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Antibodies to steroids from a small human naive IgM library

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Abstract **Human antibodies specific for digoxigenin, estradiol, testosterone and progesterone have been isolated from a small combinatorial IgM repertoire (4×10^7) of single chain antibodies (scFv). The affinities of both the anti-estradiol and anti-progesterone scFv were approximately 10^8 M^{-1}. Naive IgM genes appeared to be highly represented, since only the heavy chain variable domain of the anti estradiol antibody contained differences to corresponding germline sequences. The light chain variable domain of the progesterone receptor was also identical to a germline sequence, showing that it is possible for completely naive antibodies to bind steroids with affinities comparable to those obtained after a secondary immune response.**

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Key words: Antibody library; Human IgM repertoire; Single chain antibody; Anti-steroid antibody; Phage display

1. Introduction

Universal antibody libraries for the isolation of antibodies to any particular antigen have been generated by gene synthesis using random sequences within the hypervariable regions of the antigen binding site [1–7] or by randomly combining the heavy and light chain variable domains of the antibody gene repertoire. Using this second approach, a human antibody library was generated containing at least 10^7 members [8,9] from which antibodies were isolated to both small haptens and proteins including self-antigens [10,11] Their affinities were mainly in the micromolar range, similar to those obtained after a primary immune response. Theoretically, it should be possible to select antibodies with affinities in the nanomolar range with a sufficiently large and diverse library [12]. Vaughan et al. [13] therefore created a human scFv antibody library consisting of 1.4×10^{10} clones from the human lymphocyte repertoire of naturally-rearranged V-genes, including those derived from IgG. Antibodies with distinctly higher affinities could be isolated from this library.

We recently described a set of primers for amplifying the antibody gene repertoire from human B lymphocytes based largely on available amino acid sequences [14]. Using these primers, we generated a single chain IgG antibody library from the lymphocytes of a patient with a high titer of autoantibodies to $F(ab')_2$ fragments and isolated an anti-$F(ab')_2$ scFv [15]. In the present investigation, we employed similar conditions to generate a small naive IgM antibody library. The hormones estradiol, testosterone and progesterone were chosen as antigens for a first screening of this library, since they represent important clinical parameters and each of them shares either identical A and B rings or C and D rings with one of the other hormones, thus providing interesting comparisons of antibody specificity (Fig. 1). In addition, we included the steroid digoxigenin, which is a widely used marker. Human antibodies against this hapten are also useful for patient monitoring and provide potential therapeutic reagents for neutralising overdoses. To our knowledge, this is the first time that human antibodies to digoxigenin, testosterone and progesterone have been described.

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2. Materials and methods

2.1. Antigens

Digoxigenin-3-*O*-succinyl-ε-aminocaproic acid-*N*-hydroxy-succinimide ester (DIG-suc) and digoxigenin-3-*O*-methycarbonyl-ε-aminocaproic acid-*N*-hydroxy-succinimide ester (DIG-met) were coupled to amino groups on BSA using a DIG-protein labeling kit from Boehringer, Mannheim, FRG. Digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproyl-[5-(3-aminoallyl)-2′-desoxy-uridine-5′-triphosphate] (Dig-dUTP) was also obtained from Boehringer, Mannheim, FRG. The steroid hormones 17β-estradiol-6-CMO-BSA (1,3,5-estratriene-3,17β-diol-6-carboxymethyloxime: bovine serum albumin), testosterone-3-CMO-BSA (4-androsten-17β-ol-3-one-3-carboxymethyloxime: BSA) and progesterone-3-CMO-BSA (4-pregnen-3,20-dione-3-*O*-carboxymethyloxime: BSA) were supplied by Cortex Biochem (San Leandro, CA).

2.2. Amplification of antibody genes

Total RNA was prepared from the lymphocytes of twenty non-immunized donors as previously described [16]. Messenger RNA was prepared using the Optiprep. 2 kit from Biometra (Göttingen, FRG) and transcribed into cDNA using a kit from Amersham according to the manufacturer's instructions. The Fv-encoding regions of μ, κ and λ chains were amplified by PCR using primers and conditions similar to those described by Welschof et al. [14].

2.3. Phage display and selection for binding to antigen

The naive IgM-derived library was displayed on phage as previously described for an IgG-derived library [15]. The selection procedure was performed using Maxisorb immunotubes (Nunc, Roskilde, Denmark) that had been coated with 125 μg of antigen conjugated to BSA and then blocked with 2% skimmed milk in PBS for 2 h at room temperature. 10^{11}–10^{12} recombinant phages that had been preabsorbed with 100 μg BSA in 2% skimmed milk in PBS for 1 h were used for the screening procedure. This was essentially performed as already described [15] and repeated three times.

2.4. Phage-ELISA

Microtiter plates were coated overnight with approximately 1 μg/well of a given steroid-BSA conjugate or BSA. Following blocking with 2% (w/v) skimmed milk in PBS, between 10^9 and 10^{10} phages/well were added and incubated for 1 h at room temperature. After washing five times with 0.1% Tween in PBS, 100 μl of the mouse monoclonal antibody FE2 [17] diluted 1:5000 was added and incubated for 1 h at 37°C. After washing six times, the ELISA was developed with a monoclonal horseradish peroxidase (HRP) conjugated

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goat-anti-mouse IgG antibody (Jackson ImmunoResearch Laboratory Inc., West Grove, PA) and the substrate 3,3′,5,5′-tetramethylbenzidine (TMB; Kierkegaard and Perry, Maryland, USA). The absorption was measured at 655 nm.

2.5. Sequence analysis

Nucleotide sequencing was performed using the dideoxy termination method with the vector pBluescript II SK(+) and specific primers. The sequences were analysed using a HUSAR sequence analysis programme provided by the German Cancer Research Center and compared with the EMBL data bank.

2.6. Expression and purification of single chain antibodies in E. coli

ScFv fragments were expressed in *E. coli* XL1-Blue (Stratagene) using the secretion vector pHOG21 [18]. Antibody fragments specific to estradiol, testosterone and progesterone were isolated from soluble periplasmic extracts and culture medium by immobilized metal affinity chromatography (IMAC) essentially as described [18]. ScFv fragments specific for digoxigenin were purified by IMAC followed by ion-exchange chromatography on a Mono S HR5/5 column (Pharmacia) as described [19]. Purified antibody fragments were dialyzed against 10 mM HEPES, 0.15 M NaCl, 3.4 mM EDTA, 0.001% Tween 20, pH 7.4 (HBS) for size-exclusion chromatography and affinity measurements. The concentrations of scFvs were determined by both the Bradford dye-binding assay [20] using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany) and spectrophotometrically using the extinction coefficient $\varepsilon^{1mg/ml} = 1.66$ for anti-digoxigenin scFv, 1.26 for anti-estradiol scFv, 1.47 for anti-testosterone scFv and 1.44 for anti-progesterone scFv at 280 nm calculated according to Gill and von Hippel [21]. Analytical gel filtration of the scFv preparations was performed in HBS using a Superdex 75 HR10/30 column (Pharmacia). Sample volume and flow rate were 200 μl and 0.5 ml/min, respectively. A Low Molecular Weight Gel Filtration Calibration Kit (Pharmacia) was used to calibrate the column. The specificity of scFv preparations was analyzed by ELISA performed as previously described [22].

2.7. Affinity measurements

Antigen binding properties of scFv were characterized by surface plasmon resonance using a BIAcore 2000 instrument with a multichannel Integrated μ-Fluidic Cartridge (Pharmacia Biosensor AB, Uppsala, Sweden). The antigens were immobilized using the amine coupling kit from the manufacturer at a concentration of 100 μg/ml in 10 mM sodium acetate buffer, pH 4.5. The flow cells of the sensor chips were loaded either with estradiol-BSA to give 1631 resonance units (RU; 1000 RU correspond to a surface concentration of 1 ng/mm^2), testosterone-BSA (1858 RU), progesterone-BSA (1220 RU) or digoxigenin-BSA (3564 RU for determination of association rates and 572 RU for determination of dissociation rates). As a negative control, BSA was immobilized under the same conditions yielding 3562 RU. One channel of each sensor chip was used after activation for injection of 10 mM sodium acetate buffer, pH 4.5, followed by deactivation with ethanolamine. This channel was used to control the non-specific binding of scFv to the carboxymethylated dextran surface. All the measurements were carried out at a flow rate of 5 ml/min in HBS at 25°C. After each cycle, the surface was regenerated by a single 5 μl injection of 10 mM HCl. Analyses of scFv antibodies were performed in the concentration range 6.25–800 nM. Each injected sample of antibody fragment (25 μl) was in contact with the antigen for 5 min. The dissociation was followed for 10 min. Kinetic constants were calculated using BIAevaluation 2.1 Software (Pharmacia Biosensor AB).

3. Results

3.1. Library construction

The cloning scheme for generating a library of scFv from the lymphocytes of healthy donors is outlined in Fig. 2. After amplifying the genes coding for the variable domains of the IgM heavy chains and kappa and lambda light chains with a set of primers that we described previously [14], the light chain repertoire was cloned into the phagemid display vector pSEX81 [15]. Sublibraries of approximately 2×10^7 members for the kappa and 1.5×10^7 for the lambda chains were obtained. After combining them with the V_H DNA repertoire, the library size was determined to be 4×10^7 after serial dilution followed by plating.

3.2. Selection of antibodies to various steroids

Digoxigenin and the steroid hormones estradiol, testosterone and progesterone coupled to free amino groups on BSA were immobilised on the walls of Nunc immunotubes for panning with the naive IgM-derived antibody library displayed on phagemid particles (see Experimental Protocol). A 1000-fold increase in the titer of binding phage was found after only the second round of screening against digoxigenin. The enrichment of binding phage against the steroid hormones was more gradual but also quite significant after the third round. The specificity of clones isolated after the third round was checked by performing phage ELISAs.

Five of six clones selected at random after the third round of screening for binding to digoxigenin were found to be identical (anti-DigA). The sixth clone (anti-DigB) was more specific, although equal numbers of phagemid particles carrying either of the two scFv showed similar binding to digoxigenin in a phage-ELISA (Fig. 3a). However, after expression of their DNA in *E. coli*, the affinities of the soluble scFv in periplasmic extracts were seen to be quite different. Extracts containing anti-DigA scFv bound less well than extracts con-

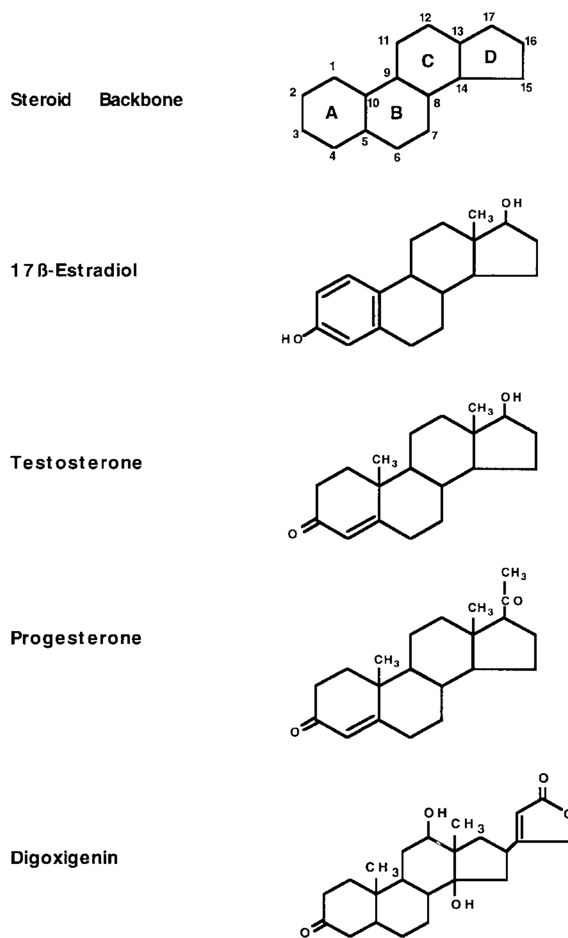

Fig. 1. Construction of the naive IgM library. For details see the Methods in Section 2.

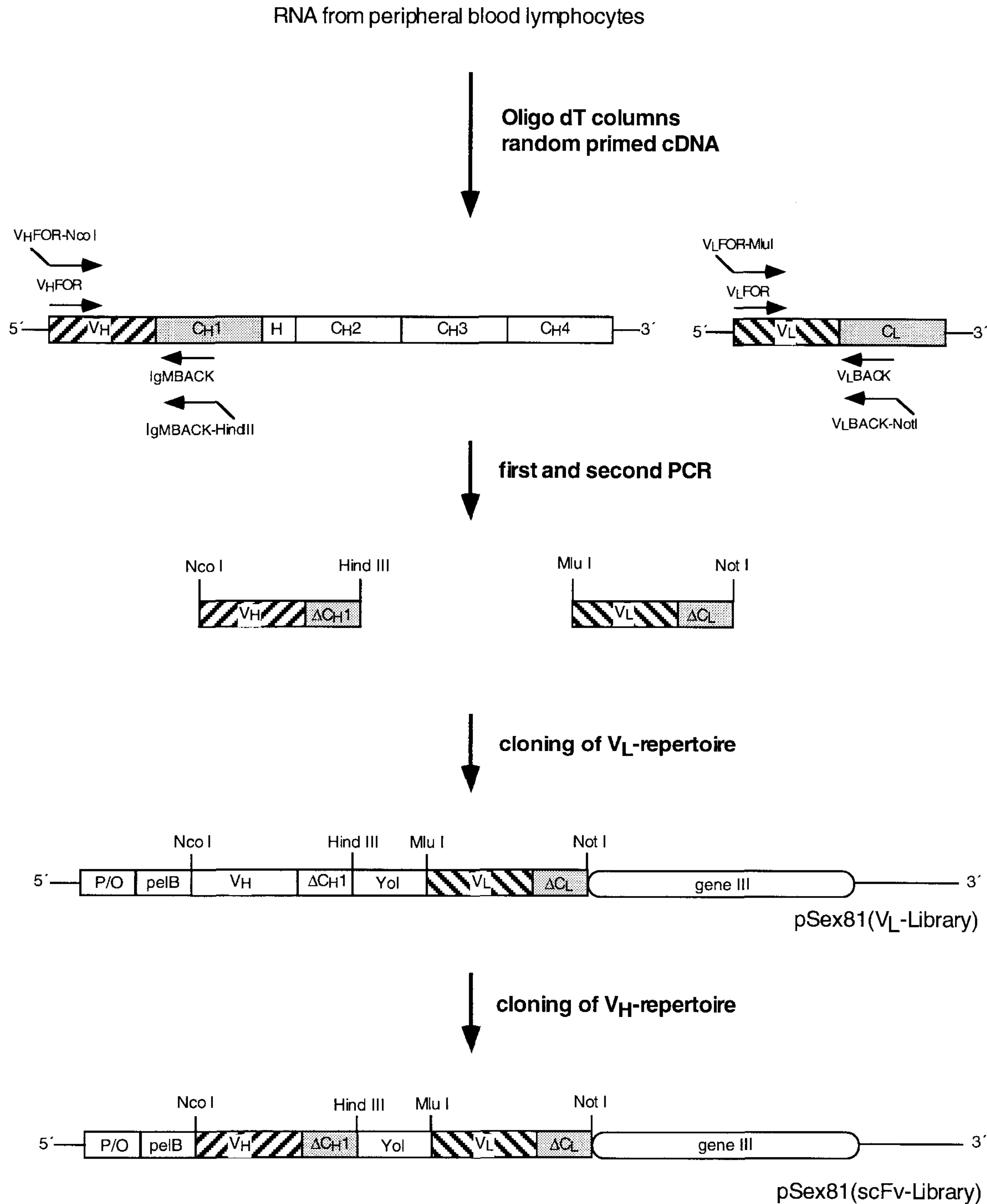

Fig. 2. Structural formulas of estradiol, testosterone, progesterone and digoxigenin.

taining anti-DigB scFv, even though the anti-DigA scFv was present in significantly higher quantities (Fig. 3b). The higher affinity anti-DigB scFv reacted equally well with digoxigenin that was coupled to BSA using either of two different linkers. Furthermore, a soluble conjugate of digoxigenin coupled to dUTP was able to competitively inhibit its binding (Fig. 3c).

To choose the best clones binding to estradiol, testosterone and progesterone, phage ELISAs of 100 randomly picked clones were performed. The DNA of fourteen anti-estradiol clones that appeared to bind best were found to be identical. In contrast, three different anti-testosterone clones were found amongst fourteen of the best binders. Of the 100 clones analysed for progesterone binding by phage ELISA, only two appeared to be specific. The most specific clones in each case were taken for further characterisation.

3.3. Purification and characterisation of scFv

Soluble scFv in periplasmic extracts were purified by metal chelate chromatography. Surprisingly, large variations were observed in the yield of purified scFv, ranging from 10.1 mg/L for the anti-estradiol scFv to only 0.74 mg/L for the anti-progesterone scFv (Table 1). Analysis of the scFv preparations by size-exclusion chromatography demonstrated that scFv specific to digoxigenin, estradiol and testosterone con-

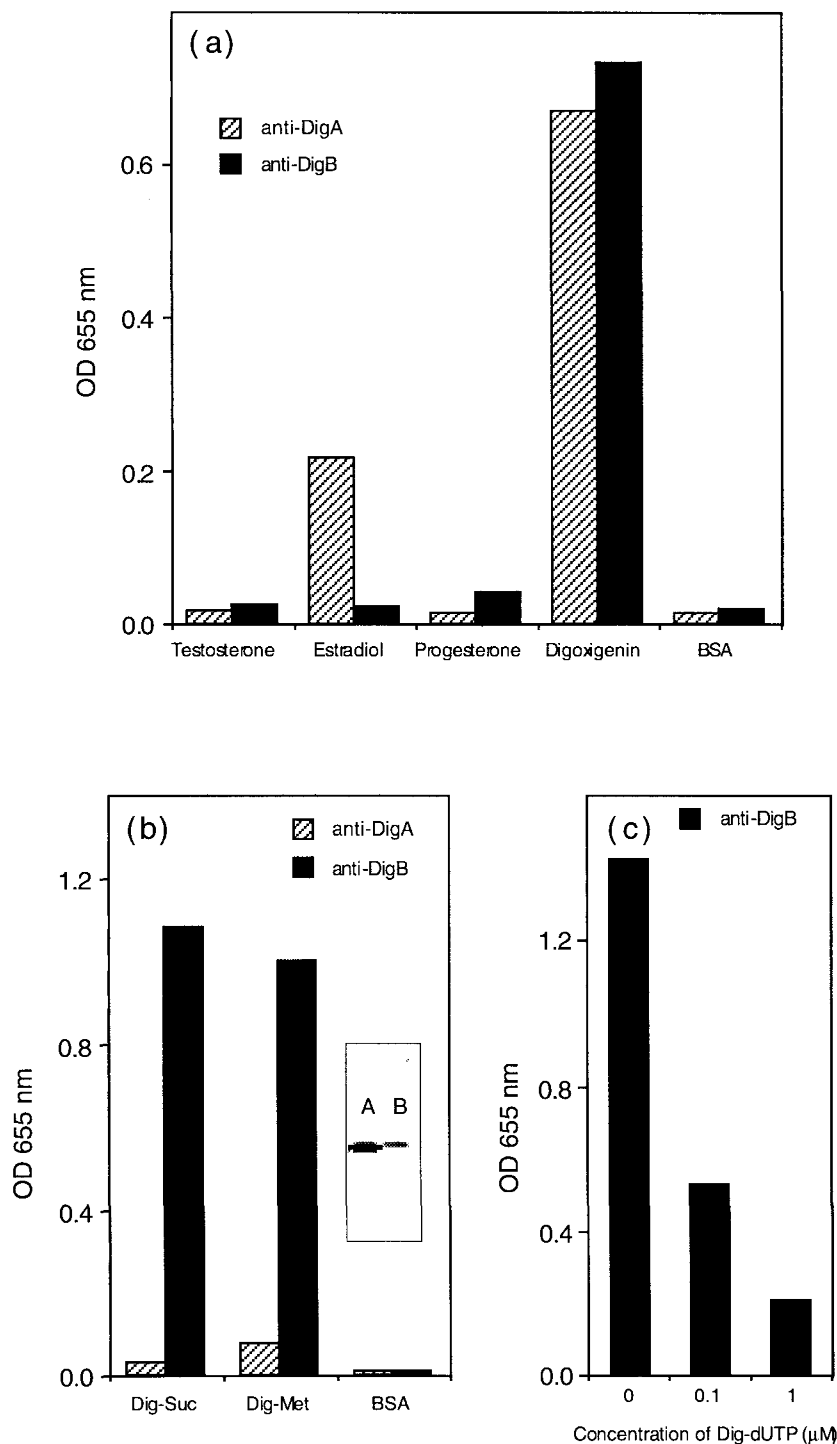

Fig. 3. Binding of phage and scFv to digoxigenin as analyzed by ELISA. Anti-DigA and anti-DigB are two different clones selected by phage display. (a) Binding of phages to digoxigenin and steroid hormones coupled to BSA. Digoxigenin was coupled to BSA with a methylcarbonyl type linker. (b) Binding of scFv (periplasmic extract) to digoxigenin coupled by a methylcarbonyl (Dig-Met) or succinyl (Dig-Suc) type linker to BSA. The insert is a Western blot of anti-DigA (A) and anti-DigB (B) using equal amounts of extract. (c) Competitive inhibition of scFv (periplasmic extract) binding to Dig-Met by a soluble Dig-dUTP conjugate.

sisted only of monomers while around 40% of the anti-progesterone scFv consisted of non-covalent dimers (data not shown). The purified antibody fragments interacted specifically with their target antigens as demonstrated by ELISA (Fig. 4) and BIAcore measurements (data not shown). The affinity constants calculated on the basis of real time antibody-antigen interaction analysis were quite high for antibodies from a naive library (Table 1).

A sequence analysis of these antibodies showed that the genes coding for the V_H domain of the anti-steroid hormones and anti-DigA, the low affinity anti-digoxogenin scFv, all belonged to the same family, namely, DP47, whereas that of

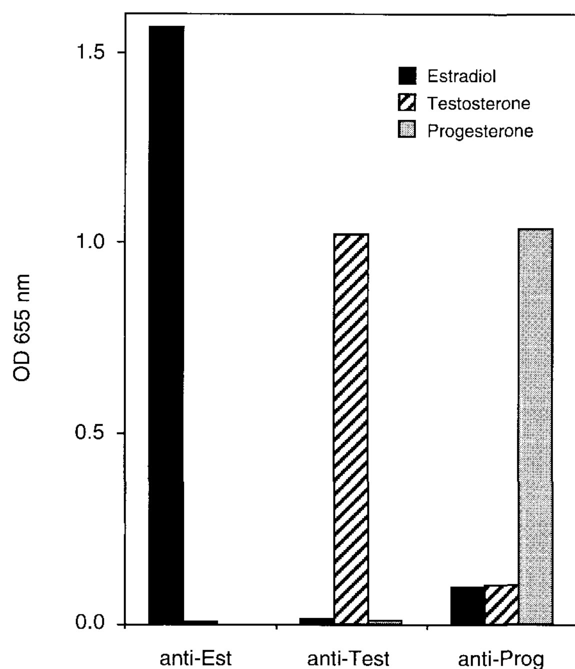

Fig. 4. Binding of purified anti-estradiol scFv, anti-testosterone scFv and anti-progesterone scFv at concentrations of 10 μg/ml to different antigens as analyzed by ELISA.

anti-DigB, the high affinity anti-digoxigenin scFv, belonged to DP71 (Table 2). Four of the five V_H gene sequences showed no differences at all to the germline sequences from which they were derived. These results suggest that the primers and conditions used for amplifying the repertoire of naive IgM genes were well chosen. All the light chains on the other hand showed significant sequence differences to the nearest germline sequence.

4. Discussion

4.1. Antibody library

Our IgM-derived antibody library contained 4×10^7 independent clones. Four of the five sequenced V_H clones described in Table 2 are completely homologous to known germline sequences, indicating that the naive IgM repertoire was indeed highly represented. In comparison, none of the published V_H sequences from a much larger lymphocyte-derived library containing 1.4×10^{10} members was completely homologous to a germline sequence [13]. The explanation could lie in the choice of antisense primers, which in the latter investigation were homologous to four J_H regions for amplifying DNA coding for all the V_H domains. However, most of the clones from a similarly derived library using IgM-specific primers for cDNA synthesis also contained a significant number of differences to the nearest germline sequence [9]. Possible explanations put forward by the authors were that somatic mutation was occurring prior to the clonal expansion of specific B cells or mutations were being introduced by PCR. The differences between the light chains and nearest germline sequences possibly reflect larger quantities of mRNA from mature versus immature B cells, since it was not possible to discriminate between these populations.

4.2. Antibody affinities

Considering the small size of the present library and the use of V_H domains that have not been matured by somatic mutation, the equilibrium association constants of the antibodies to digoxigenin, estradiol, testosterone and progesterone shown in Table 1 are quite high, approaching the affinities that one would only expect from a secondary immune response. Both the anti-estradiol and anti-progesterone clones, for example, have association constants of 1.0×10^8 M−1 and 1.3×10^8 M−1, respectively. For comparison, a value of 6×10^8 M^{-1} was recently obtained for a human scFv to estradiol that had been selected from the library of 10^{10} clones mentioned above and then reshuffled to increase its affinity [23]. Moreover, its rate of dissociation (2×10^{-3} s^{-1}), which is particularly important for the stability of binding, was not lower than that of our anti-estradiol scFv. The anti-estradiol antibody described here also appeared to be quite specific for its antigen, in contrast to the anti-progesterone antibody that showed a small amount of cross-reactivity with estradiol and testosterone.

To our knowledge, no other human anti-progesterone antibodies have been described. Murine anti-progesterone antibodies isolated from a naive IgM library of 5×10^6 members by phage display of Fab fragments fused to the major coat protein pVIII had affinities of 10^4–10^5 M^{-1}, in the range expected for a primary response [24]. One reason for the lack of binders postulated by the authors was the possible absence of particular subgroups in their library, since all or most of the anti-progesterone monoclonal antibodies are encoded by a V_H gene from the small and infrequently used VGAM3.8 family and V_L genes from the $V_\kappa5.1$ gene of the V_κII subgroup [25–27]. In contrast, the genes coding for our anti-progesterone antibody belong to commonly used families and the V_H gene DP47, in particular, is one of the most frequently found when screening antibody libraries with haptens [5,13]. The affinities

Table 1
Production levels and kinetic parameters for binding of anti-steroid scFvs to their antigens as measured by the BIAcore 2000 system

scFv	Valency[a]	Yield[b] (mg/l of culture)	k_{on} (M^{-1} s^{-1} s^{-1})	k_{off}	$K_a = k_{on}/k_{off}$ [c] (M^{-1})
anti-Est	M	10.1	$(1.78\pm0.04)\times10^5$	$(1.78\pm0.64)\times10^{-3}$	1.01×10^8
anti-Test	M	4.3	$(1.06\pm0.11)\times10^5$	$(1.96\pm0.36)\times10^{-3}$	5.41×10^7
anti-Dig	M	0.84	$(4.00\pm0.80)\times10^4$ [d]	$(3.48\pm0.63)\times10^{-3}$ [e]	1.15×10^7
anti-Prog	M+D	0.74	$(1.16\pm0.08)\times10^5$	$(8.68\pm0.48)\times10^{-4}$	1.34×10^8

[a]M, monomers; D, dimers.
[b]After purification.
[c]Affinity (equilibrium association) constants obtained directly from the ratio k_{on}/k_{off}.
[d]Kinetic association rate constants obtained using immobilized 3564 RU of Dig-BSA.
[e]Kinetic dissociation rate constants obtained using immobilized 572 RU of Dig-BSA.

Table 2
Sequences of anti-steroid antibody variable domains

scFv	Family	Germline gene	Amino acids differences from germline[a]	CDR3 aa sequence
(A) Heavy chains				
anti-DigA	VH3	DP-47	0	----D---KGSGWY
anti-DigB	VH4	DP-71	0	VSS------RGVDP
anti-Est	VH3	DP-47	5	DLGYYGSGSQPFEY
anti-Prog	VH3	DP-47	0	-WLRG---LNYFDY
anti-Test	VH3	DP-47	0	DWR------VVPAD
(B) Light chains				
anti-DigA	VL1	DPL-3	4	AAWDDS--LSGVV
anti-DigB	VL1	DPL-3	14	ASWDES--LTGVV
anti-Est	VL3	DPL-16	8	NSRDRSGNHVNVL
anti-Prog	VL2	LV2018	0	SSYTRT--STRVL
anti-Test	VL2	DPL-12	9	SSYAGR--NSFYV

[a]Excluding the primer region.
Human germline segments have been assigned according to the V-BASE Sequence Directory, Tomlinson et al., MRC Centre for Protein Engineering, Cambridge, UK.
The nucleotide sequences have been submitted to the EMBL-Data Bank with the following accession numbers: anti-DigA X98751(V_H), X98752(V_L); anti-DigB X98749(V_H), X98750(V_L); anti-Est Y09384(V_H), Y09385(V_L); anti-Test Y09386(V_H), Y09387(V_L).

of the secondary response murine monoclonal antibodies range from $2–5\times10^8$ M^{-1}, which are not much higher than the affinity of the human anti-progesterone antibody described in this paper. In particular, the anti-progesterone antibody had a low dissociation rate constant of 8.7×10^{-4} M^{-1}, probably reflecting to some extent the presence of scFv dimers. The other scFvs were present exclusively as monomers, thus demonstrating that avidity effects played no part in their relatively high affinity binding.

The affinity of the anti-testosterone antibody was about five times lower than those of the antibodies to estradiol and progesterone. Other human antibodies to testosterone have not yet been described. An ELISA of the purified scFv showed little cross-reactivity with either estradiol or progesterone, which is surprising considering that one end of the molecule resembles estradiol and the other end progesterone. Indeed, the only difference between testosterone and progesterone is at position 17, which is at the end furthest away from the linker joining position 3 to BSA. This is a strong indication that the linker makes no contribution to antibody binding. Similarly, the absence of any significant cross-reactivity of the estradiol antibody with the other steroids suggests that the carboxymethyloxime linker makes no contribution to binding.

The anti-digoxigenin scFv (anti-DigB) bound equally well to digoxigenin coupled with either one of two different linkers to BSA. Furthermore, this binding could be competitively inhibited by using a soluble conjugate of digoxigenin coupled to dUTP. The antibody anti-DigA bound digoxigenin with a much lower affinity, although equal numbers of phage particles carrying the genes for either anti-DigA or anti-DigB gave similar ELISA readings. The reason for this apparent contradiction appears to be the higher amount of soluble functional anti-DigA produced by *E. coli*. The concentration of phage particles carrying anti-DigA is therefore probably higher than those carrying anti-DigB. A fraction of the phage might also be carrying more than one anti-DigA antibody, which would increase the avidity of binding.

Interestingly, the first single chain antibody was engineered using the V_H and V_L domains of the murine anti-digoxin monoclonal antibody 26-10 [28]. It was expressed in *E. coli* and refolded from inclusion bodies. More recently, another high affinity murine anti-digoxin scFv with more specific binding characteristics was isolated in a soluble form from the periplasm of *E. coli* [29]. Both antibodies cross-reacted with the very similar digoxigenin. Human antibodies against digoxin and its active metabolites are particularly interesting because of their potential clinical application for treating overdoses of this widely prescribed heart stimulant. To our knowledge, this is the only report until now of a human anti-digoxigenin antibody.

4.3. Yields of soluble scFv

A pure preparation of the anti-estradiol scFv was obtained at the relatively high yield, for shake flask cultures, of approximately 10 mg/L. In contrast, the yield of the anti-progesterone antibody was only 0.74 mg/L. Similar variations have been observed in the yields of other recombinant antibodies [18,19,30,31]. In *E. coli*, the secretion machinery directs the recombinant scFv proteins to the periplasm where the degree of successful folding appears to depend largely on the primary structure [32,33]. The sequence of the anti-estradiol scFv might therefore provide a suitable framework for humanising murine antibodies or for generating synthetic human antibody libraries.

The V_H domain of the estradiol antibody and of the other antibodies with the exception of anti-DigB are derived from DP47, a germline sequence that is often found after selection by phage display (see above). This frequent occurrence may depend to some extent on the efficiency of folding. When paired with a light chain that also folds efficiently, high levels of soluble scFv would be secreted, as in the case of the anti-estradiol and anti-DigA antibodies.

In conclusion, we have obtained human anti-steroid scFv from a relatively small IgM-derived library with affinities similar to those obtained after a secondary immune response. Most of the V_H genes were identical to germline sequences. The anti-progesterone scFv was composed of V_H and V_L domains that were both identical to germline sequences, showing that completely naive V_H/V_L combinations can bind steroids with relatively high affinity.

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