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Fine Tuning of the Specificity of an Anti-progesterone Antibody by First and Second Sphere Residue Engineering*[§]

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The specificity of anti-progesterone P15G12C12G11 antibody was improved by combination of *in vitro* scanning saturation mutagenesis and error-prone PCR. The most evolved mutant is able to discriminate against 5 β - or 5 α -dihydroprogesterone, 23 and 15 times better than the starting antibody, while maintaining the affinity for progesterone that remains in the picomolar range. The high level of homology with anti-progesterone monoclonal antibody DB3 allowed the construction of three-dimensional models of P15G12C12G11 based on the structures of DB3 in complex with various steroids. These models together with binding data, derived from site-directed mutagenesis, were used to build a phage library in which five first sphere positions in complementarity-determining regions 2H and 3L were varied. Variants selected by an initial screening in competition against a large excess of 5 β - or 5 α -dihydroprogesterone were characterized by a convergent amino acid signature different from that of the wild-type antibody and had lower cross-reactivity. Binding properties of this first set of mutants were further improved by the addition of second sphere mutations selected independently from an error-prone library. The three-dimensional models of the best variant show changes in the antigen binding site that explain well the increase in selectivity. The improvements are partly linked to a change in the canonical class of the light chain third hypervariable loop.

Progesterone is among the most important steroid hormones required for the maintenance of pregnancy. Its dosage is currently performed by immunoassay, and its quantification needs high quality antibodies (1). However, steroids are poorly immunogenic, and thus it is difficult to obtain monoclonal antibodies, particularly with high affinity and specificity. This is complicated by the fact that immunoassays must be carried out in complex media such as blood or urine, where the compound of interest must be quantified in the presence of other chemically related molecules. The contaminating progesterone-related derivatives are characterized by modifications at posi-

tions C-3, C-5, C-11, and C-17, which induce different conformations of the steroid skeleton (2). For example, C-5 α and C-5 β are ring A reduced conformers. Although progesterone adopts the so-called conformation A, where ring A is $\sim 45^\circ$ to the plane formed by rings B, C, and D, the addition of a proton at position C-5 results in conformations B or C (Fig. 1A). In conformation B, typical of C-5 α , ring A is coplanar with the other rings, whereas in conformation C, adopted by C-5 β , ring A is at 90° to the plane of the rest of the steroid skeleton (3).

Given these structural differences, it is surprising to find that after stimulation with progesterone, the immune system responds with antibodies like DB3 which strongly cross-react with C-5 dihydro-derivatives such as 5 β -androstane-3-17-dione, aetiocholanolone (C-5 β), and 5 α -pregnane-3- β -hemisuccinate (4, 5). The structural basis for its cross-reactivity has been mapped to the binding of steroids in two different orientations using two alternative pockets P3 and P3'. The entire binding site of DB3 can be divided into four different pockets or compartments named P1, P2, P3, and P3' accommodating the different steroid rings (see Fig. 1C) (4). Rings D, C, and B of progesterone and C-5 dihydro-derivatives are fit in P1 and P2, whereas ring A of C-5 α or C-5 β derivatives uses P3 or P3', respectively. Progesterone and C-5 α analogs that interact in a *syn* conformation (with their methyl groups facing Trp^{H50}) place their ring A in pocket P3, whereas C-5 β derivatives that adopt an *anti*-orientation (with their methyl groups facing Trp^{H100}) position their ring A in P3'. The use of alternative binding pockets also comports small compensatory adjustments in the binding site residues to improve shape complementarity.

Monoclonal antibody P15G12C12G11 (referred to as C12G11 hereafter) has a high affinity for progesterone with a K_d of 20 pM. Its specificity, as evaluated in competitive immunoassays using biotinylated progesterone and various steroids analogs, includes a strong affinity for 5 β - and 5 α -dihydroprogesterone (DHP)¹ with 30 and 20% of cross-reactivity, respectively. To develop a sensitive and highly specific *in vitro* immunoassay, we initiated a protein-engineering program with the aim of reducing the cross-reaction with these two analogs while maintaining its subnanomolar affinity for progesterone. The difficulties presented by such a project are associated with the lack of distinctive chemical groups that could be used to differentiate among these three compounds. With the only distinguishing features being the three-dimensional structures that these

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¹ The abbreviations used are: DHP, dihydroprogesterone; CDR, complementarity-determining region; ELISA, enzyme-linked immunosorbent assay; H and L preceding a number indicate heavy and light chain position; His₆, hexahistidine; VH, variable heavy; VL, variable light; fd, VH and CH1 heavy domains; EMC, ethyl methyl carbonyl.

progesterone derivatives adopt, the outcome of the search for variants possessing the right binding combination appeared difficult. The high homology with DB3 allowed the construction of models of our antibody, enabling us to plan a strategy of reengineering the combining site of C12G11 using combined semirational and random mutagenesis to select by phage display mutants with the desired characteristics. We present here the strategy we used and the fine characterization of the selected mutants. Models of the best variant were built and compared with that of wild-type C12G11 to account tentatively for the structural basis for the improved binding.

EXPERIMENTAL PROCEDURES

Cloning and Construction of scFv_{C12G11}—Hybridoma cells producing C12G11 were cultured in Iscove's modified Dulbecco's medium containing 10% fetal calf serum (Invitrogen) and antibiotics (100 µg/ml) at 37 °C in a 5% CO₂ humidified chamber. Cloning and sequencing of mRNA encoding the light chain and the fd heavy chain of C12G11 were done as previously described (6). DNA encoding the scFv_{C12G11} (VL-linker-VH) was generated by PCR-based single overlap extension (7), using an 18-amino acid linker derived from the one initially described by Whitlow *et al.* (8), according to Merienne *et al.* (9).

Construction of scFv Libraries—Wild-type scFv_{C12G11} DNA was cloned into the phagemid pAK100 (10) and used as template to build the libraries. A first library named 3L/2H was designed by overlap PCR via simultaneous saturation mutagenesis at positions L94, L95, L96 (CD-R3L), H50, and H58 (CDR2H). PCR A was obtained using primer pAK100-For 5'-GTTGTGTGGAATGTGAGCGG-3' in combination with a mixture 30/70 (mol/mol) of primers CDR3L^{94/95/96}-Back 5'-GCTTGGTCCCAGCACCGAACGT(MNN)₃ATGTGTACTTTGAGAGCAGAAATAG-3' and CDR3L^{94/96}-Back 5'-GCTTGGTCCCAGCACCGAACGT(MNN)-CGGMNNTGTGTACTTTGAGAGCAGAAATAG-3', respectively. PCR B and C were, respectively, performed using the sets of primers CDR-3L-For 5'-TTCGGTGTGGGACCAAGCTGGAGCTGAAACG-3'/CDR2H-Back 5'-GTTCTCCGTTGTAGGTTTATMNNGCCCATCCACCTTAAATCC-3', and CDR2H-For 5'-ATAAACACCTACAACGGAGAACCANNKTATGTGCGATGACCTCAGAGGAC-3'/pAK100-Back 5'-CATCGGCATTTTCGGTCATAGCC-3' (N = A, C, G, or T; M = A or C; K = G or T). A second library was built independently by error-prone PCR (11) of the VH domain (epVH) via a Gene Morph PCR Mutagenesis kit (Stratagene) using a coding primer located within the linker in combination with the complementary primer pAK100-Back. The level of mutations was fixed at two or three amino acid substitutions/amplified VH domain. The DNA encoding the VL-linker-epVH scFv fragment was generated by overlap PCR. Combined variants were generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene). The GenBank accession number of the VL/CL nucleic and proteic sequences is AY854499 and of the VH/CH1 nucleic and proteic sequences is AY854500.

Screening of the Libraries and Characterization of scFv Fragments—The libraries were rescued using helper phage M13KO7, and screened following a procedure derived from that initially described by Hawkins *et al.* (12). A preselection round was carried out in the presence of 5 nM progesterone-11α-EMC-oligonucleotide-biotin. The resulting pre-screened library was then submitted to five rounds of competitive selection according to the strategy described by Saviranta *et al.* (13) using 5 nM progesterone-11α-EMC-oligonucleotide-biotin together with 5 µM 5β-DHP or 5α-DHP. Screening of the error-prone library was performed in competition as described above against 5β-DHP only.

Competition ELISA was used to characterize the selected scFv-phages. 1 × 10⁸ particles of each tested recombinant phage were added to each microtiter plate well coated with progesterone-11α-EMC-oligonucleotide and incubated for 1 h at 37 °C in the presence of decreasing concentrations (3.0 × 10⁻⁶ to 3.0 × 10⁻¹² M) of a stock solution (5 mg/ml) of progesterone, 5β-DHP, or 5α-DHP. After three washes, 100 µl/well diluted (1/2,000) biotinylated anti-fd phage antibody (Amersham Biosciences) was added and incubated for 1 h 30 min at 37 °C. Wells were washed three times, 100 µl/well diluted (1/2,000) streptavidin-peroxidase conjugate (Amersham Biosciences) was added and incubated 1 h 30 min at 37 °C. The bound scFv-phages were revealed at 405 nm in the presence of ABTS (Sigma). Descending dose-dependent inhibition curves for the binding of each scFv-phage solution to immobilized progesterone against increasing competitor concentrations were obtained. The IC₅₀ values reflect the relative affinity of the tested scFv for the competitor, defined as the concentration of a particular competitor (progesterone, 5β-DHP, or 5α-DHP) which induces a 50% drop in signal

compared with the signal obtained in the absence of competitor. The DNA encoding the best variants were subcloned into pUMR vector and expressed as scFv-His₆ fragments in the periplasmic space of MC1061 *Escherichia coli* bacteria. Resulting scFv-His₆ molecules were extracted and IMAC-purified as described previously (14). Protein concentrations were determined by amino acid analysis.

Surface plasmon resonance analysis was performed on a BIAcore 3000 biosensor optical (BIAcore, Uppsala, Sweden). Dissociation constants (*K_d*) for progesterone, 5β-DHP, and 5α-DHP were determined by competition BIAcore using a sensor chip saturated with biotinylated progesterone (≈ 690 resonance units) as described previously (15). A streptavidin surface without biotinylated progesterone was used as a control. 200-µl samples of 10 nM pure scFv variant in a buffer containing various amount of competitor, preincubated over 1 h at 37 °C, were injected through the sample loop of the system. Data were evaluated with BIAevaluation software (Amersham Biosciences) and Kaleidagraph (Synergy Software).

Molecular Modeling—Models of the Fv domain of C12G11 antibody were generated using the program MODELLER (16) with anti-progesterone antibody DB3 (PDB code 1DBA) as template. The cross-reactive steroids 5β-DHP and 5α-DHP were built using the Cschem 3Dpro program. Models of the progesterone, 5β-DHP, and 5α-DHP complexed to C12G11 were constructed using the experimental structures of the DB3 complexes with progesterone, 5α-pregnane-20-one-3β-ol-hemisuccinate, and 5β-androstane-3,17-dione (PDB codes 1DBB, 2DBL, and 1DBK, respectively). The 5β-DHP and 5α-DHP analogs were fitted in the same orientation as the progesterone analogs bound to DB3. The backbone of the mutated third hypervariable loop in the combined variant Pro^{L94}-His^{L95}-Val^{L96}/Trp^{H50}-Arg^{H58} + Lys^{H31}-His^{H32} was built as a hybrid between the same loop in DB3 and antibody J539 (PDB code 2FBJ) (17).

RESULTS

Molecular Models of C12G11—The isotype of the C12G11 is γ1, subgroup IX, D-sp2.9, JH-2 for the heavy chain and κ, subgroup I, V-Jκ5 for the light chain (18). A FASTA search on the structures deposited in the PDB identified DB3 (4, 5), another anti-progesterone antibody, as being the antibody sharing the highest degree of homology with C12G11. The variable light and heavy domains of C12G11 display, respectively, 88.3 and 79.6% of sequence identity with those of DB3, and most changes are highly conservative. The six CDRs are identical in length, and CDR2L and CDR1H have identical sequences (Table I). CDR3H, with five substitutions, is the least conserved hypervariable loop. Overall, 12 of the 16 amino acid residues described as interacting with progesterone in DB3 are conserved in C12G11. The canonical classes of hypervariable loops 1L, 2L, 3L, 1H, and 2H of C12G11 match those assigned to DB3. The theoretical model of C12G11 displays only minor deviations with the overall DB3 backbone (root mean square deviation = 0.15 Å) and no major variation within the hypervariable loops. The antibody C12G11 presents a hydrophobic cavity at the interface of the VL and VH domains, formed by CDR1L, 3L, 1H, 2H, and 3H. Models of C12G11 in complex with native progesterone, 5β-DHP, and 5α-DHP (Fig. 1B) place 7 heavy chain residues in the proximity of the steroids: Asn^{H35} (CDR1H), Trp^{H47} (FR2H), Trp^{H50} (CDR2H), and Gly^{H95}, Phe^{H97}, Trp^{H100}, and Phe^{H100k} (CDR3H). The light chain contributes with 6 residues: His^{L27d} (CDR1L), and Ser^{L91}, Thr^{L92}, His^{L93}, Val^{L94}, and Val^{L96} (CDR3L). The contacts are mainly van der Waals in nature with a strong predominance of hypervariable loops 3H and 3L. Comparing the binding site with that of DB3, we expect progesterone to interact similarly in a *syn* configuration with the methyl groups facing Trp^{H50} (CDR2H). The overall orientation of progesterone within binding pockets P1, P2, and P3 is mediated by two hydrogen bonds, the keto groups at positions 3 (ring A) and 20 (ring D) being at hydrogen bonding distances from the side chains of His^{L27d} and Asn^{H35} in binding pockets P3 and P1, respectively (Fig. 1, B and C). Compartments P1 and P2 of C12G11 are markedly hydrophobic in character with residues

TABLE I
Comparison of amino acid sequences of VL-CDRs and VH-CDRs of the anti-progesterone antibodies DB3 versus C12G11

Antibody	CDR1L		CDR2L		CDR3L	
	27abc	de 30	50		90	95
DB3	RSSQSLIHSNGNTYLH		KVSNRF		SQSSHVPPT	
C12G11	-----V-N-----		-----		---T---V-	
Antibody	CDR1H		CDR2H		CDR3H	
	31		50 52a	60		100 jk
DB3	NYGVN		WINIYTGEPTYVDDFKG		GDYVNWYFDV	
C12G11	-----		---T-N-----LR-		-VFDG----Y	

Trp^{H47}, Trp^{H50}, Phe^{H97}, Trp^{H100}, and Phe^{H100k} lining the cavity that accommodates progesterone rings D, C, and B, and more specifically, rings B and C are sandwiched between Trp^{H50} and Trp^{H100} (Fig. 1B).

Antibody C12G11 displays strong cross-reactivity toward 5 β -DHP and 5 α -DHP, two hepatic metabolites of progesterone, in the same way as DB3 interacts with high affinity with analogs such as 5 β -androstane-3-17-dione, aetiocholanolone (C-5 β), and 5 α -pregnane-3- β -hemisuccinate. The x-ray structures of these DB3-steroid complexes have been solved and detailed (4, 5). It was the similarities between the antigen binding sites of DB3 and C12G11 that allowed us to model the binding of 5 β -DHP based on the 5 β -androstane-3-17-dione and aetiocholanolone complexes, both C-5 β , and 5 α -DHP using 5 α -pregnane-3- β -hemisuccinate. Thus we modeled 5 α -DHP occupying the P1, P2, and P3 binding pockets in C12G11 in a *syn* configuration close to that of progesterone, whereas 5 β -DHP would adopt an *anti* binding mode, with its ring A positioned into the alternative pocket P3' delimited by Trp^{H50} and Val^{L94}, the methyl groups facing Trp^{H100} (Fig. 1B). We felt confident that because of the high degree of homology between C12G11 and DB3 the three-dimensional models of the steroid complexes would of sufficient accuracy for the development of a mutagenesis strategy.

Design of 3L/2H scFv-phage Library—The models emphasized the crucial role played by the binding pockets P3 and P3' in distinguishing between the binding of progesterone from its 5 α and even more strongly from the 5 β analogs. Thus, we decided to reengineer the subpockets P3 and P3' mainly through mutagenesis of the CDR3L to hinder the binding of 5 α -DHP and 5 β -DHP. Within this loop, Gln^{L90} and Pro^{L95} determine its canonical structure (19), and the side chains of residues Val^{L94}, Pro^{L95}, and Val^{L96} point out in the direction of the antigen binding cavity. The residues that delimit the binding pocket P3' are Val^{L94}, Pro^{L95}, and Val^{L96} at the extremity of CDR3L, together with Trp^{H50} and Thr^{H58} in CDR2H. Consequently, these five positions: L94, L95, L96, H50, and H58, appeared appropriate targets for substitution by combined saturation mutagenesis. Thus, to improve the binding specificity of C12G11 antibody, a first library named 3L/2H was built by simultaneous randomization of these five first sphere positions.

Because of the structural role of the canonical residue at position L95, a bias was intentionally introduced during the library construction to maintain theoretically the wild-type residue proline at 70%. The size of the library was estimated at 9.0×10^6 independent clones, with no significant bias in the generated diversity as monitored by analyzing 60 individual clones by DNA sequencing.

Screening of 3L/2H scFv-phage Library—Prior to starting the selection by competition, the naive library was voided of low affinity and nonfunctional scFv-phages by carrying out one

round of preselection with just biotinylated progesterone. The prepanned library was then subjected to five rounds of selection in the presence of a large excess of either 5 β -DHP or 5 α -DHP. After five rounds of competitive panning selection using 5 β -DHP or 5 α -DHP as competitor, we noticed that the nature of the side chains found at the degenerated positions had evolved during the panning, leading to a marked reduction of the initial diversity (Table II). Thus, position H50 totally recovered the wild-type tryptophan, and position L96 was essentially populated by hydrophobic residues with a strong preference for the wild-type valine. Despite the deliberate bias in favor of the wild-type proline at position L95, we observed a steady drop, from the 70% level forced into the library during construction, to just 21% (4/19) after five rounds of selection with the 5 β -DHP competitor with basic residues such as Arg and His becoming dominant at the 58% (11/19) level. Similarly, the wild-type residue Val^{L94} of the native C12G11 reappeared in only 21% (4/19) of the sequenced clones giving way again to basic amino acids (47%, 9/19), Lys and especially Arg, but also proline appeared in 31% (6/19) of the clones. A large majority (74%, 14/19) of basic amino acids, Arg and His, replaced the wild-type Thr^{H58}. The study with 5 α -DHP gave similar results, and the amino acid profiles for the two parallel studies were compared, showing that the diversity of the 5 α -DHP-selected clones is more limited than that retained with 5 β -DHP (Table II). The proline residue at position L95 disappeared, but proline at position L94 doubled to 74% (14/19). Finally, positions L96 and H50 completely reverted to the wild-type residues, and basic residues were found at position H58 at the same level in both cases.

After the two panning procedures, most of the selected clones were produced individually fused to the minor coat protein gIII on the surface of the phage to establish their overall binding properties (see "Experimental Procedures"). Table III gives the change in the IC₅₀ values for progesterone, 5 β -DHP, and 5 α -DHP for each mutant compared with wild-type (see "Experimental Procedures" and Table III footnotes). The cross-reaction profiles of the mutants for the two analogs were calculated as the ratios of IC₅₀ Prog/IC₅₀ 5 β -DHP, or IC₅₀ Prog/IC₅₀ 5 α -DHP, respectively. The first group of variants is characterized by basic amino acid (Lys or Arg) at position L94 and the absence of proline residue at position L95: clones B16, A23, B4, A31, B7, and B19. All but clone A31 display a decrease of their cross-reactivity for 5 β -DHP (15.9 to 22.8% versus 26% for the wild-type antibody fragment), and 5 α -DHP (4.7 to 5.9% versus 19% for the wild-type). This result suggests that in our case, functional C12G11 variants can be generated despite the absence of proline residue in the CDR3L hypervariable loop, including the canonical one at position L95. Most interestingly, four variants in particular, A3 (Pro^{L94}-Tyr^{L95}-Val^{L96}/Trp^{H50}-His^{H58}), B5 (Pro^{L94}-Arg^{L95}-Val^{L96}/Trp^{H50}-Arg^{H58}), B21 (Pro^{L94}-His^{L95}-

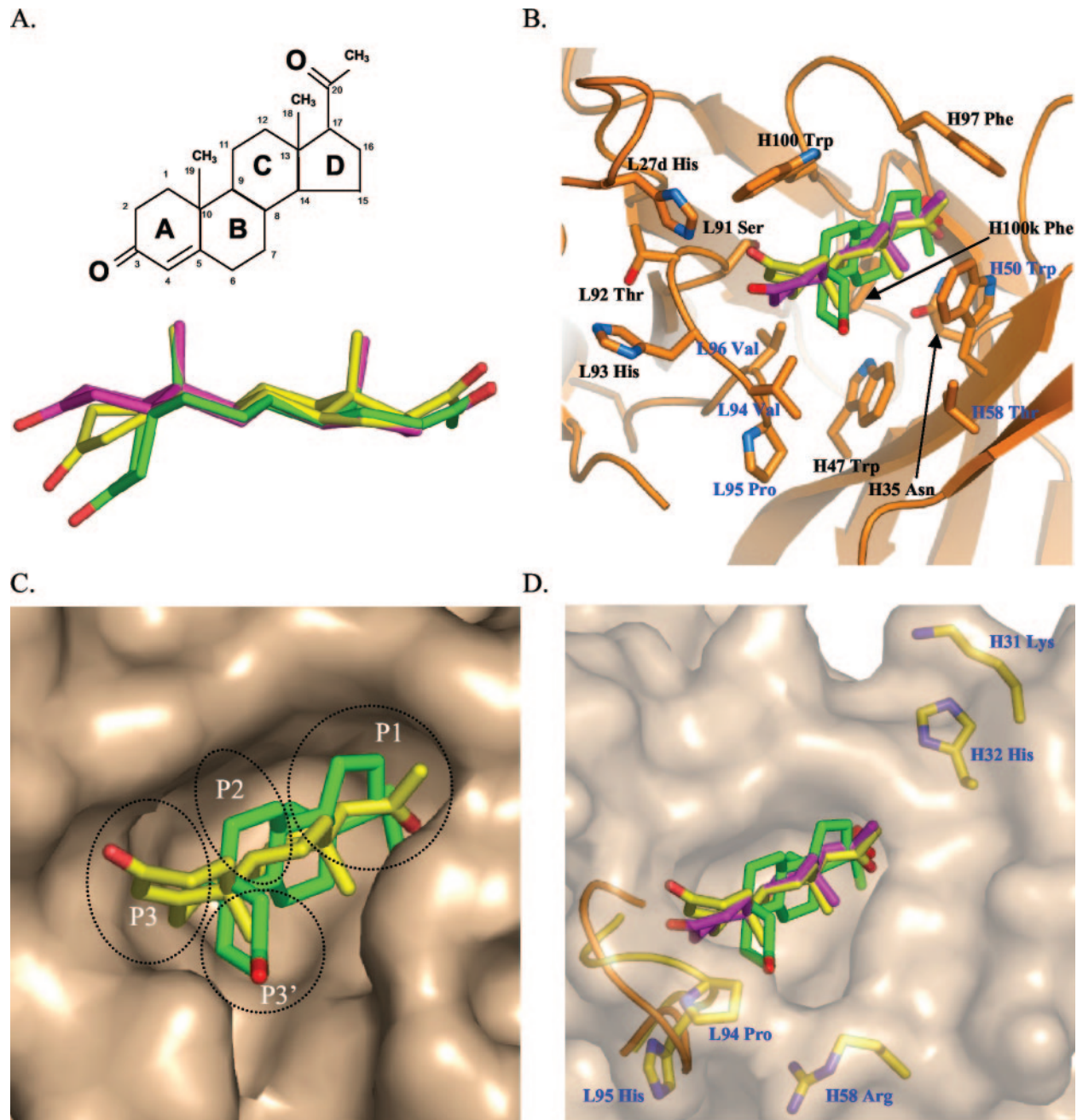


FIG. 1. Structures of progesterone, 5 β -DHP, and 5 α -DHP derivatives (A) and molecular models of native (B) and (C) and engineered (D) C12G11 binding site. A, representation of progesterone (*upper diagram*) showing the numbering scheme for the skeleton and the nomenclature of the steroid rings. Superimposing the different rings (*top diagram* onto *bottom*) shows the conformation variability of the steroid skeleton: progesterone in *yellow* (conformation A), 5 α -DHP in *magenta* (conformation B), and 5 β -DHP in *green* (conformation C). B, molecular model of the C12G11 wild-type antigen binding site with the three structurally distinct steroids: progesterone (*yellow*), 5 α -DHP (*magenta*), and 5 β -DHP (*green*). The side chains of residues important for the binding are shown. Progesterone and 5 α -DHP adopt a *syn* configuration with the methyl groups facing Trp^{H50}; ring A is positioned in pocket P3 surrounded by residues Trp^{H100}, His^{L27d}, Val^{L94}, Pro^{L95}, and Val^{L96}. 5 β -DHP adopts an *anti* conformation with its methyl groups facing Trp^{H100} and uses the alternative pocket P3' composed by Val^{L94}, Pro^{L95}, Val^{L96}, Trp^{H47}, Trp^{H50}, and Thr^{H58} for the ring A binding. Residues submitted to saturation mutagenesis are labeled in *blue*. C, location of the different binding subpockets P1, P2, P3, and P3', which form the steroid binding site of the C12G11 wild-type antigen binding site with progesterone (*yellow*) and 5 β -DHP (*green*). D, close-up view of the C12G11 mutant B14 + Lys^{H31}-His^{H32} binding site model. The CDR3L loops are shown in the backbone trace. The original canonical class 1 CDR3L loop is indicated in *orange*, and the predicted new CDR3L loop with L93-L95 in canonical class 2 is in *yellow*. The surface representation corresponds to that of the engineered scFv variant B14 + Lys^{H31}-His^{H32}. The color scheme is as in A: progesterone, *yellow*; 5 α -DHP, *magenta*; and 5 β -DHP, *green*. The five mutated residues that led to the mutant B14 + Lys^{H31}-His^{H32} are labeled in *blue*.

Val^{L96}/Trp^{H50}-Asp^{H58}), and B14 (Pro^{L94}-His^{L95}-Val^{L96}/Trp^{H50}-Arg^{H58}), are characterized by a more important reduction of cross-reactivity toward both 5 β -DHP and 5 α -DHP, than the clones of the first group. Indeed, the percentages of cross-reactivity of the latter are an average of 7.0 *versus* 26% and 3.0 *versus* 19%, for 5 β -DHP and 5 α -DHP, respectively. Further-

more, the deduced IC₅₀ values for progesterone of all selected and tested mutants remain closed to that of the wild-type scFv, with a variation comprised between 1.3 and 3.3 only.

Identification of Second Sphere Residues—Undoubtedly, the VH domain of C12G11 plays an important role both in the formation of the binding cavities and in its lack of specificity.

TABLE II
Sequence analysis of randomly picked clones after five rounds of panning selection

“—” indicates the same amino acids as those in wild-type sequence. Residue numbering is according to the kabat scheme.

	Panning selection with competitor									
	5 β -DHP					5 α -DHP				
	L94	L95	L96	H50	H58	L94	L95	L96	H50	H58
Wild type	V	P	V	W	T	V	P	V	W	T
Library	-	-	P	-	R	K	I	-	-	H
	-	-	I	-	-	K	H	-	-	-
	-	-	L	-	R	R	I	-	-	H
	-	-	-	-	H	R	L	-	-	H
	K	R	-	-	R	R	R	-	-	D
	K	I	-	-	R	P	T	-	-	S
	R	W	-	-	H	P	H	-	-	H
	R	I	-	-	R	P	H	-	-	R
	R	H	-	-	A	P	H	-	-	R
	R	R	-	-	H	P	H	-	-	R
	R	R	-	-	H	P	H	-	-	R
	R	R	-	-	R	P	Y	-	-	H
	R	R	-	-	R	P	Y	-	-	H
	P	L	-	-	A	P	Y	-	-	H
	P	R	-	-	Y	P	Y	-	-	H
	P	R	-	-	R	P	Y	-	-	H
	P	H	-	-	D	P	Y	-	-	H
	P	H	-	-	R	P	Y	-	-	H
	P	H	-	-	R	P	Y	-	-	H

Consequently, an error-prone PCR library of variants focused on the VH domain only was built in parallel to the 3L/2H library, and this was to complete tentatively with the structure-based engineering of C12G11. The resulting library was only screened against 5 β -DHP because its binding takes in particular advantage of the alternative binding pocket P3' that is mainly defined by the VH domain (Fig. 1B). After six round of panning, selected clones displayed multiple mutations, a reduced recognition of 5 β -DHP with 10 *versus* 26% of cross-reactivity, but also a significant loss of affinity for progesterone (data not shown). It is noteworthy that a majority of these multiple random mutants contained repeatedly one of following substitutions: asparagine into lysine at position 52 (CDR2H) or glutamic acid into lysine at position 56 (CDR2H) and the double mutation Asn^{H31} \rightarrow Lys/Tyr^{H32} \rightarrow His in CDR1H. Despite the lack of beneficial effect on the cross-reactivity of the corresponding single or double mutants compared with the wild-type, an improvement of specificity was systematically observed upon combination of the double substitution Asn^{H31} \rightarrow Lys/Tyr^{H32} \rightarrow His with the multiple mutants Pro^{L94}-(Leu,Arg,His)^{L95}-Val^{L96}/Trp^{H50}-(His,Arg,Asp,Ala)^{H58} (Table III). Among the bests combined mutants and compared with the wild-type scFv, clone B14 + Lys^{H31}-His^{H32} displays 3.4 \pm 0.4% *versus* 26.0 \pm 5.4% and 1.6 \pm 0.4% *versus* 19.0 \pm 1.0% of cross-reactivity toward 5 β -DHP and 5 α -DHP, respectively.

Affinity Measurements—The real affinity in solution of (i) wild-type scFv_{C12G11}, (ii) mutant B14 (Pro^{L94}-H^{L95}-Val^{L96}/Trp^{H50}-Arg^{H58}) isolated from the 3L/2H library, and (iii) combined mutant B14 + Lys^{H31}-His^{H32} for soluble and unmodified progesterone, 5 β -DHP, and 5 α -DHP, respectively, was determined by competition BIAcore experiments (15) using purified recombinant scFv-His₆ hybrids. The results clearly establish that clone B14 + Lys^{H31}-His^{H32} is much more specific than the wild-type antibody fragment with deduced K_d values in the nanomolar range (5.3 and 2.4 nM for 5 α -DHP and 5 β -DHP, respectively) *versus* 97 pM (5 α -DHP) and 28 pM (5 β -DHP) for the wild-type fragment, whereas the affinity for progesterone

remains in the picomolar range (Fig. 2). Moreover, comparison of the K_d values obtained for mutant B14 and B14 + Lys^{H31}-His^{H32} confirms the beneficial effect upon addition of the double mutation Asn^{H31} \rightarrow Lys/Tyr^{H32} \rightarrow His. The improvement in specificity for our best mutant B14 + Lys^{H31}-His^{H32} compared with the wild-type scFv_{C12G11} was calculated from measurement obtained under identical experimental conditions (Fig. 2). It shows an increase in discrimination against 5 β -DHP and 5 α -DHP, 23-fold and 15-fold, respectively, compared with the starting antibody. These improvements were calculated as cross-reaction percentages from experimentally measured K_d values (Fig. 2). Furthermore, the lower cross-reactivity of mutant B14 + Lys^{H31}-His^{H32} is associated with a moderate 3.8-fold increase in the dissociation constant for progesterone (75 pM *versus* 20 pM).

DISCUSSION

Strategies of Engineering of Anti-progesterone C12G11 Antibody—In the last decade, the ability of protein engineering to modulate the affinity and specificity of antibodies has been steadily improving, especially in the case of haptens such as steroids, sulfa-antibiotics, cardiac and cancer markers (12, 20–31). Here we describe the first successful engineering work on an anti-progesterone antibody aimed at reducing its cross-reactivity toward 5 β -DHP and 5 α -DHP, two hepatic analogs, while preserving its high affinity for progesterone. The best mutant we generated is not only more specific than the starting antibody with a 23-fold and 15-fold lower cross-reactivity for 5 β -DHP and 5 α -DHP, respectively, but it has also maintained its affinity for progesterone within the picomolar range. One should note that this was achieved progressively using a precisely targeted combination of site-directed saturation and random mutagenesis.

The binding site of DB3 was instrumental for building models of our antibody to assess first prior to this work the possibility of improving the binding specificity of C12G11 by site-directed substitutions of the main residues that define the alternative binding pockets P3' and P3. However, none of the constructed single site mutants displayed the expected specificity while still retaining the ability to bind progesterone (data not shown). This emphasized the structural plasticity and the functional adaptability of the C12G11 antigen binding site and confirmed that it is difficult to carry out successful engineering by site-directed mutagenesis alone, as was found since the first antibody engineering of an anti-digoxin binding site (32). Aware of these problems, we thus opted for a strategy of combinatorial site-directed-saturation mutagenesis aimed at modifying simultaneously positions L94, L95, L96, H50, and H58, which our models have identified as involved in the geometry of the binding pockets P3 and P3' responsible for lack of specificity of C12G11.

The resulting structure-based library was expressed and screened by phage display, using a competition panning procedure, a strategy that appears particularly well adapted to engineer the specificity of anti-hapten antibodies (13, 20, 21, 24, 33). After first screening assays using variable concentrations of ligand, we kept the concentrations of biotinylated progesterone constant at 5 nM and that of the competitor at 5 μ M throughout the five rounds of panning. A lower concentration (1 nM) of biotinylated progesterone together with 10 μ M of competitor would have been too stringent, resulting in too high a level of wild-type fragments. Because of the conformational characteristics that differentiate between the two analogs so that their A-rings occupy alternative binding pockets, we decided to carry out the competitive panning with 5 β -DHP and 5 α -DHP separately because it was not obvious at first that within the diversity generated there would yield a common

TABLE III
Characterization of mutants by competition phase-ELISA

Bold characters indicate the best combined mutant. Reported results are the means of at least three independent experiments.

Clone	Mutations									IC ₅₀ change ^a			Cross-reaction (%) ^b	
										Progesterone	5β-DHP	5α-DHP	5β-DHP	5α-DHP
Wild type	L94	L95	L96	H31	H32	H50	H52	H56	H58					
	V	P	V	N	Y	W	N	E	T	-	-	-	26.0 ± 5.4	19.0 ± 1.0
L3/H2 library :														
B16	K	I	-	-	-	-	-	-	R	2.0	2.9	6.9	20.0 ± 3.6	5.3 ± 1.6
A23	K	I	-	-	-	-	-	-	H	2.0	3.7	8.6	15.9 ± 0.3	4.7 ± 0.9
B4	K	R	-	-	-	-	-	-	R	2.5	4.1	5.6	17.5 ± 3.3	5.9 ± 1.5
A31	R	R	-	-	-	-	-	-	D	2.5	1.3	4.7	31.5 ± 6.3	7.4 ± 0.2
B7	R	R	-	-	-	-	-	-	H	2.2	3.1	6.1	20.5 ± 4.8	5.7 ± 1.3
B19	R	R	-	-	-	-	-	-	R	3.3	6.3	4.9	22.8 ± 3.6	5.4 ± 0.7
B23	P	L	-	-	-	-	-	-	A	1.4	4.3	8.4	11.4 ± 3.2	3.3 ± 0.15
A3	P	Y	-	-	-	-	-	-	H	3.0	9.6	14.8	8.2 ± 1.6	2.4 ± 1.2
B5	P	R	-	-	-	-	-	-	R	2.0	9.1	12.7	7.0 ± 1.1	2.8 ± 0.4
B21	P	H	-	-	-	-	-	-	D	1.3	4.8	8.3	7.0 ± 1.8	2.9 ± 0.1
B14	P	H	-	-	-	-	-	-	R	2.8	13.5	20.8	5.8 ± 1.9	2.2 ± 0.2
error-prone VH substitutions :														
K _{H52}	-	-	-	-	-	-	-	K	-	3.6	2.4	1.3	45.3 ± 1.4	32.2 ± 10.5
K _{H56}	-	-	-	-	-	-	-	-	K	2.4	3.3	3.5	22.4 ± 3.5	21.5 ± 3.5
K _{H31} H _{H32}	-	-	-	K	H	-	-	-	-	1.3	1.5	1.2	26.7 ± 5.4	18.0 ± 2.1
Combined :														
B23 + K _{H31} H _{H32}	P	L	-	K	H	-	-	-	A	2.0	10	27	7.6 ± 2.0	1.9 ± 0.5
B5 + K _{H31} H _{H32}	P	R	-	K	H	-	-	-	R	0.7	3.9	9.7	4.7 ± 0.6	1.9 ± 0.2
B21 + K _{H31} H _{H32}	P	H	-	K	H	-	-	-	D	2.2	13.3	21.2	5.1 ± 1.4	1.9 ± 0.5
B14 + K_{H31}H_{H32}	P	H	-	K	H	-	-	-	R	1.0	10.1	8.8	3.4 ± 0.4	1.6 ± 0.4

^a The IC₅₀ values of the wild-type are average 3.7×10^{-10} M for progesterone, 1.4×10^{-9} M for 5β-DHP, and 1.9×10^{-9} M for 5α-DHP. The relative affinity of each mutated scFv-phage was evaluated by determining the IC₅₀ change that was calculated by dividing the IC₅₀ value of the variant looked for a defined competitor by the IC₅₀ value of the wild-type for the same competitor. An IC₅₀ change greater than 1 reflects a decrease of relative affinity of the variant for the competitor compared with the wild-type.

^b The percentage of cross-reaction was calculated as follows: $(\text{IC}_{50} \text{ progesterone} / \text{IC}_{50} \text{ 5}\beta\text{-DHP or 5}\alpha\text{-DHP}) \times 100$.

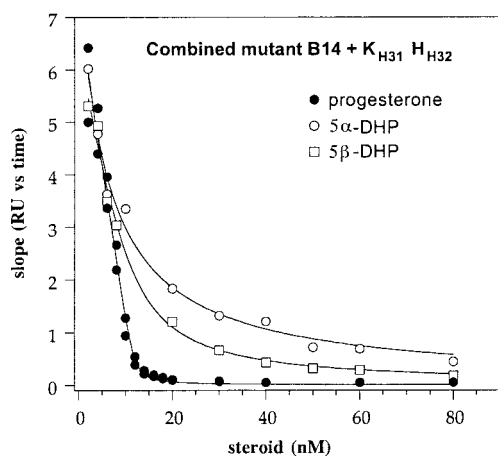
combination of mutations capable of reducing the affinity of both progesterone metabolites simultaneously. We feared that a competitive procedure with all three natural ligands of C12G11 at once would have overselected wild-type-like fragments, to the detriment of more specific variants. This was borne out by the sequence profiles that emerged from both screening procedures because no wild-type fragments were selected (Table II), all common and distinct solutions were retained, and little would have been gained from the simultaneous approach to justify the risks.

The first set of selected mutants that display the desired discriminating binding properties are characterized by the consensus sequence: Pro^{L94}-(Leu,Tyr,Arg,His)^{L95}-Val^{L96}/Trp^{H50}-(His,Arg,Asp,Ala)^{H58}. Very early during the selection procedures, positions L96 of CDR3L and H50 within CDR2H returned to the natural residues found in the initial C12G11 antibody, indicating that they are likely to be both structurally and functionally essential for high affinity recognition of progesterone. It appeared not really surprising to find a tryptophan residue at position H50 because its side chain stacks against rings B and C of the steroids, but the important role of Val^{L96} was a surprise because single site mutants Val^{L96} → Ile/Leu/Met or Asn had been found to have conserved progesterone recognition abilities (data not shown). Among the residues found at position H58, we identified a predominance (80%, 15/19) of basic side chains such as Arg and His and a minority of Tyr and Ala residues, in agreement with the unchanged binding capacities toward progesterone observed for the single site mutant Thr^{H58} → Arg (data not shown). The changes in the residues at the tip of CDR3L, L94-L95, were surprising. Sequence analysis of the most specific C12G11 variants (Table II)

showed a selection against the native canonical Pro^{L95}, which we had deliberately biased at 70% in the initial library, in favor of unrelated basic or aromatic side chains, whereas a proline residue appears one position before in the sequence, at L94. However, such “proline exchange” is not neutral as far as 5α-DHP and 5β-DHP recognition is concerned because both are now discriminated against, whereas the subnanomolar affinity for progesterone is maintained.

Interestingly, the specificity of recognition of this first set of variants was further improved by the addition of the double mutation Asn^{H31} → Lys/Tyr^{H32} → His. The latter was selected independently by screening of the error-prone VL-linker-epVH scFv library built in parallel to the structure-based one and was found interesting because it is located behind CDR3H, which contributes to the formation of the P1 pocket. However, that positive effect was not obvious because the double mutation Asn^{H31} → Lys/Tyr^{H32} → His alone was neutral upon recognition of progesterone and 5α-DHP and 5β-DHP analogs. Thus, our results show that combining mutations issued from distinct libraries and approaches may have added benefits. Similar strategies have recently been used to modulate the binding properties of two anti-hapten antibodies (23, 25). Furthermore, it is noteworthy that our best mutant associates first sphere (31, 32) and second sphere residues (29, 34, 35), confirming the importance of noncontact amino acid residues upon antigen recognition (26, 29).

In the case of the wild-type scFv_{C12G11} for 5β-DHP, we note a 2-fold discrepancy between the cross-reaction percentage deduced from the competitive phage-ELISA (Table III) and the dissociation constant value determined from competitive BIAcore experiments (Fig. 2). Such discrepancies were also re-



Clone	Dissociation constant K_D ($\times 10^9$ M)			% cross-reaction	
	Progesterone	5 β -DHP	5 α -DHP	5 β -DHP	5 α -DHP
Wild type	0.020 \pm 0.009	0.028 \pm 0.009	0.097 \pm 0.028	71%	20.6%
B14	0.068 \pm 0.020	0.670 \pm 0.200	1.5 \pm 0.34	10%	4.5%
B14 + $K_{H31}H_{H32}$	0.075 \pm 0.018	2.4 \pm 0.35	5.3 \pm 0.9	3.1%	1.4%

FIG. 2. **Competition BIAcore experiments.** Purified scFv-His₆ wild-type, B14 and B14 + Lys^{H31}-His^{H32} mutants were incubated with different concentrations of progesterone, 5 α -DHP, or 5 β -DHP for 1 h and injected over a progesterone-coated sensor chip. From the linear sensorgrams, the slopes (resonance units *versus* time in seconds) were plotted against the corresponding total competitive steroid concentration. The slopes correlating the free scFv-His₆ in the injected mixture and the dissociation constants (K_D) were calculated according to Nieba *et al.* (15). Cross-reaction percentages were determined as follows: (K_D progesterone/ K_D 5 α -DHP or 5 β -DHP) \times 100.

ported for an anti-cortisol antibody where the scFv-phage in an ELISA format *versus* the corresponding free scFv in an equilibrium dialysis procedure gave slightly discordant results (36). One must accept that there will be a difference between the scFv format of our antibody expressed on the surface of filamentous phages and the soluble version with a polyhistidine tag when used in two unrelated assays, competitive ELISA *versus* competitive BIAcore in solution. Phage-ELISA is just a first step to identify the best variants, prior their fine characterization in a soluble and purified format by a more appropriate and accurate analytical method.

Relationships between Cross-reactivity Profiles and the Structure of the Antigen Binding Pocket, as Predicted from Modeling—To explain tentatively the binding properties of our best variant, we first constructed models by energy minimization in which we kept the backbone unchanged despite the absence of the canonical Pro^{L95}. By maintaining the canonical class 1 conformation for loop CDR3L, even considering all reasonable reorientations of the Leu, Tyr, His, or Arg side chains of the variants at position L95 we fail to explain the reduced affinity for 5 α -DHP and 5 β -DHP, and this even if we add the minor structural adjustments of the loop upon binding that were described previously for DB3 (6). Only by building a second set of models in which the absence of the canonical Pro^{L95} causes a change in the loop conformation can we explain the results. Thus, to build the backbone of the Pro^{L94}-His^{L95}-Val^{L96}/Trp^{H50}-Arg^{H58} + Lys^{H31}-His^{H32} variant (clone B14 + Lys^{H31}-His^{H32}) we adopted as template for the CDR3L loop of the anti-galactan antibody J539 (17). J539 is the only antibody of known three-dimensional structure which contains, as in our case, residues Gln^{L90} and Pro^{L94} within a 6-amino acid long CDR3L. The association of these 2 residues at positions L90 and L94 is responsible for a switch in canonical structure of the

third VL hypervariable loop from class 1 to class 2 (19). Fig. 1D shows one of the models of the mutant Pro^{L94}-His^{L95}-Val^{L96}/Trp^{H50}-Arg^{H58} + Lys^{H31}-His^{H32} antigen-binding site obtained after imposing a transition of this loop toward canonical class 2. To illustrate the predicted difference in conformation of the third hypervariable loop of the VL domain, the backbone trace of the initial canonical class 1 CDR3L found in the wild-type antibody was added in Fig. 1D. According to the model, Arg^{H58} restricts the size of the P3' subpocket, and the different conformation adopted by CDR3L results in a further tightening of the two alternative binding pockets P3 and P3'. Lys^{H31}-His^{H32} is likely to have an effect on subpocket P1, although it is hard to predict in the absence of a crystal structure whether these residues affect steroid binding or restricts the adjustments in the antibody binding site. The effect in the reduction in the sizes of P3 and P3' is more easily understood (Fig. 1D): weakly affecting the binding of progesterone in its conformation A but strongly perturbing 5 α -DHP. One possible explanation is that the coplanar position adopted by rings A, B, C, and D in 5 α -DHP results in a closer proximity between the ring A of 5 α -DHP and the loop in the changed conformation, especially in the vicinity of positions L93 and L94. Also, the predicted reduction in size of the P3' alternative site is likely to be caused by the side chain of Pro^{L94} adopting the same position as in J539, possibly in combination with the side chain of Arg^{H58}. A strict grafting of the canonical class 2 CDR3L conformation would be expected to affect more strongly the binding of progesterone. Thus we expect that only the latter portion of CDR3L, mainly L94-L95 would conform strictly to the J539 conformation, whereas the unmodified early part of the loop would keep its DB3-like conformation. However, to validate these hypotheses, experimental structural analysis of these different complexes by x-ray crystallography is required.

In conclusion, we have been able to select variants of the C12G11 anti-progesterone antibody that displays improved specificity toward two structurally different competitive steroids, and this without significant loss of affinity for progesterone. More precisely, the engineering of C12G11 has resulted in the case of the variant Pro^{L94}-His^{L95}-Val^{L96}/Trp^{H50}-Arg^{H58} + Lys^{H31}-His^{H32} (clone B14 + Lys^{H31}-His^{H32}) in a simultaneous 54- and 85-fold decrease in affinity for 5 α -DHP and 5 β -DHP, respectively, together with a more limited 3.8-fold reduction in affinity for progesterone. To our knowledge this constitutes the first description of such a discriminating engineering of an anti-progesterone antibody *versus* three structurally different ligands that establish contacts via two alternative binding sites. These experimental results together with the predicted model are now exploited to design a new library of variants to assess the possibility to improve further the specificity of recognition of C12G11.

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REFERENCES

- Savouret, J. F., Misrahi, M., and Milgrom, E. (1990) *Int. J. Biochem.* **22**, 579–594
- Chen, J., Wang, R., Taussig, M., and Houk, K. N. (2001) *J. Org. Chem.* **66**, 3021–3026
- Duax, W. L., Griffin, J. F., Rohrer, D. C., and Weeks, C. M. (1980) *Lipids* **15**, 783–792
- Arevalo, J. H., Hassig, C. A., Stura, E. A., Sims, M. J., Taussig, M. J., and Wilson, I. A. (1994) *J. Mol. Biol.* **241**, 663–690
- Arevalo, J. H., Stura, E. A., Taussig, M. J., and Wilson, I. A. (1993) *J. Mol. Biol.* **231**, 103–118
- Bettsworth, F., Monnet, C., Watelet, B., Battail-Poirot, N., Gilquin, B., Jolivet, M., Ménez, A., Arnaud, M., and Ducancel, F. (2001) *J. Mol. Recognit.* **14**, 99–109
- Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989) *Gene (Amst.)* **77**, 61–68
- Whitlow, M., Bell, B. A., Feng, S. L., Filpula, D., Hardman, K. D., Hubert, S. L.,

- Rollence, M. L., Wood, J. F., Schott, M. E., and Milenic, D. E. (1993) *Protein Eng.* **6**, 989–995
9. Merienne, K., Germain, N., Zinn-Justin, S., Boulain, J. C., Ducancel, F., and Ménéz, A. (1997) *J. Biol. Chem.* **272**, 23775–23783
10. Krebber, A., Bornhauser, S., Burmester, J., Honegger, A., Willuda, J., Bosshard, H. R., and Plückthun, A. (1997) *J. Immunol. Methods* **201**, 35–55
11. Cadwell, R. C., and Joyce, G. F. (1992) *PCR Methods Applications* **2**, 28–33
12. Hawkins, R. E., Russell, S. J., and Winter, G. (1992) *J. Mol. Biol.* **226**, 889–896
13. Saviranta, P., Pajunen, M., Jauria, P., Karp, M., Pettersson, K., Mantsala, P., and Lovgren, T. (1998) *Protein Eng.* **11**, 143–152
14. Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985) *Mol. Cell. Biol.* **5**, 3610–3616
15. Nieba, L., Krebber, A., and Plückthun, A. (1996) *Anal. Biochem.* **234**, 155–165
16. Fiser, A., and Sali, A. (2003) *Methods Enzymol.* **374**, 461–491
17. Suh, S. W., Bhat, T. N., Navia, M. A., Cohen, G. H., Rao, D. N., Rudikoff, S., and Davies, D. R. (1986) *Proteins* **1**, 74–80
18. Lefranc, M. P. (2003) *Nucleic Acids Res.* **31**, 307–310
19. Chothia, C., Lesk, A. M., Tramontano, A., Levitt, M., Smith-Gill, S. J., Air, G., Sheriff, S., Padlan, E. A., Davies, D., Tulip, W. R., Colman, P. M., Spinelli, S., Alzari, P. M., and Poljak, R. J. (1989) *Nature* **342**, 877–883
20. Iba, Y., Hayashi, N., Sawada, J., Titani, K., and Kurosawa, Y. (1998) *Protein Eng.* **11**, 361–370
21. Miyazaki, C., Iba, Y., Yamada, Y., Takahashi, H., Sawada, J., and Kurosawa, Y. (1999) *Protein Eng.* **12**, 407–415
22. Chowdhury, P. S., and Pastan, I. (1999) *Nat. Biotechnol.* **17**, 568–572
23. Korpimäki, T., Rosenberg, J., Virtanen, P., Lamminmäki, U., Tuomola, M., and Saviranta, P. (2003) *Protein Eng.* **16**, 37–46
24. Lamminmäki, U., Pauperio, S., Westerlund-Karlsson, A., Karvinen, J., Virtanen, P. L., Lovgren, T., and Saviranta, P. (1999) *J. Mol. Biol.* **291**, 589–602
25. Lamminmäki, U., Westerlund-Karlsson, A., Toivola, M., and Saviranta, P. (2003) *Protein Sci.* **12**, 2549–2558
26. Zahnd, C., Spinelli, S., Luginbuhl, B., Amstutz, P., Cambillau, C., and Plückthun, A. (2004) *J. Biol. Chem.* **279**, 18870–18877
27. Adams, G. P., Schier, R., Marshall, K., Wolf, E. J., McCall, A. M., Marks, J. D., and Weiner, L. M. (1998) *Cancer Res.* **58**, 485–490
28. Hanes, J., Jeremius, L., Weber-Bornhauser, S., Bosshard, H. R., and Plückthun, A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 14130–14135
29. Boder, E. T., Midelfort, K. S., and Wittrup, K. D. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 10701–10705
30. Daugherty, P. S., Chen, G., Iverson, B. L., and Georgiou, G. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 2029–2034
31. Chen, G., Dubrawsky, I., Mendez, P., Georgiou, G., and Iverson, B. L. (1999) *Protein Eng.* **12**, 349–356
32. Burks, E. A., Chen, G., Georgiou, G., and Iverson, B. L. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 412–417
33. Chames, P., and Baty, D. (1998) *Clin. Chem. Lab. Med.* **36**, 355–359
34. Hawkins, R. E., Russell, S. J., Baier, M., and Winter, G. (1993) *J. Mol. Biol.* **234**, 958–964
35. Arkin, M. R., and Wells, J. A. (1998) *J. Mol. Biol.* **284**, 1083–1094
36. Chames, P., Coulon, S., and Baty, D. (1998) *J. Immunol.* **161**, 5421–5429