. and David States, unpublished.

[4] R.E.B., unpublished.