J. Microbiol. Biotechnol. (2011), **21**(5), 529–535
doi: 10.4014/jmb.1010.10022
First published online 18 March 2011

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Production of a Recombinant Anti-Human CD4 Single-Chain Variable-Fragment Antibody Using Phage Display Technology and Its Expression in *Escherichia coli*

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Received: October 11, 2010 / Revised: February 7, 2011 / Accepted: March 2, 2011

Single-chain variable fragment (scFv) is a fusion protein of the variable regions of the heavy (VH) and light (VL) chains of immunoglobulin, connected with a short linker peptide of 10 to about 20 amino acids. In this study, the scFv of a monoclonal antibody against the third domain of human CD4 was cloned from OKT4 hybridoma cells using the phage display technique and produced in *E. coli*. The expression, production, and purification of anti-CD4 scFv were tested using SDS–PAGE and Western blot, and the specificity of anti-CD4 scFv was examined using ELISA. A 31 kDa recombinant anti-CD4 scFv was expressed and produced in bacteria, which was confirmed by SDS–PAGE and Western blot assays. Sequence analysis proved the ScFv structure of the construct. It was able to bind to CD4 in quality ELISA assay. The canonical structure of anti-CD4 scFv antibody was obtained using the SWISS_MODEL bioinformatics tool for comparing with the scFv general structure. To the best of our knowledge, this is the first report for generating scFv against human CD4 antigen. Engineered anti-CD4 scFv could be used in immunological studies, including fluorochrome conjugation, bispecific antibody production, bifunctional protein synthesis, and other genetic engineering manipulations. Since the binding site of our product is domain 3 (D3) of the CD4 molecule and different from the CD4 immunological main domain, including D1 and D2, further studies are needed to evaluate the anti-CD4 scFv potential for diagnostic and therapeutic applications.

Keywords: ScFv, anti-CD4, phage display, recombinant antibody

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Monoclonal antibodies (MAbs) directed against cell surface targets have already shown a significant therapeutic application in oncology, cardiology, and immunology. Antibody (Ab) fragments have several advantages over monoclonal antibodies in clinical applications owing to their smaller size, which leads to better tissue penetration compared with whole antibodies. In particular, single-chain variable fragments (scFvs), at about 1/6 of the size of whole antibodies, are less immunogenic, have the potential for greater tissue penetration, and are more rapidly cleared from nonspecific tissues. ScFvs are stable at 37°C, retain the specificity and affinity of the original Fv, and are easier to express compared with recombinant Fab, where two separate proteins must be expressed and then folded together. ScFvs are produced by assembling the genes that encode the heavy-chain variable region (VH) and the light-chain variable region (VL) of an antibody using sequences encoding a short hydrophilic and flexible polypeptide linker (10–20 residues) to join the variable-region genes [3, 14]. As a result, the scFv is expressed as a single polypeptide chain.

Phage display is a selection technique in which a library of peptide variants is expressed as a genetic fusion to a bacteriophage coat protein. Each peptide variant is displayed on the surface of a virion, and allows rapid identification of variants with desired properties by multiple rounds of affinity partitioning (panning) followed by phage amplification. Individual variants with the desired binding property can then be identified by simply sequencing the DNA contained within each selected phage. Once antigen-positive clones have been identified, they can then be used to infect a nonsuppressor strain of *E. coli* for the purpose of producing soluble antibodies for use as immunological reagents.

The benefits of the *E. coli* system in this manner are the commercial availability of phage display systems using *E.*

coli as host cell, well-established protocols, relatively short duration between transformation and protein purification [36], relatively low capital costs for fermentation, simple fermentation conditions, ease of genetic manipulation [1], secretion to the periplasmic space, and that the specific release of the periplasmic protein content is simple by osmotic-shock procedures and lysozyme/EDTA treatment [20]. One of the major advantages of phage display technology of antibody fragments compared with standard hybridoma technology is that the generation of specific scFv/Fab fragments to a particular antigen can be performed within a couple of weeks [8].

CD4, also known as T4, is a 55 kDa single-chain type I transmembrane glycoprotein expressed on most thymocytes, a subset of T cells, and at low levels on monocytes/macrophages. CD4, a member of the Ig superfamily, recognizes antigens associated with MHC class II molecules and participates in cell–cell interactions, thymic differentiation, and signal transduction. CD4 acts as a primary receptor for HIV, binding to HIV gp120. Several laboratories have mapped the cellular binding site for HIV gpl20 to a short region of the CD4 receptor that resides within the first 53 amino acids of the N-terminal domain [16, 24].

The aim of the present study was to generate a monovalent anti-human CD4 scFv using phage display technology and then express the recombinant scFv in *E. coli*. The expression of anti-human CD4 scFv was checked using SDS–PAGE, Western blot, and sequence analyses. The specificity of scFv product against target antigen was analyzed in ELISA. The canonical structure anti-CD4 scFv antibody was obtained using the SWISS_MODEL bioinformatics tool.

Materials and Methods

Total RNA Extraction and cDNA Synthesis

OKT4, a mouse hybridoma cell producing MAb against human CD4 antigen, was obtained from European Collection of Cell Cultures (ECACC, UK) and maintained in Iscove's Modified Dulbecco Minimum Essential Medium (IDMEM; Gibco, UK) containing 2 mM glutamine and 20% foetal bovine serum (FBS; Gibco, UK) under standard culture condition (37°C, 95% humidity, and 5% CO2). Total RNA was extracted from 6×10^5 cells by RNeasy Mini Kit (Qiagen, UK) and free DNase Treatment and Removal Reagents (Ambion, UK) according to the manufacturer's instructions. About 2 μg of total RNA was reverse transcribed using a RETRO script Kit (Ambion, UK) with oligo(dT) primers in a final volume of 20 μl.

PCR Amplification and scFv Assembly

Mixed variable chain framework based primers (Heavy Primers and Light Primer Mix; Amersham Pharmacia, UK) and Taq Polymerase (Perkin Elmer, USA) were used to PCR amplify the VH and VL regions of the cDNA (30 cycles: 94°C for 1 min; 55°C for 2 min; 72°C for 2 min) [11, 34]. When the linkers anneal to the heavy and light chain DNA, they prime a fill-in reaction in the presence of AmpliTaq DNA polymerase and Linker Primer Mix (Amersham Pharmacia, UK). For this reaction to proceed efficiently, approximately 50 ng of heavy chain and 50 ng of light chain were added to the reaction (7 cycles as follows: 94°C for 1 min; 63°C for 4 min). The resulting diverse VH and VL regions were linked using an oligomer coding for a 15 amino acid (Gly4Ser)3 flexible linker to create the scFv DNA fragment [14, 34]. In the second PCR, the assembled scFv DNA was amplified and restriction sites (*Sfi*I and *Not*I) were added to clone the DNA into the phagemid vector using the RS Primers (Restriction Site Primers; Amersham Biotech, UK) in 30 cycles: 94°C for 1 min; 55°C for 2 min; 72°C for 2 min. The *Sfi*I and *Not*I restriction sites were annealed to the 5' end of the heavy chain and 3' end of the light chain, respectively.

Digestion and Cloning of scFv Genes

ScFv DNA fragments were ligated into the pCANTAB 5E phagemid (Amersham Pharmacia, UK) vector using *Sfi*I and *Not*I restriction enzyme sites [10]. XL1 Blue and HB2151 *E. coli* (Stratagene, USA) were used respectively for vector multiplication and protein production. The digestion conditions had been optimized for 0.25–1 μg of assembled product. The assembled scFv DNA was digested with *Sfi*I and *Not*I (New England Biolabs, UK) and purified on a 1.2% low-melting temperature agarose gel (Life Technologies Inc, UK). This digested DNA was ligated using T4 DNA ligase (New England Biolabs, UK) into pCANTAB. The ligation reaction (recombinant phagemid) was transformed into chemically competent *E. coli* XL1-Blue cells (Stratagene, USA) using a heat-shock transformation technique. The ligation efficiency was determined by plating aliquots of the transformed *E. coli* onto freshly prepared selective Luria–Bertani (LB) agar [28] containing 100 μg/ml carbenicillin or ampicillin and 12.5 μg/ml tetracycline overnight at 37°C.

Screening Clones by Colony Blot Hybridization Procedure

The colony blot procedure was used to distinguish clones that express a short tagged peptide encoded by in-frame plasmids based on the colony blot protocol from Qiagen [25]. Colonies were transferred to nitrocellulose membrane discs and subjected to immunoblot using a mouse anti-E tag antibody conjugated with horseradish peroxidase (HRP) (Amersham Pharmacia, UK). Signals were detected using enhanced chemiluminescence (ECL; Amersham Pharmacia, UK). All positive colonies were harvested for the phage display step.

Library Construction for Phage Display

To prepare the phage display library, LB medium (9 ml) was added to 1 ml of the colony blot positive culture and incubated for 1 h in a shaker incubator (37°C) until an optical density (OD) between 0.5 and 1.0 was reached at 600 nm. After adding 15 μl (10^{12} pfu/ml) of helper phage VCSM13 (Stratagene, USA), the cells were left to incubate at room temperature for 30 min and grown overnight in 100 ml of LB [28] supplemented with 10 μg/ml tetracycline, 100 μg/ml ampicillin, and 10 μg/ml kanamycin. After overnight incubation at 200 rpm and 37°C for phage rescue, the culture was centrifuged at 10,000 ×*g* for 20 min at 4°C and phage particles were precipitated from the supernatant by the addition of 0.2 volumes of 2.5 M sodium chloride/20% polyethylene glycol (PEG) 4000. To precipitate phage particles, the supernatant was incubated overnight at 4°C. Phage was harvested by centrifugation at 10,000 ×*g* for 20 min at 4°C and the pelleted phage was resuspended in 2–3 ml of phosphate-buffered saline (PBS) and used for subsequent panning rounds. The phage titer was determined by infecting 2 ml of log phase *E. coli* XL1-Blue with an

aliquot of the phage display library for 30 min at room temperature and then plating out samples of the culture onto selective LB agar.

Isolation of Phage Displaying scFv-Binding Peptides (Biopanning)

The screening of phage display anti-CD4 scFv was carried out on recombinant human soluble CD4 (R&D Systems, USA). Recombinant human soluble CD4 (20 μg/ml in 100 μl of coating buffer; 0.05 M carbonate buffer, pH 9.6) was coated to ELISA wells (Costar, UK) overnight at 4°C. Coated wells were washed 3 times with PBS/0.05% Tween. To block any nonspecific phage binding to the well later in the procedure, 300 μl of blocking buffer, containing 2% dried milk powder and 10% glycerol in PBS, was added to the wells and incubation at 4°C continued for 2 h. The wells were washed twice with 150 μl of PBS/0.05% Tween before the addition of a 100 μl sample of phage display library containing 1×10^{10} CFU. The wells were then incubated overnight at 4°C to allow interaction of the antibodybead complexes with peptides displayed on the surface of the phage particles. The wells were washed extensively (about 10 times) with PBS/0.05% Tween to remove any unbound phage. Bound phages were eluted from the well with 150 μl of 100 mM hydrochloric acid (adjusted to pH 2.2 with solid glycine). The elution buffer was pipetted up and down several times, and neutralization of the phage suspension was accomplished by the addition of 9 μl of 2 M Tris buffer, pH 8. The phages were subsequently used to infect 2 ml of exponentially growing *E. coli* XL1-Blue (Stratagene, USA) for 30 min at room temperature. Aliquots of the infected cells were plated onto selective LB agar containing 10 μg/ml tetracycline and 100 μg/ml ampicillin, in order to determine the number of phage eluted and to allow the isolation of pCANTAB 5E phagemid containing cDNA inserts. To make sure of no phage contamination, 1 μl and 10 μl of XL1-Blue infected with the screened library and 50 μl of the host cell were plated on selective LB agar and grown overnight at 37°C. To generate phage for a further round of selection, the infected *E. coli* XL1-Blue culture was superinfected with VCSM13 as detailed above. Phages were prepared from the infected culture, titered as described above, and stored at −20°C. This first-round library enriched in phage displaying scFv-binding peptides was used in a second round of selective enrichment as detailed above. In all, four rounds of biopanning were undertaken. Glycerol stocks were prepared and miniprep DNA extracted was subjected to sequence analysis.

Plasmid Purification and Sequencing

A single colony was picked from a freshly streaked selective plate and inoculated in a culture of 1−5 ml LB medium containing the appropriate selective antibiotic for 12−16 h at 37°C with vigorous shaking. The bacterial cells were harvested by centrifugation at 6,800 ×*g* for 3 min at room temperature. The amplified vectors were extracted with a QIAprep spin miniprep kit (Qiagen, UK) according to the manufacturer's instructions and were used for sequencing and transformation of *E. coli* strain HB2151 for expression. Sequencing of the anti-CD4 scFv gene was performed by the University of Sheffield at the Davis Sequencing and Synthesis Laboratory on an ABI automated sequencer using the Big Dye fluorescent dideoxy chain terminator Mix v1.1 with sequencing primers set (Amersham Pharmacia, UK).

Expression of Single-Chain Antibodies

Once an antigen-positive clone has been isolated using phage display, soluble antibodies can be produced in *E. coli* and detected using the anti-E Tag antibody. Antigen-positive phage was used to infect HB2151 cells, which were then plated overnight onto solid medium containing glucose. To obtain actively growing cells, the culture was shaken at 37°C until an OD of about 0.8 was reached at 600 nm. The glucose was then removed by centrifuging the cells, decanting the medium, and adding fresh medium containing isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce the lac promoter to produce soluble scFv protein. The culture was grown for at least 3 h at 23°C to allow accumulation of expressed soluble antibodies [7].

Electrophoresis and Immunoblotting

SDS−PAGE [19] and Western blot [31] analyses of periplasmic extracts of anti-CD4 scFv were performed using mouse anti-E tag antibody conjugated with horseradish peroxidase (Amersham Pharmacia, UK). Signals were detected using enhanced chemiluminescence (Amersham Pharmacia, UK).

ScFv Purification and Protein Concentration

The recombinant scFv was purified from the culture supernatant of transformed HB2151 strain of *E. coli* using a HiTrapTM anti-E Tag column (Amersham Pharmacia, UK). The concentration of scFv was determined according to the Bradford method, using bovine serum albumin as a standard [4].

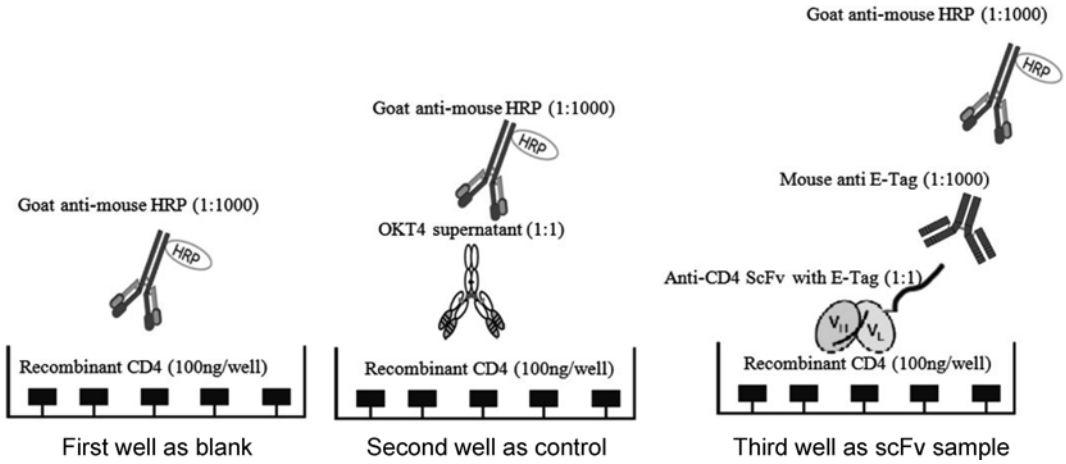

Fig. 1. A schematic diagram showing scFv ELISA procedure.

ScFv ELISA

A schematic diagram showing scFv ELISA procedure is shown in Fig. 1. ELISA plates (Costar, UK) were coated with 100 µl of recombinant human soluble CD4 (R&D Systems, USA) at 100 ng/well in coating buffer and incubated at 4°C overnight. After washing 3 times with 300 µl of washing solution, blocking was performed with 200 µl of 2% BSA/PBS and incubation allowed for 2 h at 37°C. Then, for negative control, 2% BSA/PBS, and for positive control, supernatant of OKT4 hybridoma were added to coated wells, and anti-CD4 scFv was added to the test wells. After incubating at 37°C for 1 h and washing with PBS/Tween (0.05%), 100 µl of 2% BSA/PBS was added to negative and positive samples, and mouse anti-E tag antibody was added to test wells. After incubating at 37°C for 1 h and washing, 100 µl of goat anti-mouse HRP-conjugated was added to all wells at 37°C for 1 h. The wells were washed 3 times with washing solution. Tetramethylbenzemidine (TMB) substrate solution was added to each well and incubated in the dark and at room temperature for 10 min. Sulfuric acid (1 M) stopping solution was added to each well and the plates were read with an ELISA reader at 450 nm wavelength with a 630 nm diffraction filter.

Homology Modeling

SWISS-MODEL was used to model the anti-CD4 scFv chain [22, 23, 29] and the images were represented in cartoon ribbons and space-fill models using RasMol software, version 2.6.

Homology modeling was used only for comparing the structure of final product with expectance models in references.

Results

Amplification of the VH and VL Regions and Construction of the scFv

When the cDNA was synthesized from hybridoma cell line OKT4, total RNA was amplified by PCR with oligo(dT) primers for human VH and VL regions. The use of the whole cDNA reaction mixture for PCR amplification of the variable light and heavy chain genes allowed sufficient amounts of products to be obtained for cloning. The first-strand antibody cDNA was used as a template for PCR amplification to generate 340 bp and 325 bp PCR products for heavy and light chains, respectively. The VH and VL DNA fragments were then linked with the flexible peptide $(Gly4\ Ser)_3$ sequences, as described in Materials and Methods (Fig. 2).

Cloning and Expression of the scFv Gene in *E. coli*

The assembled scFv DNA was cloned and ligated into a purified pCANTAB 5E phagemid vector and transformed to chemically competent cells of XL1-Blue *E. coli*. The colony blot of the various clones was determined to verify that the construction was in-frame. Phage display was performed for all positive clones, and the sequences of the positive clones were determined and proved the scFv structure of the construct. Following amplification with helper phage, the titer of the stock phage display library was assessed by infecting *E. coli* XL1-Blue and plating aliquots of the culture onto LB agar containing ampicillin and tetracycline. A titer of 1×10^{11} CFU/ml was estimated. The scFv was found to accumulate in the *E. coli* periplasm and was also released into the culture medium (data not shown). After purification using a HiTrap anti-E Tag column, yields of 0.8–1.2 mg/l were achieved. Purified antibody fragments were analyzed by electrophoresis on 12% SDS–polyacrylamide gels. The molecular mass of anti-CD4 scFv from gene 3 signal sequences until the end of E-tag with 290 amino acid was 31 kDa. ScFv was secreted from *E. coli* cells transformed with the pCANTAB 5E phagemid and was detected in crude periplasmic extracts by Western blot analysis using HRP-conjugated anti-E tag antibody (Fig. 3).

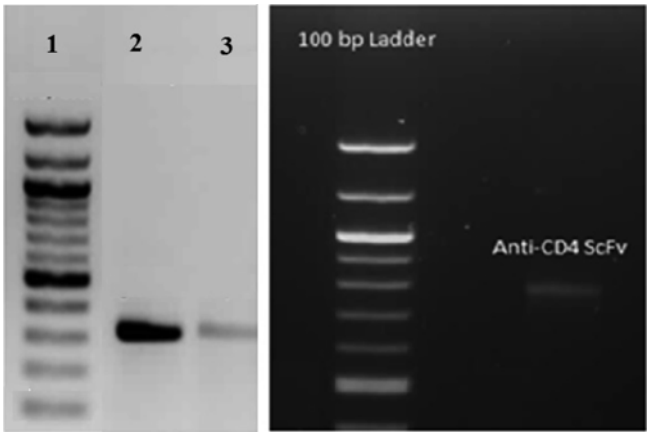

Fig. 2. Electrophoresis of PCR products from light chain, heavy chain, and anti-CD4 scFv on a 1% agarose gel.
Left: lane 1, 100 bp ladder; lane 2, light chain; lane 3: heavy chain. Right, PCR product for anti-CD4 scFv.

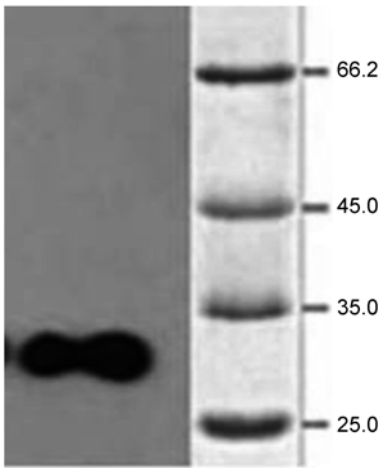

Fig. 3. Western blot analysis of anti-CD4 scFv.
ScFv was produced by E. coli transformed with the pCANTAB 5E phagemid and were detected in crude periplasmic extracts by western blot analysis using HRP conjugated anti-E tag antibody.

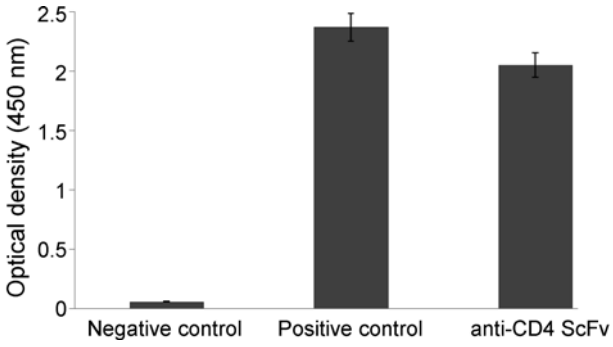

Fig. 4. Quality ELISA analysis of anti-CD4 scFv binding to recombinant human soluble CD4.
ELISA wells were coated with recombinant human soluble CD4. Negative control: BSA/PBS; Positive control: supernatant of OKT4 hybridoma.

Antigen-Binding Activity of the Secreted scFv

ELISA assay was performed to verify the reactivity of the *E. coli*-expressed scFv against recombinant human soluble CD4 antigen. As shown in Fig. 4, the OD_{450} obtained with *E. coli*-produced scFv (2.05) was significantly higher than negative control (OD_{450}=0.05), indicating strong antigen-binding activity of the secreted scFv, which was comparable with supernatant of OKT4 hybridoma as a positive control (OD_{450}=2.37).

Homology Modeling

The 3D canonical structures for heavy chain, light chain, and anti-CD4 scFv are presented in ribbons models using RasMol software (Fig. 5).

DISCUSSION

The CD4 molecule contains four immunoglobulin-like domains. The binding site for MHC class II molecules is thought to involve both the D1 and D2 domains (Fig. 6) [17]. Anti-CD4 antibodies have long been proposed and used as immune suppressors, for example in clinical trials for the treatment of human autoimmune diseases [13]. Anti-CD4 mAbs were found capable of inducing functional inactivation of T cells [15, 33], although activation of T-cell functions was also reported [9]. For instance, it has long been known that anti-CD4 monoclonal antibodies are immune suppressive or tolerogenic depending on the circumstances of their administration [2, 12, 26]. Moreover, CD4 is a primary receptor used by HIV-1 to gain entry into host T cells, and the region containing residues 40–60 in the first domain of CD4 is the primary binding site for gp120 [32]. Burkly *et al.* [6] have tested an anti-CD4 antibody that recognizes a CD4 second domain epitope and does not

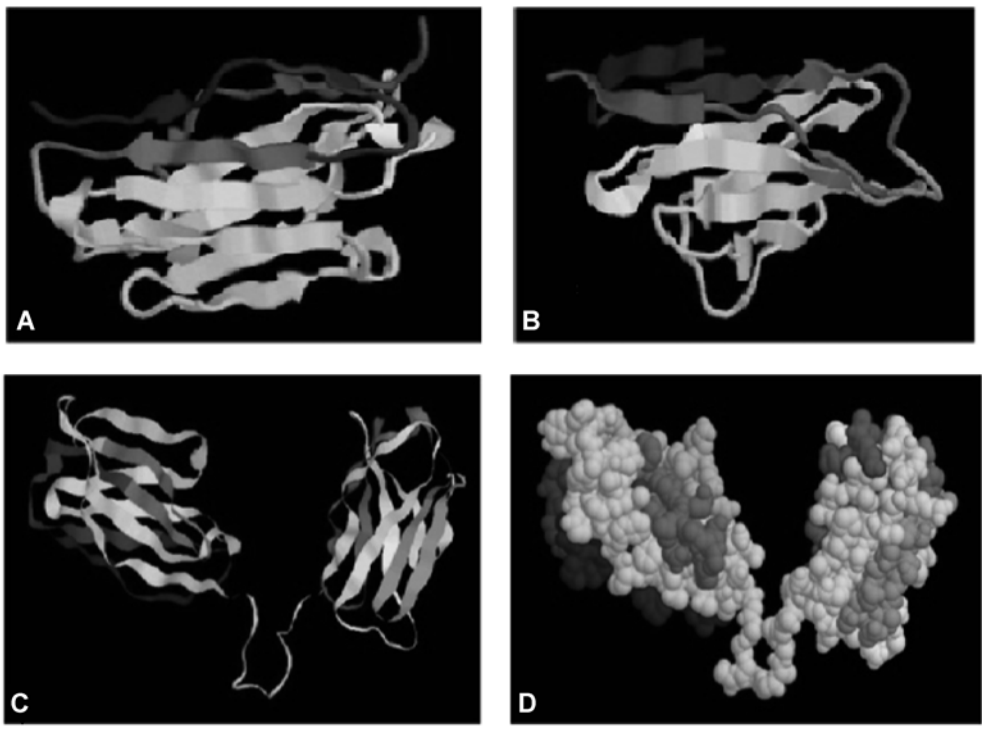

Fig. 5. Swiss model canonical structures.
Canonical structures for heavy and light chains of scFv (**A**, **B**). Swiss model: Cartoon ribbons automated protein homology-modelling (**A**) Heavy chain of anti-CD4 ScFv (**B**) Light chain of anti-CD4 scFv. Canonical structures for anti-CD4 scFv (**C**, **D**). Swiss model: Ribbons automated protein homology-modelling (**C**), Space fill model in group colours (**D**). Each chain is drawn as a smooth spectrum from blue through green, yellow and orange to red. Hence the N terminus of proteins and 5' terminus of nucleic acids are coloured red and the C terminus of proteins and 3' terminus of nucleic acids are drawn in blue.

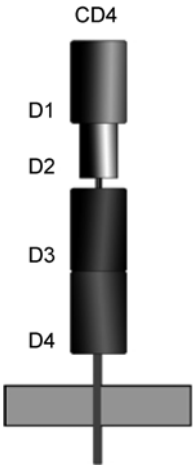

Fig. 6. The outline structures of the CD4 domains.

significantly interfere with HIV-1 docking on the cell, but the antiviral activity of that antibody was explained as a consequence of the interference on conformational changes taking place on the cellular HIV-1 receptor at the post-binding level membrane [6].

Several researches have shown that anti-CD4 MAbs are capable of abrogating HIV virus attachment to $CD4^+$ T cells. The whole OKT4 monoclonal antibody specifically binds to the gp120 binding site of CD4, and displays, as expected, a remarkable anti-HIV activity *in vitro* [5]. However, this reagent is also extremely immune suppressive owing to interference on the physiological CD4 function [5].

The effect of anti-CD4 in human therapy is far from being a straightforward immune suppression and is influenced by many different factors, including epitope specificity, isotype, and number of binding sites. Thus, in engineered derivatives, variation in the number of binding sites (*e.g.*, single-chain constructs, Fc, scFv, or Fab fragments) implies modification of functional effects of the original reagent [30].

There are many advantages in using scFv, particularly in the fields of therapeutics and diagnostics. ScFv offers several advantages over monoclonal antibodies as a carrier of radionuclei and drugs to tumors, including greater tumor penetration owing to their small size, low kidney uptake, rapid blood clearance, and a lower negative response by the human immune system. The key advantage of using scFv antibody fragment is that the recognition element can be produced and genetically engineered into a ready-to-use form by expressing inexpensively in bacteria. Apart from reductions in costs, scFv can be produced without the use of animals and can be readily selected for antigen specificity through the use of phage display antibody libraries [18, 27, 35]. The usage of human derivatives of mouse monoclonal antibodies also reduces the generation of xenogeneic reactivity of rodent antibodies.

In the present study, scFv of the monoclonal antibody was generated by phage display that specifically binds to recombinant human CD4 antigen. This is the first study that used this technique for generating scFv against human CD4 antigen. One of the biggest advantages of phage display technology is avoiding denaturation of the polypeptides that are displayed on the surface of the phage. This may be important in detecting antibody binding to conformational epitopes [21]. In addition, immobilization of the scFv instead of the cDNA library allows the specific enrichment of phage particles that interact with the antibody immobilized onto a solid phase. Antigens that are present at low abundance can therefore be enriched, increasing the possibility of detection and identification.

Engineered anti-CD4 scFv could be used in immunological studies, including fluorochrome conjugation, bispecific antibody production, bifunctional protein synthesis, and other genetic engineering manipulations. Further studies are needed to evaluate the use of recombinant anti-CD4 scFv for diagnostic and therapeutic applications.

Acknowledgments

This study was supported by the Graduate Office of the University of Isfahan and was carried out in the laboratory of Professor R.J. Ross (University of Sheffield, United Kingdom) as a joint project for PhD student. The authors are very grateful to them for their support. The authors also would like to thank Ian Wilkinson, Philip F. Watson, Sue Justice, Byron Carpenter, Sarbendra Pradhanaga, and Alia Munir at the University of Sheffield and also Malayer University of Iran for their help and support. Special thanks to Mr. Michael Copeland for his work on the manuscript English editing.

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