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(54) Titre : CLONAGE, EXPRESSION, SEQUENCAGE ET AMELIORATION DE L'EFFICACITE D'UN ANTICORPS MONOCLONAL CONTRE LE VIRUS DE L'ENCEPHALOMYELITE EQUINE DU VENEZUELA

(54) Title: CLONING, EXPRESSION, SEQUENCING, AND FUNCTIONAL ENHANCEMENT OF MONOCLONAL SCFV ANTIBODY AGAINST VENEZUELAN EQUINE ENCEPHALITIS VIRUS(VEE)

(57) Abrégé/Abstract:
The invention discloses generation of a single chain variable fragment (ScFv) antibody from a well-characterized monoclonal antibody (Mab) against Venezuelan equine encephalitis (VEE) virus, by cloning variable regions of the heavy (V_H) and the light (V_L) chain antibody genes, connected by a DNA linker, in phagemid expression vector pCANTAB 5 E. Mab clone $1A_4A_1$ was successfully cloned as ScFv in Escherichia coli strain TG-1 and expressed as a ~30 KDa ScFv protein which was functional in recognizing VEE by ELISA. Results were reproduced in Escherichia coli strain HB2151, where the same clone, designated A_{116}, was expressed mainly as soluble periplasmic protein. The 30 KDa A116 displayed weak binding specificity to VEE. Sequence analysis revealed a frame shift in the N-terminal region of the V_L domain, upstream to the complementarity-determining region 1, as the probable cause of reduced activity. A PCR-based site-directed mutagenesis approach was adopted to re-introduce the three single-base deletions in the 5 prime region of the V_L gene of A116, corresponding to framework-1 region. The mutagenized A116 was designated as MA116. The introduction of these three bases corrected a localized frame-shift in framework-1 region to consensus framework-1 amino acid sequence. Five MA116 clones (MA116-4, MA116-6, MA116-14, MA116-15, and MA116-16) have been analyzed for their reactivity to VEE antigen, with MA116-15 showing comparable reactivity to the parental 1A4A-1 Mab in recognizing VEE antigen. Sequence data revealed that only MA116-15 had incorporated the three intended base insertions without accumulating any other mutational changes.

ABSTRACT

The invention discloses generation of a single chain variable fragment (ScFv) antibody from a well-characterized monoclonal antibody (Mab) against Venezuelan equine encephalitis (VEE) virus, by cloning variable regions of the heavy (V_H) and the light (V_L) chain antibody genes, connected by a DNA linker, in phagemid expression vector pCANTAB 5 E. Mab clone $1A_4A_1$ was successfully cloned as ScFv in *Escherichia coli* strain TG-1 and expressed as a ~30 KDa ScFv protein which was functional in recognizing VEE by ELISA. Results were reproduced in *Escherichia coli* strain HB2151, where the same clone, designated A_{116}, was expressed mainly as soluble periplasmic protein. The 30 KDa A116 displayed weak binding specificity to VEE. Sequence analysis revealed a frame shift in the N-terminal region of the V_L domain, upstream to the complementarity-determining region 1, as the probable cause of reduced activity. A PCR-based site-directed mutagenesis approach was adopted to re-introduce the three single-base deletions in the 5 prime region of the V_L gene of A116, corresponding to framework-1 region. The mutagenized A116 was designated as MA116. The introduction of these three bases corrected a localized frame-shift in framework-1 region to consensus framework-1 amino acid sequence. Five MA116 clones (MA116-4, MA116-6, MA116-14, MA116-15, and MA116-16) have been analyzed for their reactivity to VEE antigen, with MA116-15 showing comparable reactivity to the parental 1A4A-1 Mab in recognizing VEE antigen. Sequence data revealed that only MA116-15 had incorporated the three intended base insertions without accumulating any other mutational changes.

CLONING, EXPRESSION, SEQUENCING, AND FUNCTIONAL ENHANCEMENT OF MONOCLONAL ScFv ANTIBODY AGAINST VENEZUELAN EQUINE ENCEPHALITIS VIRUS (VEE)

FIELD OF INVENTION

This invention relates to the cloning, expression, sequencing, mutagenesis and functional enhancement of reactivity of recombinant monoclonal single-chain variable fragment (ScFv) antibodies against Venezuelan equine encephalitis (VEE) virus antigens.

BACKGROUND OF THE INVENTION

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The alphavirus family comprises of a large number of viruses that are closely related in their molecular structure but cause a variety of different diseases in humans and other animals [1]. Some alphaviruses, upon infection, enter the central nervous system (CNS) and lead to encephalitis. A New World Alpha virus of particular importance in this regard is Venezuelan equine encephalitis (VEE) virus. VEE virus infections mainly target the CNS and lymphoid tissues causing severe encephalitis in equines and systemic febrile infection with occasional encephalitis in humans. VEE virus is highly infectious by aerosol inhalation for humans [1].

Serologically the VEE complex of viruses can be subdivided into six subtypes (I - VI), with subtype I exhibiting five variants (I_{AB}, I_C, I_D, I_E, I_F) [1]. VEE epizootics are associated with members of subtypes I_{AB} or I_C. The other subtype I variants (I_D, I_E and I_F) and subtypes II - VI have been associated with enzootic VEE transmission [2].

The molecular structure of the VEE virion consists of a plus sense RNA genome encapsulated in an enveloped icosahedral nucleocapsid [3]. The envelope contains two important structural glycoproteins (gp), E_2 (56KDa) and E_1 (50KDa) [4]. The viral neutralization sites reside in the E_2 envelope protein [5]. Thus the E_2 protein of VEE is an important target for immunodetection/protection studies.

Hybridoma technology [6] made it possible to generate monoclonal antibodies (Mab) directed against viruses. The disadvantages of using monoclonal antibodies (Mabs) as immunodiagnostic or immunotherapeutic reagents are known. The cost of large-scale production of Mabs is excessive. The potential for genetic variations introduced during repeated cycles of cell growth make Mabs difficult to handle and potentially unreliable. In addition, antigenicity of the complete antibody molecule, when administered as therapeutic reagent, is associated with "serum sickness" in recipients.

Furthermore, due to the large size of the whole antibody molecule, there is low penetrability of administered antibody into target tissues. These features make the complete antibody molecule unattractive for use as therapeutic reagent [19, 20].

However, with the development of recombinant antibody technology, where functional antibody fragments can be produced in bacteria, the application of antibodies as immunodiagnostic and/or immunotherapeutic reagents has become more feasible [21, 22, 23, 24].

To develop a monoclonal recombinant antibody from 1A4A-1 Mab, the inventors chose the Recombinant Phage Antibody System (RPAS). Briefly, in RPAS technology, the Variable heavy (V_H) and Variable light chain (V_L) domains of an antibody are cloned and covalently joined by a polypeptide linker in bacterial expression vectors generating single chain Variable fragment (ScFv) antibodies [8, 9, 10]. This results in expression in bacteria of the antigenic recognition domain of an antibody as a single chain, which can fold itself into a functional molecule. The attractiveness of this system is in the relative ease with which huge quantities of functionally active molecules can be obtained in a very short time. Since these ScFv lack the constant region of the antibody molecule, they have very low antigenicity. The low antigenicity of ScFv, coupled with their small size (~30 KDa) and hence the ease with which they can penetrate tissues, makes these molecules attractive alternatives to whole antibody molecules as therapeutic materials.

Roehrig et al. [7] generated Mab directed against VEE viruses. Using these Mab, the antigenic structure of the envelope glycoprotein E_2 of VEE virus was analyzed. Protection studies using anti-E_2 VEE virus Mab revealed a critical neutralization epitope (E_2^c). One of the Mab that recognized this neutralization epitope (E_2^c) was 1A4A-1. Mab 1A4A-1 not only recognizes VEE virus serological subtypes I_A - I_D and II_{EVE} in enzyme-

linked immunosorbant assay (ELISA) but also can neutralize all of these subtypes [7], making it a very good candidate for development of second generation antibody with both diagnostic and immunotherapeutic application potential.

SUMMARY OF THE INVENTION

The present invention is directed to the cloning, expression, sequencing, mutagensis, and functional enhancement and of ScFv from hybridoma cell line 1A4A1.

The initial results of generating a recombinant 1A4A-1 ScFv antibody are described elsewhere [11]. Briefly, 1A4A-1 Mab was cloned as a ScFv antibody at Defence Research Establishment Suffield (DRES). The functional analysis of the 1A4A-1 ScFv (A116) by ELISA and western blotting revealed low reactivity to VEE whole virus antigen. Sequence analysis of A116 revealed three base deletions in the 5 prime end of the A116 V_L gene. At the protein level, these mutations resided in the framework-1 region of V_L of A116. The three individual base deletions introduced a localized frame shift in the first 20 amino acids of the conserved framework-1 region of V_L of A116, resulting in a partially functional protein [11].

Framework-1 region of the light chain is highly conserved in antibody molecules. It adopts an anti-parallel beta pleated sheet conformation. β-strands A and B of the antibody light chain stabilize each other through hydrogen bonding and ultimately place complementarity determining region (CDR) L1 in proper context in relation to the other two CDRs for antigen interaction. Since the frame shift in V_L of A116 occurred in a region that is conserved in antibody molecules, it is possible that the frame-shift affected the overall conformation of the antibody molecule, thus rendering it partially inactive. A repair strategy was conceived whereby the framework-1 region would be restored to

consensus amino acid sequence, and such repaired clones could be analysed for restored reactivity to VEE whole virus antigen. In this invention, the inventors describe the successful isolation of MA116 clones with enhanced reactivity to VEE whole virus antigen and discuss the molecular changes that restored the full functionality of original A116 ScFv protein.

Through repairing the genetic mutation of the ScFv antibody gene, the present invention provides enhanced reactivity of the cloned ScFv antibody with homologous VEE antigen.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of steps in development of recombinant ScFv antibodies: A. Cloning and PCR; B. Expression and analysis.

Figure 2A shows Western blot of cell lystates from 1A4A1 clones exhibiting full length ScFv fragments.

Figure 2B shows screening of selected ScFv protein lysates from *E. coli* TG-1 cells for functional reactivity by ELISA.

Figure 3A shows screening of A116 ScFv protein in lysates of *E. coli* HB2151 cells for expression of soluble ScFv antibody by western blotting.

Figure 3B shows screening of soluble ScFv protein in lysates from *E. coli* HB2151 cells for functional reactivity by ELISA.

Figure 4A shows Western blot of soluble A116 ScFv protein in periplasmic extract (PE) of *E. coli* HB2151 cells.

Figure 4B shows functional analysis of A116 ScFv protein from periplasmic extract (PE) of *E. coli* HB2151 cells by ELISA.

Figure 5A shows V_H amino acid sequence comparisons.

Figure 5B shows V_L amino acid sequence comparisons.

Figure 6 shows comparison of nucleotide sequences in the framework-1 region of V_L of the six 1A4A1 ScFv clones shown to carry full length ScFv fragments.

Figure 7 shows PCR based site directed mutagenesis strategy for repair of A116 ScFv gene.

Figure 8 shows isolation of DNA and Xho I restriction enzyme digestion of MA116 clones.

Figure 9 shows Western blot analysis of selected lysates from MA116 clones.

Figure 10 shows ELISA to confirm functionality of selected MA116 clones.

Figure 11 shows the nucleotide sequence in framework-1 of the light chain of A116-16 and the MA116 clones.

Figure 12 shows a comparison of the reduced amino acid sequence of A116 and the MA116 clones.

Figure 13A shows SDS-PAGE analysis of MA116-15 ScFv purification - Coomassie stained SDS-PAGE gel.

Figure 13B shows SDS-PAGE analysis of MA116-15 ScFv purification - Westerm blot analysis of the MA116-15 samples.

Figure 13C shows ELISA analysis of the ScFv-positive purified fractions of Clone MA116-15.

Figure 14 shows functional comparison of purified MA116-15 (F6) ScFv and parent 1A4A-1 Mab at equi-molar concentration.

DETAILED DESCRIPTION OF THE INVENTION

Part I. Cloning, Expression, Sequencing and Functional Analysis of ScFv from Hybridoma 1A4A1 MATERIALS AND METHODS

The Recombinant Phage Antibody System (RPAS), consisting of mouse ScFv module, expression module, and detection module, mRNA Quickprep™ kit, and anti-E Tag antibody labelled with horseradish peroxidase (HRP), was purchased from Amersham Pharmacia Biotech (Baie d'Urfé, QC). Taq polymerase used in polymerase chain reaction (PCR) was from Boehringer Mannheim (Laval, QC). Restriction enzymes were purchased from Gibco/BRL (Burlington, ON), Amersham Pharmacia Biotech, or New England Biolabs (Beverly, MA). Unless otherwise specified, chemical reagents were purchased from Sigma chemical company (St. Louis, MO) and tissue culture reagents were purchased from Gibco/BRL. Where possible, high grade reagents, nuclease-free water (Promega Inc., Madison, WI), siliconized microfuge tubes, pre-sterilized solutions, and disposable labware were used. Standard methods were used for the manipulation and cloning of cDNAs [12].

Cobalt-irradiated VEE strain TC83 was a gift from Dr. Jonathan Smith, U. S. Army Medical Research Institute of Infectious Diseases, Frederick, MD.

Growth and Maintenance of Hybridoma Cell Lines

VEE hybridoma clone 1A4A-1 was kindly provided by Dr. J.T. Roehrig, Division of Vector-borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO. The hybridoma cells were grown and maintained in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories,

Logan, UT), 2 mM L-glutamine, 1 mM sodium pyruvate, antibiotic/antimycotic supplement (100 units/mL penicillin G, 100 µg/mL streptomycin, and 25 µg/mL amphotericin B), 1x Vitamins solution, and 100 µM non-essential amino acids. The cells were maintained at a density of ~$1x10^5$/mL.

Construction of ScFv Antibody

A scheme representing the procedure adopted for cDNA cloning and construction of recombinant ScFv antibody is shown in Figure 1 and described elsewhere (25). Messenger RNA was isolated from $1x10^7$ hybridoma cells by use of a mRNA Quickprep™ kit (Amersham Pharmacia Biotech), in accordance with the manufacturer's recommended protocol. The final yield of mRNA was 8 µg. Two µg of mRNA was used as template for the reverse transcriptase reactions for each V_H and V_L chain. First strand cDNAs were synthesized by using primed, first strand reaction mixtures. The cDNAs coding for the respective V_H and V_L regions were then amplified by PCR by use of a set of primers included in the mouse ScFv module of the RPAS. PCR amplification was carried out for 30 cycles (94°C for 1 min; 55°C for 2 min; 72°C for 2 min). Amplified DNAs of V_H (~340-350 bp) and V_L (~325 bp) were purified from incomplete products and primers by agarose gel electrophoresis. The purified V_H and V_L cDNAs (50 ng of each) were then mixed with linker DNA fragment. Assembly PCR was carried out for seven cycles (94°C for 1 min; 63°C for 4 min), thus connecting the two cDNAs in the correct reading frame. The assembled fragments were then amplified using primers with a *Sfi I* restriction site at the 5' end and a *Not I* site at the 3' end, to facilitate the cloning of the PCR products into the phagemid pCANTAB 5 E vector (Amersham Pharmacia Biotech). Phagemid pCANTAB 5 E is designed in such a way that the ScFv fragment can

be cloned downstream to the leader peptide of the M13 gene 3 but upstream to the E Tag peptide, followed by an amber translational stop codon and the main body of the remaining M13 gene 3. The ligation mixture for the recombinant ScFv antibody was transformed into *E. coli* TG-1 competent cells (Amersham Pharmacia Biotech), an amber stop codon suppresser strain, and the transformed cells were subsequently plated on Luria Bertani (LB) agar plates containing 2% glucose and 100 μg/mL ampicillin. The plates were incubated overnight at 30°C.

Screening of ScFv Clones for Expression by Western Blotting

Individual phagemid clones of 1A4A1 ScFv recombinant antibody were picked and grown overnight in LB broth containing 2% glucose and 100 μg/mL ampicillin. Fresh 1.5 mL cultures were prepared the following morning at a starting A_{600nm} of 0.05 and grown at 30°C with shaking to a density of 0.5. Cells were then pelleted by centrifugation and resuspended in 1.5 mL fresh LB broth containing 100 μg/mL ampicillin and 3 mM iso-propyl β-D-thiogalactoside (IPTG). Subsequently, the cells were grown at 37°C to an A_{600nm} of 1.5 (~2 hours), pelleted again, resuspended in 0.6 mL of phosphate buffered saline (PBS), pH 7.5, and lysed by boiling. The boiled lysates were microfuged to remove cellular debris and the supernatants were frozen at -70°C. Three to six μL of each of the lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels. The separated proteins were transferred to Immobilon™-P membranes (Millipore Corp., Bedford, MA) by use of Towbin buffer (25 mM Tris/HCl, pH 8.3, 192 mM glycine, and 20% methanol). Membranes were blocked for one hour with blocking buffer (PBS containing 3% non-fat

skim milk). HRP-labelled anti-E Tag antibody, diluted 1:2000 in blocking buffer, was then added and the membranes incubated in this solution for a period of 45-60 minutes. Specific binding to expressed ScFv was detected by use of an enhanced chemiluminescence (ECL) kit purchased from Amersham Pharmacia Biotech. For all washing steps, PBS containing 0.1 % Tween-20 was used.

Expression of Soluble ScFv Antibody Molecules

DNA clone 1A4A1-16 was transformed into *E. coli* HB2151 cells (Amersham Pharmacia Biotech), an amber stop codon non-suppresser strain. The resulting transformants were referred to as A116. As described above for TG-1 cells, transformed HB2151 clones were grown in LB broth containing 2% glucose and 100 μg/mL ampicillin, were induced with IPTG, lysed by boiling, and the lysates submitted to SDS-PAGE and Western blot analysis.

Preparation of Periplasmic Extracts

Periplasmic extracts were made according to the protocol provided by Pharmacia Biotech, with the following variations. Overnight cultures were grown at 30°C with shaking in 3 mL LB broth containing 2% glucose and 100 μg/mL ampicillin. Fresh 50 mL LB broth cultures, containing 100 μg/mL ampicillin, were prepared the following morning at a starting A_{600nm} of 0.025. When the cultures had reached an A_{600nm} of 0.1 (~1 hour), they were induced with 2 mM IPTG. The induced cultures were grown at 30°C until they reached an A_{600nm} of 0.5 (~2 hours). The cells were then harvested by centrifugation at 1500 x g for 10 minutes and the pellet gently resuspended in 20 mL of ice cold 1x periplasmic extraction buffer (0.2 M Tris/HCl, 0.5 mM EDTA, and 0.5 M

sucrose, pH 8.0) per liter of cell culture. Thirty-three mL of 0.2x periplasmic extraction buffer was added per liter of cell culture and the solution mixed. Cells were incubated on ice for 20 minutes with gentle agitation, then centrifuged at 1500 x g for 10 minutes, and the supernatant collected and filtered through a 0.45μ filter (Millipore Corp.). Filtered supernatants were stored at -70°C.

ScFv Sequence Analysis

Plasmid DNA from 1A4A1-10, 1A4A1-12, 1A4A1-16, 1A4A1-17, 1A4A1-24, and 1A4A1-30 were used in double-stranded dideoxynucleotide sequencing, in both directions, using a pCANTAB 5 E sequencing primer set purchased from Amersham Pharmacia Biotech. Sequencing reactions were performed using a Big Dye™ Terminator Cycle Sequencing kit (Perkin Elmer Applied Biosystems, Mississauga, ON), as per the manufacturer's recommendations. The reaction products were purified from unincorporated dye molecules using Centri-Sep™ columns (Princeton Separations Inc., Adelphi, NJ). The nucleotide sequence data was generated using an automated Prism™ 310 genetic analyzer system (Applied Biosystems, Foster City, CA). Results were analyzed on Lasergene DNA analysis software (DNA Star, Madison, WI). Protein search and analysis was performed on the PIR Release 58.0 (September 1998) and SWISS-PROT Release 36.0 (July 1998) databases, using Wisconsin package version 9.0 (Genetics Computer Group, Madison, WI). Subgrouping and family grouping were performed using the Kabat Database of Sequences of Proteins of Immunological Interest through Internet (February 1999 dataset) [15].

Enzyme-linked Immunosorbant Assay

The enzyme-linked immunosorbant assay (ELISA) was carried out in 96-well Nunc Maxisorb™ flat-bottom plates (GIBCO/BRL, Bethesda, MD). Inactivated VEE (strain TC-83) was used as antigen, at concentrations of 4 or 10 μg/mL. Wells were coated overnight at 4°C with 100 μL VEE antigen prepared in 2x carbonate bicarbonate buffer, pH 9.6, containing 0.02% sodium azide. Plates were washed with PBS containing 0.05% Tween-20. Plates were blocked twice with blocking buffer (PBS containing 2% BSA and 0.05% Tween-20). Unless otherwise specified, primary antibody (ScFv lysate or periplasmic fraction) was diluted in antibody dilution buffer (PBS containing 2% BSA) and incubated for one hour at 37°C. HRP-labelled anti-E Tag antibody was used as indicator antibody and diluted 1:4000 or 1:6000 in antibody dilution buffer. Plates were developed with ABTS substrate solution (2, 2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt/hydrogen peroxide 1:1 mixture) (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The plate blanks received blocking buffer and substrate only. Other appropriate controls were included in each assay.

RESULTS

Cloning of ScFv Genes

Messenger RNA was isolated from hybridoma cell line 1A4A1 and cloned as ScFv by procedures outlined in Figure 1. Distinct bands of ~340 bp for V_H and ~325 bp for V_L were detected by agarose gel electrophoresis after the first PCR amplification. The amount of V_H product was estimated to be greater than the amount of V_L product (data not shown). Gel purified cDNAs of V_H and V_L were assembled into their respective

single gene 750 bp fragments and amplified by PCR. The gel purified 750 bp fragments, following digestion with the restriction enzymes *SfiI* and *NotI,* were ligated to phagemid vector pCANTAB 5 E and the ligation reaction transformed into *E. coli* TG-1 competent cells. Forty transformants of 1A4A1 ScFv were picked and grown individually for screening by "miniprep" DNA analysis. The remaining transformants were pooled and made into a 1A4A1 library.

Screening of Transformants for the Presence of Full Length ScFv Fragments

In order to screen for the presence of full length 750 bp ScFv inserts, "miniprep" DNA was prepared from the 40 individual transformants and restriction enzyme analysis was performed. From the 40 transformants, a total of six (1A4A1-10, 1A4A1-12, 1A4A1-16, 1A4A1-17, 1A4A1-24, and 1A4A1-30) were found to carry a full length 750 bp ScFv fragment (data not shown). These six transformants were selected for further analysis by western blotting, to evaluate for expression of the ScFv product.

Screening of Clones with Full Length Fragments for Expression in E. coli TG-1

Following IPTG induction, cell lystates of the six TG-1 clones containing full length 1A4A1 ScFv were screened for expression of product by western blot analysis. *E. coli* strain TG-1 carries a suppressor transfer RNA for amber translational stop codons which allows suppression (readthrough) of the amber stop codon, present between the ScFv and gene 3 sequences of pCANTAB 5 E, at a frequency of about 20%. Therefore, both the ~70 KDa ScFv-gp3 fusion protein and the ~30 KDa ScFv soluble protein products were expected to be produced. Figure 2A represents a western blot of cell lysates from the six 1A4A1 ScFv clones exhibiting full length ScFv fragments.

Surprisingly, only clone 1A4A1-16 expressed protein products that were detected by anti-E Tag antibody. The other five 1A4A1 clones (1A4A1-10, 1A4A1-12, 1A4A1-17, 1A4A1-24, and 1A4A1-30) showed no expressed product. Since no background material was detected in the control TG-1 lysate by anti-E Tag antibody, it was deemed likely that the expression products seen in 1A4A1-16 lysate were from the ScFv full length fragment. As expected, both the soluble 30 KDa ScFv product and the ~70 KDa ScFv-gp3 fusion product were detected in the 1A4A1-16 lysate, as well as intermediate products that could have resulted either from degradation or incomplete synthesis.

Screening of 1A4A1-16 ScFv Lysate from E. coli TG-1 for Recognition of VEE Antigen

To assess the functionality of the expressed 1A4A1-16 ScFv products present in TG-1 lysates, ELISA was performed to test for reactivity with inactivated VEE antigen. TG-1 control cell lysates and lysates from 1A4A1-12 were included as negative controls, while 5B4D6 lysate, another VEE ScFv shown previously to react with VEE antigen [25], was used as positive control (Figure 2B). Only 1A4A1-16 ScFv clone gave positive ELISA results, as indicated by retention of ScFv products in the presence of VEE antigen. Wells with VEE antigen specifically retained the expressed ScFv products at absorbance levels at least twice as high as that observed in wells without VEE antigen, indicating that 1A4A1-16 ScFv antibody was functionally active. It may also be observed from Figure 2B that the 1A4A1-16 ScFv clone product, although functional, was less active than the 5B4D6 ScFv positive control lysate. The results of this ELISA complemented the expression results shown in Figure 2A, with no activity demonstrated for the other 1A4A1 clones..

Expression of Functional Soluble ScFv Proteins in E. coli HB2151 Cells

To demonstrate the functionality of the soluble 30KDa ScFv products when expressed in the absence of the ~70 KDa ScFv-gp3 fusion protein, 1A4A1-16 DNA was transformed into a non-suppressor background cell type, *E. coli* HB2151. To distinguish between TG-1 and HB2151 transformants, transformants obtained as a result of HB2151 transformations were referred to as A116, while TG-1 transformants were called 1A4A1-16.

To establish that A116 clones expressed soluble 30 KDa ScFv protein, western blot analysis was performed on a number of different transformants. The cells were grown, induced with IPTG, and lysates made, as described above. The results of western blot analysis performed on the lysates of A116 transformants clearly showed the expression of 30 KDa soluble ScFv protein (Figure 3A). Control *E. coli* HB2151 lysates did not show any detectable background. These results suggested that the A116 ScFv terminated correctly at the amber translational stop codon at the end of the E Tag. To show that the expressed soluble ScFv proteins in the A116 lysates were also functional, ELISA was performed with inactivated VEE, as described above for TG-1 lysates. HB2151 control lysates were included as negative controls (Figure 3B). As observed in the ELISA of TG-1 lysates, A116 ScFv 30 KDa soluble proteins were functional in recognizing VEE antigen, with an observed approximate four-fold enhancement in absorbance over the HB2151 negative control lysate. Although functional, A116 ScFv 30 KDa soluble protein exhibited weaker interaction with the antigen when compared to an approximately equivalent concentration of D66 ScFv protein.

Analysis of Expressed ScFv Proteins from the Periplasm of E. coli HB2151 Cells

It has been well documented that ScFv protein isolated from the periplasm is more active than ScFv protein present in the cytoplasm, because periplasmic protein is properly folded and in functional conformation. Conversely, most cytoplasmic ScFv protein is non-functional because it has not yet been properly folded. Activity assays reported in previous sections of this paper were performed using expressed ScFv extracted from whole cell lysates. It was hypothesized that ScFv A116 might be more sensitive to proper folding than was D66 and, for this reason, exhibited lower activity than D66.

To determine if the functional reactivity of A116 ScFv could be enhanced by expression in the periplasm, A116 and D66 ScFv (positive control) proteins were extracted from periplasmic fractions and examined for expression by western blot and reactivity with VEE antigen by ELISA. Figure 4A shows a Western blot demonstrating the presence of the 30 KDa expressed ScFv protein from both A116 and D66 periplasmic extracts analyzed. To test the functionality of the A116 ScFv expressed in the periplasm, an ELISA was performed (Figure 4B). It was clear from the results of this ELISA that there was little difference in the activity of the A116 whether extracted from the periplasm (Figure 4B) or from the whole cell lysate (Figure 3B). This result suggested that the weak reactivity of A116 ScFv with VEE antigen was due to factors inherent in the primary structure of the protein rather than to issues relating to protein folding in the periplasm.

Nucleotide Sequence Analysis of 1A4A1 ScFv Clones

To investigate the reasons why the five full length 1A4A1 ScFv clones (1A4A1-10, 1A4A1-12, 1A4A1-17, 1A4A1-24, and 1A4A1-30) did not express ScFv products, their DNA was sequenced. In addition, DNA from clone 1A4A1-16 was sequenced, to

identify the gene families for the V_H and V_L regions, as well as to elucidate potential mutations that might have contributed to its weak activity. It was also reasoned that, by knowing the nucleotide and deduced protein sequences, possible sequence defects could be identified and experiments could be designed to repair these defects and potentially re-engineer the antibody for improved activity.

Nucleotide sequences of the six 1A4A1 ScFv clones were generated, as described above, and amino acid sequences were deduced from the nucleotide sequence data generated (data not shown). The deduced amino acid sequences of the six 1A4A1 clones confirmed the expected protein structure, where V_H and V_L regions were connected by a linker region composed of $(Gly_4\ Ser)^3$. It was further revealed that three of the five non-expressive 1A4A1 clones (1A4A1-10, 1A4A1-17, 1A4A1-24) had in-frame stop codons in the V_H, as well as in the V_L genes and, therefore, could not express a protein product. The remaining two non-expressive clones (1A4A1-12 and 1A4A1-30), although free of any in-frame stop codons, were frame shifted, which resulted in an out-of-frame E-peptide sequence. Since anti-E Tag antibody was used for western blot detection of ScFv expression, this accounts for why the protein products expressed by these clones could not be detected.

Detailed analysis was performed on the nucleotide and amino acid sequences of clone 1A4A1-16 (A116). A comparison of the deduced amino acid sequences of the V_H and V_L regions of A116, as determined from the nucleotide sequence data, with other ScFv antibody amino acid sequences of murine origin available in the GCG protein database. Amino acid sequence analysis of A116 revealed what had been suspected i.e., that mutations had been introduced which resulted in a weakly active clone. The V_H framework regions of A116 showed a high degree of homology with D66 and with the

other murine ScFv used for sequence comparison. As expected, the sequence divergence was seen mainly in the three CDR regions (Figure 5A). The C-terminal V_L region also showed a high degree of homology in the framework regions and divergence in the three CDR regions, when compared to D66 or the published murine ScFv protein sequences (Figure 5B). The protein sequence also diverged in the N-terminal first framework region of the V_L chain of A116, located between the end of the linker and just before the beginning of the CDR1.

The nucleotide and amino acid sequences of the ScFv A116 were analyzed, using the Kabat Database of Sequences of Proteins of Immunological Interest (February 1999 Dataset), to determine the family grouping and subgrouping of the V_H and the V_L genes, respectively. To be included in a gene family, the database allows up to 34 base mismatches with existing sequences. The family grouping results for ScFv A116 V_H were indeterminate, indicating that A116 V_H is a unique sequence. This result could mean that either the V_H gene belongs to a family that is under-represented in the database collection or, that the V_H nucleotide sequence had mutated due to the accumulation of random single base changes during the PCR amplification steps. The V_L nucleotide sequence was identified as belonging to the family XX, with 26 base mismatches. The amino acid sequences were analyzed for subgrouping and it was found that V_H belongs to subgroup IA while V_L belongs to subgroup V.

To determine the similarity of A116 ScFv V_H and V_L proteins with other murine antibodies, a similarity search of A116 sequence was performed using the GCG Word search program and two protein databases, PIR version 58.0 and SWISS-PROT version 36.0. Sequences showing a high degree of similarity were aligned for comparison. The ScFv A116 V_H, including the CDR regions, was compared and, on average, there was

>75% similarity in the amino acids (Figure 5A). This level of similarity is striking, considering that the CDR regions were included in the comparison. For the V_L region, similarity was ~80% in the C-terminal proximal two-thirds portion of the V_L protein (Figure 5B).

Figure 6 is a comparison of the nucleotide sequence of all six A116 clones in the region of the frame shift (Framework-1 of V_L). Deletions of three nucleotide bases at different positions in the N-terminal region of the V_L of A116 were observed. Theoretically, if these deleted bases were to be introduced back into the nucleotide sequence and the amino acid sequence deduced, the frame-shifted region would be corrected to the consensus framework-1 region. This suggests that the deduced localized frame shift in the framework-1 region of V_L is, indeed, genuine and was probably responsible for the observed weak binding of A116 ScFv antibody to the VEE antigen.

DISCUSSION

ScFv antibodies offer several advantages over monoclonal antibodies generated by hybridoma technology, in that ScFv antibodies can be rapidly and economically produced, resulting in antibodies that are both functionally active and genetically stable (26. 27). In a previous publication, the present inventors reported the cloning and expression of D66 ScFv, a functionally active ScFv antibody produced from VEE hybridoma 5B4D6 (25). In this present study, the cloning and expression of another functionally active anti-VEE ScFv, A116, prepared from VEE hybridoma, 1A4A1, is disclosed.

VEE Mab 1A4A1, from which ScFv A116 was cloned in this study, has been previously well characterized by Roehrig et al. [18]. Parental Mab 1A4A1 is a

neutralizing antibody, thus the cloning of this antibody as ScFv was of particular interest due to its potential usefulness as an immunotherapeutic reagent. In addition, the recombinant ScFv form of 1A4A1 MAb would be expected to be genetically more stable than its parental MAb. Furthermore, expression of recombinant ScFv in bacteria and purification therefrom could constitute a cost-effective alternative approach for the large-scale production of antibodies for ultimate use as VEE immunodetection reagents and/or immunotherapeutic reagents for the treatment of VEE-induced encephalitis.

Initial screening of the ScFv clones, to find full length 750 bp ScFv DNA, was performed by restriction enzyme analysis. Since the starting material for cloning had been a hybridoma cell line, our screening strategy relied on the fact that, theoretically, all clones were expected to contain the same ScFv, or fragments thereof. Thus a screening protocol was adopted that consisted of picking clones that contained 750 bp full length fragments rather than panning against antigen by phage display, a technique that is tedious and time-consuming. Clones containing full length ScFv DNA were then subsequently screened by western blot, to select for ScFv expression-positive clones. Initial screening of a total of 40 ScFv clones, by restriction digest analysis, revealed six clones with 750 bp inserts. Surprisingly, when probed with HRP-labeled anti-E Tag indictor antibody, only one of these clones, 1A4A1-16, showed expression of the desired size protein products. The E Tag peptide is expressed as an integral part of the soluble (30 KDa) ScFv, as well as the gp-3-fused (70 KDa) ScFv product, and ScFv molecules expressed in the correct frame can be immunodetected by using anti-E Tag antibody. By DNA sequencing, it was shown that three out of the six full length clones expressed ScFv products but, because of frame shifts, internal to the V_H or the V_L genes, the E-peptide was out-of-frame. Thus, the ScFv products expressed from clones 1A4A1-12 and 1A4A1-

30 with an out-of-frame E-peptide could not be detected in the western blot analysis. The observed increase in mutagenesis i.e., frame-shifts and introduction of nonsense codons seen in ScFv genes, can be explained by an inherent default in the cloning process. PCR reactions are known to introduce mutations due to the low fidelity of the proof-reading function of the thermo-polymerases used in the reaction. As both the V_H and V_L genes collectively undergo 60 cycles of PCR amplifications before cloning as ScFv genes, it is not surprising that increased mutagenesis was observed.

Functional analysis of the A116 ScFv antibodies by ELISA against VEE antigen showed that the A116 ScFv was functional in specifically recognizing VEE. The fact that A116 ScFv was not as reactive as D66 ScFv, a previously cloned anti-VEE ScFv, led the present inventors to further investigate the A116 clone from the perspective of its protein sequence. The A116 ScFv amino acid sequence was compared with that of D66 ScFv, as well as with ScFv sequences of murine origin. Amino acid sequence comparisons clearly indicated that the sequences were >75% homologous in all regions of both the V_H and V_L proteins, except in the CDR regions and the framework-1 region of the V_L protein. The CDR regions are expected to be non-homologous, as they are responsible for antibody diversity and specificity. Framework regions of the antibody, on the other hand, are known to be highly conserved. However, framework-1 region of the V_L protein of ScFv A116 was not conserved when compared to other murine V_L amino acid sequences available in the database. Antibody specificity is attributed primarily to the V_H protein, which confers the recognition of and crude binding to the target antigen. This initial interaction is fine-tuned by the V_L protein, resulting in strong recognition and binding of the antigen. Additionally, framework regions of the antibody contribute to its function by putting the CDR regions in the proper configuration to interact optimally with the epitope

on the target antigen. To explain the lower reactivity of A116 ScFv antibody compared to D66 ScFv, the present inventors propose that, because of a localized frame shift in the framework-1 region of the A116 V_L, the antibody was unable to assume a functional conformation that would allow optimal interaction with the epitope on VEE antigen. The lack of homology in the framework-1 region of A116 ScFv, supports this hypothesis. By comparing the nucleotide sequences of the six 1A4A1 ScFv clones, the three individual nucleotide deletions that could have caused the proposed localized frame shift were located. It is likely that, if the deleted nucleotides were to be added back to the A116 nucleotide sequence, in the proper context, the consensus framework-1 region amino acid sequence would be restored.

In summary, a recombinant ScFv antibody has been generated from a well-characterized MAb to VEE virus. This ScFv, designated A116, was shown to be minimally reactive with the target VEE antigen when compared to another VEE ScFv previously cloned by this laboratory. By investigation of the nucleotide and deduced amino acid sequences of the V_H and V_L chains of A116 ScFv, mutations were identified that could have accounted for its reduced reactivity with antigen.

Part II. Functional Enhancement of Reactivity of ScFv

MATERIALS AND METHODS

Restriction enzymes and polynucleotide kinase were purchased from Amersham Pharmacia Biotech. PCR extra long kit was purchased from Perkin-Elmer/Applied Biosystems (Mississauga, ON). Unless otherwise specified, chemical reagents were purchased from Sigma Chemical Company (St. Louis, MO). Where possible, high-grade reagents, nuclease-free water (Promega Inc., Madison, WI), siliconized microfuge tubes,

pre-sterilized solutions, and disposable labware were used. Standard methods were used for the isolation and manipulation of DNA [12].

Synthesis of Primers

Two primers were synthesized for this study. A116 forward primer was a 20mer with a sense polarity and carried the 5 prime distal-most insertion of a "T" (denoted as third insertion). The sequence of forward primer synthesised was 5'caa-att-cgt-Tgt-cca-cat-ca 3'(with upper case T indicating the third base insertion). A116 reverse primer was a 30mer with anti-sense polarity and carried the first two 5 prime proximal "T" insertions which would appear on the sense strand as "A" (denoted as first and second insertions). The sequence of reverse primer synthesised was 5' gag-acT-gag-tga-gct-cga-Tgt-ccg-atc-c 3' (with upper case T indicating the first and the second base insertions).

PCR-Based Site Directed Mutagenesis and Amplification of A116 DNA

The strategy for PCR-based site directed mutagenesis and DNA amplification is outlined in Figure 7. Two and 5 ng of A116 DNA were used in the PCR reactions. Final concentration of the primers in the reaction was 800nM. The reaction conditions and other ingredients were as recommended by the manufacturer of GeneAMP^{R} XL PCR kit (Perkin-Elmer/Applied Biosystems). Special polymerase was used for this particular PCR reaction. The enzyme rTth DNA polymerase, XL (Perkin-Elmer/Applied Biosystems) has been genetically modified to give better performance in PCR reactions where extra long DNA synthesis is required. Four units of this polymerase were added per reaction. The PCR reaction (denaturation at 94 ^{0}C for one minute, annealing at 56 ^{0}C for two minutes, extension at 72 ^{0}C for five minutes) was performed for 25 cycles. PCR reactions amplified a 5100 bp band, that was subsequently purified by standard DNA purification methods. The purified PCR-amplified DNA was subjected to reaction with polynucleotide kinase (Amersham Pharmacia Biotech), in accordance with the manufacturer's recommendations. The end phosphorylated PCR-amplified DNA was gel purified again and ligated, overnight at 15 °C, with T4 DNA ligase (Amersham Pharmacia Biotech). The ligation reaction product was used to transform *E. coli* HB2151 competent cells, and the resulting transformation reaction was plated on Luria-Bertani

(LB) agar containing 100 ug/mL ampicillin and incubated overnight at 37 ^{0}C. The individual colonies that grew were treated as individual clones. Mini preparation of DNA was prepared from individual colonies after growth in LB broth media by standard protocols (12). The restriction digestions were performed according to standard protocols (12).

Preparation of Whole Cell Lysates of MA116 ScFv Clones

Individual re-engineered clones of A116 ScFv recombinant antibody were grown overnight in LB broth containing 2% glucose and 100 μg/mL ampicillin. Fresh 1.5 mL cultures were prepared the following morning at a starting A_{600nm} of 0.05 and grown at 30°C with shaking to a density of 0.5. Cells were then pelleted and resuspended in 1.5 mL fresh LB broth containing 100 μg/mL ampicillin and 3 mM iso-propyl β-D-thiogalactoside (IPTG). Subsequently, the cells were grown to an A_{600nm} of 1.5 (~2 hours) at 37°C and were pelleted again and resuspended in 0.6 mL of phosphate-buffered saline (PBS), pH 7.5, and lysed by boiling. The boiled lysates were microfuged to remove cellular debris and the supernatants were frozen at -70°C. Nine μL of each of the lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels followed by western blotting (see below).

Preparation of Periplasmic Extracts

Periplasmic extracts were made according to the manufacturer's protocol (Amersham Pharmacia Biotech, Baie d'Urfé, QC), with the following variations. Overnight cultures were grown at 30°C with shaking in 30 mL LB broth containing 2% glucose and 100 μg/mL ampicillin. Two 1 Litre LB broth cultures containing 100 μg/mL ampicillin, were prepared in the morning at a starting A_{600nm} of 0.05 and grown at 37°C.

When the cultures had reached an A_{600nm} of 0.25 (~2 hour), they were induced with 2 mM IPTG. The cultures were grown at 37°C for three hours. The cells were harvested by centrifugation at 1500 x g for 10 minutes. The pellet was gently resuspended in 20 mL of ice cold 1x periplasmic extraction buffer (0.2 M Tris/HCl, 0.5 mM EDTA, 0.5 M sucrose, pH 8.0) per litre of cell culture. Thirty-three mL of 0.2x periplasmic extraction buffer was added per litre of cell culture and the solution mixed. Cells were then incubated on ice for 30 minutes with gentle agitation, then centrifuged at 10,000 x g for 10 minutes and the supernatant collected and filtered through a 0.45µ filter (Millipore Corp., Bedford, MA). Filtered supernatants were stored at -70°C.

Purification of MA116-15 ScFv from Periplasmic Extract

Purification of MA116-15 ScFv was carried out by use of a RPAS antibody purification kit from (Amersham Pharmacia Biotech), according to the manufacturer's recommendations, with the following variation. The periplasmic extract (100 mL) was passed through the anti- E tag affinity column at a much slower constant rate of ~ 1 mL per minute, by use of a peristaltic pump. Ten fractions (1 mL each) were collected at the elution step.

Analysis of ScFv Proteins by Coomassie Staining and Western Blot

Ten µL of each purified MA116-15 protein fraction was analyzed by SDS-PAGE on duplicate 10% gels. The separated proteins on one gel were stained by Coomassie brilliant blue R-250 stain [12]. The separated proteins on the second gel were transferred to Immobilon™-P membranes (Millipore Corp.) by use of Towbin buffer (25 mM Tris/HCl pH 8.3, 192 mM glycine, 20% methanol). Membranes were blocked for one

hour with blocking buffer (PBS containing 3% non-fat skim milk). HRP-labeled anti-Etag antibody (Amersham Pharmacia Biotech), diluted 1:1000 in blocking buffer, was then added and the membranes incubated in this solution for a period of 60 minutes. Specific binding to expressed ScFv was detected by use of an enhanced chemiluminescence (ECL) kit purchased from Amersham Pharmacia Biotech. For all washing steps, PBS containing 0.1% Tween-20 was used.

Sequence Analysis of ScFv MA116 Re-engineered Clones

Purified plasmids of four MA116 clones (MA116-2, MA116-4, MA116-6, MA116-14, MA116-15, and MA116-16) were used in double-stranded dideoxynucleotide sequencing in both directions using a *pCANTAB5E* sequencing primer set purchased from Amersham Pharmacia Biotech. Sequencing reactions were performed using the Big Dye™ Terminator Cycle Sequencing kit (Perkin Elmer/Applied Biosystems), according to the manufacturer's recommendations. The reaction products were purified from unincorporated dye molecules using Centri-Sep™ columns (Princeton Separations Inc., Adelphi, NJ). The nucleotide sequence data was generated using an automated Prism™ 310 genetic analyzer system (Perkin Elmer/Applied Biosystems). Results were analyzed on Lasergene DNA analysis software (DNA Star, Madison, WI).

VEE Virus

Gamma-irradiated, purified VEE virus, strain TC83, was a gift from the United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Frederick, MD.

Enzyme-Linked Immunosorbant Assay

Enzyme linked immunosorbant assay (ELISA) was carried out in 96-well Nunc Maxisorb™ flat bottom plates (GIBCO/BRL, Bethesda, MD). Inactivated whole VEE virus (strain TC-83) was used as antigen, at a concentration of 10 μg/mL. Wells were coated overnight at 4°C with 100 μL VEE antigen prepared in 2X carbonate bicarbonate buffer, pH 9.6, containing 0.02% sodium azide. Plates were washed with PBS containing 0.05% Tween-20. Plates were blocked twice with blocking buffer (PBS containing 2% BSA and 0.05% Tween-20). Unless otherwise specified, primary antibody (ScFv lysate) was diluted in antibody dilution buffer (PBS containing 2% BSA and 0.1% Tween-20) and incubated for one hour at 37°C. HRP-labeled anti-E tag antibody (Amersham Pharmacia Biotech), diluted 1:6000 in antibody dilution buffer, was used for the detection of MA116 ScFv molecules. HRP-labeled anti-mouse antibody (Jackson ImmunoResearch Laboratories) (Bio/Can Scientific, Mississauga, ON), diluted 1:3000 in antibody dilution buffer, was used for the detection of MAb. Plates were developed with ABTS substrate solution (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt / hydrogen peroxide, 1:1 mixture) (Kirkgaard and Perry Laboratories) (Canada Life Technologies, Burlington, ON). The plate blanks received blocking buffer and substrate only. Other appropriate controls were included in each assay.

RESULTS

Following PCR-based site directed mutagenesis and amplification of the mutagenized A116 plasmid (MA116), a distinct linearized DNA band of ~5100 bp was detected by agarose gel electrophoresis (data not shown). The gel purified 5100 bp MA116 linear DNA was phosphorylated at the ends with polynucleotide kinase to make it

ligation competent. The phosphorylated MA116 linear DNA was gel purified and ligated. This ligated MA116 circular DNA was then transformed into *E. coli* HB2151 competent cells. Approximately 70 transformants were obtained.

Screening of Transformants for the Loss of XhoI Restriction Enzyme Site

To screen MA116 transformants, 16 individual colonies were picked and mini-preparation DNA was isolated for XhoI restriction enzyme digestion analysis. A116 ScFv plasmid contains two XhoI restriction enzyme sites. One of these sites is present on the plasmid outside of the ScFv gene while the second XhoI site resides in the 5 prime end of VL gene. It was reasoned that incorporation of the first "A" into the A116 sequence would destroy the second XhoI site. Thus MA116 clones that gave linear 5100 bp DNA bands upon digestion with XhoI, would have incorporated at least one of the three intended insertions. From XhoI restriction enzyme digestion analysis of 16 MA116 transformants (Figure 2), all except two showed a distinct 5100 bp linear DNA band, indicating that PCR site directed mutagenesis had been successful. The two MA116 clones (MA116-2, and MA116-6) showed digestion patterns which were identical to the wild type A116 un-mutagenized DNA (Figure 8).

Screening of MA116 Clones for Expression and Function in E.coli HB2151 Cell Lysates

Since *E. coli* HB2151 has a non-suppresser background, only the 30 KDa soluble ScFv product would be synthesized if the ScFv open reading frames (ORF) were free of amber stop codons.

To establish that MA116 clones expressed soluble 30 KDa ScFv protein, Western blot analysis was performed on a number of different transformants. The cells were grown, induced with IPTG, and lysates were prepared, as described above. Western blot performed on the lysates of selected MA116 clones showed the expression of 30 KDa soluble ScFv proteins for clones MA116-4, MA116-6, MA116-14, MA116-15, and MA116-16, while lysates of MA116 clones MA116-1, MA116-2, MA116-3, and MA116-8 were negative for protein expression (Figure 9). Control *E. coli* HB2151 lysates did not show detectable background. These results strongly suggested that clones MA116-4, MA116-6, MA116-14, MA116-15, and MA116-16 were free of amber translational stop codons in their ORF.

Functional Confirmation of Five MA116 Clones by ELISA

Fresh whole cell lysates were made from the parental A116 clone and five MA116 clones (MA116-4, MA116-6, MA116-14, MA116-15, and MA116-16) and induced with 2 mM IPTG for expression of ScFv antibody. These lysates were then used to evaluate their relative reactivity to VEE antigen, in relation to parental A116, by ELISA. The ELISA reactivity of the ScFv was determined, in triplicate, in the presence and absence of VEE whole virus antigen, for each of the five MA116 re-engineered MA116 clones and parental A116 clone (Figure 10). MA116-15 was the most reactive clone, being four times more active than the original A116 clone in recognizing VEE antigen. MA116-14 and MA116-16 also showed good reactivity, being two to two and one-helf times more reactive than the original A116 clone. MA116-4 and MA116-6, although active, were less active than the parental A116 clone. All five MA116 clones showed specific retention, at

least two and one-half-fold greater, in the presence of VEE antigen as compared to non-specific retention in the absence of VEE antigen.

Sequencing of MA116 Re-engineered Clones

Sequencing of selected MA116 clones (MA116-4, MA116-6, MA116-14, MA116-15) was carried out to establish the incorporation of the three intended base insertions in the proper context of the re-engineered MA116 genes. Furthermore, sequencing was performed to rule out random mutations that might have accumulated in the PCR mutagenesis step. The nucleotide sequences of MA116 and A116 clones, as determined by sequencing, compared to the consensus sequence in the region of intended base insertions, is shown in Figure 11. As deduced from Figure 11, in clone MA116-15, all three bases were inserted at the pre-defined target positions, making the sequence of this re-engineered clone identical to that of the consensus sequence. MA116-4 also showed insertion of all three nucleotide bases at the intended positions but, there were accumulated additional mutations also noted i.e., deletion of A at nt 398 and insertion of T after nt 419 . MA116-6 did not incorporate any intended changes, but additional mutations were observed. MA116-14 incorporated the expected insertions, similar to MA116-15, however, an additional insertion of a C occurred after nt 419, and a deletion at nt 433 frame-shifted the reading frame back to the MA116-15 reading frame. MA116-16 also incorporated the expected changes of MA116-15, but a point mutation at nt 46 (C replaced by a T) caused an amino acid change.

The results of the mutagenesis are best illustrated in the deduced amino acid sequence alignment performed using the Clustal W program of DNASTAR. As illustrated in Figure 12, A116-16 has a major alteration in sequence in the Framework-1

region of the light chain (residues 134-142), when compared with the MA116 sequences. In addition, A116-16 contained additional difference at residues 77 and 79 of the heavy chain, and residue 226 of CDR-L3 were also noted. MA116-6 was identical to A116-16 in Framework-1, and had one addition change at residue 59 in CDR-H2. MA116-4 was changed from the MA116-15 from residues 133-140, as well as a conserved change at residue 213. MA116-14 contained a 4 amino acid change from MA116-15 in Framework-1 (residues 141-144), and a conserved change at residue 210. Lastly, MA116-16 was identical to MA116-15, except for a radical substitution at residue 16. Interestingly, the deduced amino acid changes follow the reactivity of the expressed protein in ELISA (Figure 10). The most altered Frame-work 1 clones: A116-16, MA116-6 and MA116-4 (Figure 12), showed the lowest reactivity. MA116-14 and MA116-16 demonstrated intermediate activity in recognition of VEE on ELISA (Figure 10), one in Framework-1 (MA116-14) and one outside Framework –1 (MA116-16). The E-tag was identical for all 6 clones, and was not included for comparison in Figure 12. As MA116-15 was the correctly repaired, showed no additional mutations from the MA116 consensus and demonstrated the highest activity, it was chosen for further evaluation.

Purification and Functional Analysis of Ppurified MA116-15 ScFv Antibody

MA116-15 was chosen for purification, based on this clone being the most active and the most molecularly "correct" of the MA116 clones. An anti-E tag affinity column (Amersham Pharmacia Biotech.) was used to purify MA116-15 ScFv antibody from the periplasmic extract of MA116-15 clone. To determine the level of purification and to estimate the protein concentration of the purified ScFv, the collected fractions were analyzed by SDS-PAGE (Figure 11).. For estimation of the protein concentration of

purified MA116-15 ScFv, a known concentration (400 ng) of another purified ScFv, D66 (25) was also loaded on the gels. Figure 13A represents a Coomassie stained gel. The D66 ScFv protein was detected on the gel at a molecular weight of approximately 32 Kda. Higher molecular weight bands were seen in all the lanes of Coomassie stained gel but fraction six of MA116-15 showed a faint band at a similar molecular weight to the D66 ScFv. Figure 13B is a western blot of a gel, loaded identically to the Coomassie stained gel; the presence of MA116-15 ScFv in fractions six, seven and eight is visible. This western blot confirmed that the faint band seen in purified fraction six of MA116-15 ScFv (Figure 13A) was, indeed, the purified MA116-15 single chain antibody. Fraction 6 appeared to be the most concentrated fraction of the MA116-15 ScFv protein, followed by fraction seven. Fraction eight is the least concentrated fraction of MA116-15 ScFv protein. In addition, from Figure 13B, it was evident that the higher molecular weight bands, as seen in Figure 13A, were not multimers of MA116-15 ScFv, as those bands were not recognized by the anti-E tag antibody.

To further confirm that fractions six, seven and eight contained purified MA116-15 ScFv antibody and that this was, indeed, functional, ELISA was performed.Equal volumes of purified MA116-15 fractions six, seven, and eight were analysed by ELISA, in triplicate, in the presence and absence of VEE antigen.,. The ELISA results are depicted in Figure 13C. ELISA results were consistent with the western blot data in Figure 13B. The fractions most reactive with the VEE antigen by ELISA were, in order of decreasing magnitude, fraction six, followed by fractions 7 and 8. Similarly, by western blot analysis, fraction 6 was the most concentrated of the three ScFv fractions, followed by fraction seven; fraction 8 contained the least amount of purified MA116-15 protein.

Functional Comparison of Purified MA116-15 ScFv with the Pparent 1A4A-1 MAb

To compare the reactivity of the purified, functionally enhanced MA116-15 ScFv with the 1A4A-1 MAb in recognizing VEE whole virus antigen, an ELISA was performed. Since a functional comparison of the two species of antibodies was at issue, it was important that functionally equimolar quantities of the two proteins must be used in ELISA. Equimolar calculations were based on the fact that the ScFv molecule is approximately five times smaller than the monoclonal antibody molecule in terms of molecular weight. Thus, at a given concentration of protein, there would be five times more molecules of ScFv than Mab. Since there are two reactive sites on a Mab and only one reactive site on the ScFv molecule, the functional ratio of ScFv to Mab would be 1:2.5. Thus, for every given unit of concentration of MA116-15 ScFv by ELISA, the corresponding equimolar amount of 1A4A-1 Mab would be 2.5 times greater.

Figure 14 represents the results of ELISA based on the calculations discussed above. The ELISA data showed that MA116-15 ScFv was at least as reactive as the 1A4A-1 Mab in recognizing VEE antigen. Indeed, at higher protein concentrations, MA116-15 ScFv appeared to be more reactive than the 1A4A-1 Mab. , Mab 1A4A1 reached its maximum reactivity at approximately 315 ng protein; with further additions of Mab protein, reactivity with the VEE antigen appeared to be inhibited.

DISCUSSION

In this invention, the inventors report successful utilization of a molecular engineering approach to enhance the functional reactivity of a partially active ScFv antibody. The reactivity of VEE ScFv A116 was enhanced at least four-fold in

recognition of VEE whole virus antigen. The engineering strategy employed PCR-based site directed mutagenesis to re-introduce three single bases, identified previously as deletions in the 5 prime end of the V_L gene of A116 clone. Introduction of these bases was predicted to repair a localized frame-shift in the N-terminal framework-1 region of the light chain of A116 ScFv antibody. Five clones (MA116-4, MA116-6, MA116-14, MA116-15, and MA116-16) were identified with potential enhanced reactivity to VEE whole virus antigen. These five MA116 clones were analyzed in detail to elucidate the molecular changes responsible for enhanced reactivity with target VEE antigen.

The strategy employed PCR-based site directed mutagenesis to re-introduce the three deleted bases. Two primers were synthesised for this purpose in the region of repair, located adjacent to each other but in opposite directions i.e., one forward and one reverse. The primers carried the bases to be inserted in the designated regions. The whole plasmid carrying A116 ScFv was PCR-amplified using the synthesized primers with the three insertions, thereby incorporating the three bases in the sequence of A116 gene. The introduction of the three bases and the repair of the framework-1 region was determined by DNA sequencing of the re-engineered A116 clones.

The PCR-based site directed mutagenesis of A116 ScFv gene was successfully accomplished as evidenced by the fact that only two out of the 16 MA116 clones analyzed, showed XhoI digestion patterns which were identical to the un-mutagenized parental A116 clone. The remaining 14 MA116 clones showed incorporation of at least one base insertion as indicated by the loss of XhoI site in the 5 prime end of the VL geneThis corresponds to a mutagenesis rate of ~ 90%.

Since the mutagenesis strategy included PCR amplifications, the chance the unintended changes might occur, leading to nonsense mutations in the ORF was high.

For this reason, it was important to screen the MA116 re-engineered clones by Western blot, to select those clones that were expressing the ScFv. Out of the 14 mutagenized MA116 clones, as demonstrated by XhoI digestion, only five actually expressed a full length 30 KDa ScFv product (Figure 9). Our interpretation of these results is that there was introduction of unintented nucleotide changes, leading to nonsense mutations, as opposed to non-insertion of intended mutations. Since base insertions were incorporated into the primers which become part of every PCR amplified DNA molecule, it would be a rare event where the insertions would be deleted. A more common occurrence would be the introduction of unintented changes due to low fidelity of the proof reading ability of thermal polymerases. Some of these unintended changes would lead to nonsense or frameshift mutations, resulting in lack of expression of protein product. This interpretation of events is strengthened by observations made from clones MA116-2 and MA116-6. Although both of these clones exhibited XhoI digestion patterns identical to the non-mutagenized A116, MA116-2 did not express ScFv protein, probably due to introduction of nonsense mutations in the ORF. On the other hand, MA116-6 showed expression of normal ScFv protein.

ELISA results (Figure 10) indicated that the MA116 clones showed variability in reactivity with the VEE antigen. Based on the reactivity with VEE antigen by ELISA, MA116 clones could be placed into three groups relative to the ELISA reactivity demonstrated by the parental A116 clone. MA116-15 was in Group A (functionally the most reactive group), with at least four-fold higher reactivity with VEE antigen than the parental A116 clone. MA116-14 and MA116-16 were in Group B, with moderately higher reactivity (~ 2.5 times higher than A116). MA116-4 and MA116-6 were in Group C (the least reactive group), with reactivity less than A116. Since equal amounts of total

cellular proteins were added in ELISA for each of these clones, it is likely that the variable reactivities of Groups A, B, and C, as defined above, resulted from molecular differences at the gene level. However, to prove this assumption, sequencing of the complete ScFv gene for each of these clones was essential.

Complete nucleotide sequencing of the five MA116 clones was undertaken to determine the nature of the changes that had occurred at the gene level and to relate these changes to the observed functional reactivity of the ScFv produced.The sequencing data presented in Figure 11 clearly demonstrates that there were significant molecular changes at the gene level that could, theoretically, be responsible for differences in reactivity at the protein level.

To explore the structure to function relationship in MA116 clones, Figure 12 was generated from the information reduced from the complete nucleotide sequences of A116-16 and the five MA116 clones. The first 13 amino acids from the light chain of each of the clones belonging to reactive Groups A, B, and C were compared to the consensus amino acid sequence of the framework-1 region. The consensus sequence was developed by Kabat and Wu [15] after analyzing more than 200 light chain antibody sequences. From analysis of the data in Figure 12, it can be proposed that the level of reactivity of the re-engineered ScFv proteins would be directly proportional to the percent identity with the consensus mouse amino acid sequence, with the exception of MA116-16. Thus, it can be observed that the amino acid sequence of MA116-15 (reactive Group A) (the most reactive in function) was 90% identical to the consensus amino acid sequence. On the other hand, the amino acid sequence of the parental ScFv A116, which was minimally active, was only 40% identical with the consensus sequence. MA116-6 and MA116-4 were also significantly altered and framework-1, and showed minimal

activity in ELISA. MA116-14 demonstrated moderate functional activity, as only 4 amino acids were altered. Although MA116-16 had only a point mutation, this mutation was located in Framework-1 of the heavy chain, and disrupted the structure in this region by replacement of the P with a S at residue 16, reducing the binding activity to a moderate level (Figure 10, 12).

Since MA116-15 ScFv was found to be the most reactive clone, the MA116-15 ScFv product was purified. The purified protein was used to confirm the earlier results from whole cell lysates and to do a functional comparison with the parental 1A4A-1 MAb. The purification of MA116-15 ScFv protein, with minimal contaminating proteins, was clearly demonstrated (Figures 13A and 13B). Calculations of the total MA116-15 ScFv protein purified suggested a concentration of between 50 - 80 μg, with the highest concentration being in fraction six at roughly 6 - 10 ng/µL. This concentration is consistent with the results obtained by Coomassie staining and western blot (Figure 13A). The sensitivity limit of Coomassie staining is ~ 100 ng of protein. Since 10 µL were loaded on the gel and the band was faintly visible, the concentration would fall within the range of 6 - 10 ng/µL. That purified MA116-15 ScFv protein was functional in recognizing VEE whole virus antigen, was clearly demonstrated (Figure 13C). The ELISA interaction was highly specific to the presence of VEE whole virus antigen, as demonstrated by an 8 - 10-fold difference between VEE antigen-specific interaction and non-specific background interaction.

The most important result of this study was that the MA116-15 ScFv protein was shown by ELISA to be functionally as reactive with VEE antigen as the parent 1A4A-1 Mab, when functionally equimolar quantities of the two proteins were used (Figure 14).

Further experiments are needed to answer certain ambiguities of this result. One ambiguity, for example, is that at concentrations lower than 375 ng, 1A4A-1 MAb appeared to be as reactive as the MA116-15 ScFv, but at higher concentrations, the reactivity of 1A4A-1 Mab seemed to be inhibited. A number of explanations for this observation are plausable. It is possible that the antigen concentration was limiting for the 1A4A-1 MAb i.e. once the antigen was depleted and antibody was in excess, no further antigen-antibody interaction could occur with increasing additions of antibody. A second possibility is that ScFv molecules, being five times smaller than MAb molecules, had better accessibility to plate-bound antigen than did larger MAb molecules that were physically hindered. Future experiments are planned to examine and resolve this observed ambiguity.

CONCLUSIONS

A116 ScFv has been successfully re-engineered for enhanced reactivity with VEE whole virus antigen. MA116-15, an extremely reactive clone has been isolated. In the process of defining molecular changes responsible for the enhanced reactivity of this MA116-15 ScFv and other MA116 clones, amino acids key to ScFv functionality have been identified. These amino acids may play a significant role in the conformational and functional stability of the framework-1 region of the antibody light chain, which in turn may affect the overall function of the antibody molecule. MA116-15 ScFv protein has been purified and found to be highly reactive to VEE whole virus antigen. Initial results suggest that MA116-15 ScFv antibody is as reactive in recognizing VEE antigen as is the parent 1A4A-1 MAb.

The importance of this reactive MA116-15 ScFv clone is emphasized by the fact that the parent 1A4A-1 Mab is neutralizing for a number of different pathogenic VEE isolates. Future experiments planned include detailed characterization of this MA116-15 ScFv and determination of the neutralizing capability of this re-engineered ScFv. This invention is also important in that it opens the door for future molecular modelling studies on different MA116 clones. Such studies would define the role played by some of the key amino acid residues in antibody stability and function. This MA116-15 ScFv can also be a good candidate for animal protection studies to evaluate the usefulness of ScFv antibodies as anti-viral therapeutic agents *in vivo*.

It is to be understood that the embodiments and variations shown and described herein are merely illustrative of the principles of this invention and that various modifications may be implemented by those skilled in the art without departing from the scope and spirit of the invention.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The Minister of National Defence, Government of Canada

(ii) TITLE OF INVENTION: Cloning, expression, sequencing, and functional enhancement of monoclonal scFv antibody against Venezuelan equine encephalitis virus (VEE)

(iii) NUMBER OF SEQUENCES: 7

(iv) CORRESPONDENCE ADDRESS:
1900 - 66 Slater Street, Ottawa, ON K1P 5H1

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC Compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PC-DOS/MS-DOS

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Nelligan O'Brien Payne LLP
(C) REFERENCE/DOCKET NUMBER:

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 774
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: mouse mouse hybridoma cell line 1A4A1

(vii) IMMEDIATE SOURCE: VH and VH antibody fragments directly cloned using polymerase chain reaction as a single chain fragment into the expression vector pCANTAB 5E

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1 .. 774
(D) OTHER INFORMATION: scFv protein A116-16 1 - 717
e-tag 718 - 774

(x) PUBLICATION INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 257
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: mouse mouse hybridoma cell line 1A4A1

(vii) IMMEDIATE SOURCE: VH and VH antibody fragments directly cloned using polymerase chain reaction as a single chain fragment into the expression vector pCANTAB 5E, clone A116-16

(ix) FEATURE:
(A) NAME/KEY:
(B) LOCATION: 1 .. 257
(D) OTHER INFORMATION: scFv protein A116-16 1 - 239
e-tag 240 - 257

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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        35                  40                  45
Gly Leu Glu Trp Ile Gly Met Thr Tyr Pro Gly Phe Asp Asn Thr
    50                  55                  60
Asn Tyr Ser Glu Thr Phe Lys Gly Lys Ala Thr Leu Thr Val Asp
65                  70                  75
Thr Phe Ser Thr Thr Val Tyr Met Gln Leu Ser Ser Leu Thr Ser
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Glu Asp Thr Val Val Tyr Phe Cys Ala Arg Gly Val Gly Leu Asp
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Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly
        110                 115                 120
Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ser Ser
    125                 130                 135
Ser Leu Val Ser Lys Phe Val Ser Thr Ser Ile Gly Asp Arg Ile
140                 145                 150
Arg Ile Thr Cys Lys Ala Ser Gln Asp Val Asp Thr Ala Val Gly
155                 160                 165
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        185                 190                 195
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Asn Val Gln
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215                 220                 225
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 774
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: mouse mouse hybridoma cell line 1A4A1

(vii) IMMEDIATE SOURCE: VH and VH antibody fragments directly cloned using polymerase chain reaction as a single chain fragment into the expression vector pCANTAB 5E

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1 - 774
(D) OTHER INFORMATION: scFv protein MA116-6 1 - 717
e-tag 718 - 774

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 777
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: mouse mouse hybridoma cell line 1A4A1

(vii) IMMEDIATE SOURCE: VH and VH antibody fragments directly cloned using polymerase chain reaction as a single chain fragment into the expression vector pCANTAB 5E

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1 .. 777
(D) OTHER INFORMATION: scFv protein MA116-4 1 - 720
e-tag 721 - 777

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 777
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: mouse mouse hybridoma cell line 1A4A1

(vii) IMMEDIATE SOURCE: VH and VH antibody fragments directly cloned using polymerase chain reaction as a single chain fragment into the expression vector pCANTAB 5E

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1 .. 777
(D) OTHER INFORMATION: scFv protein MA116-14 1 - 720
e-tag 721 - 777

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 777
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: mouse mouse hybridoma cell line 1A4A1

(vii) IMMEDIATE SOURCE: VH and VH antibody fragments directly cloned using polymerase chain reaction as a single chain fragment

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into the expression vector pCANTAB 5E

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1 .. 777
(D) OTHER INFORMATION: scFv protein MA116-16 1 - 720
e-tag 721 - 777

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 777
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: mouse mouse hybridoma cell line 1A4A1

(vii) IMMEDIATE SOURCE: VH and VH antibody fragments directly cloned using polymerase chain reaction as a single chain fragment into the expression vector pCANTAB 5E

(ix) FEATURE:
(A) NAME/KEY: CMV promoter
(B) LOCATION: 1 .. 1260
(D) OTHER INFORMATION: scFv protein MA116-15 1 - 720
e-tag 721 - 777

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

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ggtgtgggcc ttgactactg gggccaaggg accacggtca ccgtctcctc aggtggaggc 360
ggttcaggcg gaggtggctc tggcggtggc ggatcggaca tcgagctcac tcagtctcca 420
aattcgttgt ccacatcaat aggagacagg atcagaatca cctgcaaggc cagtcaggat 480
gtggatactg ctgtaggctg gtatcaacag agaccagggc aatctcctaa actactgatt 540
ttctggtcat ccacccggca cactggagtc cctgatcgct tcacaggcag tggatctggg 600
acagatttca ctctcaccat tagcaatgtg cagtctgaag acttggcaga ttatttctgt 660
caccaatata gcagctatcc attcacgttc ggctcgggga caaagttgga aataaaacgg 720
gcggccgcag gtgcgccggt gccgtatccg gatccgctgg aaccgcgtgc cgcatag 777
//

We claim:

1. A recombinant A116-16 ScFv antibody against Venezuelan equine encephalitis (VEE) comprising nucleotide sequence shown in SEQ ID NO: 1.

2. A recombinant A116 ScFv antibody against VEE comprising amino acid sequence shown in SEQ ID NO: 2.

3. A recombinant A116 ScFv antibody expressed from cloned gene sequences of Mab 1A4A1 hybridoma cells.

4. A recombinant A116 ScFv antibody of claim 3, wherein said antibody has mutated region located at framework-1 with three individual nucleotide deletions.

5. A recombinant A116 ScFv antibody of claim 4, wherein said nucleotide deletions take place in the N-terminal region of the V_L.

6. A recombinant MA116-6 ScFv antibody against Venezuelan equine encephalitis (VEE) comprising nucleotide sequence shown in SEQ ID NO: 3.

7. A recombinant MA116-4 ScFv antibody against VEE comprising nucleotide sequence shown in SEQ ID NO: 4.

8. A recombinant MA116-14 ScFv antibody against VEE comprising nucleotide sequence shown in SEQ ID NO: 5.

9. A recombinant MA116-16 ScFv antibody against VEE comprising nucleotide sequence shown in SEQ ID NO: 6.

10. A recombinant MA116-15 ScFv antibody against VEE comprising nucleotide sequence shown in SEQ ID NO: 7.

11. The nucleotide sequence of MA116-15 ScFv antibody of claim 10, wherein said sequence is 90% identical to the consensus amino acid sequence in mouse variable light chain framework-1 region.

12. The nucleotide sequence of MA116-15 ScFv antibody of claim 10, whereby said sequence displays high degree of functional reactivity in the mouse variable light chain framework-1 region.

13. The nucleotide sequence of MA116-15 ScFv antibody of claim 12, wherein said amino acid sequence of framework-1 is DIELTQSPNL at residues 133 to 143, respectively.

14. A recombinant MA116-15 ScFv antibody whereby said antibody is functionally as reactive with VEE antigen as its parental 1A4A-1 monoclonal antibody provided that functionally equimolar quantities of the two proteins were used.

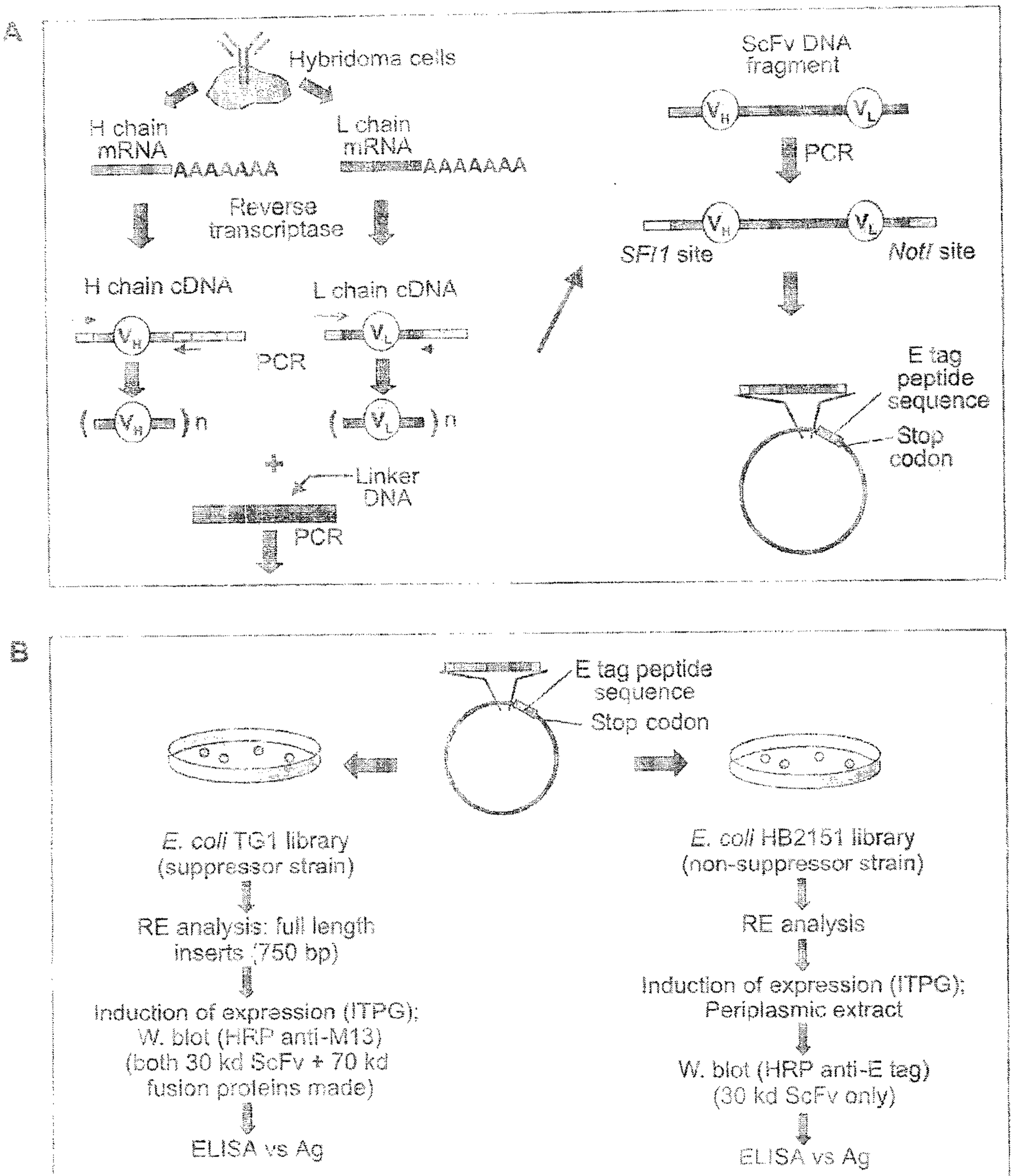
15. Use of the recombinant MA116-15 ScFv antibody of claim 14 as a diagnostic reagent for detection and identification of VEE.

Application number/ Numéro de demande : 2374027

Documents of poor quality scanned
(request original documents in File Prep. Section on the 10th floor)

Documents de pièrre qualité numérisés
(Pour obtenir les documents originaux, veuillez vous adresser à la Section de préparation des dossiers, située au 10e étage)

Figure 1

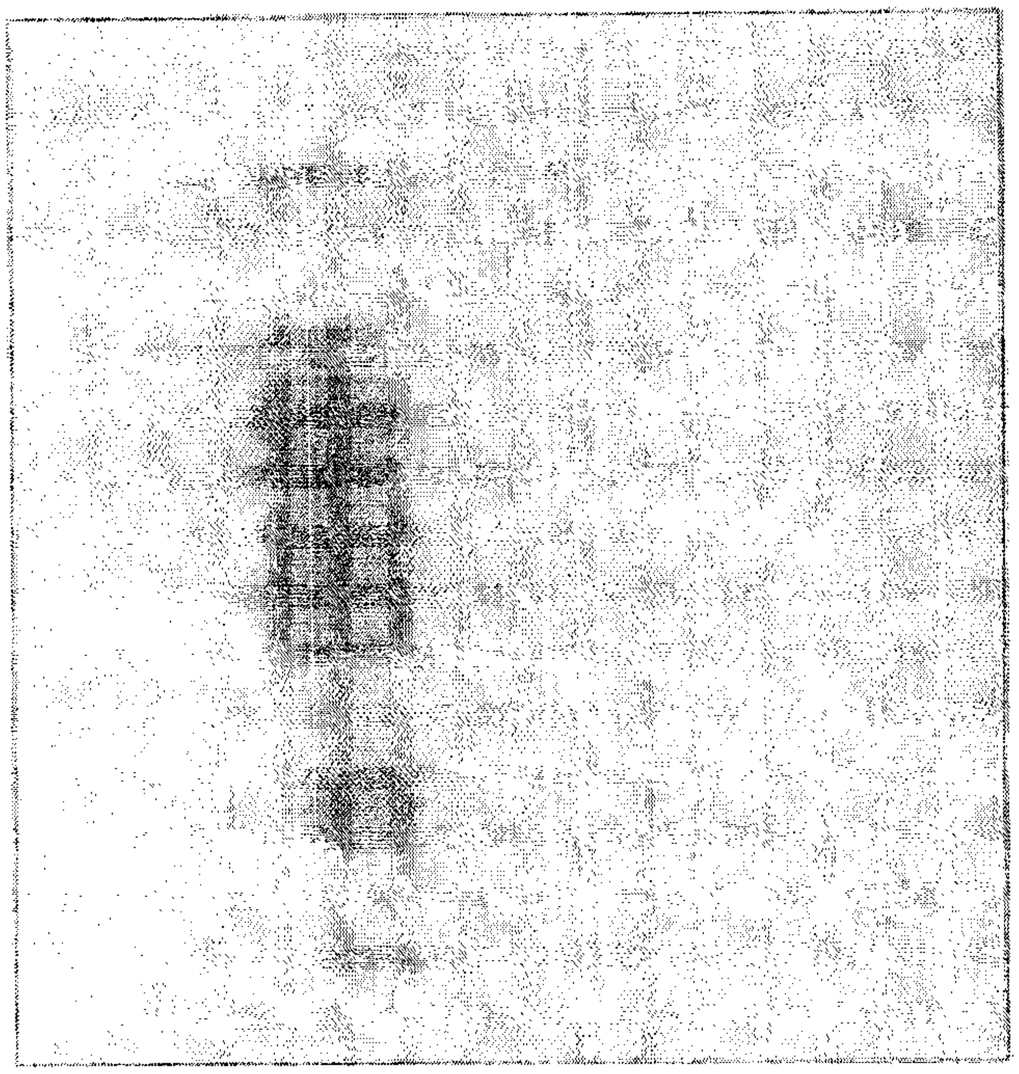


Schematic representation of steps in development of recombinant scFv antibodies:

A. Cloning and PCR

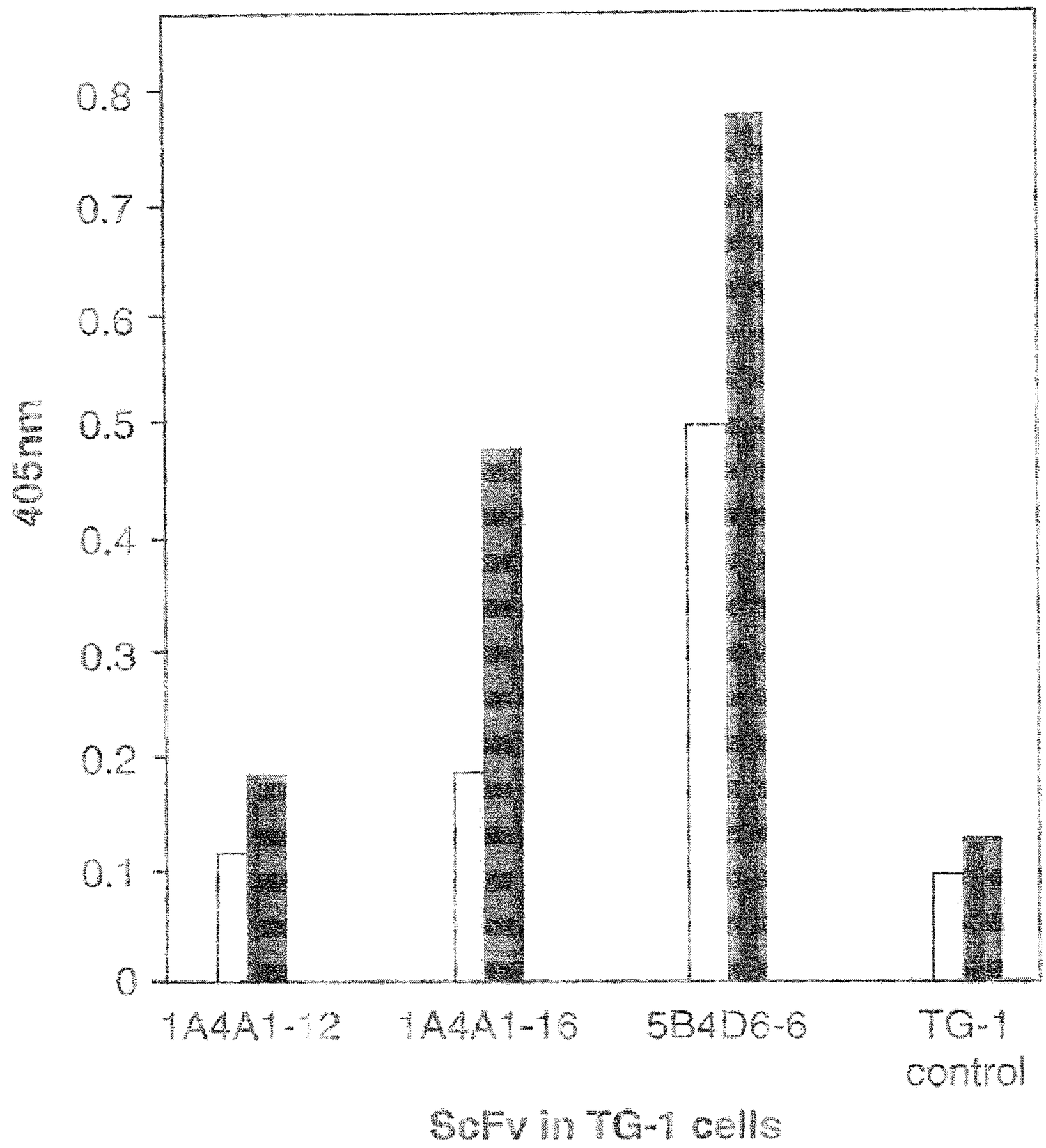
B. Expression and analysis

Figure 2A



Western blot of cell lystates from 1A4A1 clones exhibiting full length scFv fragments. Fifteen μL of lysate in a final volume of 25 μL was loaded per well on a 12% SDS-PAGE gel. Indicator antibody was HRP-labelled anti-E Tag antibody. Position of molecular weight markers is indicated on the right hand side of the gel.

Figure 2B

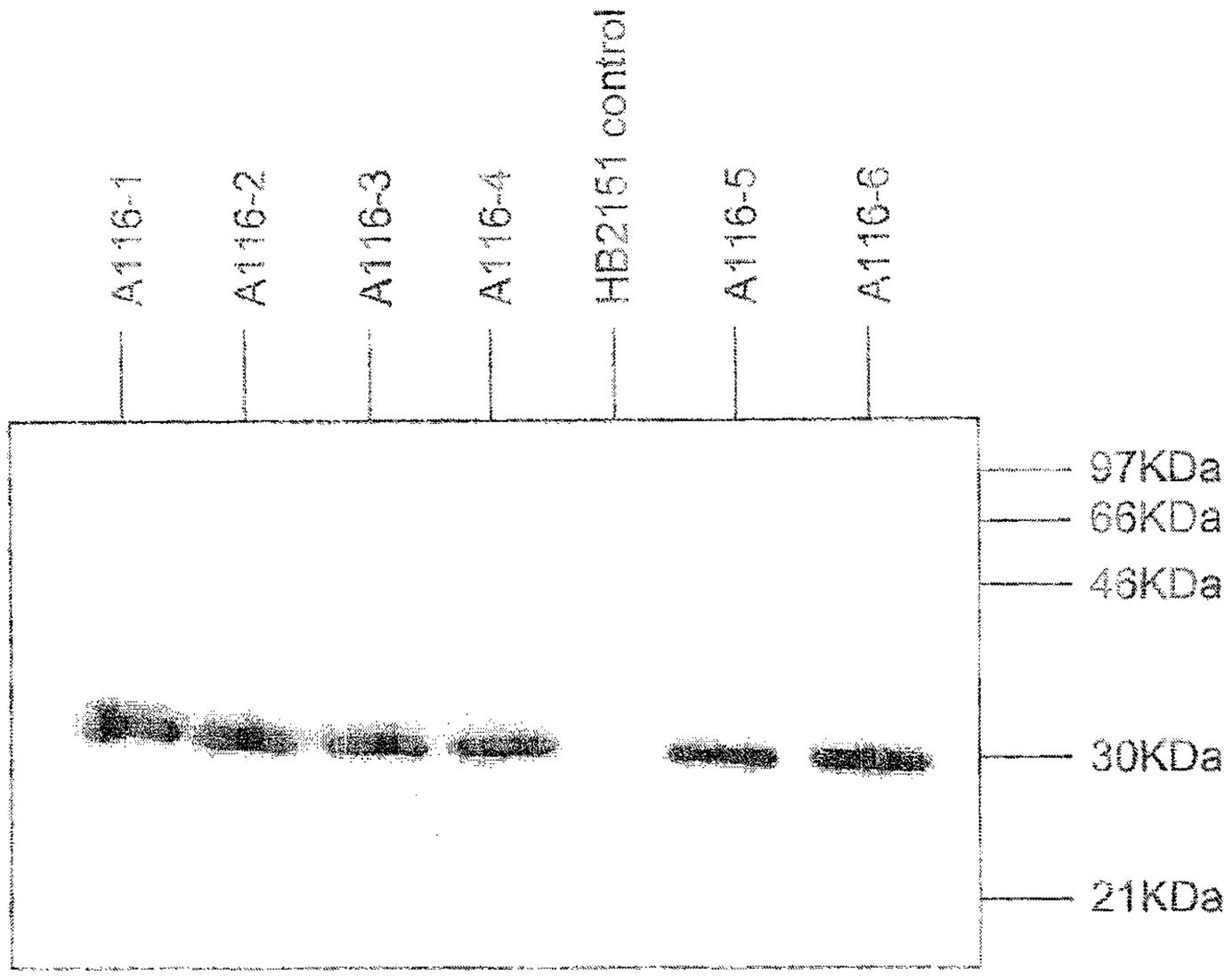


Screening of expression-positive scFv clone lysates from *E. coli* TG-1 cells for functional reactivity by ELISA. Wells were coated with 100 µL inactivated VEE virus at a concentration of 4 µg/mL. Fifty µL of each test lysate was added per well. Indicator antibody was HRP-labelled anti-E Tag antibody, diluted 1:4000 in antibody diluation buffer. Plates were read 30 minutes following addition of substrate solution. Data points are the mean of triplicate determinations.

■ With antigen

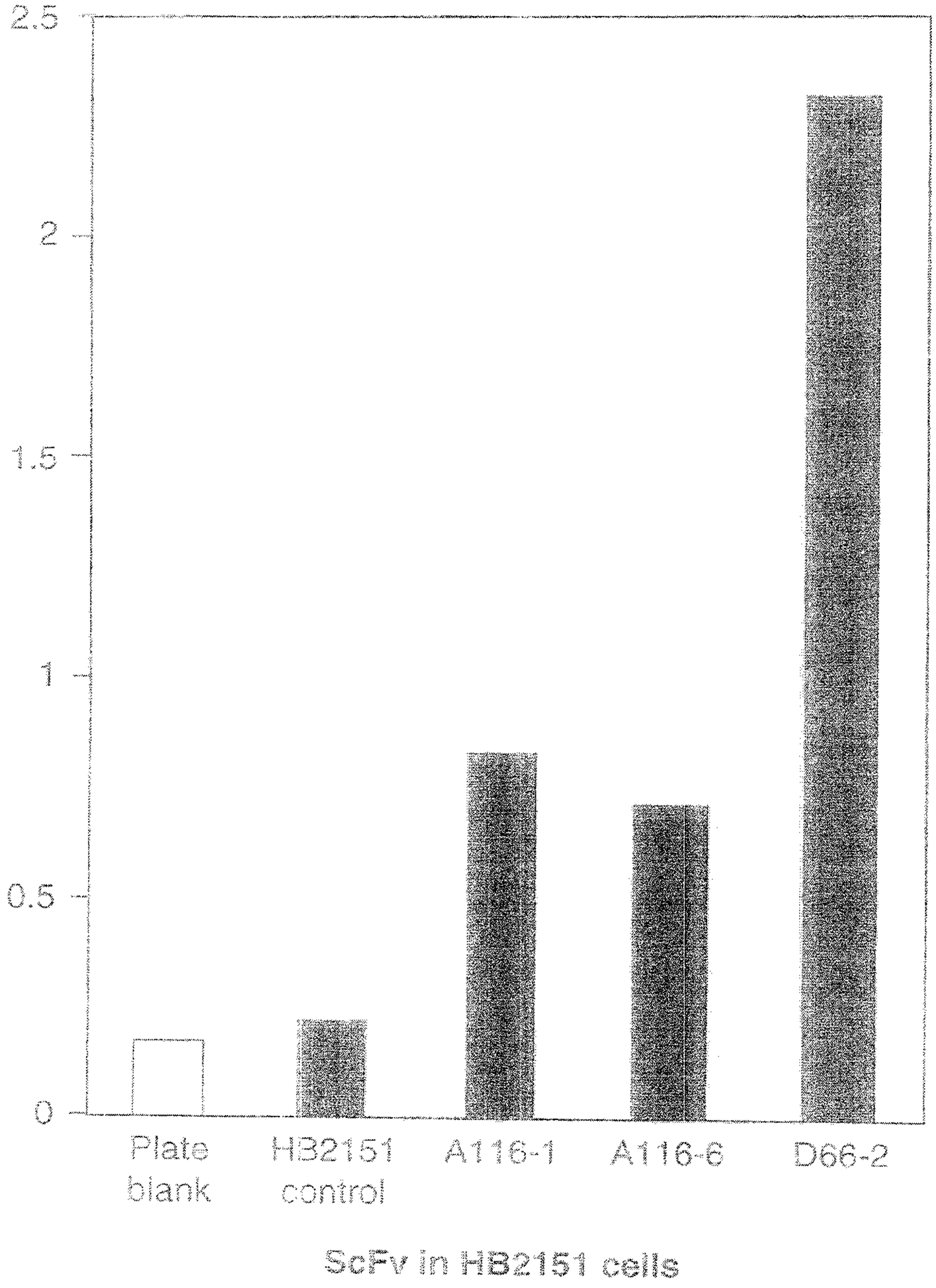
□ Without antigen

Figure 3A



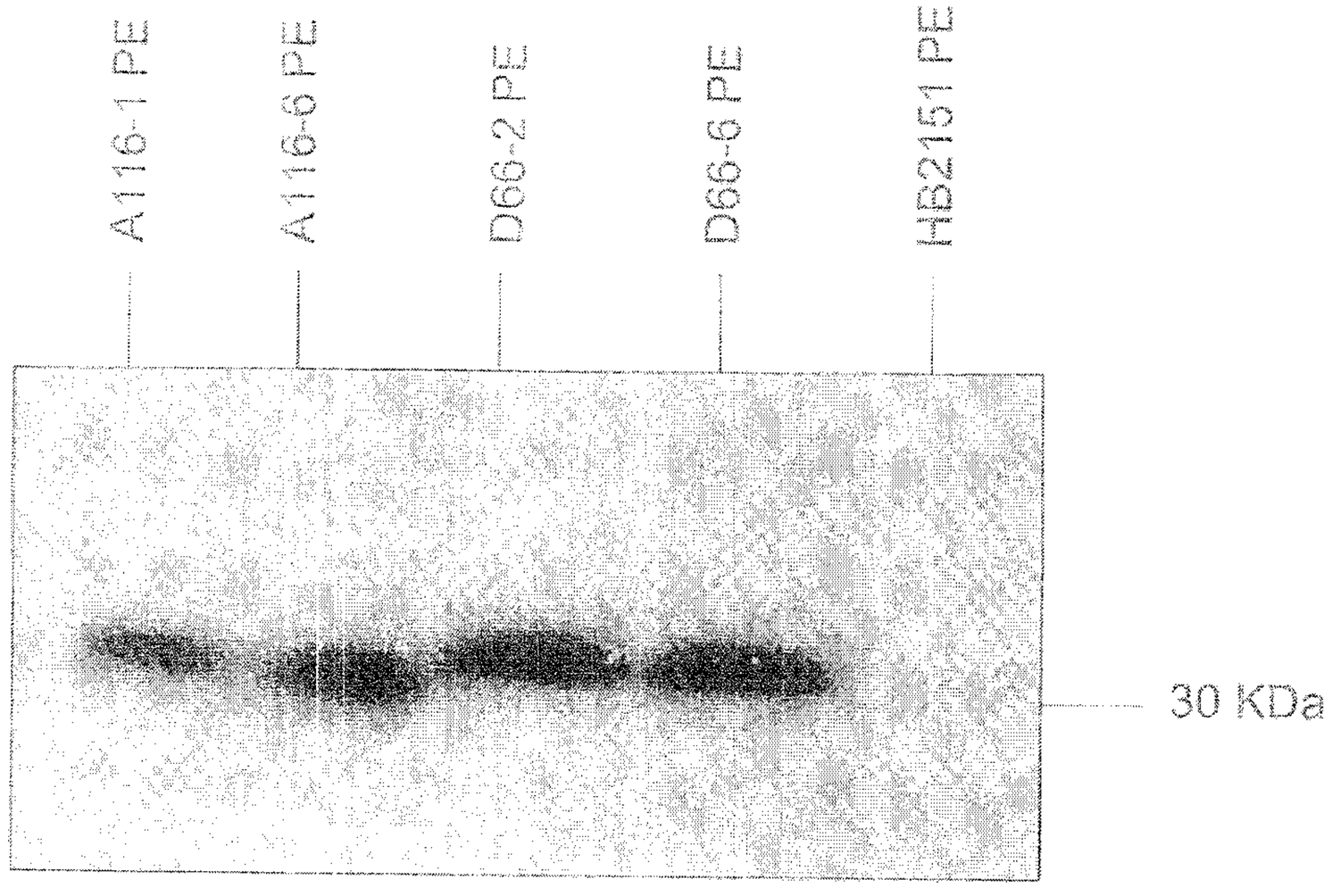
Screening of A116 scFv clones in lysates of *E. coli* HB2151 cells for expression of soluble scFv antibody by western blotting. Six µL of lysate in a final volume of 24 µL was loaded per well and electrophoresed on 12% SDS-PAGE gels. HRP-labelled anti-E Tag antibody was used at 1:2000 dilution. Position of molecular weight markers is indicated on the right hand side of the gel.

Figure 3B



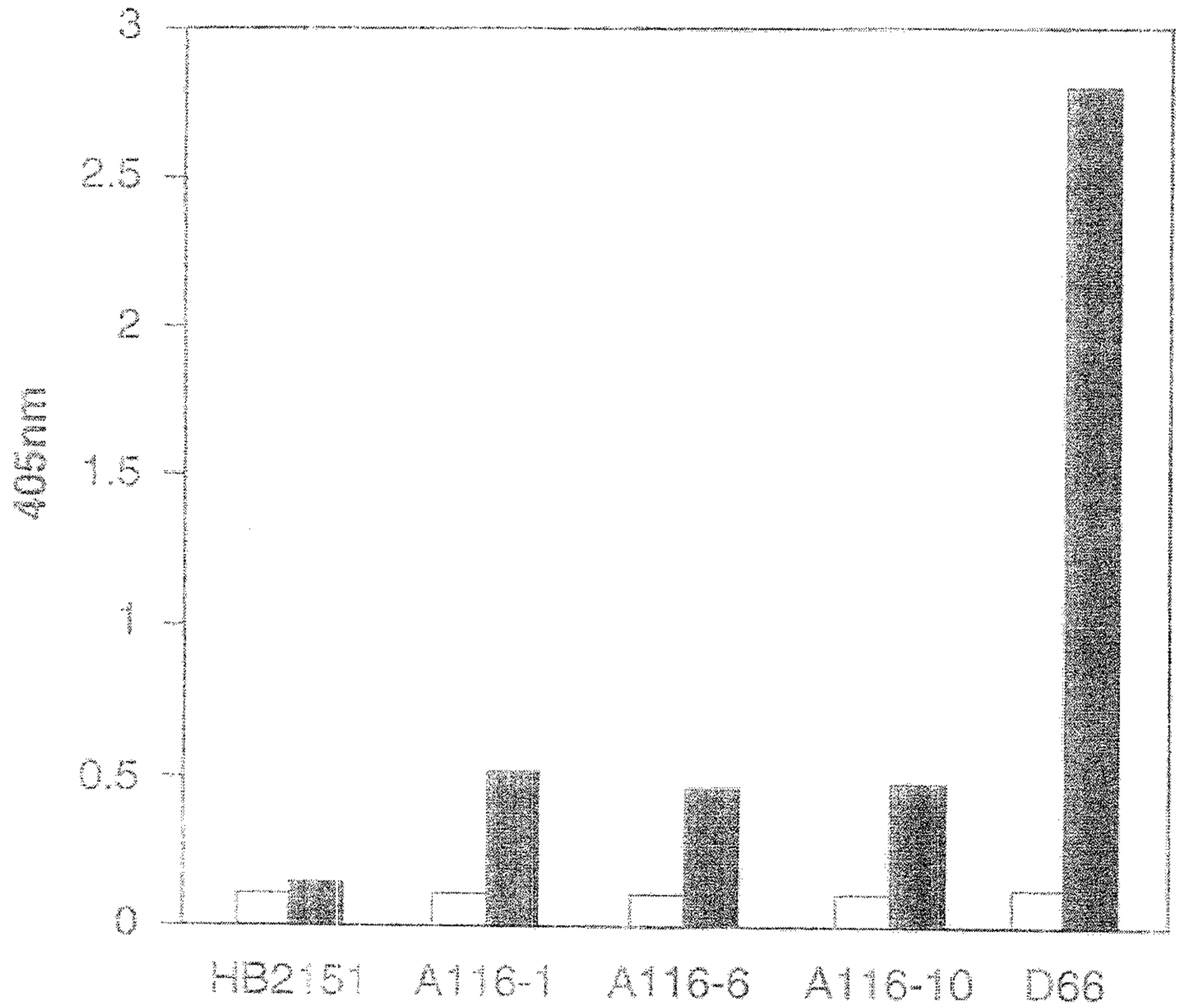
Screening of soluble scFv protein in lysates from *E. coli* HB2151 cells for functional reactivity by ELISA. Wells were coated with 100 µL inactivated VEE virus at a concentration of 4 µg/mL. Fifty µL of each test lysate was added per well. Indicator antibody was HRP-labelled anti-E Tag antibody, diluted 1:4000 in antibody dilution buffer. Plates were read 30 minutes following addition of substrate solution. Data points represent the mean of triplicate determinations. Position of molecular weight markers is indicated on the right hand side of the gel.

Figure 4A



Western blot of soluble A116 scFv protein in periplasmic extract (PE) of *E. coli* HB2151 cells. Fifteen μL of PE in a final volume of 25 μL was loaded per well and electrophoresed on a 12% SDS-PAGE gel. Indicator antibody was HRP-labelled anti-E Tag antibody. Position of the 30 KDa molecular marker is indicated on the right hand side of the gel.

Figure 4B



Functional analysis of A116 scFv protein from periplasmic extract (PE) of *E. coli* HB2151 cells by ELISA. Wells were coated with 100 µL inactivated VEE virus at a concentration of 10 µg/mL. Thirty µg of PE was diluted in antibody dilution buffer (PBS containing 0.1% Tween-20) to a final volume of 100 µL and added to the wells. Indicator antibody was HRP-labelled anti-E Tag antibody, diluted 1:5000 in antibody dilution buffer. Plates were read 30 minutes following addition of the substrate. Data points are the mean of triplicate determinations.

▒ With antigen

□ Without antigen

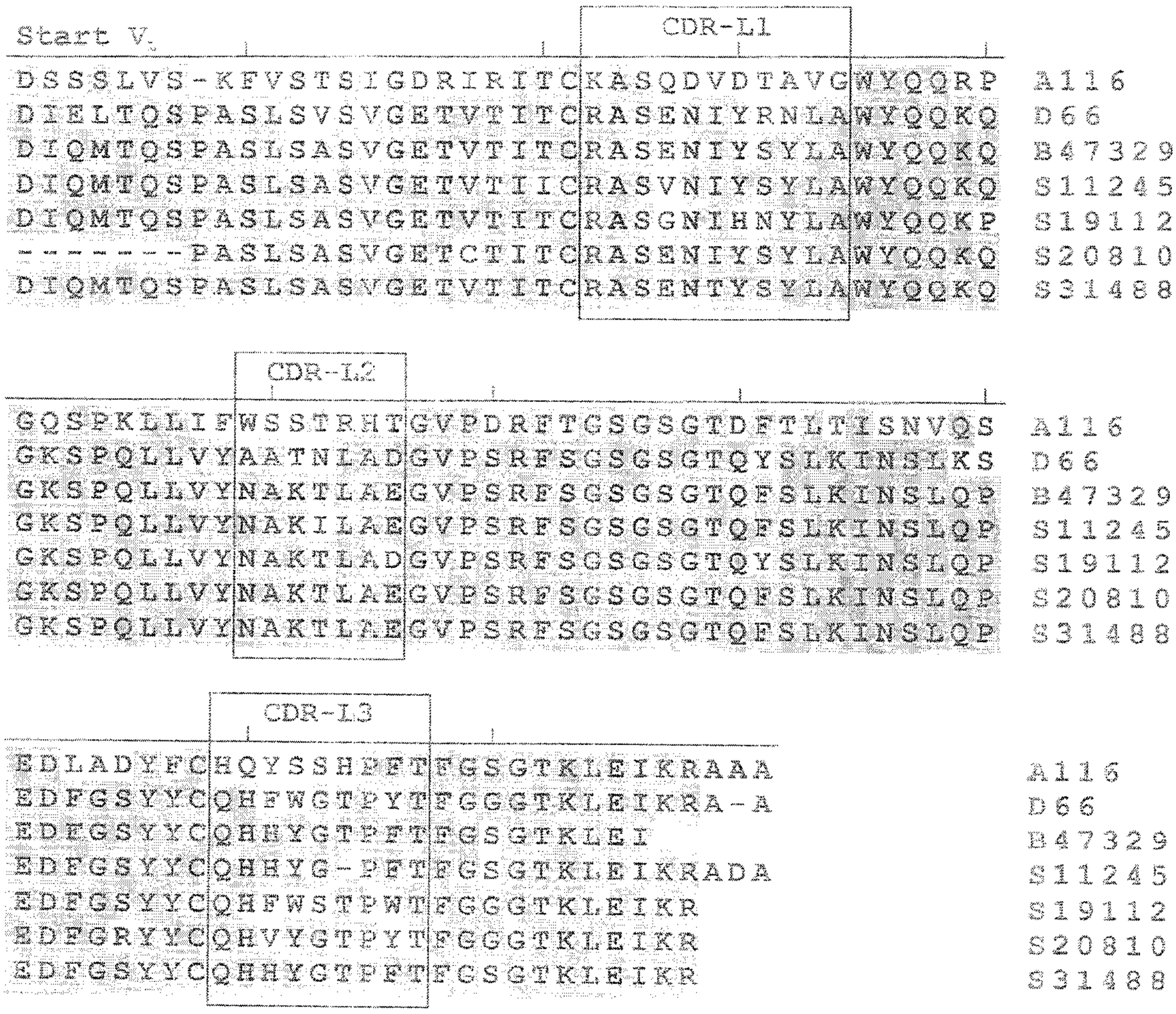
Figure 5A



V_H amino acid comparisons

The deduced amino acid sequence of A116 V_H and V_L regions from the determined nucleotide sequence of the A116 clone is shown in the top lane. The A116 amino acid sequence is compared to the amino acid sequence of D66 scFv antibody and to other murine scFv antibodies from the GCG PIR database which match closest to A116 V_H and V_L amino acid sequences. Hypervariable CDR regions are boxed and labelled. The PIR database accession numbers of the compared sequences are indicated on the right hand side of the sequence array; the amino acid number is shown on the left hand side. The V_H and V_L chains are joined by a peptide linker (amino acid sequence not shown). Amino acid sequences that are identical are shaded; residues differing from the consensus are clear.

Figure 5B



V_L amino acid comparisons

The deduced amino acid sequence of A116 V_H and V_L regions from the determined nucleotide sequence of the A116 clone is shown in the top lane. The A116 amino acid sequence is compared to the amino acid sequence of D66 scFv antibody and to other murine scFv antibodies from the GCG PIR database which match closest to A116 V_H and V_L amino acid sequences. Hypervariable CDR regions are boxed and labelled. The PIR database accession numbers of the compared sequences are indicated on the right hand side of the sequence array; the amino acid number is shown on the left hand side. The V_H and V_L chains are joined by a peptide linker (amino acid sequence not shown). Amino acid sequences that are identical are shaded; residues differing from the consensus are clear.

Figure 6



Comparison of nucleotide sequences in the framework-1 region of V_L of the six 1A4A1 scFv clones shown to carry full length scFv fragments. These sequences were used to identify predicted deletions in 1A4A1-16 that led to a localized frame shift. Nucleotide sequences that are identical are shaded; sequences differing from the consensus sequence are clear. Nucleotide insertions suggested for correction of frame shift are indicated.

Figure 7

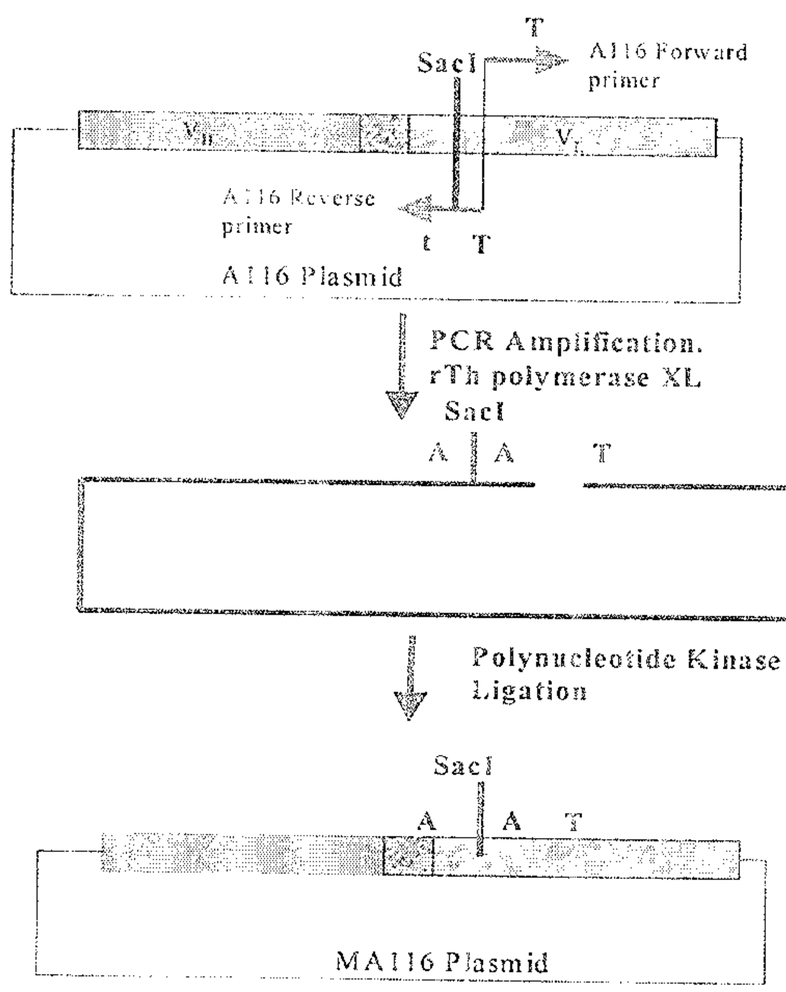


Figure 7: PCR based site directed mutagenesis strategy for repair of A116 ScFv gene: A cartoon presentation of steps involved in re-engineering of A116 ScFv clone for repair of framework one region of VL gene. The lower case "t" on reverse primer indicates the first insertion which, upon incorporation, destroys the XhoI site within the VL gene. Note that the primers lie adjacent to each other and pointed away from each other in the direction of DNA synthesis.

Figure 8

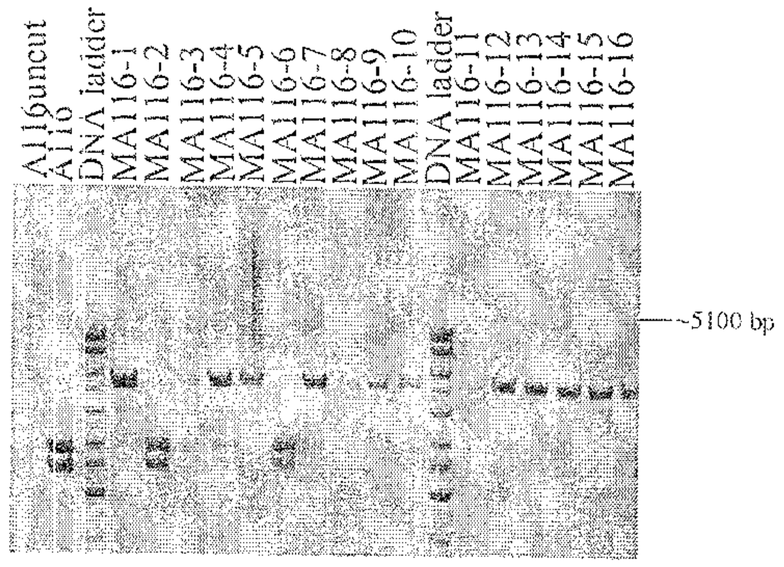


Figure 8: Isolation of DNA and Xho I restriction enzyme digestion of MA116 clones.
Mini preparation DNA of MA116 transformant clones was isolated and run on a 1% agarose gel. Figure 2 represents the gel run with Xho I digested DNA from MA116 clones.

Figure 9

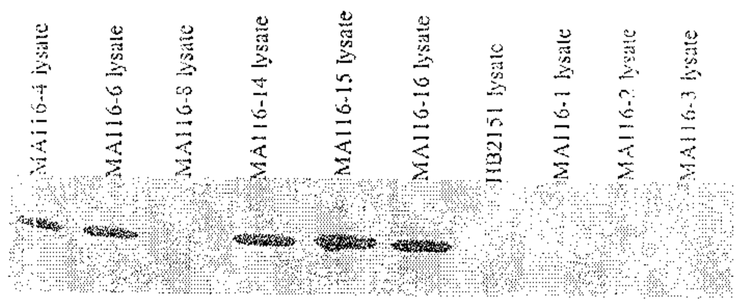


Figure 9: Western blot analysis of selected lysates from MA116 clones
Lysates from selected MA116 clones were run on a 10% SDS-Page gel. 9 uL of each individual lysate were run. HRP labelled anti-E tag antibody was used as secondary antibody at a dilution of 1:1000. Image was captured on a X-ray film using enhanced chemilumeniscence substrate from Amersham Pharmacia.

Figure 10

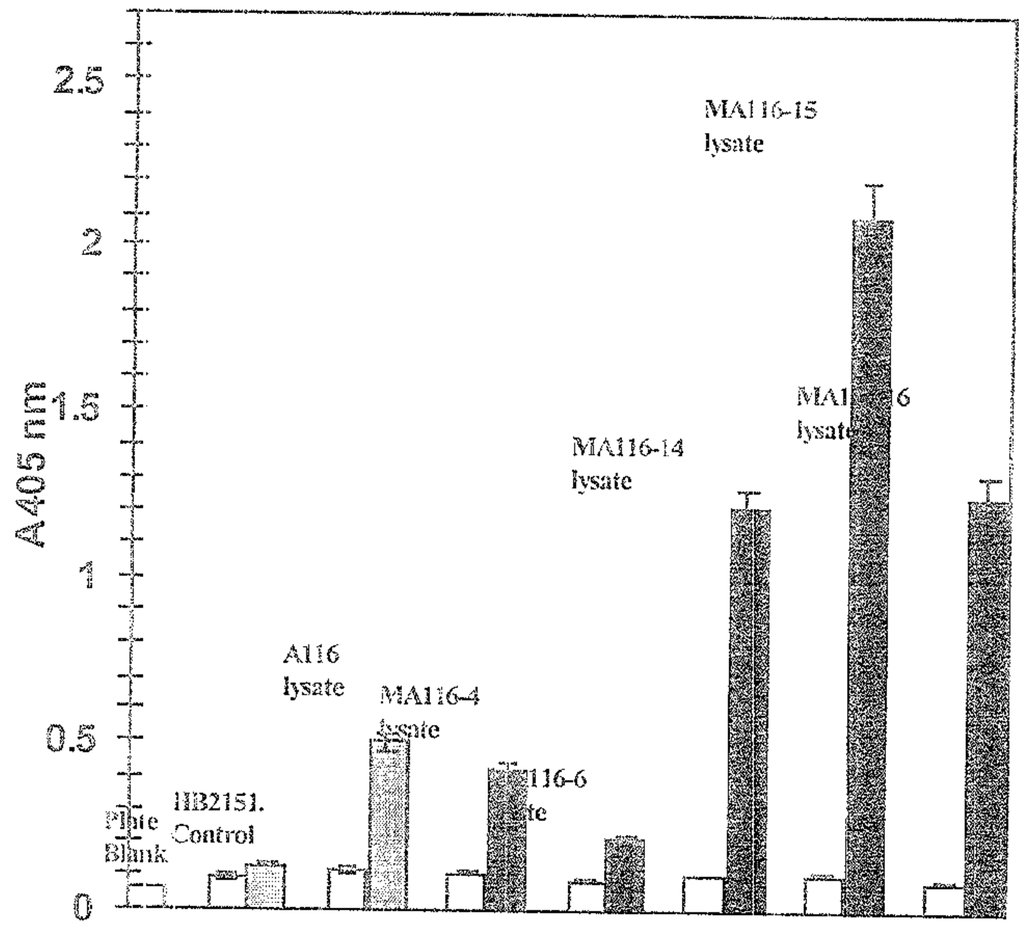


Figure 10: ELISA to confirm functionality of selected MA116 clones
VEE purified whole virus antigen (320 ug/ml) was used to coat ELISA plates at a concentration of 10 ug/mL (1 ug/well). Thirty uL of individual ScFv expressing MA116 whole cell lysates were used per well and each clone was tested in the presence and absence of bound VEE antigen in triplicate. Clear bars represent ScFv retention in the absence of VEE whole virus antigen. Solid bars represent retention of ScFv in the presence of VEE whole virus antigen. HRP-labeled anti-E tag antibody was used as indicator antibody. Results represent 30 minute reading after the addition of substrate. To distinguish MA116 clones, A116 and control lysates in the presence of VEE antigen are represented by gray bars.

Figure 11

```
397                                                                  436
GAC–TCGAGC TCACTC–GTC CTC–CAAATTC GT–GTCCACA                    A116-16
GAC–TCGAGC TCACTC–GTC CTC–CAAATTC GT–GTCCACA                    MA116-6
G–CATCGAGC TCACTCAGTC CTCTCAAATTC GTTGTCCACA                    MA116-4
GACATCGAGC TCACTCAGTC CTCCCAAATTC GTTGTC–ACA                    MA116-14
GACATCGAGC TCACTCAGTC CTC–CAAATTC GTTGTCCACA                    MA116-16
GACATCGAGC TCACTCAGTC CTC–CAAATTC GTTGTCCACA                    MA116-15
```

Figure 11 Nucleotide base composition of the framework-1 region of the light chain. Nucleotide base sequence generated from A116-16 and the five MA116 clones are compared in the region of nt 397 to 436. This region comprises the first half of framework-1 of the mouse IgG light chain.

Figure 12

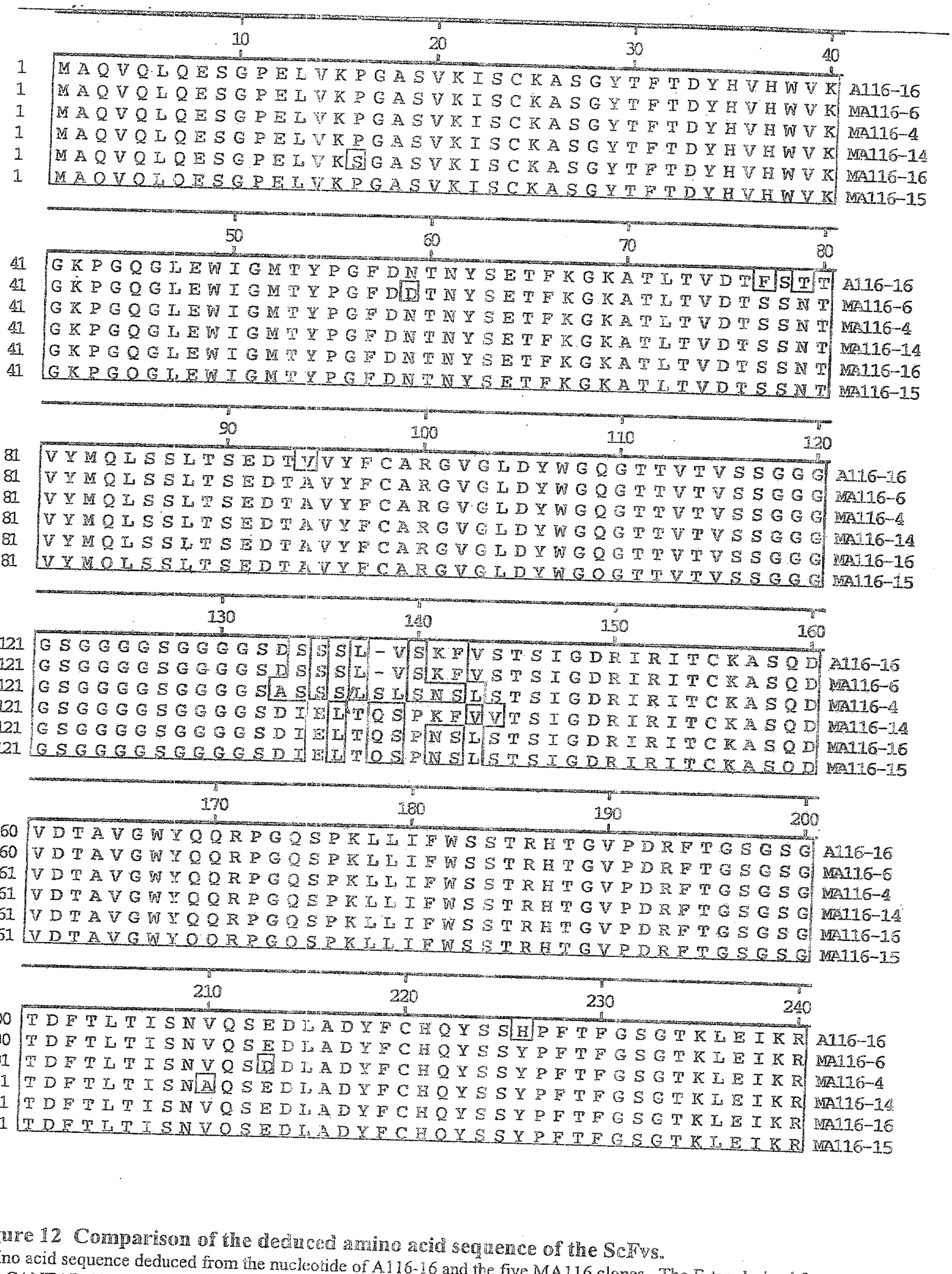


Figure 12 Comparison of the deduced amino acid sequence of the ScFvs.
Amino acid sequence deduced from the nucleotide of A116-16 and the five MA116 clones. The E-tag derived from the pCANTAB 5 E vector was identical for all clones and was not included. Area of similarity are boxed.

Figure 13A & 13B

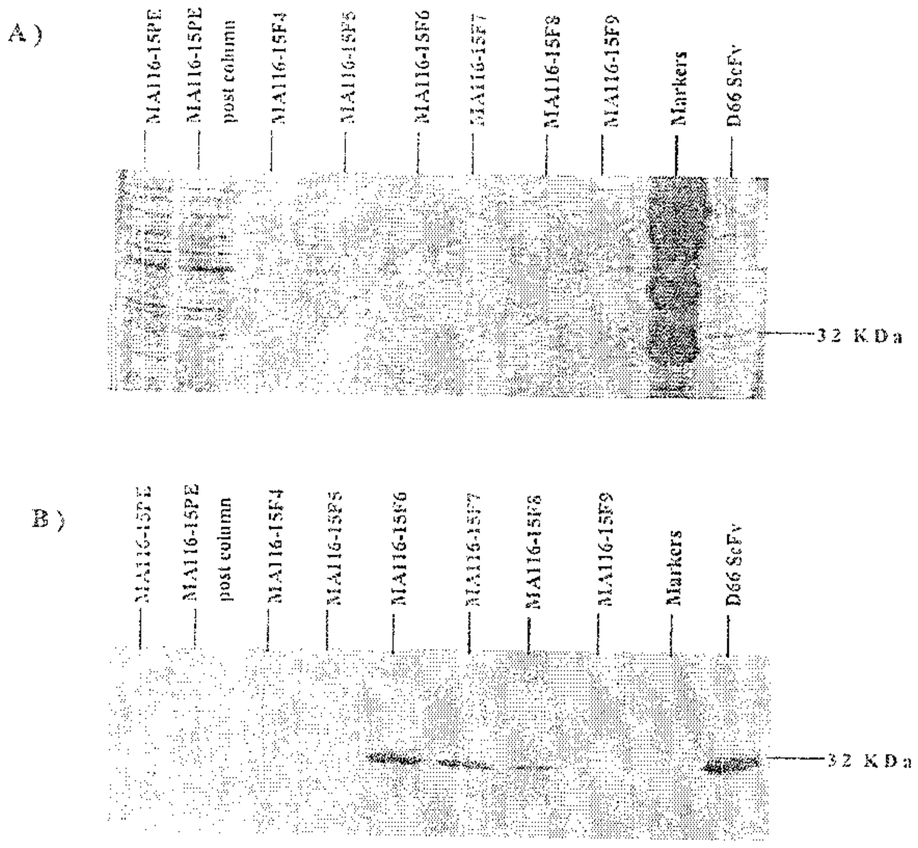


Figure 13: SDS-PAGE analysis of MA116-15 ScFv purification
Selected purified fractions, along with the crude PE and post column PE from MA116-15 purification, were separated on 10% SDS-PAGE gels. Ten ul of each sample was loaded per gel.

A) Coomassie stained SDS-PAGE gel.

B) Western blot analysis of the MA116-15 samples. HRP-labeled anti-E tag antibody was used as secondary antibody at a dilution of 1:1000. Image was captured on a X-ray film using enhanced chemiluminescence substrate from Amersham Pharmacia Biotech.

Figure 13C

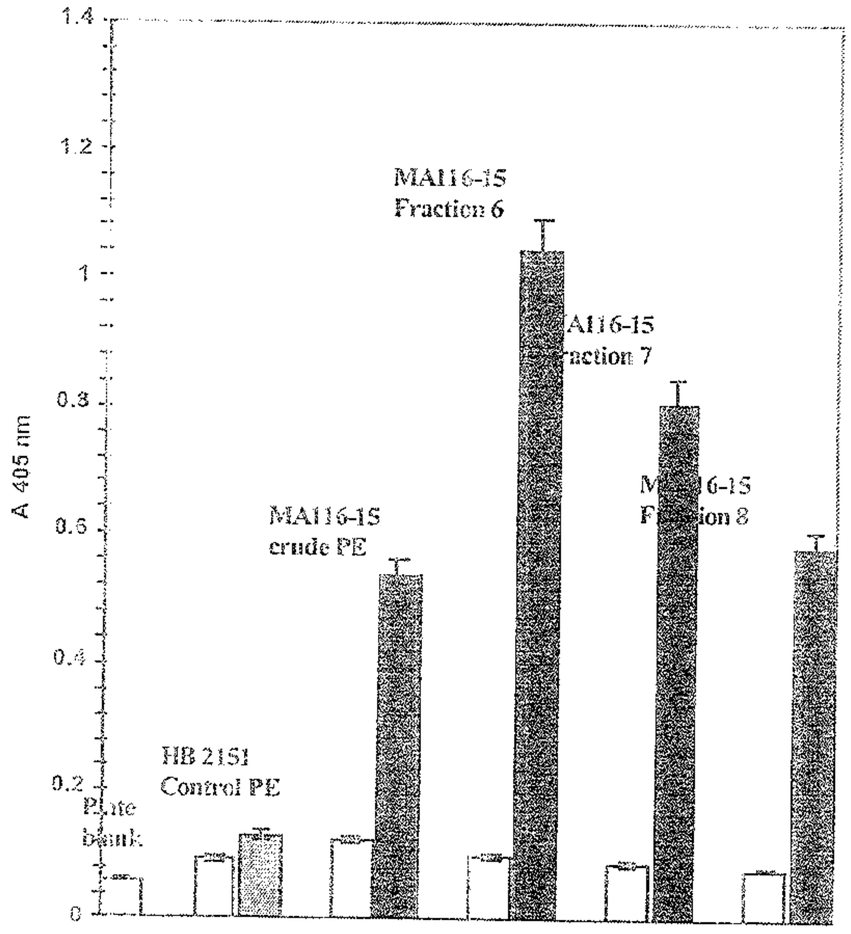


Figure 13C: ELISA analysis of the ScFv-positive purified fractions of Clone MA116-15

VEE purified whole virus antigen (10ug/mL) was bound per well. Crude periplasmic extract (PE) was used for HB2151 control. MA116-15 PE (50 uL/well) was included in the analysis. Twenty uL/well of purified fractions were used. All samples were tested in the presence and absence of bound VEE antigen, in triplicate. Clear bars represent ScFv retention in the absence of VEE whole virus antigen. Solid bars represent retention of ScFv in the presence of VEE whole virus antigen. Gray Bar is control PE in presence of VEE antigen. Results represent 30 minute readings after the addition of substrate.

Figure 14

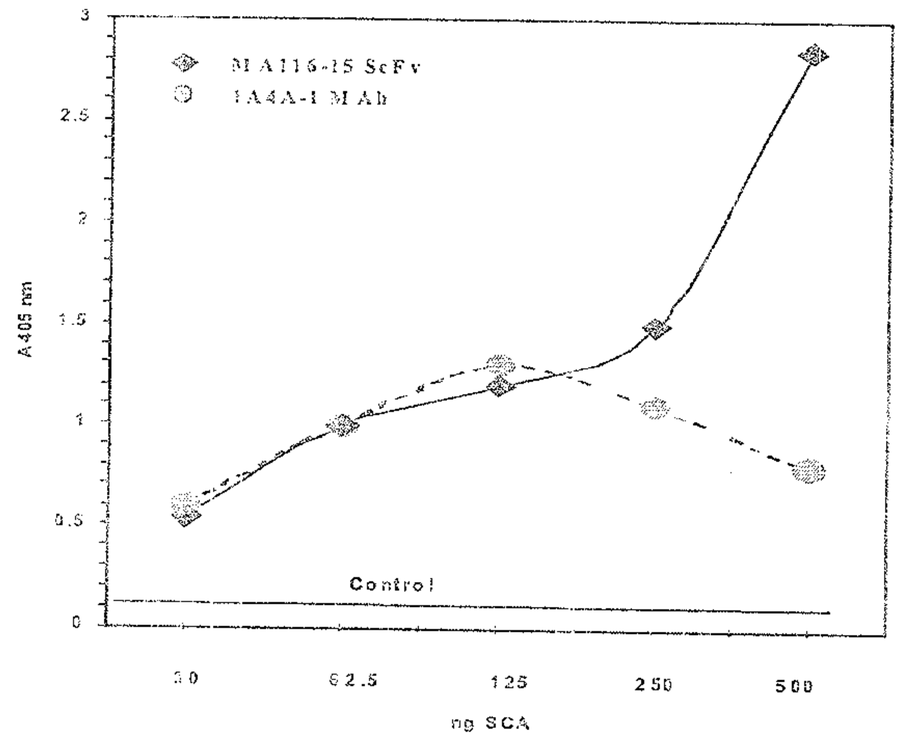


Figure 14: Functional comparison of purified MA116-15 (F6) ScFv and parent 1A4A-1 MAb at equi-molar concentration.
ELISA analysis of MA116-15 ScFv and 1A4A-1 MAb was performed by adding increasing equimolar amounts of the respective proteins. X-axis represents the amount of ScFv added. The amount of MAb at each variable pouint is 2.5X higher (e.g. at 30 ng ScFv, the amount of MAb is 75 ng). VEE purified whole virus antigen was coated on the plate at 10ug/mL concentration. The signal was detected using HRP-labeled anti-E tag antibody and HRP-labeled anti-mouse antibody for ScFv and Mab, respectively. Results represent 30 minute readings after the addition of substrate.