J.Sci.Soc.Thailand, 23(1997) 159-170

EXPRESSION OF A RECOMBINANT SINGLE CHAIN Fv ANTIBODY FRAGMENT IN MAMMALIAN CELLS

WILAIWAN CHOTIGEAT[a] AND STEPHEN M MAHLER[b]

[a] *Biochemistry Department, Prince of Songkla University, Hat Yai, Songkla, Thailand.*

[b] *Biotechnology Department, University of New South Wales, Sydney, Australia 2052.*

(Received 30 January, 1997)

ABSTRACT

Expression of recombinant antibodies and antibody fragments in procaryotic production systems is characterized by low yields and sometimes a reduced affinity for antigen. These problems have been attributed in part to folding constraints in procaryotes, and may be overcome by expressing in eucaryotic production systems. This work is an attempt to produce scFvs of WM65 in mammalian expression system. Since the scFvs of WM65 recognizes a cell surface antigen on normal leukocytes and leukemic cells therefore it could have potential therapeutic application for treatment of chronic lymphocytic leukemia (CLL) and for T-cell depletion of bone marrow to prevent the graft rejection in bone marrow transplantation. The cDNA for scFvs of WM65 in the pHEN1 bacterial expression vector was amplified and modified using polymerase chain reaction, and subcloned into the mammalian expression vector pSVL. The recombinant vector was transfected into the COS cell line by the calcium phosphate technique for transient expression of the scFvs. Immunoaffinity purification (via the C-terminal c-Myc tag) of the scFvs from the cell culture supernatant yielded a protein with molecular weight 28.5 kDa (corresponding to the molecular weight of the scFvs). Two other protein contaminants co-purified with the scFvs, which were tentatively identified by molecular weight as host c-Myc proteins. Purification of the scFvs will allow a comparison of the scFvs produced by mammalian cells and bacteria with respect to the affinity of the scFvs for antigen.

INTRODUCTION

Immunoglobulins or antibodies are proteins synthesized by plasma cells in response to foreign antigens invading the body. However, these antibodies to an antigen are derived from many clones of antibody-secreting cells. The development of hybridoma techniques[1] allows the *in vitro* production of antibody molecules from the one cell clone, namely monoclonal antibody. Monoclonal antibodies have been widely used in several areas for clinical and diagnostic purposes. Mouse or rat monoclonal antibodies are suitable for analytical or diagnostic applications, however their use as therapeutic reagents has not met with wide success. Administration of rodent antibodies to human patients results in a human anti-mouse antibody response (HAMA) since the rodent antibodies are foreign proteins. The HAMA reduces the efficacy of the rodent antibodies in therapeutic applications.[2]

Since the production of intact human antibodies for therapeutic purpose has been relatively unsuccessful, rodent Fab fragments containing the variable region and the first constant region have had some success as therapeutics. Eliminating some of the constant domains of the heavy chain has been found to reduce the problems of the HAMA response.[3] Perhaps an even better molecule to use as a therapeutics agent would be the Fv fragment, which consists of only the V_L and V_H portion. Currently, this Fv fragment was produced as single chain antibody variable (scFvs) regions which are composed of two antibody variable regions, one from the variable light chain (V_L) and the other from variable heavy chain (V_H).

The two variable regions are linked to each other by a peptide of approximately 15 to 25 amino acid residues such that a continuous polypeptide chain is formed.[4-5] Since the scFvs have a small size and lack the Fc portion of an antibody, they are expected to have advantages in clinical applications in being able to penetrate the microcirculation surrounding solid tumors better than whole antibodies, and also being less immunogenic.[4] In addition, the specificity of the variable region of scFvs could be used in diagnosis and treatment of diseases. The idea for treatment of diseases is using the scFvs as carriers linked to cytotoxins, so that they would either bind to the target tumor or alternatively clear quickly from the body, thereby reducing the toxic side effects.[6-8] As a result of the potential of the scFvs in medical and commercial applications, there is much interest in the production of antibody fragments. Both eucaryotic and procaryotic production systems are being evaluated for the production of antibodies and antibody fragments. Presently, the scFvs antibody of different types have been successfully produced and in the trial process.[9-11]

Several scFvs genes have been produced efficiently in the *E. coli* system.[5,12-13] However, most recombinant proteins produced in *E. coli* are insoluble which make complicated downstream processing of proteins and recovery is variable. Soluble scFvs have been produced in some experiments, for example, *B. subtilis*[14] and *E. coli*[15] but the yield was relatively low. Moreover a number of produced scFvs have affinities lower than the parent antibody.[16-17] Therefore, systems for the production of scFvs, antibody fragments or whole antibodies which have exactly the same properties as the natural antibodies are being researched.

Eucaryotic expression systems, especially mammalian cell system in an alternative mean to produce therapeutic proteins since they can synthesized soluble proteins, can performed post-translational modification and can perform folding like natural proteins.[18] The antibody Fv fragment and human anti-tetanus toxoid antibody have been successfully expressed in myeloma cells, with the yield for the antibody Fv fragment being 8 mg/ml.[19-20] Therefore, eucaryotic expression systems, especially mammalian cells present a challenge to other more simple systems for expressing antibodies or antibody fragments which mimic the natural antibodies.

This work was a trial to expressed scFvs WM65 in COS cells. The WM65 monoclonal antibody reacts specifically with a cell surface antigen on virtually all normal leukocytes and leukemic cells. This monoclonal antibody recognizes a unique 40-50 kilodalton leukocyte surface membrane antigen, so that it could have potential therapeutic application for treatment of chronic lymphocytic leukemia (CLL) and for T-cell depletion of bone marrow to prevent the graft rejection in bone marrow transplantation. However, the scFvs WM65 has been expressed *in E. coli*, and it has been found that the affinity of the scFvs proteins are lower than the native monoclonal antibody. Probably the reasons of decreased affinity of the proteins have been discussed above. Therefore , the expression of scFvs WM65 in mammalian system may clarify the problems and pave the way to express other antibody fragments.

MATERIALS AND METHODS

1. Subcloning of scFvs of WM65

The cDNA encoding scFvs of WM65 (V_H-linker-V_L) linked with c-Myc tag expressed in the recombinant pHEN1 vector were obtained from Dr. Nick Hawkins and Ms. Robyn Ward, School of Pathology, University of New South Wales, Sydney, Australia and the sequence was shown in Fig 1. The polymerase chain reaction (PCR) was performed to amplify the scFvs

[1]GGAGACAGTC/ATAATGAAAT/ACCTATTGCC/TACGGCAGCC/GCTGGATTG/
TATTACTCGC/GGCCCAGCCG/GCCATGGCCC/AGGTGCAGCT/GCAGCAGTCT/
GGGGCAGAGC/TTGTGAAGCC/AGGGGCCTCA/GTCAAGTTGT/CCTGCACAGC/
TTCTGGCTTC/AACATTAAAG/ACACCTATAT/GCACTGGGTG/GAGCAGAGGC/
CTAAACAGGG/CCTGGAGTGG/ATTGGAAGGA/TTGATCCTGC/GAATGGTTAT/
ACTGAATATG/ACCCGAAGTT/CCAGGGCAAG/GCCACTATAA/CAGCAGACAC/
ATCCACCAAC/ACAGCCTACC/TGCAGCTCAG/CAGCCTGACA/TCTGAGGACA/
CTGCTGTCTA/TTACTGTACT/GGGGGTAACT/ACGCCTATGG/TATGGACTAC/
TGGGGTCAAG/GAACCTCAGT/CACCGTCTCC/TCAGGTGGAG/GCGGTTCAGG/
CGGAGGTGGC/TCTGGCGGTG/GCGGATCGGA/CCTGGAGAGT/CAGCATCCAT/
CTCCTGCAGG/TCTAGTAAGA/GTCTCCTGCA/TAGTAATGGC/GACACTTACT/
TGTATTGGTT/CCTGCAGAGG/CCAGGCCAGT/CTCCTCAGCT/CCTGATATAT/
CGGATGTCCA/ACCTTGCCTC/AGGAGTCCCA/GACAGGTTAC/GTGGCAGTGG/
GTCAGGAACT/TCTTTCACAC/TGAGAATCAG/TAGAGTGGAG/GCTGAGGATG/
TGGGTTTTTA/TTTCTGTATG/CAACATCTAG/ATTATCCGTACACGTTCGGA/
GGGGGGACCA/AGCTGGAAAT/AAACGTGCG/GCCGCAGAAC/AAAAACTCAT/
CTCAGAAGAG/GATCTGAATG/GGGCCGCATA/GACTGTTGAA/AGTTGTTAG/
CAAAACCTCA/TACAGAAAAT/TCATTTACTA/ACGTCTGGAA/[930]

Fig.1 The cDNA sequence of scFVs WM65 gene linked with c-Myc gene (Copied from Dr. Nick Hawkins and Ms. Robyn Ward, School of Pathology, University of New South Wales, Sydney, Australia).

of WM65 linked with c-Myc tag gene. The condition was as follows; 95°C for 2 minutes (1 cycle), 95°C for 5 seconds, 55°C for 15 seconds,72°C for 30 seconds (30 cycle) and 72°C for 2 minutes (1 cycle). Forward and backward primers were designed so as to create *Xho* I and *Bam* HI terminal restriction sites on the insert to facilitate cloning into the expression vector pSVL. In addition, the primers were included a start and stop codon to allow expression of scFvs gene in mammalian cell properly as shown below.

Polymerase chain reaction primers

Backward primer

5'CCC**GAGCTC**CTCGAGCC**ATG**GCCCAGGTGCAGCTGCAC 3'

*Sac*I *Xho*I Start

Forward primer

5'AACC**GGATCC**CTATGCGGCCCCATTCAGATC 3'

*Bam*HI Stop

2. Expression of scFvs of WM65 in COS cell

The cDNA of Fv of WM65 ligated in pSVL vector was transfected into COS cells (donated by CSIRO, Division of Biomolecular Engineering, Australia) by the calcium phosphate-DNA coprecipitate technique.[21] Briefly, DNA was precipitated with calcium phosphate to form fine particles then was transferred into the medium above the cell monolayer. The transfected cells were incubated for 24 hours at 37°C in an atmosphere of 5-7% CO_2; the

medium and precipitate were removed by aspiration and the cells were washed with phosphate buffer saline (PBS). Prewarmed fresh medium was added, and supernatant of the transfected cells were screened for transient expression after the transfected culture was incubated for 24-60 hours.

Cell culture supernatant for scFvs WM65 was screened by dot blotting. Briefly, a sample of supernatant was applied to nitrocellulose strips and dried by air for 5 minutes. The nitrocellulose was blocked with 1% BSA in PBST (PBS+0.5%Tween) for 15 minutes and washed twice with PBST (5 minutes each wash with shaking). The strips were incubated with anti-c-Myc tag antibody (1mg/ml) for 20 minutes with shaking at 37°C, and washed three times in PBST (5 minutes each wash). The strips were then incubated with rabbit anti-mouse horse radish peroxidase conjugated antibody (Dako corporation, Australia), washed three times in PBST and incubated in ECL developing solution (Amersham, Australia) for 1 minute. The strips were then exposed to Hyperfilm for 30 seconds, and the film was developed.

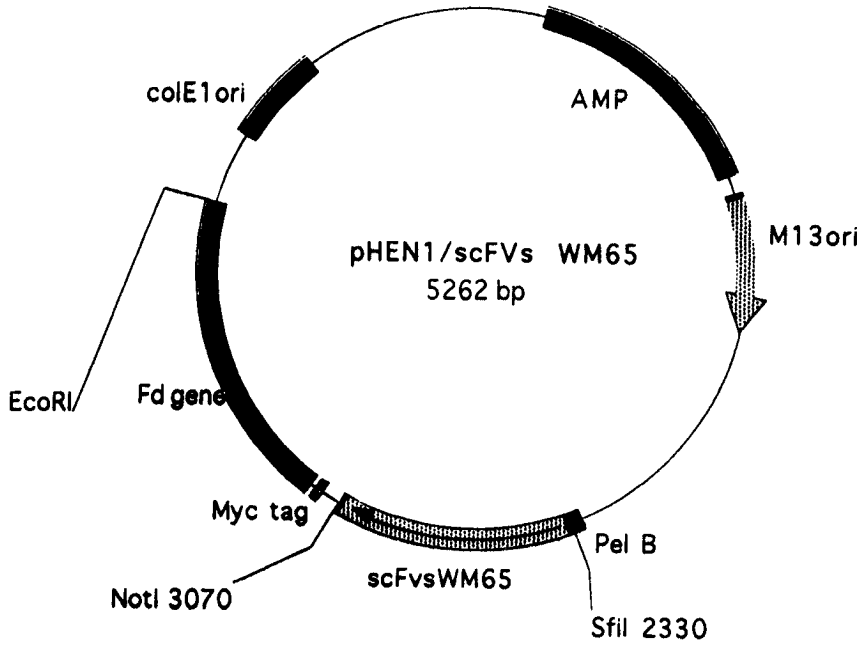
3. Purification of the scFvs WM65 by immunoaffinity chromatography

Purification of scFvs was performed by immunoaffinity chromatography using the c-Myc tag antibody. The c-Myc tag antibody was purified from hybridoma cell line (ATCC 9E10). The antibody recognizes an epitope at the C-terminal end of the peptide tag.[22-23] The immunoaffinity column was prepared by binding the c -Myc tag antibody to Protein G Sepharose according to the method described by Harlow and Lane, 1988.[24] The purified samples were loaded onto an 8-25% SDS- PAGE gradient gel, and electrophoresis was performed using the Pharmacia Phast system unit (Pharmacia, Upsula, Sweden) under the following condition; 250V, 10mA, 3W, 15°C, 60Vh. The gels were then silver stained using the automated development chamber.

RESULTS

1. Subcloning of scFvs into pSVL

The pHEN1 bacterial expression vector has expressed protein being a fusion protein made up of Pel B leader, the scFvs, a c-Myc protein tag and the g3p phage coat protein for display on the surface of filamentous phage (Figure 2). For expression in mammalian cells, the Pel B leader (for periplasmic expression) is not required. Thus a translation start codon must be positioned at the beginning of the scFvs sequence. The g3p protein is also not required, however the c-Myc tag is required for detection the expression of scFv gene so that a translational stop codon must be positioned after the end of the c-Myc tag sequence. Therefore , the primers for amplification of the cDNA encoding scFvs were designed to incorporate translational start, stop codons and appropriate terminal restriction sites on the insert to facilitate cloning into the expression vector. The cDNA encoding scFvs was amplified by polymerase chain reaction (PCR) using the recombinant pHEN1 vector as template. Forward and backward primers were designed so as to create *Xho* I and *Bam*HI terminal restriction sites on the insert to facilitate cloning into the expression vector pSVL (Figure 2B). The PCR product was approximately 800 bp in size (Figure3A lane 2), corresponding to the size of the cDNA encoding scFv WM65. The inserted pSVL plasmid was digested with *Xho*I and *Bam*HI and showed the inserted cDNA being 800 bp in Figure 3B.



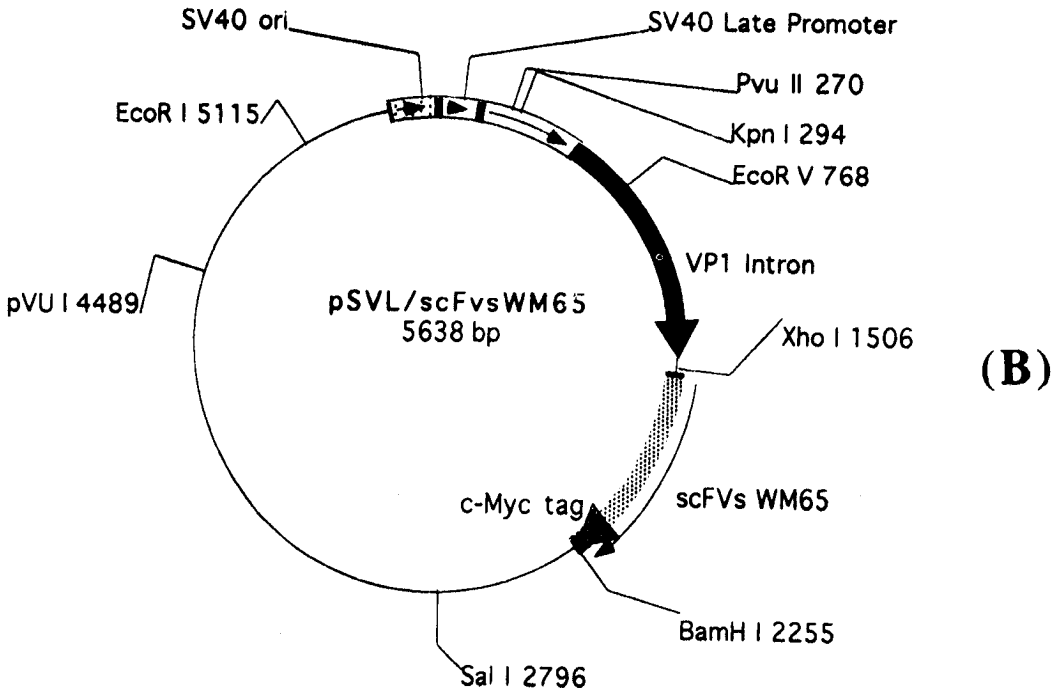


Fig.2 Diagram of scFVs WM65 gene in pHEN1 vector (A) and pSVL vector (B).

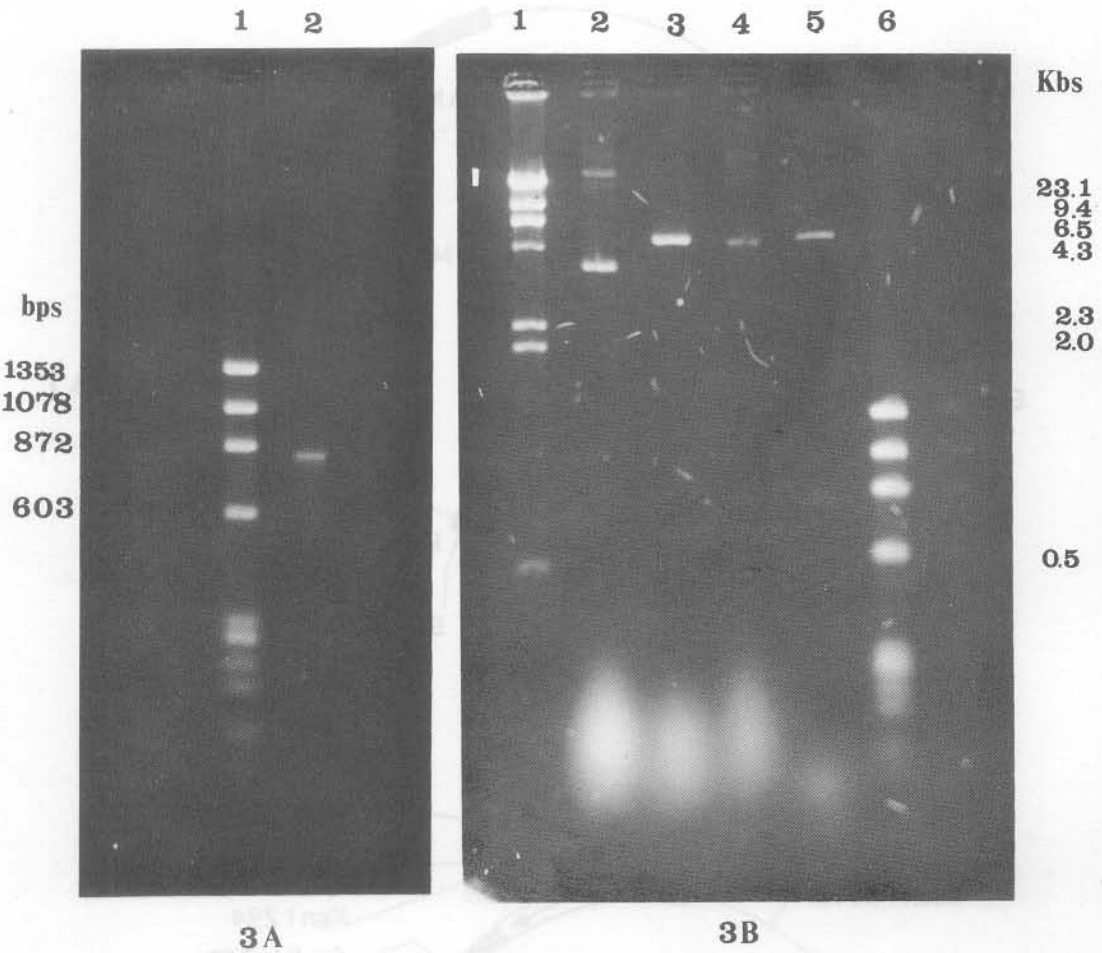


Fig.3A The Polymerase Chain Reaction product of scFvs WM65 on 2% agarose

Lane 1= Standard phi X 174 digested by *Hae*III

Lane 2= The Polymerase Chain Reaction product of scFvs gene

Fig.3B The recombinant scFvs in plasmid pSVL

Lane 1= Standard l DNA digested with *Hind*III

Lane 2= Undigested pSVL transformed into *E.coli* strain DH5

Lane 3= Digested pSVL with *Xho*I&*Bam*HI

Lane 4= Undigested recombinant plasmid

Lane 5= Digested recombinat plasmid

Lane 6= Standard phiX 174 digested with *Hae*III

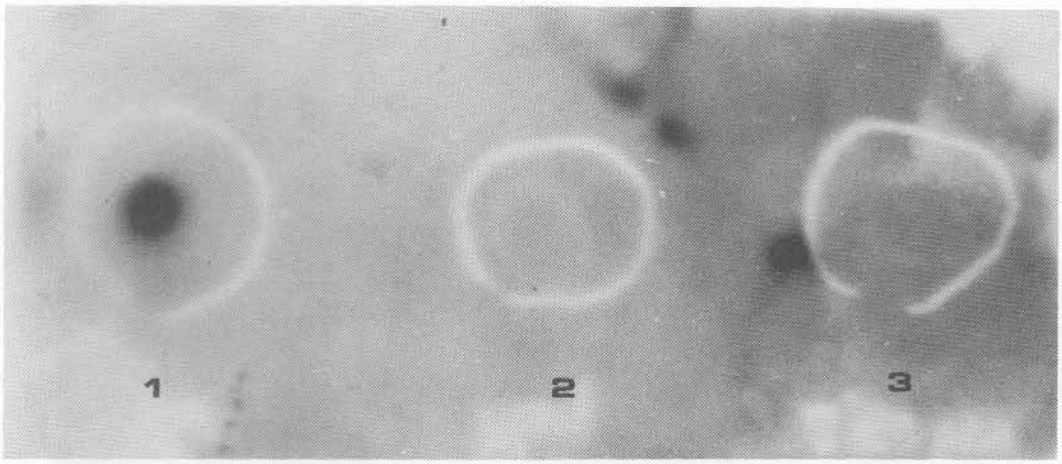


Fig.4 Screening the scFvs WM65 protein in the supernatant of transfected COS cells culture by dot (western) blot
1= Positive control of scFvs WM65 protein
2= Supernatant of the normal COS cells culture
3= Supernatant of the transfected COS cells culture

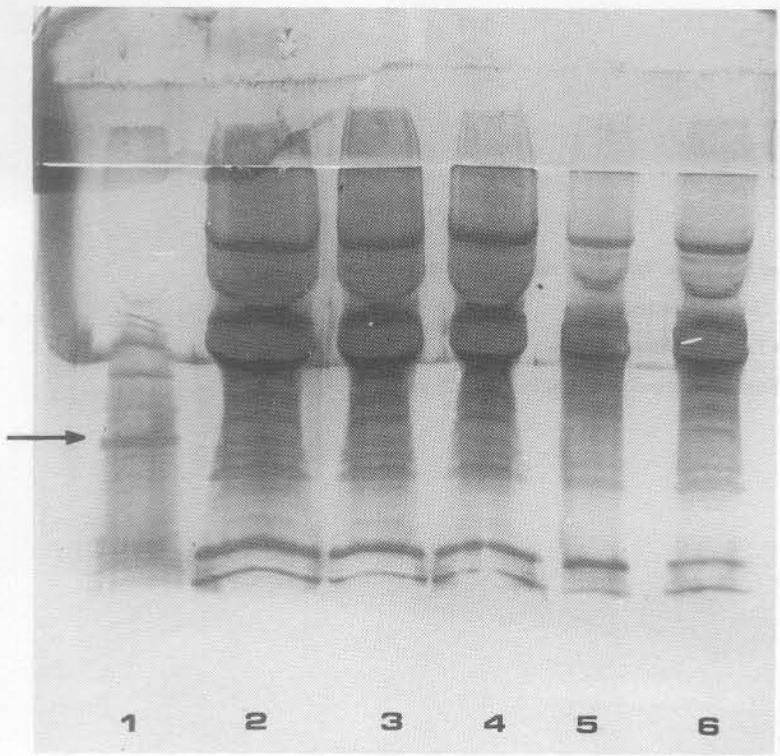


Fig.5 Separation of proteins in supernatant of transfected COS cell culture on 8-25% SDS-PAGE
Lane 1 = *E.coli* lysate of scFvs WM65
Lane 2,3= Supernatant of the transfected COS cell culture after 50 hours (culture 2 and 1 respectively)
Lane 4,5= Supernatant of the transfected COS cell culture after 20 hours (culture 2 and 1 respectively)
Lane 6 = Supernatant of normal COS cell culture

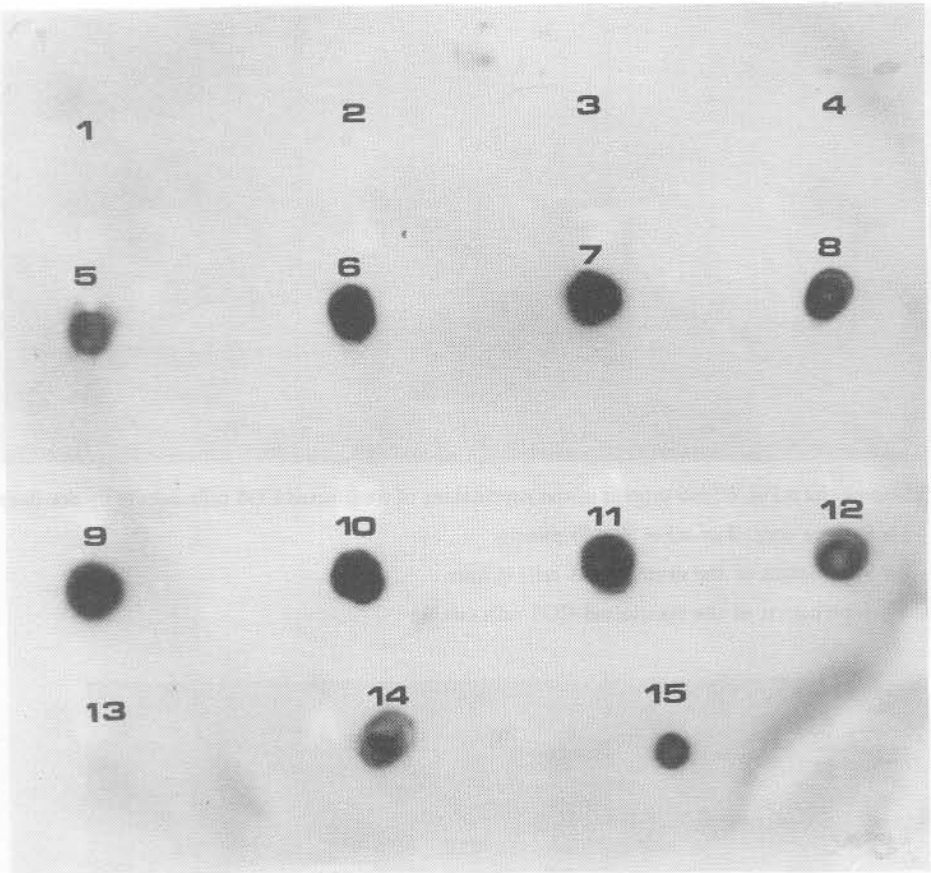


Fig.6 Screening of the scFvs WM65 protein in the elution fraction

1-12 = Elution fractions

13 = Fraction before elution

14,15= Positive control of the scFvs WM65 protein

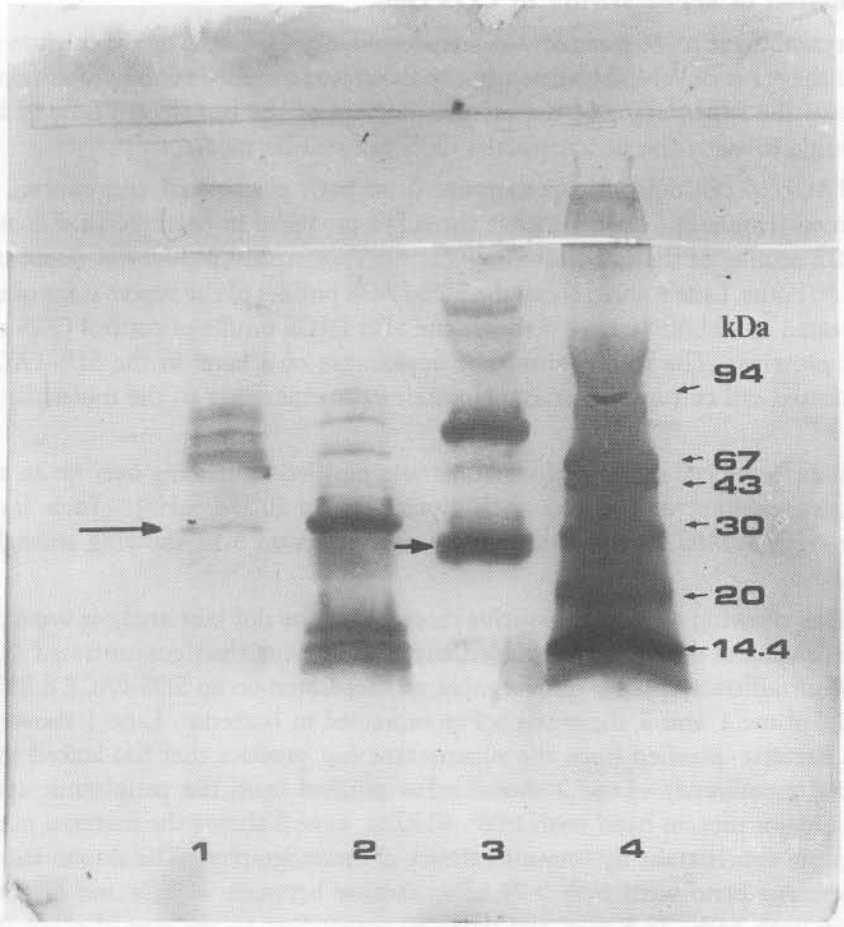


Fig.7 Separation of immunoaffinity purified scFvs on 8-25% SDS-PAGE

Lane 1,2 = scFvs WM65 protein from *E.coli* supernatant and lysate respectively

Lane 3 = Pooled elution fraction from immunoaffinity column

Lane 4 = Standard low molecular weight proteins

2. Expression of scFvs WM65 in COS cells

The recombinant pSVL plasmid was transfected into COS cells by the calcium phosphate method, and the scFvs of WM65 transiently expressed was screened for by protein dot blotting. Figure 4 shows the supernatant of the culture medium of the transfected cells to be positive for scFvs compared with the untransfected COS cell culture media.

SDS-PAGE of cell culture supernatants from both transfected and control COS cells were performed (Figure 5). Lane 1 shows the scFvs produced in bacteria. Lane 2 and 3 show the SDS-PAGE profiles of the transfected cell culture supernatant proteins of duplicate cultures harvested at 50 hours. Lane 4 and 5 show the SDS-PAGE profiles of the supernatant of transfected culture harvested at 20 hours. Lane 6 shows the SDS-PAGE profile of control COS cell culture supernatant proteins. The results show the appearance of a band in the SDS-PAGE profiles of the transfected cell culture supernatant proteins corresponding to the molecular weight of the scFvs.

The transfected cell culture supernatant was purified by passing over an an anti c-Myc immunoaffinity column, and eluted with glycine buffer (0.2M, pH3). Each fraction was screened for scFvs WM65 by dot blot analysis, with fraction 5-12 showing strongly positive results (Figure 6).

Fractions showing a strongly positive reaction for the dot blot analysis were pooled and diluted with 3 volume of PBS. The pooled supernatant was then concentrated (x6) using a Centricon 10 ultrafiltration tube. This sample was separated on an SDS-PAGE 8-25% gradient gel (Figure 7). Lane 1 and 2 show the scFvs expressed in bacteria. Lane 1 shows the scFvs produced in bacteria purified from the supernatant (i.e. product that has leaked through the outer bacterial membrane). Lane 2 shows scFvs purified from the periplasmic space. Both lanes show a major protein band with MW. 30 kDa. Lane 3 shows the material purified from COS cell culture supernatant by immunoaffinity chromatography. The results show 3 major protein bands; one band with MW >94 kDa, another between 94 kDa and 67 Kda and the smallest band with MW. 28.5 kDa, the latter corresponding to the size of scFvs WM65.

DISCUSSION

One of the areas where treatment with monoclonal antibodies have shown great promise is in the treatment of leukemia. Current treatment for leukemia incorporates allogeneic bone marrow transplantation (using a matched sibling as donor) in conjunction with chemotherapy. This treatment regime may result in remission in up to 50% of sufferers of chronic and acute myeloid leukemia and acute lymphoblastic leukemia, however only 25 % of the population have a matched sibling donor, and relapse is common. It is envisaged that new treatments for leukemia will require reagents that interact specifically with cell surface structures, such as monoclonal antibodies and growth factors. one such reagent is the immunotoxin, which combines the specificity of an antibody for its antigen, with the cytotoxicity of compounds such as ricin, diptheria toxin and pseudomonas exotoxin.

Recent developments in the production of recombinant antibodies and antibody fragments have created new interest in the use of these reagents as therapeutics. The construction of human antibody libraries for selection of novel antibodies (thus bypassing immunization) has alleviated the main problem with the use of mouse antibodies as therapeutic reagents, that is the human anti-mouse antibody response (HAMA). As previously discussed above, production of recombinant antibody fragments in procaryotic production systems may lead to decreased

yields due to protein folding constraints. Also, it is not uncommon for the antibody fragments produced in bacteria to show a decreased affinity for antigen. These problems can be overcome by production in mammalian cell systems. By optimising cell culture parameters, production costs in eucaryotic system can be lower if not competitive with their procaryotic counterparts.

For initial evaluation of the scFvs of WM65 produced in mammalian cells, the COS cell line was used. The COS cell line is an SV40-transformed monkey cell line which constitutively expresses large T-antigen, required for initiation of SV40 viral DNA replication . Transfection of COS cells with expression vectors carrying the SV40 origin of replication results in extrachromosomal gene amplification, resulting in transient high level expression of recombinant protein.

The scFvs WM65 (V_H-linker-V_L) cDNA was amplified with modified terminal restriction sites by the polymerase chain reaction and was successfully cloned into the pSVL expression vector. The recombinant pSVL plasmid containing the scFVs WM65 was transfected into COS cells. Due to high level of extrachromosomal DNA replication after transfection, transfected COS cells have limited lifetime, with transient expression occurring in the first 48-72 hours.[25-27] The supernatant of transfected culture was harvested at 50 hours, and screened for the c-Myc tag protein which was fused to the scFvs of WM65. Dot blot analysis of the supernatant showed a weak positive signal for scFvs.

The transfected supernatant was purified by immunoaffinity chromatography. Fractions eluted from the column showed a strong positive signal by dot blot analysis. SDS-PAGE analysis on the pooled fractions showed three major protein band on the gel, with MW. of >94 kDa, >67 kDa and approximately 28.5 kDa, the latter corresponding to the molecular weight of scFvs WM65 with the c-Myc protein tag. The molecular weights of the larger proteins were not accurately determined, since low molecular standards were used. Although the two large protein bands have not been characterized in this experiment, the two proteins is likely to be c-Myc proteins from COS cells which are transformed cells. Additionally, Wurm *et al.* (1986)[28] expressed recombinant c-Myc in CHO cell and found three forms with molecular weights of 75 kDa, 66 kDa, 64 kDa. Therefore, the scFvs WM65 protein need to be further purified which will allow to study on specificity and affinity to leucocytes. Since it has been shown that CHO cells have very low level of c-Myc[28] therefore the scFvs could be expressed in CHO cells, which would have advantages in stable expression of the protein, however selection of completely stable transfectants is time consuming.

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