Anti-digoxin scFv fragments expressed in bacteria and in insect cells have different antigen binding properties

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Abstract A gene encoding a single-chain antibody fragment directed against digoxin (named 1C10 scFv) was cloned in two expression systems. For this purpose, a new baculovirus transfer cassette fully compatible with the procaryotic pHEN vector was constructed. Baculovirus production led to higher yield than did *Escherichia coli* expression. The procaryotic fragment showed variations in the fine specificity profile but an affinity constant nearly identical to that of the 1C10 F_{ab}, whereas the eucaryotic scFv fragment had a lower affinity with a specificity profile identical to original mAb. The half-lives of the digoxin:scFv complexes and the global specificity are compatible with therapeutic use of this antibody fragment.

Key words: Single-chain variable fragment; Digoxin; Affinity; Specificity; Baculovirus; *Escherichia coli*; Protein expression; SPR analysis

1. Introduction

In the past decade, the development of immunoglobulin (Ig) gene manipulation and expression systems has provided a large number of recombinant antibodies (Abs) and Ab fragments [1]. These engineered Abs have applications in many fields such as immunodiagnosis [2,3], research [4], immunoscintigraphy [5], and mainly immunotherapy [6–8]. The small Ab fragment scFv (single-chain Fv) is of considerable interest in these fields. scFv is a unique polypeptide composed of the heavy chain variable region (V_H) covalently linked to the light chain variable region (V_L) by a short flexible peptide of about 15 residues. It can be derived from phage-display libraries [9] or hybridoma [10]. Furthermore, an scFv of desired specificity can be engineered to modify its properties, e.g. affinity [11]. Numerous scFvs have been produced for therapeutic needs, including cancer treatment [12,13] and detoxication [14,15]. For detoxication, as well as for immunoscintigraphy, the small size of the scFv (27 kDa) permits good tissue penetration and rapid diffusion in the body. Furthermore, the absence of constant domains decreases the risk of immunogenicity [8]. In cancerology, scFvs are frequently produced as fusion proteins with an effector [16] or as bivalent or bispecific Ab fragments [17].

Bacterial expression systems are currently used for scFv production. High expression levels can be achieved in *Escherichia coli* (up to several hundred milligrams), but the counterpart is an accumulation of aggregated material in the cytoplasm (inclusion bodies); a subsequent 'tricky' step of refolding is then necessary [18]. Cell secretion methods, using a signal peptide directing the scFv protein to the periplasmic space, can obviate this [19]. The signal sequence is cleaved during transport through the cytoplasmic membrane. Generally the secreted protein is then correctly folded and has a homogeneous N-terminus [1]; nevertheless, the yield is often low and periplasmic inclusion bodies and dimers or higher polymers can occur [20]. Alternative expression systems have been used for scFv, including yeast [21–23], mammalian cells [24], plants [25,26], and insect cells with the baculovirus vector [27,28]. The latter offers theoretically a strong advantage over the other systems for therapeutic purposes. As far as it is known, insect cells and baculovirus are devoid of pathogenic or toxic compounds for humans; moreover, insect cells can be grown in serum-free medium, i.e. without mammalian contaminants, leading to easy and safe purification. Chimeric whole Ab, with mouse variable regions and human constant regions, have been produced in insect cells for use in human therapy [29,30].

We previously produced a monoclonal anti-digoxin antibody named 1C10 which has a high affinity for digoxin (0.23 nM) and broad specificity [31]. In particular, it recognizes digoxin analogs (lanatoside C, deslanoside, digitoxin, digitoxigenin) and all its active metabolites. This monoclonal antibody (mAb) has been described as a powerful tool in the treatment of life-threatening digoxin intoxications [32,33]. To improve its kinetics in vivo, we have genetically engineered a reduced sized mAb. We produced the 1C10 scFv in *E. coli* and in the baculovirus/insect cell system. After amplification of the V_H and V_L genes of the 1C10 hybridoma, the scFv was assembled through the $(Gly_4Ser)_3$ linker and cloned either in the pHEN-His vector or in an original baculovirus vector named pBFv, each of which contains a signal peptide and a *myc*-hexahistidine (His_6) tag. The same *Sfi*I and *Not*I cloning sites were used in both vectors, leading to complete compatibility between the phagemid and the baculovirus system.

We report here that the 1C10 scFv was produced in an

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Abbreviations: BCA, bicinchoninic acid; CNBr, cyanogen bromide; EDTA, ethylenediaminetetraacetic acid; IMAC, ion metal chelate affinity chromatography; IPTG, isopropyl-thiogalactoside; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SN, supernatant

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active form in both *E. coli* and in the baculovirus/insect cell system. The 1C10 scFv expressed in bacteria showed a different fine specificity profile compared with the parental mAb. The same scFv expressed in the baculovirus system showed intact specificity but had lower affinity for the hapten.

2. Materials and methods

2.1. Cell lines, bacterial strains and vectors

The 1C10 hybridoma and 1C10 mAb have been described previously [31]. The 1C10 F_{ab} used as reference was obtained by papain digestion [34]. Anti-*myc* tag 9E10 mAb, HB2151 *E. coli* strain and pHEN1 phagemid [35] were kind gifts from Dr. G. Winter. *Spodoptera frugiperda* Sf9 insect cells were purchased from ATCC (CRL 1711); p118 plasmid and AcSLP10 baculovirus have been described previously [36].

2.2. Cloning and sequencing of V_H and V_L 1C10 genes

Total RNA was extracted from 6×10^8 1C10 hybridoma cells using the guanidium thiocyanate method as described by Chomczynski and Sacchi [37]. Reverse transcription was performed with 2 μg of total RNA, the reverse transcriptase Superscript (GibcoBRL) and primers OPP-SoFdγ1-3′ (5′ CAAAGATCTCAACCACAATCCCTGGGCA 3′) for cDNA heavy chain synthesis and OPP-SoCκ3′ (5′ CGCGCAGATCTAACACTCATTCCTGTTGAAGC 3′) for cDNA light chain synthesis. One μl of first strand cDNA was used as matrix for the PCR to amplify the 1C10 V_H-C_{H1} and V_K-C_K genes in two separate reactions using VentDNA polymerase (New England Biolabs) and the following degenerate primers: for V_H-C_{H1} amplification, OPP-SoFdγ1-3′ and OPP-SoVH-5′ (5′ GAGGT(C/G)CAGCTGCAG(C/G)AGTC(T/A)GG 3′); for V_K-C_K amplification, OPP-SoCκ-3′ and OPP-SoVκ-5′ (5′ GA(C/T/A)ATTGAGCTCAC(C/A)CAG(T/A)CTCCA 3′). The amplified fragments were cloned in pUC19 in the *Sma*I site and sequenced using the dideoxy chain termination method [38] with the T7 sequencing kit (Pharmacia).

2.3. Construction of the baculovirus pBFv cassette

As described in Fig. 1A, a modified pUC19 plasmid containing a unique *Bgl*II site in the *Hin*cII site of the polylinker was linearized with *Bam*HI and *Bgl*II. A *Bam*HI/*Bgl*II oligonucleotide of 83 bp (Fig. 1B) was cloned into the digested pUC19 to obtain the pUC19/insert I construction. This oligonucleotide contains the first 13 codons of the cDNA of the mouse V47 VH gene signal sequence [39] followed by *Sfi*I and *Not*I sites. The 71 bp oligonucleotide (Fig. 1B) was then inserted into pUC19/insert I, previously digested with *Not*I and *Bgl*II. This oligonucleotide contains the *myc* tag and His_6 tag sequences between *Not*I and *Bgl*II sites. To construct the pBFv cassette, pUC19/insert I+II was linearized with *Bam*HI and *Bgl*II; the insert I+II was then purified using the PCRprep Wizard purification kit (Promega). The p118 plasmid, a p10 transfer vector containing a unique *Bgl*II site downstream of the baculovirus p10 promoter [36], was linearized with *Bgl*II. The pBFv transfer cassette was obtained by cloning the insert I+II in the *Bgl*II site. Sequences of all the constructions were verified on both strands.

2.4. Construction and cloning of the 1C10 scFv

V_H and V_K DNA were separately amplified using cloned Pfu DNA polymerase (Stratagene) with specific sets of primers: VHBack1C10 (5′ CATGCCATGACTCGC*GGCCCAGCCGGCC*ATGGCCCAGGTCCAAGCCGCAGAGTCCGG 3′), VHFor1C10 (5′ GCCAGAGCCACCTCCGCCTGAACCGCCTCCACCTGCAGAGACAGTGACCAGAGTCC 3′), VκBack1C10 (5′ TCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGACATCCAGATGACCCAGACTCCATCCTCCTTATCT 3′) and VκFor1C10 (5′ GAGTCATTCT*GCGGCCGC*CCGTTTGATTTCCAGCTTGGTGCCTCC 3′). VHFor1C10 and VκBack1C10 are overlap primers containing sequences encoding the $(Gly_4Ser)_3$ linker peptide (underlined), which were designed to be complementary at their extremities, thus allowing subsequent annealing of the amplified V_H and V_L regions. VHBack1C10 and VκFor1C10 contain the *Sfi*I and *Not*I restriction sites, respectively, for the scFv cloning (italics). The V_H and V_κ genes obtained after 30 cycles of PCR (94°C 1 min, 55°C 1 min, 72° 2 min) were gel purified; next, an overlapping PCR was performed to construct the scFv gene under the same conditions, but with the VHBack1C10 and VκFor1C10 primers. The scFv DNA was gel purified, cleaved successively with *Sfi*I and *Not*I, and inserted into pHEN-His linearized using the same restriction enzymes. pHEN-His was derived from pHEN1, through addition of a hexahistidine peptide (His_6) just after the *myc* sequence using site-directed mutagenesis. The recombinant pHEN-His (BM28) was electroporated into competent *E. coli* HB2151 cells and positive clones were checked by PCR using VHBack1C10 and VκFor1C10 oligonucleotides.

The scFv DNA was cloned into the pBFv vector using the same *Sfi*I and *Not*I restriction sites, yielding pBFv1C10. The nucleotide sequence of 1C10 scFv cloned either in pHEN-His or in pBFv was verified and is presented in Fig. 2.

2.5. Expression and purification of 1C10 scFv

For procaryotic expression, 1:50 dilution of an overnight culture of HB2151 transfected with the BM28 vector was grown at 37°C to an OD_{600nm} of 0.6 in 2×TY medium containing 100 μg/ml ampicillin and 5% glycerol, induced with 0.5 mM final IPTG and shaken for 2 h at 22°C. The periplasm containing soluble scFv was extracted according to the procedure described by Skerra and Plückthun [40] and stored at +4°C. Periplasmic scFv was purified on an anti-*myc* affinity column using 9E10 mAb coupled to a CNBr-activated Sepharose 4B (Pharmacia). Bound scFv was eluted with 3 M glycine pH 2.2, 0.5 M NaCl, and the pH was immediately neutralized with a solution of 1 M Tris. Fractions were assayed by ELISA (described below) and their purity verified on a 15% SDS-PAGE using the procedure of Laemmli [41]. The purified scFv was dialyzed at +4°C against water containing 5% saccharose.

Insect cell expression of recombinant scFv was performed as previously described [30]. Briefly, Sf9 cells were cotranfected with 5 μg of pBFv1C10 and 500 ng of the purified DNA of the AcSLP10 baculovirus, yielding after the recombination event a baculovirus expressing the 1C10 scFv under the control of the p10 promoter. This virus was selected by plaque assay as an occlusion body-negative virus. Sf9 cells grown in serum-free medium were then infected with the recombinant baculovirus at a multiplicity of infection of 2 in a spinner culture (10^6 cells/ml). Supernatant (500 ml) was harvested 10 days post-infection. This supernatant (SN) was purified by IMAC on a nickel-NTA agarose gel (Qiagen) using a batch procedure. Ten ml of SN were incubated for 1 h at +4°C on 0.5 ml of nickel-NTA gel equilibrated twice with 5 ml of a 25 mM Tris, 500 mM NaCl, pH 7.5 buffer (TN) containing 5 mM imidazole. After centrifugation at 700×*g* and two washes in 5 ml of TN, 25 mM imidazole, the scFv was eluted twice with 0.5 ml of TN, 500 mM imidazole, dialyzed in water containing 5% saccharose, and checked by ELISA for activity and electrophoresis for purity.

Protein content was determined by the microBCA method (Pierce) using bovine serum albumin as standard, and by measuring the absorbance at 280 nm. A 1 mg/ml solution of the 1C10 scFv was found to have an absorbance of 1.9, calculated according to Mach et al. [42]. Both methods gave the same value.

2.6. Detection of scFv anti-digoxin activity and determination of specificity

An indirect ELISA procedure was performed as previously described [43] to detect the scFv anti-digoxin activity in periplasm, Sf9 supernatant, or pure preparations. Briefly, microtiter plates coated with 5 μg/ml of digoxin-BSA conjugate (Dig-BSA) were incubated with different dilutions of scFv preparations in PBS-0.1% Tween for 2 h at 37°C. Bound scFv was detected using the 9E10 anti-*myc* mAb and a horseradish peroxidase-labeled anti-mouse IgG conjugate (Sigma).

The specificity of the scFv was determined by inhibition ELISA as follows: various concentrations (from 10^{-3} to 10^{-11} M) of digoxin analogs (Sigma) were preincubated overnight at +4°C with periplasm or Sf9 supernatant. Digoxin, other cardiac glycosides (digitoxin, gitoxin, lanatoside C, acetylstrophantidin, ouabain), active metabolites (digoxigenin, digitoxigenin), and endogenous steroids (progesterone, testosterone, cortisone) were tested. The dilution of each scFv sample was chosen so as to give a final absorbance of approximately 1.0 in absence of inhibitor. One hundred μl of each mixture was added to the Dig-BSA-coated wells and the procedure described above was followed. The ratio of the analog concentration giving 50% inhibition to the digoxin concentration required to give 50% inhibition reflects the relative affinity of each analog [31]. The specificity profile of the purified 1C10 mAb obtained in the same experiment served as refer-

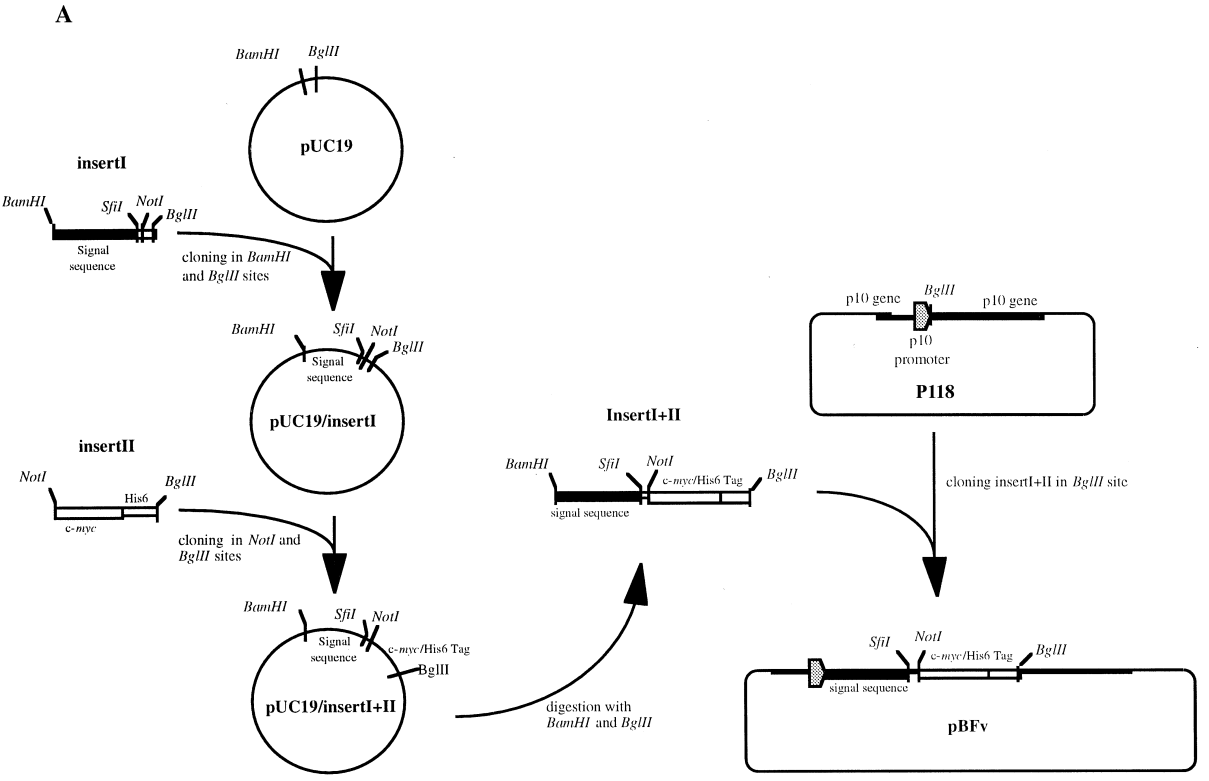

Fig. 1. A: Construction of the pUC19/insert I+II plasmid and cloning of insert I+II into the *Bgl*II site of p118, leading to the scFv cassette transfer vector pBFv. B: Sequence of the 83 bp oligonucleotide inserted in the *Bam*HI/*Bgl*II site of pUC19 for the construction of pUC19/insert I; sequence of the 71 bp oligonucleotide inserted in the *Not*I/*Bgl*II site of pUC19/insert I for the construction of pUC19/insert I+II.

ence; the 1C10 mAb was detected directly with the horseradish peroxidase conjugate.

2.7. Affinity determination

The kinetic parameters, association rate constant (k_a), and dissociation rate constant (k_d), were determined by surface plasmon resonance (SPR) analysis, using a BIAcore 1000 instrument (Biacore AB). The apparent equilibrium dissociation constant K_d, which is the ratio of k_d/k_a, was calculated using the BIAevaluation 3.0 software. We used a sensor chip surface covered with the NTA ligand for nickel (Biacore AB) to immobilize the 1C10 scFv through its His_6 tail: after washing the sensor chip surface with 350 mM EDTA, nickel chloride (0.5 mM in PBS) was added, and then the pure scFv sample was injected. Sensorgrams representative of the fixation of the Dig-BSA conjugate on the immobilized scFv were analyzed. It should be noted that digoxin alone could not be used because its low molecular mass (781 Da) does not give any registrable refraction index variation on the BIAcore 1000. To validate the nickel-NTA sensor chip, one of the *E. coli* samples was analyzed through a conventional procedure: digoxin was immobilized on a CM5 sensor chip (Biacore AB) through its oxidized sugar moiety as reported earlier [44], then pure scFv was added, and its binding was analyzed. The K_d value was exactly the same as that found when the nickel-NTA sensor chip was used (data not shown). The F_{ab}, obtained by cleavage of the parental mAb, was analyzed on the digoxin-coupled sensor chip, and served as reference.

3. Results

3.1. Sequence of the 1C10 scFv gene

The nucleotide sequences of 1C10 scFv cloned in pHEN-His and in pBFv were identical and the V_H and V_K sequences were the same as those obtained after cloning of V_H-C_{H1} and V_K-C_K in pUC19 (data not shown). As described in Fig. 2, sequence comparison using the Kabat database showed that the V_H region of the 1C10 Ab belongs to the subgroup IIB and the V_H gene to the J558 family (91.1% homologous to the closest J558-197 germline gene). The V_H region has accumulated a total of 30 somatic mutations, leading to 14 amino acid changes, during the immunization process. The H chain CDR3 is composed of the DSP2.2 D segment and the addition of 12 nucleotides, 9 upstream from the D gene and 3 downstream. The 1C10 Ab uses the J_{H3} gene with 2 muta-

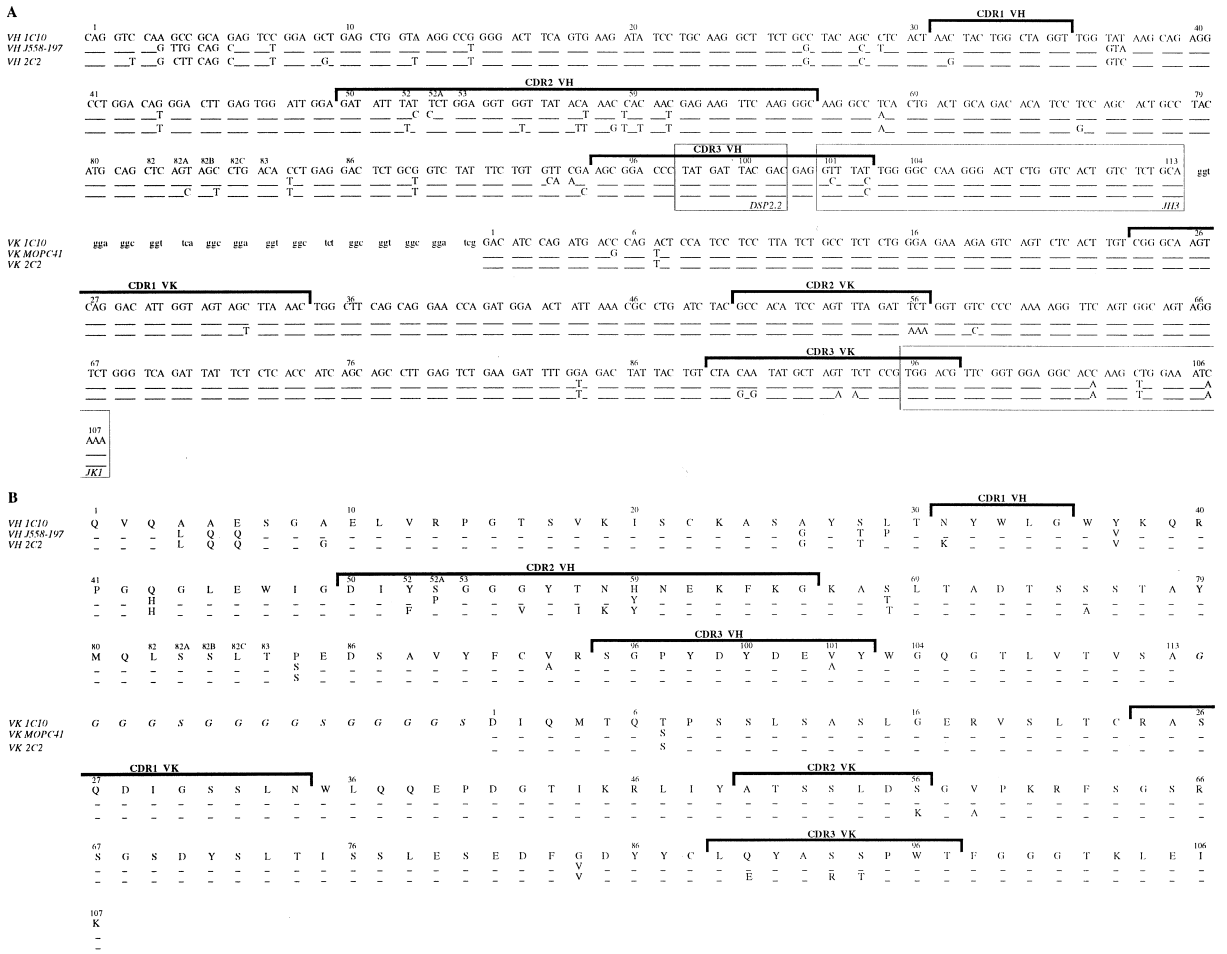

Fig. 2. A: Nucleotide and B: predicted amino acid sequences of the 1C10 anti-digoxin scFv compared with the most homologous germline gene and the 2C2 anti-digoxin scFv. Amino acid sequences are given in the one letter code. The complementarity determining regions (CDR) of V_H and V_L are highlighted and the linker peptide $(Gly_4Ser)_3$ is in italics. Dashes in sequences indicate identity with the residues given in the top line. J segments and D genes are boxed.

tions. The V_L region belongs to the V_K subgroup V (family V_{K9}), showing 99.1% homology with the MOPC41 germline gene, and uses the J_{K1} gene. The V_K region accumulated only 6 somatic mutations, leading to 2 amino acid changes localized in the framework regions.

Sequence comparison of the 1C10 variable regions and other anti-digoxin antibodies shows that significant homology can only be found with the 2C2 Ab, another anti-digoxin mAb generated in our lab from the same fusion experiment as 1C10 mAb [31,43]. The V_H regions of these Abs show 92.7% homology, corresponding to 17 amino acid differences, 5 of them being concentrated in the CDR2 region. Surprisingly, the CDR3-J_{H3} segment of 1C10 V_H is totally identical, except one silent mutation, to the corresponding segment of the 2C2 Ab. The V_L regions of 1C10 and 2C2 Ab have 95.6% homology, corresponding to 7 amino acid differences, 4 of them being concentrated in the CDR regions.

Other anti-digoxin antibody V_H regions show less than 70% homology with the 1C10 V_H, even the 26.10 Ab described by Near et al. [45], which belongs to the same J558 family. It should be noted that among the antibodies that match with the 1C10 CDR3, and a fortiori with the 2C2 CDR3, several Abs directed against haptens have been described such as anti-alprenolol [46] and anti-haloperidol [47].

3.2. Cloning and expression of 1C10 scFv

The 1C10 scFv was constructed using specific primers and overlapping PCR to incorporate the $(Gly_4Ser)_3$ linker. It was cloned in a procaryotic vector, the phagemid pHEN-His, or in an original baculovirus transfer vector named pBFv (Fig. 1). This pBFv vector contains a P10 promoter, a signal sequence for secretion, tags for detection and purification, and unique restriction sites *Sfi*I and *Not*I. These characteristics allow easy cloning of a PCR-amplified scFv obtained from a hybridoma or a bacterial library into this vector. Furthermore, the transfer from the procaryotic to the eucaryotic expression systems is facilitated using the pHEN/pBFv compatibility. The 1C10 scFv was easily transferred from pHEN-His to pBFv in a single cloning step, then recombination of 1C10 pBFv with baculovirus DNA containing the polyhedrine gene under the control of P10 promoter was performed in Sf9 cells. The scFv was then secreted into serum-free culture medium of the Sf9 cells. After purification of 200 ml Sf9 SN on a nickel column, 2 mg of pure scFv was obtained, corresponding to a yield of 10 mg/l. Purification of the SN by affinity chromatography on an anti-*myc* column yielded a pure scFv fragment but without any binding activity to dig-BSA (data not shown).

In pHEN-His, the PelB signal peptide upstream from the scFv directs the expression to the periplasmic compartment.

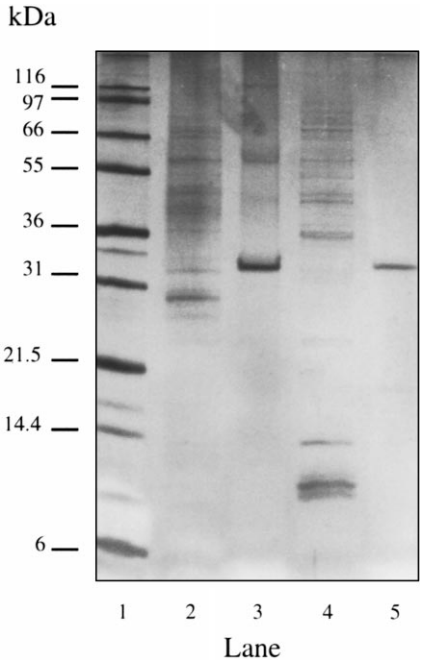

Fig. 3. Silver-stained SDS-PAGE of sf3 culture supernatant containing 1C10 scFv (lane 2), purified 1C10 scFv (lane 3) from baculovirus-infected insect cells, periplasmic extract containing 1C10 scFv (lane 4) and purified 1C10 scFv from bacteria (lane 5). Molecular weight markers are presented on lane 1.

Affinity chromatography on an anti-*myc* column yielded to 200 μg pure scFv per 20 ml periplasm (corresponding to 1 l of culture). Purification on a nickel column was tested but did not lead to enough pure fractions (data not shown).

Both pure scFvs were controlled on 15% SDS-PAGE (Fig. 3) and migrated with a higher apparent molecular mass (33 kDa) than the calculated one (28 212 Da), as already reported [48]. A very low quantity of dimer is present in pure preparations. As demonstrated in Fig. 4, pure scFv expressed either in bacteria or in baculovirus retained the digoxin binding activity.

3.3. Affinity

Affinity constants were determined on BIAcore 1000 by real-time interaction analysis, with the enzymatically cleaved F_{ab} as reference and are presented in Table 1. Using the BIAevaluation 3.0 software, the global fit analysis [49] showed that the experimental and theoretical curves can be superimposed, with most of the residuals being less than 0.5 resonance unit, indicating that the curves are monoexponential (data not shown). These observations suggest that the scFv preparations are mainly composed of monomers. The affinity of the 1C10 *E. coli* secreted scFv was close to the affinity of the F_{ab}. The insect cell secreted 1C10 scFv had a 8.5-fold lower affinity than that of the F_{ab}, mainly due to a faster dissociation.

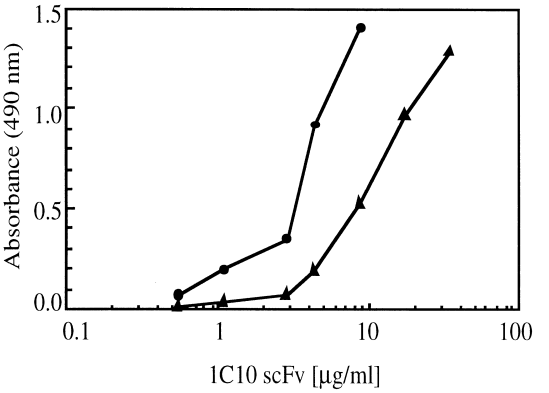

Fig. 4. ELISA binding activity of purified baculovirus- (▲) and *E. coli*- (●) expressed 1C10 scFv on an adsorbed Dig-BSA conjugate.

The 4.5-fold difference between the scFv K_d values can be explained by a lower association rate between the hapten and the baculovirus fragment than with the *E. coli* secreted fragment. This difference is in agreement with the ELISA curve (Fig. 4), where equal amounts of pure scFv are shown to give a lower detection signal in the baculovirus system than in the bacterial one.

Calculated half-lives of the scFv:digoxin complexes from the k_d values showed that they are still compatible with a pharmacological use of the scFv (52 and 40 min for the procaryotic and eucaryotic fragments, respectively), even though they are lower than that of the F_{ab} (170 min).

3.4. Specificity

Table 2 shows the fine specificity properties of the recombinant scFv fragments and the parental mAb. As described previously [31], 1C10 mAb recognizes digoxin, digitoxin and their active deglycosylated metabolites, but does not bind endogenous steroid analogs nor other cardiac glycosides. This profile is maintained when the 1C10 mAb is diluted either in periplasm obtained after induction of bacteria transfected with pHEN-His alone or in supernatant of Sf9 cells infected with wild-type baculovirus (data not shown), suggesting that the binding characteristics are not influenced by the media. The overall specificity of the parental mAb is conserved in both scFvs, i.e. binding to the related glycosides and metabolites, absence of binding to the endogenous steroid analogs and the more structurally distant glycosides. The baculovirus-expressed scFv has the same fine specificity profile as that of the 1C10 mAb, whereas the 1C10 scFv from the *E. coli* periplasmic extract shows significant changes in its fine specificity properties, in particular with digitoxin and the deglycosylated metabolites (digoxigenin, digitoxigenin). Firstly, the *E. coli* scFv binds to digitoxin with lower affinity than to digoxin. Secondly, the procaryotic scFv binds the aglycone molecules

Table 1
Affinity constants of the recombinant 1C10 scFv expressed in *E. coli* and insect cells and the 1C10 F_{ab}, as determined by BIAcore analysis

Antibody fragment	Association rate constant (k_a, M^{-1} s^{-1})	Dissociation rate constant (k_d, s^{-1})	Apparent equilibrium dissociation constant (K_d, M)
1C10 F_{ab}	$(2.48 \pm 0.05) \times 10^{4}$[a]	$(7.00 \pm 0.03) \times 10^{-5}$	$(2.82 \pm 0.07) \times 10^{-9}$
1C10 scFv expressed in *E. coli*	$(2.40 \pm 0.15) \times 10^{4}$	$(1.22 \pm 0.25) \times 10^{-4}$	$(5.04 \pm 1.03) \times 10^{-9}$
1C10 scFv expressed in insect cells	$(2.09 \pm 0.04) \times 10^{4}$	$(5.13 \pm 0.03) \times 10^{-4}$	$(24.5 \pm 1.15) \times 10^{-9}$

[a]Results are the means ± S.E. obtained from three separate experiments, using purified samples.

Table 2
Specificity profile of 1C10 scFv expressed in *E. coli* and insect cells

Analog	1C10 scFv expressed in *E. coli*	1C10 scFv expressed in insect cells	1C10 mAb
Cardiac glycosides			
Digoxin	1.0[a]	1.0	1.0
Digitoxin	4.7 ± 0.7	0.3 ± 0.04	0.9
Lanatoside C	0.5 ± 0.1	0.3 ± 0.1	0.2
Gitoxin	116.0 ± 14.0	47.4 ± 26.0	40.0
Acetylstrophantidin	ND[c]	> 1000	> 1000
Ouabaïn	> 1000	> 1000	> 1000
Active metabolites			
Digoxigenin	0.3 ± 0.04[b]	1.1 ± 0.4	2.1
Digitoxigenin	1.2 ± 1.0	16.2 ± 4.9	17.4
Endogenous steroids			
Progesterone	> 1000	> 1000	> 1000
Testosterone	> 1000	> 1000	> 1000
Cortisone	ND	> 1000	> 1000

[a]Results are expressed as the ratio of analog concentration giving 50% inhibition of scFv binding to digoxin, to the digoxin concentration giving 50% inhibition (arbitrarily set at 1.0).
[b]Each value is the mean ± S.E. of ratios obtained from triplicate wells in three separate experiments.
[c]ND, not determined.

with higher affinity than the corresponding glycosides, suggesting that the specificity properties of the *E. coli*-expressed fragment is influenced by the sugar moiety, unlike the intact mAb [31]. Although the two scFv preparations were diluted to give a final absorbance of 1.0 in the inhibition ELISA experiments, the different protein content of these preparations might have influenced their specificity.

4. Discussion

1C10 mAb is a high affinity monoclonal antibody whose specificity is of great interest for medical purposes, namely, detoxication [31]: this Ab recognizes the cardiac glycosides digoxin and digitoxin, and their active metabolites, the more active ones being also highly toxic. The in vitro and in vivo efficiency of this mAb in digoxin neutralization has been previously demonstrated by Scherrmann et al. [32,33].

Since mutations resulting from antigen-driven maturation provide information on the parts of the Ab variable regions important in affinity and specificity, we analyzed the nucleotide sequence of the variable regions of this particular mAb. Sequence comparison shows that this Ab is close to another anti-digoxin mAb named 2C2 generated from the same fusion (95% homology). These two mAbs use identical germline genes, J558-197 for the V_H region and MOPC41 for the V_L region. They recognize digoxin with comparable affinities (K_d: 0.23 and 0.17 nM for 1C10 and 2C2, respectively), but their fine specificity properties, i.e. binding to digoxin metabolites and analogs, are completely distinct [31]. The other anti-digoxin antibodies described in the literature [45,50,51] do not match significantly with the 1C10 mAb.

Many authors have demonstrated that the fine specificity properties of antibodies are mostly due to mutations in the heavy chain variable region [52–54] and that the affinity is also often borne by this part of the molecule [55]. In particular the CDR3 of the V_H region (CDR3H) is described to play a major role in Ab specificity, not only for anti-digoxin or anti-hapten Abs [52,56], but also for many other Abs [53,54,57]. The light chain might nevertheless contribute to specificity and affinity; in particular the VJ junction seems to be critical [51,54]. In our case, the 1C10 V_H gene is highly mutated (14 amino acid changes compared with the germline gene), whereas V_L region has only 2 amino acid changes in the framework segments, suggesting that the V_L chain has little influence on the specificity and affinity of this Ab. The 1C10 CDRH3 has two point mutations, only one leading to an amino acid change, and shares exactly the same J_H heavy chain segment as the 2C2 mAb in spite of distinct specificities, strongly suggesting that this CDR3 gene segment is not involved in fine discrimination between the digoxin analogs. Interestingly, the CDR2 heavy chain genes of V_H 1C10 and 2C2 are the most mutated CDRs, with 5 different residues out of 17 between these two mAbs. This particular CDR might thus be involved in the fine specificity of these two mAbs. The role of mutations in CDRH2 on Ab specificity has already been proposed by Jahn et al. [53]. However, we cannot exclude that the framework1 and CDR1 are also implicated, as described previously [53,57]. Mutagenesis analysis as well as structural studies on the scFv:digoxin complexes should shed light on this issue.

In this study, we used a eucaryotic expression system adapted to a procaryotic vector for Ab fragment production and phage display. This allowed a facile transfer of the scFv genes and the expression of scFv in insect cells. The baculovirus expression system seems to be a promising way to produce molecules for therapeutic purposes because of its capability for a high production level [58] and absence of known intrinsic or secreted molecules toxic for man. This is in contrast with *E. coli* which can release endotoxins, or plants which contain toxic or allergenic compounds. Furthermore, Sf9 cells can be accustomed to grow without serum thus avoiding the presence of mammalian contaminants [58]. Only few scFv Ab fragments have been expressed until now with this system [27,28]. To have complete compatibility between the procaryotic and baculovirus vectors for scFv cloning, we constructed an original baculovirus transfer cassette named pBFv, containing the same *Sfi*I and *Not*I sites as the pHEN vector, a phagemid often described in the literature to construct Ab libraries [35]. After selection on antigen, the scFv cloned in the pHEN vector could then be easily trans-

ferred to pBFv for expression in the baculovirus system. To evaluate the potentialities of these two systems of production, we constructed and cloned an anti-digoxin scFv either in pHEN or in the new pBFv.

We chose to directly express the 1C10 scFv in a functional form, i.e. secreted either in the supernatant of Sf9 or in the *E. coli* periplasm and to compare these two systems of production. The yield of pure 1C10 scFv was higher in the baculovirus expression system than in the bacteria (10 and 0.2 mg/l culture, respectively) and was also higher than other scFv flask productions in baculovirus described until now [27,28]. In addition, the affinities of these two genetically identical scFvs were different: 1C10 scFv from baculovirus showed 5-fold lower binding to digoxin than that from *E. coli*, essentially because of a lower association rate constant. It should be noted that the affinity of 1C10 *E. coli*-expressed scFv was in the same order of magnitude as that of the 1C10 F_{ab}. To our knowledge, this is the first time that binding of an Ab to a steroid hapten is analyzed by BIAcore. It is also the first time that an NTA sensor chip is used to determine the kinetics of an scFv binding to antigen. This format is a fast and convenient way of affinity determination because it permits calculation of the binding constants of a His-fusion protein in a complex medium without any knowledge of its concentration.

Surprisingly, 1C10 scFv expressed in bacteria had a fine specificity profile different from that of the scFv and the parental mAb produced in eucaryotic systems. Furthermore, we found an apparent conflicting result since the scFv expressed in baculovirus had a specificity profile identical to that of the parental mAb but with lower affinity for digoxin than the scFv expressed in the procaryotic system. This suggests that the eucaryotic fragment binding site folding is closely related to the parental binding site, the lower affinity being partly a consequence of the absence of constant domains [59,60]. The changes in binding of the *E. coli* scFv fragment to digitoxin and the aglycone molecules might be due to slight variations in the fitting to carbon-12 in the digoxin molecule and to the presence of the digitoxoses, resulting in an increase of the association rate constant for digoxin. Slight conformational changes of this protein produced in the two expression systems might have several origins: (i) the different procedures used to purify the scFv may have affected their affinity, even though preliminary equilibrium dialysis indicated that the fraction of active binding sites in each scFv preparation was nearly identical (J-M. Scherrmann, personal communication), (ii) changes in the post-translational status are probably involved, e.g. glycosylations which can occur in the baculovirus system, but not in the bacterial system; there is no potential *N*-glycosylation site in the amino acid sequence, but *O*-glycosylation sites are present; (iii) influence of an incompletely cleaved signal peptide cannot be excluded, (iv) our results can also be a consequence of distinct chaperoning between the eucaryotic and procaryotic cells, leading to changes in the 1C10 binding site conformation, (v) influence of the expression temperature (28°C in Sf9 cells, 22°C in *E. coli*) on the folding is also possible.

A previous report described a different specificity profile between the scFv expressed in bacteria and the F_{ab} obtained from the same mAb [10], but no specificity difference has been described until now between scFvs showing the same nucleotidic sequence and expressed in eucaryotic and procaryotic vectors [27,28]. Nevertheless, the majority of scFvs described in the literature do not show any change in the specificity profile compared with the parental mAb [60,61]. The possibility cannot excluded however that fine variations do exist but have not yet been identified. It should be noted that the digoxin model is interesting for this purpose because of the existence of a large panel of close analogs differing by a unique substitution on the steroid moiety.

The choice of the expression system appears to be a key factor in the production of functional scFv fragments of therapeutic interest. Information on the affinity and specificity of the antibody fragment is necessary to validate the choice. For the 1C10 scFv, we demonstrated that the baculovirus/insect cell system offers advantages in comparison with the *E. coli* system in terms of yield and specificity. In addition, our new baculovirus vector, using the same cloning sites as the pHEN phagemid, proved to be very efficient for rapidly producing antibody fragments in insect cells.

1C10 mAb has been shown to be of great therapeutic interest [31], but it cannot be used because of its large size. The results obtained here with the 1C10 scFv, namely, the half-life of the digoxin:scFv complex and the fine specificity profile, indicate that this Ab fragment could be a powerful tool for immunodetoxication of patients who have absorbed lethal doses of digoxin.

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