Contributions of Antibody Heavy Chain CDR1 to Digoxin Binding Analyzed by Random Mutagenesis of Phage-displayed Fab 26–10*

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We constructed a bacteriophage-displayed library containing randomized mutations at H chain residues 30–35 of the anti-digoxin antibody 26–10 Fab to investigate sequence constraints necessary for high affinity binding in an antibody of known crystal structure. Phage were selected by panning against digoxin and three C-16-substituted analogues. All antigen-positive mutants selected using other analogues also bound digoxin. Among 73 antigen-positive clones, 26 different nucleotide sequences were found. The majority of Fabs had high affinity for digoxin (\(K_d = 3.4 \times 10^9\) M\(^{-1}\)) despite wide sequence diversity. Two mutants displayed affinities 2- and 4-fold higher than the parental antibody.

Analysis of the statistical distribution of sequences showed that highest affinity binding occurred with a restricted set of amino acid substitutions at positions H33–35. All clones save two retained the parental Asn-H35, which contacts hapten and hydrogen bonds to other binding site residues in the parental structure. Positions H30–32 display remarkable diversity, with 10–14 different substitutions for each residue, consistent with high affinity binding. Thus complementarity can be retained and even improved despite diversity in the conformation of the N-terminal portion of the H-CDR1 loop.

Although our understanding of the mechanisms for antibody (Ab)\(^*\) diversification has increased rapidly from studies at the DNA level using monoclonal antibodies (mAbs), we have insufficient tools to accurately relate the primary structure of hypervariable regions to the three-dimensional structure dictating binding specificity. Wu and Kabat (1) proposed that the hypervariable regions located within variable (V) regions of heavy (H) and light (L) chains were the sites of amino acid residues conferring Ab specificity and folded together to form Ab combining sites. Subsequently, x-ray crystallographic analyses on hapten binding and anti-protein Fab fragments (reviewed in Refs. 2–5) revealed that the antigen combining cavities are indeed composed of the H and L chain hypervariable or complementarity-determining regions (CDRs). Certain CDR sequences have been shown, in general, to function in the context of different framework sequences, as the framework three-dimensional folding is remarkably uniform (6, 7). Moreover, there is evidence for a small repertoire of homologous “canonical” CDR loop conformations (8, 9). The correlation between antibody-combining site structure and binding function has been studied intensively using mutagenesis, modeling, and three-dimensional structure determination.

The model system we use for studies of antigen-Ab complementarity through protein engineering is based on a set of murine mAbs to the cardiac glycoside digoxin. Several features of these antibodies and the hapten lend themselves to this objective. Cardiac glycosides consist of a cardenolide steroid ring structure with a C-14 β-OH, attached β-glycosidic moieties at C-3 and a 17β unsaturated five-membered lactone ring (Fig. 1). Digoxin is a relatively rigid large hydrophobic hapten without charged groups. There are hundreds of structurally related natural and synthetic cardenolide analogues of known crystal structure or stereochemistry (reviewed in Ref. 10), permitting studies of fine specificity. In addition, anti-digoxin antibodies have relatively high affinity compared to antibodies against smaller hapten.

The three-dimensional structure of the high affinity anti-digoxin 26–10 Fab in the uncomplexed state and complexed with digoxin has been determined (11). Neither the antibody nor digoxin undergoes any significant conformational change upon forming the complex. Neither hydrogen bonds nor salt bridges are formed between 26–10 and digoxin. High affinity binding occurs solely by shape complementarity involving extensive nonpolar interactions between antibody and the hydrophobic hapten.

We used in vitro selection to isolate variants of 26–10 with altered affinity or hapten binding specificity (12, 13). Among a set of 11 independent spontaneous mutants of 26–10, mutation at several H chain residues affected affinity. Some of these proved to be hapten contact residues, as shown by the crystal structure (11). Among these spontaneous variants, recurrent mutations at H chain residue 35 were observed (13).\(^*\) The complementarity between the 26–10 Asn-H35 residue and digoxin was analyzed further by constructing mutants using site-directed mutagenesis in a hybridoma system (13). In order to advance our understanding of the complementarity between Ab 26-10 and digoxin, we turned to a phage display system.

The demonstration that both Fv (14) and Fab (15) can be produced correctly folded in bacteria by expression in the periplasmic space was a critical step, which has accelerated experimental antibody-combining site engineering. Display of

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The abbreviations used are: Ab, antibody; mAb, monoclonal antibody; Fab, antigen-binding fragment of antibody; Fd, H chain variable region and first H chain constant region; V, variable region; Fv, antibody fragment including heavy and light chain variable regions only; sFv, single-chain Fv; CDR, complementarity-determining region; bp, nucleotide base pair; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; LB, Luria-Bertani medium.

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\*S.-Y. Shaw, D. Parks, L. Herzenberg, and M. M. Margolies, unpublished observations.
Anti-digoxin Antibody Mutagenesis

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C-16-substituted digoxin analogues, to probe the 

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divere libraries of V regions as Fab or single-chain Fv (ScFv) on 

the surface of filamentous bacteriophage has permitted selec- 

tion of rare antibodies with altered binding affinity (16–18). 

Here we use a phage-displayed 26–10 Fab library, randomized 

in the region of H chain CDR1 and selection with digoxin and 

C-16-substituted digoxin analogues, to probe the 26–10 comb- 

ing site.

MATERIALS AND METHODS

Vector Construction—Sequences encoding the 26–10 Fab, from H 

chain position Glu-H1 to Arg-H228 in the hinge region (12, 19), and the 

entire L chain (Asp-L1 to Cys-L214), were introduced into the pComb3 

vector (20) for expression on the surface of bacteriophage M13 and 

altering the amino acid sequence. Primer 1 introduces an 

Aat II site in the 5’ terminus of the 26–10 H chain and the MunI site (Table 

1, Fig. 2B). The 147-bp product of this reaction encodes the 5’ V region 

of 26–10 H chain through CDR1 and the adjacent 

nucleotide position 504 bp. 

This 147-bp fragment was digested with Ncol and MunI, and the 

resulting 114-bp fragment was dialyzed and concentrated using a Cen-

tricon 30 filter unit (Amicon, Beverly, MA), ligated into a Ncol-MunI-
digested pComb3–26–10–2 vector, and introduced by electroporation into E. coli XL1-Blue F’ cells. Inflection of the XL1-Blue library of CDR1 

mutants with VCSM13 helper phage generated a library of phage with surface Fab (XL1-Blue’ F’) and VCSM13 are from Stratagene, LaJolla, 

CA). Phage were recovered and concentrated by polyethylene glycol/ 

NaCl precipitation from bacterial supernatants (20). Bacteriophage 

yield was quantitated by titration on lawns of XL1-Blue F’ bacteria, and 

phagemid was quantitated by postinfection XL1-Blue F’ colony forma-

tion on LB (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, pH 7.0)/agar/ 

carbonic acid plates (22, 24).

Biopanning—Bacteriophage were panned on glycoside-BSA-coated 

microtiter wells (Costar 3690, Cambridge, MA) according to Barbas and 

Lerner (24). In preliminary experiments, phage encoding an anti-teta-

nus toxoid Fab (kind gift of C. F. Barbas and D. R. Burton) were used as 

negative control. 

Phage were analyzed after four successive rounds of biopanning. 

Phagemid was grown without VCSM13 helper, and DNA was isolated 

from a 50-ml culture using a Plasmid Midi Kit (Qiagen, Chatsworth, 

CA). An aliquot was sequenced to estimate the complexity of the se-

quences in the mutated CDR1. A 1-μg sample was cut with Ndel and 

SspI and religated (see Fig. 2A) to remove the gene III sequence that 

serves as a membrane anchor (20, 24). Fab secreted from cells contain-

ing these phagemids terminate at Arg-228 in the hinge region of 26–10 

and contain a gene III-encoded Thr and Ser just 5’ to the stop codon. 

After religation, the DNA was transformed into XL1-Blue cells and 

placed on LB/carbonic acid plates. Bacterial colonies were isolated and 

stained for Fab production and antigen binding.

Fab Production—Each colony was grown in superbroth (3% tryptone, 

2% yeast extract, 1% 3-(N-morpholino)propanesulfonic acid, pH 7.0) 

containing carbenicillin at 37 °C to an A595 of 1.0. Isopropyl b-thio-

galactopyranoside (Sigma) was added to the culture, and incubation con-

tinued overnight. Culture supernatants and bacterial periplasmic space 

extracts prepared by osmotic lysis (26) were tested in an ELISA for Fab 

production level and binding to cardiac glycoside-BSA conjugates (see 

below). If a majority of the clones were Fab positive/glycoside negative,
additional rounds of panning were performed, and the output was analyzed. Yield of Fab was determined by ELISA titration on goat anti-mouse Fab-coated plates using enzymatically prepared 26–10 Fab of known concentration as a standard. The DNA sequences of cardiac glycoside ELISA-positive clones were determined using the dideoxy sequencing method.

**ELISA**—Bacterial supernatants were tested in a direct binding ELISA in a 96-well microtiter plate (Falcon 3911, Becton Dickinson, Oxnard, CA) coated with BSA alone, cardiac glycoside-BSA, or goat anti-mouse Fab (ICN, Costa Mesa, CA). Wells were coated with 50 μl of 100 μg/ml antigen in PBSA and incubated at 4 °C overnight. Wells were washed with water several times, blocked with 100 μl of 10% horse serum in PBSA, and incubated at room temperature for 2 h. After another wash, 50 μl of bacterial supernatant was added, and incubated for 3 h at room temperature. After washing to remove free Fab, bound Fab was detected after a 2-h room temperature incubation with peroxidase-labeled F(ab′)2 fragment of goat anti-mouse IgG and IgM (Jackson ImmunoResearch, West Grove, PA) using the TMB microwell peroxidase substrate system (KPL, Gaithersburg, MD) and neutralization with 1.0M phosphoric acid. Color development was measured at 450 nM in a Bio-Tek ELISA reader (Bio-Tek, Winooski, VT). The 26–10 Fab, prepared by papain digestion of affinity-purified 26–10 Ig (27), was used as a positive control and standard for estimation of Fab concentration and digoxin binding activity of the bacterial

### Table I

| Location       | Nucleotides              | Sequence (5'-3')       |
|----------------|--------------------------|------------------------|
| 1. pdB & 5' H chain | GCCCAACCGAGTGCCGAGTGGCTGCAATGCACAGGTCCGGACCTG | MunI                  |
| 2. 3'H chain & M13 gene III | AGAGCCACCGCCACACTAGTTCGTCCTGCAATTTTCTT | SpeI                  |
| 3. 5'L chain    | GCCAAGACCGGATGGCCGAGTGGCTGATGACCCACGACCTCCA | AatII                 |
| 4. 3'L chain & stop codon | CTAAACCTCTAGAAATTACACTCATCTCTGATGAA | XbaI                  |
| 5. pdB & 5'H chain | GCCAAGACCGGATGGCCGAGTGGCTGATGACCCACGACCTCCA | MunI                  |
| 6. 3'H chain & M13 gene III | CAGAAGACTGIGGTGCTGCTGGCTCT | AatII                 |
| 7. 5'L chain    | TCACAGACGTCGCCATCGCTGGTGCCC | AatII                 |
| 8. 3'L chain & stop codon | AATGAGTGTTAATTTGTTAGGATTAAG | XbaI                  |
| 9. 26-10 H chain CDR1 | ATTCACTGACCTACAGGCTGGTGAGGCGGACAG | XbaI                  |
| 10. 26-10-H-CDR1 NNS randomized | CTTCCATGGCTGCTGCTAGCCA(SNN)GAAATATCTACGAGACTG | NcoI                  |

* Newly generated restriction enzyme sites are indicated below the underlined sequences.

**Fig. 2.** Diagram of the construction of the vector pComb3-26-10 and generation of randomly mutated residues H30–35. A, the portion of the pComb3 bacterial expression vector (20) containing the lacZ promoters, pelB leader sequences, insertion sites for the Fd portion of the H chain and the L chain, and the GGGS linker at the M13 coat protein III (cpIII) fusion site. The MunI and AatII restriction enzyme sites were introduced to provide sites for insertion of 26–10 H and L chains that would not alter the wild type amino acid sequence. The resulting first four residues of the H chain (EVQL) and the first two of the L chain (DV) and Ala(A) encoded by the pelB leader are indicated with the single letter codes. Numbered arrows refer to the oligonucleotides used in these manipulations (see Table I). B, the 26–10 H chain V region with the oligonucleotides used for alteration of the polymerase chain reaction template and randomization of amino acids H30 to H35. Nucleotide numbering is in italics.
supernatant Fab. The anti-arsonate Fab 36–71 (28) was used as a negative control. ELISA was also performed on phage-displayed Fab using the same reagents and methods but detected with peroxidase-labeled anti-M13 (Pharacia Biotech Inc.).

SDS-Polyacrylamide Gel Electrophoresis, Western Blot, and Protein Sequence Analysis—Bacterial supernatants and enzymatically prepared Fab were analyzed using Crossmas Blue-stained, SDS, 10–17% polyacrylamide gel electrophoresis under reducing and nonreducing conditions. For Western blot analysis following electrophoresis, the proteins were electroblotted onto polyvinylidene fluoride membranes (Millipore, Bedford, MA) and developed using peroxidase-labeled goat anti-mouse antibody and enhanced chemiluminescence reagents (Amersham). An aliquot of purified bacterial 26–10 Fab was filtered through a ProSpin column (Applied Biosystems, Foster City, CA), and protein sequence was determined using an Applied Biosystems gas phase sequencer.

Affinity and Specificity Determination—Affinities for digoxin were measured on dilute bacterial supernatants with a saturation equilibrium assay using filtration through glass fiber filters to separate bound and free [3H]digoxin (DuPont NEN). This method differs from a previously published assay (12, 29), which used intact anti-digoxin antibodies, by the addition of goat anti-mouse Fab antibody (ICN) to retain the Fab[3H]digoxin complex on the surface of the filter, as Fab was not otherwise efficiently retained during the separation step. Each tube contained an amount of Fab, one of 2 serial dilutions of [3H]digoxin (1.1 × 10−7 M to 6.7 × 10−11 M, depending on preliminary screening), and 0.5 μg/ml goat anti-mouse Fab antibody. Scatchard analysis on data from two or more independent experiments was used to determine affinities, except for done D4–3.

The specificity of Fab for different cardiac glycosides was determined using a competition radioimmunoassay based on the affinity assay described above (12, 13, 29), with the addition of 0.5 μg/ml goat anti-mouse Fab antibody to each sample to retain Fab on the filter. Stock solutions of cardiac glycosides (10−2 M in pyridine) were diluted in PBSA to result in 10−5 to 10−12 M in the final mixture. Each mutant Fab was competed with digoxin as an internal control. The values reported are ratios of molar concentrations of inhibitor required to give 50% inhibition relative to the molar concentration of digoxin that gave 50% inhibition.

Mixing Experiments—In order to determine whether clones that dominated in panning using one glycoside could be selected by another glycoside if present in equal proportions in the original phage preparation, mixing experiments were performed. Individual clones were grown separately with helper phage using the same method employed for eluted phage during panning. Each preparation was titrated independently, and a mixture was prepared with equal amounts of phage from several clones. This mixture was panned three times against BSA conjugates of digoxin, gitoxin, 16-formylgitoxin, and 16-acetylgitoxin. An unpanned aliquot of the mixture was expanded and processed each day in parallel with the phage eluted from the panning wells to control for selection of a clone due to growth advantage. The outputs were first analyzed by DNA sequence of a batch DNA preparation of phagemid proving contaminated with unmutated phagemid having XbaI SpeI I boundaries were verified as correct. When transduced the gene III coding fragment of the phagemid by XL1-Blue. Bacterial Fab was indistinguishable from enzymatically prepared 26–10 Fab by Western blot using isotype-specific reagents and by ELISA utilizing digoxin-BSA conjugates (data not shown). Because periplasmic space preparations and secreted preparations both yielded about 1 mg/liter of 26–10 Fab, the more easily prepared secreted samples were used thereafter.

Bacterial 26–10 Fab was purified on a ouabain-amine-Sepharose column (32). This preparation was indistinguishable from enzymatically prepared 26–10 Fab by SDS-polyacrylamide gel electrophoresis. Amino acid sequence analysis of bacterial 26–10 Fab resulted in a mixture of H and L chain amino acids in a 1:1 ratio with residues identical to the bona fide hybridoma antibody 26–10 (12). This result indicated that cleavage of the pelB leader did not result in a product of different length and that the N-terminal sequences of 26–10 H chain and L chain in pComb3 had been correctly modified.

In preliminary biopanning experiments the phagemid library proved contaminated with unmutated phagemid having the same nucleotide sequence as wild-type 26–10. In order to avoid contamination, a nonfunctional template designated pComb3–26–10–2 was designed. pComb3–26–10–2 was used to create a library of mutants randomized at H chain positions 30–35 inclusive. We randomized the H chain CDR1 amino acids 31–35 as well as the nominal framework residue 30. Randomization was achieved using nucleic acid mixtures containing all four dNTPs (N) or dCTP and dGTP (S), in the order NNS at the three codon positions of the H chain 30–35 amino acids. This results in a complexity of 6.4 × 103 different codon combinations.

Phage aliquots were panned against four cardiac glycosides differing at position 16 (Fig. 1) on the cardenolide moiety (16-H, digoxin; 16-OH, gitoxin; 16-OCHO, formylgitoxin or gitaloxin; and 16-OCOCH3, 16-acetylgitoxin) (Table IV). These analogues were chosen because HCDR1 residues are close to position 16 in the co-crystal structure of 26–10-digoxin (Fig. 3 (11)).

Initially, the library was panned against digoxin-BSA and 16-acetylgitoxin-BSA. Following four rounds of panning, 20 clones for each cardiac glycoside were isolated, and the secreted products were tested for binding to digoxin, 16-acetylgitoxin, and goat anti-mouse Fab (Table II). Using an ELISA, all digoxin-selected clones secreted Fab and bound digoxin-BSA. All digoxin-selected mutants except mutants D4–3 (see Table II, footnote a, for nomenclature) and D4–12 bound 16-acetylgitoxin and digoxin, with the most potent clones binding 16-acetylgitoxin and digoxin at 16-fold lower concentrations than digoxin. This is consistent with a competition ELISA performed on dilute bacterial supernatants with a saturation equilibrium assay using filtration through glass fiber filters to separate bound and free [3H]digoxin (DuPont NEN). This method differs from a previously published assay (12, 12), which used intact anti-digoxin antibodies, by the addition of goat anti-mouse Fab antibody (ICN) to retain the Fab[3H]digoxin complex on the surface of the filter, as Fab was not otherwise efficiently retained during the separation step. Each tube contained an amount of Fab, one of 2 serial dilutions of [3H]digoxin (1.1 × 10−7 M to 6.7 × 10−11 M, depending on preliminary screening), and 0.5 μg/ml goat anti-mouse Fab antibody. Scatchard analysis on data from two or more independent experiments was used to determine affinities, except for done D4–3.

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d$^*$ M. Sullivan, personal communication.
toxin. Nineteen of 20 of the 16-acetylgitoxin-selected clones contained Fab and bound both cardiac glycoside conjugates. DNA sequence was obtained for the entire H chain V region of each mutant Fab. There were no sequence alterations outside the mutated region, and all selected clones had G or C in the third codon position. After four rounds of panning, 15 unique sequences were present in the 20 clones selected by digoxin-BSA and only two unique sequences in the 19 clones selected by 16-acetylgitoxin. In every instance, clones selected more than once had identical nucleic acid sequences.

After four rounds of panning, the Asn (N) residue at position H35 was present in 13 of 15 unique sequences selected by digoxin-BSA and both sequences selected by 16-acetylgitoxin (Table II). The exceptions were Val-H35 in clone D4–3 and Ser-H35 in clone D4–15. Clone D4–3 differs from clone D4–11 only at position H35. Clone D4–15 has Asn at position H34, while other digoxin selected clones have Phe, Ile, Val, or Tyr at H34. The wild-type Met-H34 was not found in any mutant. Position H33 was predominantly Phe, Tyr, or Trp. Positions H30–32 demonstrated a less restricted set of amino acids. At position H32 we observed Phe, Gln, Arg, Ser, Thr, Gly, Met, Ala, or Leu. Positions H30 and H31 contained the most varied sets of amino acids found in the mutated segment, although there is some preference for the non-wild-type Pro at position H30 and the wild-type Asp at H31.

The parental 26–10 sequence was not panned from this library, although the sequence chosen five times by digoxin-BSA selection (RDFYYN) differs by only two residues from 26–10. The wild-type residues Thr-H30 and Met-H34 do not appear in any of the mutants selected by these two cardiac glycosides.

Another aliquot of the same library was grown and selected against gitoxin-BSA and 16-formylgitoxin-BSA. Four rounds of panning produced 6/20 clones ELISA-positive for gitoxin binding and 0/20-positive for 16-formylgitoxin binding. All were producing approximately 1 µg of Fab/ml of media. Two additional rounds of panning were carried out, and 10 clones were analyzed for each analogue from pan 6. All pan 6 gitoxin-BSA-selected clones were positive for Fab production and bound both gitoxin and digoxin. Eight of 10 16-formylgitoxin-BSA clones selected from pan 6 were positive for Fab production, 16-formylgitoxin binding, and digoxin binding.

DNA sequence analysis of the gitoxin-BSA and 16-formylgitoxin-BSA pan 6 clones revealed a limited set of sequences. Seven of 10 of the gitoxin-selected clones were RDFYYN, two contained GERFFN, and one was SKRYIN. Of the 16-formylgitoxin clones sequenced, six of eight had the sequence SHSYIN and two were TRYWFN. Using 16-formylgitoxin-BSA, we did not observe the mutant RDFYYN, which dominated the output of the final pans in the experiments using the other three cardiac glycosides, even though the phage preparation used was an aliquot of the same library.

To seek a more diverse sample of positive clones, 10 clones from pan 5 on gitoxin-BSA and 16-formylgitoxin-BSA were analyzed. All 10 gitoxin-BSA-selected clones were ELISA-positive for gitoxin and digoxin, while six of 10 16-formylgitoxin-BSA clones were positive for 16-formylgitoxin and digoxin binding (Table II). The corresponding amino acid sequences are shown in Table II. Pan 5 contained a more diverse group of clones than pan 6. Two of the gitoxin-selected clones from pan 6 were also selected in pan 5 (shown by asterisk), and four additional pan 5 sequences were found. All antigen-binding clones had Asn-H35. The distributions of amino acids at positions 33 and 34 were similar to those in the digoxin-BSA-selected group. Clones from the 16-formylgitoxin pan 5 group contained both clones seen in pan 6 and two additional unique clones. Shown for comparison in the table are sequences from clones that did not bind either digoxin or 16-formylgitoxin. Two of these (mutants F5–1 and F5–7) had a residue other than Asn at position H35, and two others (mutants F5–2 and F5–8) had a pair of adjacent prolines.

The most prevalent amino acids at each position in unique clones selected by all four analogues shown in Table II are (H30–35) PD(F/Y)(F/W/Y)(I/F/Y)(N). This “consensus” is based...
whether the clone binds to the congener used in selection.

**Table II**

| Cardiac glycoside | Fab | H chain amino acid sequence (positions 30–35) | Frequency of selection | Antigen binding
|-------------------|-----|-----------------------------------------------|-----------------------|-----------------|
| 26–10             | D4–1 | W D L W Y N                                  | 1                     |                 |
| Digoxin           | D4–2 | P G R Y I N                                  | 1                     |                 |
|                  | D4–3 | N N Y F F V                                  | 1                     |                 |
|                  | D4–4 | G G S G I N                                  | 1                     |                 |
|                  | D4–5 | M P S Y I N                                  | 1                     |                 |
|                  | D4–6 | P P A F Y N                                  | 1                     |                 |
|                  | D4–7 | R D F Y Y N \(N^{*}\)                        | 5                     |                 |
|                  | D4–8 | P D L Y I N                                  | 1                     |                 |
|                  | D4–9 | M P Q Y V N                                  | 1                     |                 |
|                  | D4–10| R G F Y Y N                                  | 1                     |                 |
|                  | D4–11| N N Y F F N                                  | 1                     |                 |
|                  | D4–12| A R M Y I N                                  | 1                     |                 |
|                  | D4–15| D D T Y N S                                  | 1                     |                 |
|                  | D4–17| H D F F F N                                  | 2                     |                 |
|                  | D4–18| R G L Y Y N                                  | 1                     |                 |
|                  | A4–19| P S F Y Y N                                  | 1                     |                 |
|                  | A4–20| R D F Y Y N \(N^{*}\)                        | 18                    |                 |
| 16-Acetylgitoxin  | G6–1 | G E R F F N                                  | 2                     |                 |
|                  | G6–2 | S K R Y N \(N^{*}\)                          | 4                     |                 |
|                  | G6–3 | R D F Y Y N \(N^{*}\)                        | 7                     |                 |
|                  | G5–1 | R D F Y Y N \(N^{*}\)                        | 4                     |                 |
|                  | G5–2 | S M Q W F N                                  | 1                     |                 |
|                  | G5–3 | R R G I V N                                  | 2                     |                 |
|                  | G5–4 | K D M Y I N                                  | 1                     |                 |
|                  | G5–6 | S K R Y I N \(N^{*}\)                        | 1                     |                 |
|                  | G5–7 | P F R F I N                                  | 1                     |                 |
| 16-formylgitoxin  | F6–1 | T R Y W F N \(N^{*}\)                        | 2                     |                 |
|                  | F6–2 | S H S Y I N \(N^{*}\)                        | 6                     |                 |
|                  | F5–3 | R T R Y I N                                  | 1                     |                 |
|                  | F5–4 | S P W F N \(N^{*}\)                          | 1                     |                 |
|                  | F5–5 | S H S Y I N \(N^{*}\)                        | 3                     |                 |
|                  | F5–10| P W T L L N                                  | 1                     |                 |
|                  | F5–1 | S N L E D S                                  | 1                     |                 |
|                  | F5–2 | S G R F Y N                                  | 1                     |                 |
|                  | F5–7 | Y N R S Q A                                  | 1                     |                 |
|                  | F5–8 | L P P T S N                                  | 1                     |                 |

*a* Clones are listed with a single initial for the glycoside used in selection: D, digoxin; A, 16-acetylgitoxin; G, gitoxin; F, 16-formylgitoxin. The first number represents the panning cycle at which the clone was obtained. The number following the dash is the sample number for each clone rescued from a given panning round.

*b* Secreted Fab was induced using an ELISA with goat anti-mouse Fab, digoxin-BSA, and the congener-BSA used for selection as coating antigens. All clones were positive for Fab. The designation + or − refers to whether the clone binds to the congener used in selection.

Amino acid sequences of clones selected by different cardiac glycosides from a 26–10 Fab phage library randomized at H chain positions 30–35 and of an antigen binding ELISA are listed.

**Table III**

| Fab | Affinity\(^{a}\) | Heavy chain amino acid sequence (positions 30–35) |
|-----|------------------|-----------------------------------------------|
| 26–10 E \(^{b}\) | 5.4 ± 0.6 | T D F Y M N |
| 26–10 B | 5.3 ± 1.9 | T D F Y M N |
| A4–19 | 21.7 ± 8.1 | P S F Y Y N |
| A4–20 | 9.7 ± 3.8 | R D F Y Y N |
| D4–1 | 3.4 ± 0.2 | W D L W Y N |
| D4–2 | 5.7 ± 1.1 | P G R Y I N |
| D4–3 | 0.006 | N N Y F F V |
| D4–4 | 0.9 ± 0.1 | G G S G I N |
| D4–5 | 3.5 ± 0.2 | M P S Y I N |
| D4–6 | 1.8 ± 0.5 | P P A F Y N |
| D4–8 | 4.5 ± 0.1 | P D L Y I N |
| D4–9 | 2.0 ± 0.6 | M P Q Y V N |
| D4–10 | 6.6 ± 1.6 | R G F Y Y N |
| D4–11 | 6.6 ± 1.0 | N N Y F F N |
| D4–12 | 4.0 ± 0.2 | A R M Y I N |
| D4–15 | 1.8 ± 0.2 | D D T Y N S |
| D4–17 | 5.1 ± 0.3 | H D F F F N |
| D4–18 | 5.6 ± 0.1 | R G L Y Y N |
| G6–1 | 4.3 ± 0.6 | G E R F F N |
| G6–2 | 0.3 ± 0.033 | S K R Y I N |
| F6–1 | 3.9 ± 0.6 | T R Y W F N |
| F6–2 | 5.6 ± 1.7 | S H S Y I N |

\(^{a}\) Affinities (\(K_{d}\)) for digoxin of Fab 26–10 and mutants (randomly mutated at positions H30–35 inclusive) selected from the bacteriophage library by digoxin (D), gitoxin (G), 16-acetylgitoxin (A), and 16-formylgitoxin (F). Affinities were measured using an equilibrium saturation method with filtration through glass fiber filters for separation of bound and free ligand (33), except that Fab was immobilized on the filter using goat anti-mouse Fab.

26–10E refers to Fab enzymically prepared from hybridoma protein while 26–10B was prepared from bacterial supernatants in a manner identical to that for the mutant samples.

with affinities in the range of that of 26–10 had Asn-H35. Clone D4–3 had an affinity reduced 5000-fold compared with parental and a Val at position H35. Clone G6–2 had reduced affinity (3.0 × 10\(^{-9}\) M \(^{-1}\)) for digoxin of 26–10had Asn-H33 instead of Tyr compared with the parental and had reduced affinity for digoxin (9 × 10\(^{-8}\) M \(^{-1}\)). Among 20 different mutant clones analyzed, 17 had affinities for digoxin equal to or greater than 1.8 × 10\(^{-9}\) M \(^{-1}\). All mutants with Tyr-H34 had affinities for digoxin equal to or greater than parental 26–10.

The specificity of mutant Fab for different cardiac glycosides was compared using a competition assay (Table IV). Binding to the other cardiac glycosides was not enhanced compared with digoxin. Binding of mutants F6–1 and F6–2 to gitoxin was reduced 4–9-fold compared with wild-type 26–10, and binding of mutant G6–2 to 16-acetylgitoxin was reduced 30-fold.

To determine whether there was another characteristic besides relative avidity for cardiac glycoside-BSA that served as a basis for mutant selection, a series of mixing experiments were performed (Table V). In the first experiment, A4–20 phage was mixed with wild-type 26–10. Following a single round of panning, the only sequence detectable was that of A4–20 (data not shown). In a second experiment, we mixed clones A4–19, G6–1, G6–2, F6–1, F6–2, and 26–10. After three rounds of panning, the batch sequences from digoxin-BSA, 16-acetylgitoxin-BSA, and 16-formylgitoxin-BSA pannings were exclusively A4–19. The only exception was the sample panned against gitoxin-BSA, which showed a mixture of G6–2 and A4–19. The un-
The importance of the identity of position H35 (Asn) to digoxin binding characteristics of a set of spontaneous variants and engineered mutants of 26–10 were determined (12, 13, 29, 33).

Parental 26–10 was observed only twice among 10 sequences selected by digoxin and 16-acetylgitoxin showed no predominant sequence (data not shown). In a third experiment, G6–1, G6–2, F6–1, F6–2, and 26–10 were mixed. DNA sequences from the pooled output of the third pan against each glycoside revealed selection bias, prompting sequencing of 10 individual clones from each analogue (Table V). None of the unselected clones were G6–1, suggesting that a growth advantage exists for G6–1. Seven of 10 clones selected by gitoxin-BSA and 16-formylgitoxin-BSA were G6–2 despite the low affinity for digoxin of G6–2 (Table III). Clones selected by digoxin and 16-acetylgitoxin showed no predominant sequence. Parental 26–10 was observed only twice among 10 sequences selected by digoxin-BSA. A4–19 and A4–20 dominated in mixing experiments and had the highest affinities for digoxin. However, certain clones, such as G6–2, were able to outcompete the others in panning against specific analogues.

To determine panning efficiency, the number of colonies for digoxin BSA alone (output) divided by the number of colonies in the aliquot of input bacteriophage was measured. The 26–10 phage and the negative control 36–65 phage bound gitoxin, 16-acetylgitoxin, and 16-acetylgitoxin conjugates nearly equal to BSA and then the negative control 36–65 phage bound gitoxin, 16-acetylgitoxin, and 16-acetylgitoxin conjugates. The 26–10 phage bound to the glycoside-BSA well minus the number bound to BSA alone. (Table II). Phage 26–10 bound digoxin-BSA with a panning efficiency of 2.8 \times 10^3. In contrast, A4–3, A4–19, G6–1, G6–2, F6–1, and F6–2 all showed panning efficiencies for digoxin at least an order of magnitude higher than 26–10 phage.

In order to examine a wider variety of mutants that potentially alter digoxin binding, we used phage display of 26–10 Fab and randomized H chain resin 30–35 including H chain CDR1 (residues 31–35). By enriching and selecting mutants by panning on antigen-coated surfaces, we attempted to investigate several questions. To what degree do structural constraints on digoxin binding limit sequence diversity in the CDR loop? Is there more than one solution for sequences in this segment consistent with high affinity digoxin binding? Are there mutants with enhanced affinity or altered specificity relative to the wild-type 26–10?

Bacteriophage libraries were selected by binding to digoxin-protein conjugates and analogues of digoxin conjugated to proteins, to probe the structural requirements for antigen binding. We chose three digoxin analogues that bear substitutions at the cardenolide C-16 position (see Fig. 1) of varying size, with corresponding effects on binding to 26–10 as compared with digoxin (12, 13, 29). We displayed Fab rather than sFv, as reduced binding may be observed for certain sFv constructs, which perforce contain a synthetic interdomain peptide linker (34).

The pComb3 vector (20, 24) dictates certain amino acids at the N terminus of both H and L chains when DNA coding for Fab is inserted at the existing restriction sites. These sequences differ from those of 26–10 nucleotides encoding the first four amino acids of the H chain and first two of the L chain (Fig. 2). We previously demonstrated that H chain N-terminal sequence differences of anti-digoxin antibodies cause reduction in affinity for digoxin (35, 36). These results were confirmed by mutagenesis in which short truncations and point mutations of the N terminus altered affinity for digoxin. A single engineered mutation in the H chain signal peptide sequence at the –2 position of 26–10 resulted in an antibody with three additional residues at the N terminus associated with a 100-fold reduction in affinity for digoxin.
Anti-digoxin Antibody Mutagenesis

![Stereo views of H-CDR1 and digoxin from the x-ray crystal structure of the 26–10 Fab-digoxin complex (11).](image)

FIG. 4. Stereo views of H-CDR1 and digoxin from the x-ray crystal structure of the 26–10 Fab-digoxin complex (11). The digoxin molecule is on the left with the lactone at the bottom. At the top is shown a single attached sugar (digoxigenin monodigitoxoside). Tyr-H33 and Asn-H35 of 26–10 CDR1 make van der Waals contact with the hapten.

The H-CDR1 loop of antibody 26–10 contains residues at positions H26, H27, H29, and H34, also identified in a canonical H-CDR1 loop described by Chothia et al. (8) that are thought to be important for packing H-CDR1 against the framework region. However, 26–10 lacks Arg at position H94, which is presumed to be important in maintaining the H-CDR1 loop structure. In addition, such a canonical loop conformation would be incompatible with the structure of 26–10 (11) because of interactions of H-CDR1 with residues in H-CDR2 and H-CDR3. Pro-H52 in H-CDR2 would clash with the conserved aromatic at H29 in the “canonical” structure, Tyr-H53 would clash with the side chain of Thr-H30, and Ser-H96 in H-CDR3 would clash with the side chain of Tyr-H32 (Phe in 26–10).

There is no evidence from the electron density maps of a well defined conformation for residues H27–30, although the possible backbone conformations are somewhat restricted by the end point connections at H26 and H31. This disorder is also evident in the structure of the uncomplexed 26–10 Fab (11) as well as in the structure of the 26–10 nonbinding mutant R9.5 The crystallographic disorder indicates either that there are several different conformations for that region or that the region is highly mobile.

We analyzed 26 unique mutants obtained from the library by panning against digoxin and three analogues (Table II). All mutants specifically bound digoxin in an ELISA, and were therefore included in a statistical analysis of amino acids at each position (Fig. 3). As 26 unique clones constitute a relatively small sample size, the absence of a particular amino acid at any position is not statistically significant. One hundred fifty different clones are the minimum needed for the absence of an amino acid designated by 1 in 32 codons to be significant (greater than 2\(\Delta\alpha\)). On the other hand, the frequent presence of a given amino acid at a particular position can be significant. For a sample including 26 clones, an amino acid must be present at least three, five, or six times to differ from the mean by greater than 2\(\Delta\alpha\), depending on whether it is encoded by one, two, or three codons, respectively.

A distinct pattern of amino acid usage emerged from the statistical distribution of amino acids at positions H30–35 (Fig. 3). The most dramatic was the conservation at position H35 of Asn, which contacts digoxin in the crystal structure. This result was not entirely unexpected, as in earlier experiments employing site-directed mutagenesis at position H35, all substitutions tested had significantly lower affinity for digoxin, indicating that Asn was essential for optimal complementarity at this position (13).

In the co-crystal structure of 26–10 with digoxin (11), the side chain of Asn-H35 contacts the digoxin D ring atoms C-16 and C-17 and the lactone (atoms C-20, C-21, and C-22) (Fig. 1). In addition, Asn-H35 forms hydrogen bonds with Tyr-H47 and Ser-H95, each of which also contacts hapten. Asn was found at position H35 in every high affinity clone isolated from the phage libraries. Clone D4–3 (Val-H35) had an affinity of \(6 \times 10^6 \text{M}^{-1}\), and clone D4–15 (Ser-H35) had an affinity for digoxin of \(1.8 \times 10^6 \text{M}^{-1}\). Clone D4–15 is the only mutant with Asn at position H34. Asn-H34 cannot functionally replace Asn-H35 and maintain loop integrity, but Ser-H35 could partially fulfill the role of Asn-H35 by making a hydrogen bond with either Ser-H95 or Tyr-H47.

At position H34, the wild-type residue methionine was not observed in any mutant. The predominant residues were Ile and the aromatic residues Phe and Tyr. In the wild-type structure, the side chain at residue H34 points away from the binding cavity and packs into the interior. Aromatic substitution at this position (particularly Tyr) would clash with Pro-H52 and Trp-H32, resulting in some rearrangement of the local structure. However, all four mutants containing Tyr-H34 (Table III) had affinities equal to or greater than parental 26–10, suggesting that such rearrangement can enhance complementarity. The phage-selected consensus sequence (see below) differs from the parental 26–10 (TDFYMN) only at positions 34 and 30. Met-H34 and Thr-H30 occur in more than 50% of murine H chains (39) and may reflect conserved germ line residues. Three base changes are required to convert Met-H34 (ATG) to either codon for Tyr (TAT, TAC). The affinity maturation process in vivo that results in high affinity Abs such as 26–10 is constrained by the germ line V region gene sequences, which are subjected to random somatic mutation and antigen-driven selection. Such putative constraints are not operative during in vitro selection of phage-displayed mutants (such as A4–19 and A-20), which can display even higher affinities for digoxin. Enhancement of affinity of high affinity Abs using phage display selection was previously reported (40), as was

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5 R. K. Strong, P. D. Jeffrey, G. A. Petsko, S. Sheriff, and M. N. Margolies, unpublished observations.
improvement in affinity using site-directed mutagenesis based on crystal structures of secondary response mAb (41).

The other contact residue in CDR1, Tyr-H33 (Fig. 4), is predominantly selected among the mutants (Fig. 3), but can be replaced with Phe or Trp, consistent with the importance of a planar hydrophobic (aromatic) residue at this position. The side chain of Tyr-H33 contacts the C-3, C-4, C-7, and C-15 atoms of digoxin.

Amino acid residues at positions H30–32 demonstrate much greater sequence variability than those at positions H33–35 (Table II). The statistically significant predominant amino acids are Pro-H30, Asp-H31, and Phe- or Tyr-H32 (Fig. 3). However, 12 different amino acids are found at position H30, 14 at position H31, and 10 at position H32, all of which are permissible with retention of digoxin binding. Residues H27–32 exhibit disorder in the crystal structure of 26–10-digoxin (11). This disorder is paralleled by the promiscuity in amino acid sequence usage at residues H30–32 among the mutants. For most Fabs, the side chains at positions H30 and H31 are solvent-exposed and have wide variations in their conformation. Since this part of H-CDR1 is not well-defined in the 26–10 crystal structure, one might not expect substitution at these positions to have much effect on affinity. Nonetheless, the observation that mutant G6–2 (SKRY1N) has an affinity for digoxin reduced by more than an order of magnitude compared with mutant D4–2 (PGRY1N), which differs only at positions H30 and H31, suggests that there are restrictions upon certain substitutions or sets of substitutions at these positions. Position H32 is partially solvent-exposed, with side chain conformations clustered in other Fab structures. In 26–10, the wild-type type Phe and also Tyr occur frequently, but many other substitutions are allowed.

Although we found a consensus sequence for positions H30–35 (PD(F/Y)(F/W/Y)(F/I/Y)N) (Fig. 3), no single mutant Fab exhibits the entire consensus sequence (Table II). Several mutants differ from the consensus by only a single amino acid within positions 30–32 (mutants D4–8, D4–17, A4–19, and A4–20). Such a consensus sequence accounts only for the prevalence of amino acids at individual positions, independent of possible effects of each upon the other.

Among digoxin-binding clones, proline was not observed at positions H32–35 inclusive, yet it occurs nine times at positions H30 and H31 (Table II). Proline residues restrict backbone conformation, and may disturb backbone folding necessary for complementarity at positions H33–35. The permissiveness at residues H30 and H31 is illustrated by mutant D4–6, which binds digoxin despite proline at both positions. However, a double proline present in the mutants F5–2 at H33–34 and F5–8 at H33–32 (Table II) is associated with failure to bind digoxin, despite the presence of Asn at H35.

The data indicate that H-CDR1 is more plastic than we anticipated. Although the diversity of side chain use is consistent with the disordered crystal structure in this region of 26–10, it is remarkable that there are many amino acid combinations that result in Fabs with similar affinities for digoxin. Two Fabs (A19 and A20) have affinities greater than 26–10 (4.1- and 1.8-fold, respectively), and 14 of 20 mutants (Table II) have affinities $\geq 3.4 \times 10^9$ M$^{-1}$. Thus, the variability at positions H30–32 does not prevent digoxin binding but must provide a stable framework for the contacting residues H33–35, which are the main arbiters of affinity and specificity in this region.

We wondered whether the absence of the wild-type 26–10 sequence among selected mutants could be accounted for through considerations of library size, preferential growth of certain mutants, or difference in panning efficiency. Not all amino acid sequences are represented equally in an NNS-substituted library, with the ratio of various amino acids being 1:2:3, depending on codon usage. In a library with six randomized positions, the likelihood of finding an amino acid encoded by 1 of 32 codons at all positions is 1 in $1.1 \times 2^9 = 5.4 \times 10^9$ clones. Because the library contains $1 \times 10^7$ different nucleotide sequences, it is not surprising that 26–10 was not observed. The probability of identifying 26–10 among the panned mutants may be further reduced by lack of selection due to inefficient expression of 26–10 on phage as a fusion protein or uneven clone distribution due to growth advantages of individual clones. Mixing experiments revealed that 26–10 does not compete as well with some other mutants with similar or even lower affinities for digoxin in competitive binding to antigen-coated surfaces (Table V), although 26–10 was readily enriched from mixtures (1:1000) with an anti-tetanus clone. Fab 26–10 showed a panning efficiency at least an order of magnitude lower than the other tested phage, sufficient to account for its absence after panning against digoxin-BSA. These observations raise the possibility that clones of equal or higher affinity for cardiac glycosides may not have been recruited from libraries panned against cardiac glycoside-BSA conjugates.

It is also possible that carrier effects during panning are operative in selecting certain mutants. However, as digoxin is coupled to BSA through the terminal digitoxose moiety, and the crystal structures of the 26–10-digoxin complex have shown that the sugars are not involved in binding, a role for carrier in affecting the panning results is unlikely. The selection of clone G6–2 from the bacteriophage library, despite its low affinity, is reproduced in the mixing experiment (Table V), and therefore does not directly reflect the affinity of this mutant for digoxin or gitoxin. Clone G6–2 predominates in selection from the mixture of five clones, and clone G6–1 appears to display a distinct growth advantage based on its predominance in the unpanned control. The specificity experiments (Table IV) indicate that G6–1 and G6–2 Fab, both isolated following panning against gitoxin-BSA, bind gitoxin in the same range as native 26–10, A4–19, and A4–20, but better than the 16-formylgitoxin-selected clones. A4–19 has higher affinity for digoxin (Table III) and 16-acetylgitoxin (Table IV) than A4–20, yet it was not among the clones selected following panning against digoxin-BSA and occurs in only one of 19 clones selected in pans against 16-acetylgitoxin (while A4–20 accounts for 18 of 19). Furthermore, A4–19 predominated in mixing experiments. Perhaps A4–19 may not have been selected as frequently as A4–20 due to underrepresentation in the original library. The close complementarity between antibody 26–10 and digoxin in the region of the 16 position in the D ring of digoxin (Fig. 4) results in 26–10 distinguishing sub- stituents of different sizes at C-16. Digoxin C-16 contacts H-CDR1 as well as H-CDR3 (11). The 26–10 antibody binds gitoxin (16-$\beta$-OH) 3–5-fold more weakly than digoxin. Larger substitutions at C-16 would cause more disruption of the combining site, and analogues with formyl and acetyl groups at C-16 were shown to demonstrate further reductions in affinity for wild-type 26–10 (Table IV) (29).

All 26–10 mutants bound digoxin best and bound the other three analogues in the same decreasing order: gitoxin, 16-formylgitoxin, and 16-acetylgitoxin (Table IV). Three of six mutants tested demonstrated specificity shifts. Clone G6–2 binds 16-acetylgitoxin 30-fold more weakly than digoxin as compared with wild-type 26–10. However, Fab G6–2 has reduced affinity for digoxin. Clones F6–1 and F6–2 selected by 16-formylgitoxin had moderate reduction in relative affinity for gitoxin. The affinity data (Table II) indicating the frequent
occurrence of high affinity clones of diverse sequences, taken together with the modest specificity shifts, indicate that, at least in experiments employing random mutagenesis limited to H-CDR3, no mutants with significant improvement in relative binding for C-16-substituted analogues can be obtained without concomitant drastic reduction of affinity for hapten. Thus, antibody 26–10 is relatively “optimized” in this region to bind digoxin. However, the results for a few mutants (A419 and A420) indicate that this affinity can be improved.

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