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Engineering of an Anti-Steroid Antibody: Amino Acid Substitutions Change Antibody Fine Specificity from Cortisol to Estradiol

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Immunoassays are widely used for determination of the concentration of steroid hormones. However, obtaining specific anti-steroid monoclonal antibodies remains difficult. We used antibody engineering and phage display methods to change the specificity of an anti-cortisol monoclonal antibody towards estradiol. This work demonstrates that production of recombinant antibodies may be a valuable way of obtaining the high-specificity antibodies required for steroid immunoassays.

Key words: Antibody; Cross-reactivity; Phage display; Single-chain variable fragment (scFv); Steroid.

Introduction

Steroid hormones have a number of different effects on their target tissues. They are involved in many cellular processes controlling metabolism, reproduction, differentiation and behaviour (1). Many clinical symptoms are associated with abnormal steroid levels. Synthetic steroids are widely used as agonists or antagonists for therapy.

Steroids are small molecules (300–400 Da) biosynthesized from cholesterol. Their molecular structures are very similar. Estradiol has a hydroxyl group at C17, whereas estrone has a ketone group. Cortisol is produced from its precursor, 11-deoxycortisol, by hydroxylation at C11. Measurement of steroid concentration is very useful during therapy and for clinical diagnosis. It is difficult to measure hormone concentrations in target cells, but blood hormone concentrations and hormone metabolites in urine can be determined. A competitive binding assay, using specific antibodies with high affinity and radioactive steroids, is the most commonly used method for measuring steroid concentrations.

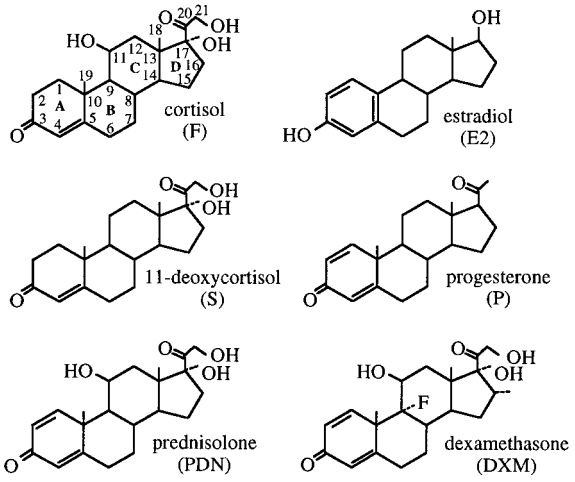
However, the structural similarities of steroids often cause problems with cross-reaction in immunoassay. Plasma and urine both contain substances that cross-react with anti-steroid antibodies. The steroids can be extracted with an organic solvent and separated by chromatography before measuring their concentration by immunoassay (2–4). This method is laborious and direct immunoassay on plasma or urine extracts is preferable for multiple determinations. Unfortunately, the method used for immunoassay can significantly affect the results obtained (5). Moreover, steroids cannot themselves cause an immunological reaction and must be coupled to immunogenic proteins. The specificity of an anti-steroid antibody depends on the position of coupling to the carrier protein (6). The ring coupling position determines the cross-reactivity of the antibody (7). Cross-reactivity with different steroids may result from small but significant conformational changes in the antibody which allow it to accommodate different ligand orientations in the binding site (8). For all these reasons, no anti-estradiol antibody able to detect picogrammes of estradiol in plasma and with no cross-reaction against related steroids, has ever been produced.

Remodelling the antibody-combining site could be used to increase the specificity of an antibody. Genetic engineering techniques may make it possible to improve the binding properties of potentially useful antibodies by using mutagenesis to modify the amino acid sequence within the antibody-combining site. Substitutions in only a few amino acids can increase affinity (9, 10), decrease cross-reactivity with closely-related antigens (9) or change specificity (11). The use of phage display technology has made possible the genetic engineering of immunoglobulins. It is possible to mimic the immune system *in vitro* by expressing antibody fragments on the surface of filamentous bacteriophages (12). Antibody fragments expressed at the phage surface can be selected on the basis of specificity, affinity, avidity, or binding kinetics. Genes coding for antibody fragments can be mutated by PCR methods to increase affinity (13, 14), change specificity (15) or modify cross-reactivity (16, 17). Short *et al.* obtained two anti-digoxin Fab mutants with affinities two and four times higher than that of the parental antibody from a phage display library containing randomized mutations of heavy chain residues (18).

X-ray crystallographic data from antibody fragments indicate that only a few residues of the antibody-combining site contribute significantly to affinity. The binding site must have some plasticity, particularly when the antigens themselves are rigid, as is the case for steroids (19). The cross-reactivity of an anti-progesterone Fab for steroid analogues may be due to flexibility of the antibody-combining site (20). Molecular modelling of the antibody-combining site may be a valuable approach for identifying residues in contact with the steroid where the X-ray structure is not known (21).

We have described the production in *Escherichia coli (E. coli)* of an anti-cortisol single-chain variable fragment (scFv 5A4; where the variable domains V_H and V_L are covalently associated by a flexible peptide) (22). The corresponding 5A4 monoclonal antibody (mAb) recognizes cortisol with a dissociation constant of 10^{-8} M (22). Like most anti-steroid antibodies, this mAb cross-reacts with steroid analogues but does not rec-

ognize estradiol (Fig. 1). We have also described the identification of the residues of the antibody-combining site and those which are likely to interact with cortisol (22). In the present study, we used mutagenesis to change the specificity of this anti-cortisol antibody into an anti-estradiol antibody.



F	E2	S	P	PDN	DXM
100%	<0.01%	<0.01%	0.08%	33.3%	20%

Fig. 1 Structures of cortisol (F), estradiol (E2), 11-deoxycortisol (S), progesterone (P), prednisolone (PDN), and dexamethasone (DXM) and their cross-reactivity with monoclonal antibody 5A4. The cross-reactivities determined by equilibrium dialysis are given as the percentage of cortisol concentration divided by steroid concentration at 50% binding of the zero standard (22).

Materials and Methods

Bacterial strain

The *E. coli* strain TG1 was used as the bacterial host for the preparation of phagemids, and as the host of bacteriophage M13K07.

Construction of the wild-type anti-cortisol scFv

To construct the 5A4 scFv, V_H and V_L genes were amplified from the plasmid pscFv5A4 by PCR (22). The 3' primer used to amplify V_H contained part of the coding sequence for the linker $(Gly_3Ser)_4$ and the 5' primer used to amplify V_L contained the rest of the sequence (with 15 overlapping bases). The scFv was assembled by splice overlap extension PCR (23). At the same time, two restriction enzyme sites (*Nco*I and *Eag*I) were introduced at the 5' and 3' ends, respectively, of the scFv gene to facilitate cloning into the pHENI phagemid (24) to give pHENscFv5A4.

Construction of the mutant library

We constructed the mutant V_H library using recursive PCR methods (25) and a set of overlapping oligonucleotides (Fig. 2). The mutant V_L library was constructed with a new PCR-based site-directed mutagenesis method using magnetic beads (26).

The oligonucleotides used for PCR are: oligo 1: 5' CTCGC *K*GC*S*CAGCCGGCCATGGC 3'; oligo 2: 5' CCCAGGCTTCACC AGCTCAGTTCCTGACTGCTGCAGCTGGACCTCGGCCATGGC CGGCTG 3'; oligo 3: 5' GAGCTGGTGAAGCCTGGGGCTTCA GTGAAGCTGTCCTGCAAGACTTCTGGCTTCACCTTCAGCAG TAGG 3'; oligo 4: 5' CCAGACTTTTGCTTCAACCA*VNN*CAT*VN N*CCTACTGCTGAAGGTG 3'; oligo 5: 5' GGTTGAAGCAAAAGT CTGGACAGAGTCTTGAGTGGATTGC 3'; oligo 6: 5' GCCTGTG

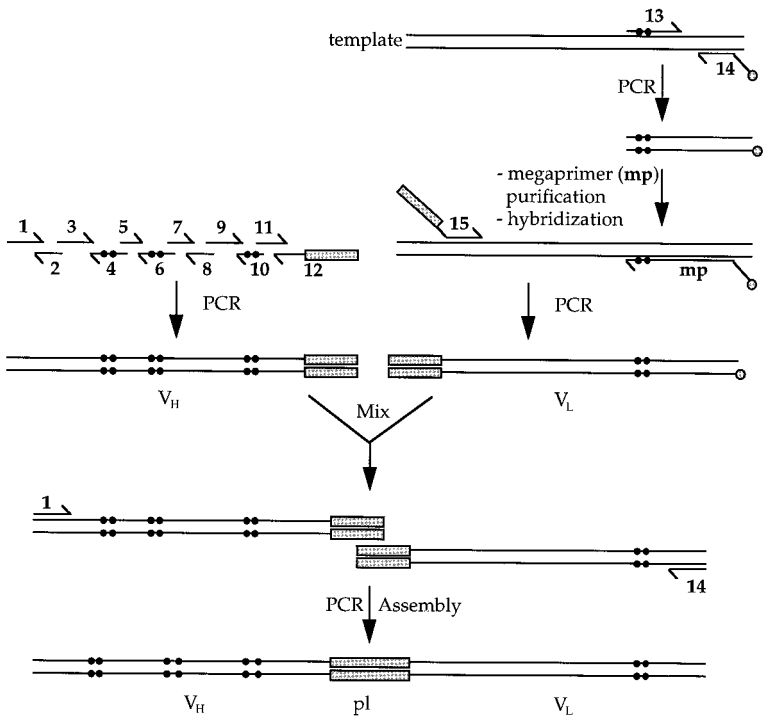


Fig. 2 PCR assembly of the scFv library. Oligonucleotides 1–12 were mixed and subjected to PCR amplification to create the mutated V_H library. The mutated V_L library was constructed using the wild-type phagemid template pHENscFv5A4 and oligonucleotides 13 and 14 for the first PCR amplification. After purification, the biotinylated single stranded megaprimer (mp) was mixed with the template and oligonucleotide 15 for a second PCR amplification. The two mutated PCR fragment libraries (V_H and V_L) were mixed with oligonucleotides 1 and 14 and amplified by PCR to give the mutated scFv library. The dots mark the mutated positions. The boxes represent the coding sequence for the peptide linker (pl).

AACTTCTGATTATA*VNN*AGAACCACCAGTTCCACCA T AAAT*VNN*TGCAATCCACTCAAGACTC 3'; oligo 7: 5' AATCA GAAGTTCACAGGCAAGGCCCAAGTGACTGTAGACACATC 3'; oligo 8: 5' GCTGAATTGCATGTAGACTGTGCTGGAGGATGTG TCTACAGTCAC 3'; oligo 9: 5' CTACATGCAATTCAGCAGCCT-GACAACTGAGGACTCTGCCATCTATTACTGTGCAAG 3'; oligo 10: 5' GGCCCCAGGAATCAAAGGG*VNN*GCTGCTACCATAG ACACT*VNN*TCTTGCACAGTAATAGATGG 3'; oligo 11: 5' TT GATTCCTGGGGCCAAGGCACCTCAGTCACCGTCTCCTCA 3'; oligo 12: 5' TCACCTGAACCAGGTTTACCAGAACCTGAGGTA-GAACCTGAGGAGACGGGTGAC 3'; oligo 13: 5' GCCAGCAGT GGAGTAGT*NNB*CCA*NNB*ACGTTCGGTGCTGG*SAC*N*A* AG*B*TGGA 3'; oligo 14: 5' CAAGCTTACTAGTTTATGCGGCCC-CATTCAGATCC 3'; oligo 15: 5' CTGGTAAACCTGGTTCAGGT GAAGGTAGTACTAAAGGTGACATTGTGCTGAC 3'. The mutated positions are indicated by italics (*B* = G, T or C; *K* = G or T; *N* = A, C, G or T; *S* = G or C; and *V* = G, A or C). The codon *NNB* was used to reduce the number of stop codons. The PCR products were purified by electrophoresis in polyacrylamide gels and the two libraries were assembled by splice overlap extension. *Nco*I and *Eag*I sites (underlined) were introduced at the same time. The final, purified PCR product (5 µg) was inserted into the pHENI phagemid (2 µg). Fifty electroporations were used to produce a library of 10^8 clones. Some of these clones were tested by miniprep and restriction analysis for the presence of an insert of expected size. These clones were then tested for expression of a 30 kDa product as previously described (27). The library was rescued as previously described (28) using helper phage M13KO7.

Selection of phage library

The antigen, estradiol-6-O-carboxymethyloxime-bovine serum albumin (E2-6-CMO-BSA; 10 µg/ml in phosphate buffered saline (PBS) solution) was incubated in maxisorp immunotubes (Nalge Nunc International, Naperville, IL, USA) overnight at 4 °C. The tubes were then saturated with 2 % milk PBS (MPBS) and incubated for one hour at 37 °C. We used 10^{13} titered units of library phages in a total volume of 2 ml MPBS per immunotube for the first round of selection. Immunotubes were washed and bound phages were eluted as previously described (28). After each round of selection, *E. coli* TG1 were reinfected with eluted phages and rescued to provide phages for the next round of selection. Selection was stopped when the recovery of eluted phages increased significantly (>10 fold, round 4 in this case).

Screening of clones using enzyme-linked immunosorbent assay

Single ampicillin-resistant colonies, resulting from infection of *E. coli* TG1 with eluted phages were used to inoculate 150 µl of 2YT medium containing ampicillin 100 µg/ml and glucose 2 %, in 96-well plates. Overnight production of phages was as previously described (28). Supernatants containing phages were tested for binding by enzyme-linked immunosorbent assay (ELISA) in Falcon 96-well plates coated with antigens (incubated at 10 µg/ml in PBS overnight) and saturated with MPBS. Binding of phages was detected with a horseradish peroxidase-anti-M13 antibody conjugate (Amersham Pharmacia Biotech, Uppsala, Sweden).

Specificity of selected clones

Analysis of phage binding to steroid-BSA was performed using bacterial supernatants containing phage, essentially as previously described (29, 30), with 10 µg/ml antigen in PBS in all cases, in Falcon 96-well plates.

Results and Discussion

We used the 5A4 antibody to create a library of anti-steroid antibody fragments by site-directed mutagenesis. The objective was to use phage display to select from the mutants the antibody fragment with the required specificity.

We have previously cloned the scFv fragment (heavy and light variable domains linked by a flexible peptide linker) of the 5A4 mAb and sequenced its gene (22). We expressed scFv fused to the g3p protein at the M13 phage surface, and used phage ELISA experiments to show that scFv had the same pattern of cross-reaction. The phage antibody immunoassay is a highly sensitive detection system. It facilitates the characterization of antibody fragments and eliminates the need for purification of antibodies (30). The 5A4 phage antibody (Phab) had very similar cross-reactivity to the parental monoclonal antibody, as is often the case (31). We have previously built a molecular model of the 5A4 paratope complexed with cortisol from the primary sequence and information about recognition of cortisol derivatives and analogues (22). This model allowed us to identify amino acids putatively in contact with the steroid. We found that the steroid nucleus may be stacked between the side chains of two tryptophans. We decided to conserve these two amino acids that could interact with any steroid, and all amino acids close to the steroid were randomized. Eight different positions were chosen in four complementarity-determining regions (CDR) (Tab. 1). The creation of a large library required large quantities of insert coding for scFv. These mutations were mapped to sites along the entire length of the gene. Therefore, we used recursive PCR methods (25) to introduce six mutations in the V_H gene and a new PCR-based method using magnetic beads to improve the mutagenesis efficiency in V_L (26). Splice overlap extension (23) was then used to assemble the V_H and V_L library (Fig. 2). The DNA was purified, digested, ligated, and electroporated to produce a library of 10^8 different clones. Library quality was tested by DNA restriction analysis and Western blot, and 96 % of clones contained a full length insert and 36 % of clones expressed scFv detected by Western blotting using IPTG induction and the 9E10 antibody to detect the c-myc tag. Vaughan *et al.* obtained similar results from their library (32). The low percentage of scFv-expressing clones may be due to frame-shifts, deletions or creation of stop codons introduced during the three successive PCR steps.

Tab. 1 Residues forming the antibody-combining site in the 5A4 scFv model. The mutated residues of the library are in bold script.

Chain segment	Residues
CDR-H1	Y33, S35
FR2-H	W47
CDR-H2	W50, N58
CDR-H3	H95, T100B, F100D
CDR-L1	H34
CDR-L3	W91, N94, P96

The 5A4 Phab was not cross-reactive with estradiol, a steroid very different in structure to cortisol. So, after rescue using a phage helper, the library was selected against estradiol-6-CMO-BSA. Positive Phabs were detected by ELISA after the third round of selection, and almost all Phabs eluted after the fourth round were positive (Tab. 2). Sequencing of 18 positive Phabs showed that a single clone had been selected. The V_H sequence of this clone (named 1B8) was identical to that of the wild-type scFv. However, the V_L gene had two mutations (Asn94Arg and Pro96Asp) in the CDR3. The 1B8 Phab was further characterized by ELISA (Fig. 3). Cross-reaction of the 5A4 Phab with prednisolone was not detectable with the 1B8 Phab. The 1B8 Phab gave a strong signal with estradiol but had residual binding to cortisol. Estradiol has a hydroxyl group at position C3. This position is close to the two mutations in the mutant Phab in our model. These new amino acids may form a hydrogen bond at this position, allowing recognition of estradiol. However, only part of the original paratope was modified and this may explain the residual binding to cortisol. It is highly improbable that selection led to conservation of wild-type codons in the V_H gene. The selected wild-type V_H gene may result from contamination during the construction of the mutant library. It may also indicate that the gene coding for the V_H domain in the original library may have been mutated too much, destabilizing the paratope scaffold. This problem could be overcome by using the parsimonious mutagenesis method that limits the number of amino acids that do not retain parental structural features (33). The mutation rate is controlled and can be limited to two or three mutations per clone over the eight degenerated positions.

Tab. 2 Frequency of binding clones from the scFv-phage library. The number of phage-transfecting units (T. U.) is given before (input) and after (output) each round of selection. Forty eight clones in each round of selection were tested by ELISA for their capacity to bind estradiol-6-CMO-BSA.

	Round of selection			
	1	2	3	4
Input phages (T.U.)	10^{13}	5.10^{12}	5.10^{12}	5.10^{12}
Output phages (T.U.)	10^{5}	10^{5}	5.10^{5}	5.10^{8}
Binders	0/48	0/48	4/48	46/48

We selected a new anti-estradiol antibody fragment by molecular modelling, site-directed randomization and phage display. The specificity of this clone could be improved. For example, chain-shuffling (34) could be used to select a V_H partner that specifically interacts with another site of the steroid. The resulting clone may have higher affinity for estradiol and may lose its affinity for cortisol. This work demonstrates that antibody engineering is a valuable technique for obtaining specific anti-steroid antibodies for immunoassays.

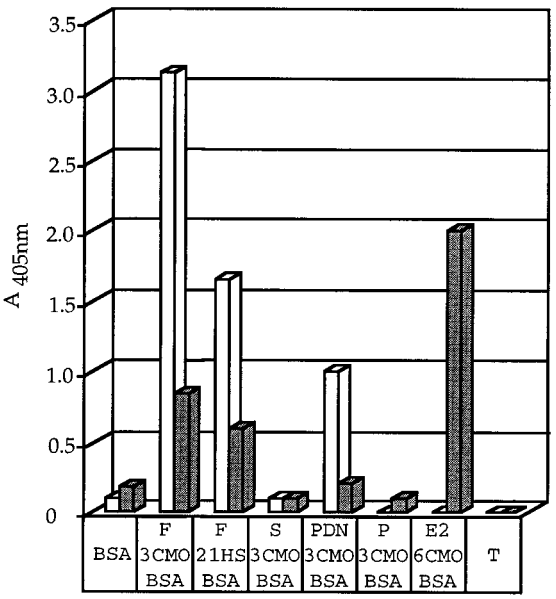


Fig. 3 Specificity of 5A4 and 1B8 Phabs. ELISA was used to test Phabs for their ability to bind directly to steroid-BSA. Cortisol-3-O-carboxymethyloxime-BSA (F-3-CMO-BSA), cortisol-21-hemisuccinate-BSA (F-21-HS-BSA), 11-deoxycortisol-3-CMO-BSA (S), prednisolone-3-CMO-BSA (PDN), progesterone-3-CMO-BSA (P), estradiol-6-CMO-BSA (E2), BSA or milk (T) were used to coat 96-well plates. Culture supernatants containing Phabs were incubated with the plates for one hour at room temperature. The plates were washed and bound Phabs were detected by addition of horseradish peroxidase/anti-M13 conjugate. The absorbance, indicated by bars, was measured at 405 nm. □ 5A4 Phab, ■ 1B8 Phab

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