YBRIDOMA AND HYBRIDOMICS
olume 21, Number 3, 2002
Mary Ann Liebert, Inc.

SL-2001-156

Development of a Second Generation Monoclonal Single Chain Variable Fragment Antibody against Venezuelan Equine Encephalitis Virus: Expression and Functional Analysis

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ABSTRACT

We have generated a single chain variable fragment (ScFv) antibody from a well-characterized monoclonal antibody (MAb) against Venezuelan equine encephalitis virus (VEE), 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 5E. MAb 1A4A1 was successfully cloned as a 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 A116, was expressed primarily as soluble periplasmic protein. The 30 kDa A116 antibody displayed weak binding specificity to VEE antigen. Sequence analysis revealed a frame shift in the N-terminal region of the V_L domain, upstream to the complementarity-determining region 1 (CDR1), as the probable cause of reduced activity. The protein sequence of A116 was highly homologous to published murine ScFv protein sequences except in the region of the identified frame shift.

INTRODUCTION

VENEZUELAN EQUINE ENCEPHALITIS VIRUS (VEE) is an alphavirus that causes encephalitis in horses and humans. A characteristic VEE infection in humans is targeted to lymphoid tissues, causing a systemic febrile infection with occasional encephalitis. The host range of VEE is quite wide, including both vertebrates (rodents, equines, and humans) and invertebrates (a variety of mosquito species.[1] VEE has caused major epizootics in North America in the 1960s and 1970s[2] and, more recently, in South America (1995).[3]

The epidemiology, serology, and molecular structure of VEE have been studied extensively. Six serological subtypes (I–VI) of VEE have been identified, with subtype I exhibiting five variants (IAB, IC, ID, IE, and IF).[1] VEE epizootics are associated with members of subtypes IAB or IC. The other subtype I variants (ID, IE, and IF) and subtypes II–VI have been associated with enzootic VEE transmission.[4,5]

The VEE virion consists of an enveloped nucleocapsid containing a single-stranded, non-segmented, positive sense RNA genome. The important viral encoded structural glycoproteins. E2 (56 kDa) and E1 (50 kDa) protrude from the envelope.[6] The E2 protein has become a major target for immunodetection and immunoprophylaxis research, since it contains the viral neutralization and hemagglutination sites, as well as epitopes that elicit the protective antiviral antibody response.[7,8,9]

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 mouse 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.[10,11] 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.[12,13,14,15]

One approach to the development of recombinant antibodies is the generation of single chain variable fragment (ScFv)

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antibodies in which the variable heavy (V_H) and variable light (V_L) chain antibody domains are covalently joined by a polypeptide linker and cloned in bacterial expression vectors.[16,17,18] This results in expression in bacteria of the antigenic recognition domain of an antibody as a single chain that can fold itself into a functional molecule. The attractiveness of this system lies in the relative ease with which huge quantities of functionally active antibody can be obtained in a very short time. Since these ScFvs lack the constant region of the complete antibody molecule, they induce only low levels of human anti-mouse antibodies. The low antigenicity of ScFv coupled with their small size (~30 kDa) makes these molecules attractive alternatives to whole antibody molecules as therapeutic materials.

Roehrig et al.[9] have generated and characterized a number of MAbs directed against the E_2 protein of VEE.[7,8] These MAbs have been shown to provide good protection against challenge virus in animal protection studies, indicating their usefulness in providing passive immunity.[7] One such MAb, 1A4A1, not only recognizes VEE subtypes I_A–I_D and II_{EVE} by ELISA, but also neutralizes all of these subtypes. The hybridoma producing this MAb is, therefore, a very good candidate for use in the development of second generation antibodies with potential for diagnostic and therapeutic applications.

In this report we describe the cloning, sequencing, and expression of functional ScFv from hybridoma cell line 1A4A1. In addition, we have predicted how the antibody activity of this ScFv could be improved by the localization and repair of mutations identified in the gene sequence.

MATERIALS AND METHODS

Materials

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, Canada). Taq polymerase used in polymerase chain reaction (PCR) was from Boehringer Mannheim (Laval, QC, Canada). Restriction enzymes were purchased from Gibco/BRL (Burlington, ON, Canada), Amersham Pharmacia Biotech, or New England Biolabs (Beverly, MA), and nuclease-free water was purchased from Promega Inc. (Madison, WI). Unless otherwise specified, chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and tissue culture reagents were purchased from Gibco/BRL. Standard methods were used for the manipulation and cloning of cDNAs.[19]

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

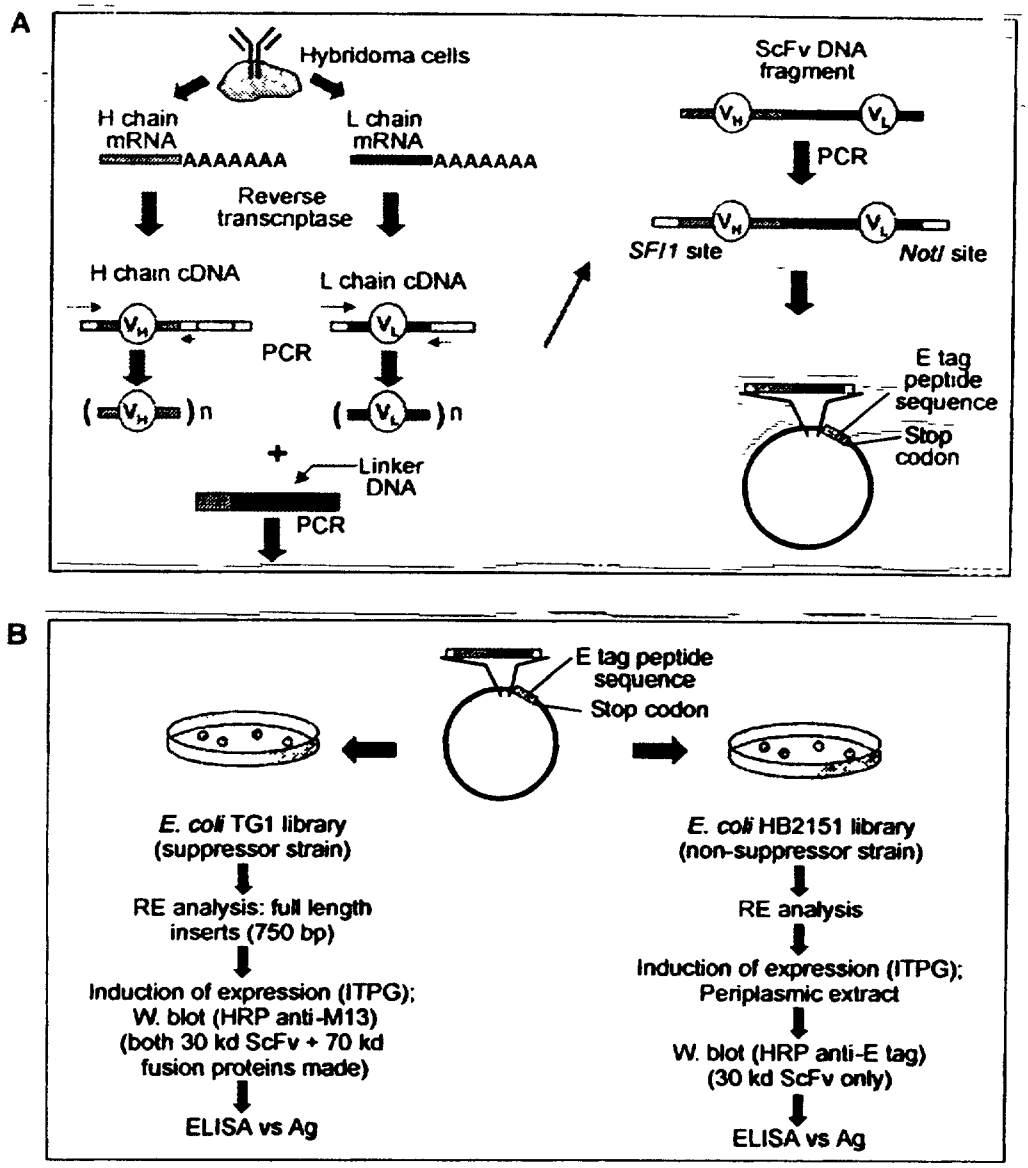
VEE hybridoma cell line 1A4A-1 was kindly provided by Dr. J.T. Roehrig, Division of Vector-borne Infectious Diseases, Centers for Disease Control and Prevention (Fort Collins, CO). The hybridoma cells were grown and maintained in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum (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), 1× Vitamins solution, and 100 μM non-essential amino acids. The cells were maintained at a density of ~1×10^5/mL.

Construction of ScFv antibody

A scheme representing the procedure adopted for cDNA cloning and construction of recombinant ScFv antibody is shown in Fig. 1 and described elsewhere.[20] Messenger RNA was isolated from 1×10^7 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 for the ScFv fragment to be cloned downstream of the leader peptide of the M13 gene 3, and upstream of 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 microliters of each of the lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels. The separated pro-



IG. 1. Schematic representation of steps in development of recombinant ScFv antibodies: (A) cloning and PCR; (B) expression and analysis.

ins were transferred to Immobilon™-P membranes (Millipore orp., Bedford, MA) by use of Towbin buffer (25 mM ris/HCl, pH 8.3, 192 mM glycine, and 20% methanol). Membranes were blocked for one hour with blocking buffer (PBS ontaining 3% non-fat skim milk). HRP-labelled anti-E Tag antibody, diluted 1:2000 in blocking buffer, was then added and e membranes incubated in this solution for a period of 45–60 inutes. Specific binding to expressed ScFv was detected by e of an enhanced chemilumeniscence (ECL) kit purchased om Amersham Pharmacia Biotech. For all washing steps, PBS ontaining 0.1% Tween-20 was used.

xpression of soluble ScFv antibody molecules

DNA clone 1A4A1-16 was transformed into *E. coli* HB2151 lls (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 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 h), they were induced with 2 mM IPTG. The induced cultures were grown at 30°C until they reached in A_{600nm} of 0.5 (~2 h). The cells were then harvested by centrifugation at 1500 × g for 10 min and the pellet gently resuspended in 20 mL of ice cold 1× 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 microliters of 0.2× 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 × g for 10 min, 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, Canada), 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).[21]

Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent 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 2× 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 Fig. 1. Distinct bands of ~340 bp for V_H and ~325 bp for V_L were detected by agarose gel electrophoresis after initial 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.

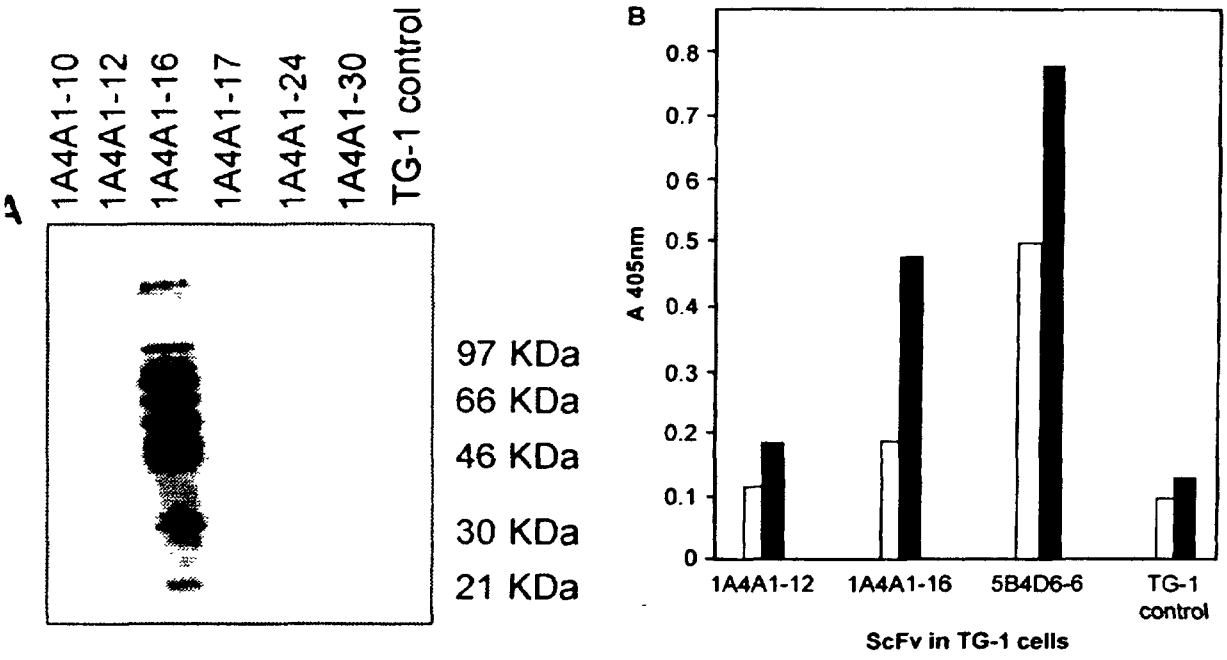
In order to screen for the presence of full length 750 bp ScFv inserts, "miniprep" DNA was prepared from the 40 individual transformants and restriction enzyme analysis was performed. From the 40 transformants, a total of six (1A4A1-10, 1A4A1-12, 1A4A1-16, 1A4A1-17, 1A4A1-24, and 1A4A1-30) were found 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 for expression in E. coli TG-1

Following IPTG induction, cell lysates 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 percent. 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.

Functional characterization of 1A4A1-16 ScFv lysate from E. coli TG-1

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



(G. 2. (A) Western blot of cell lysates from 1A4A1 clones exhibiting full length ScFv fragments. Fifteen uL of lysate in a fi- l volume of 25 uL was loaded per well on a 12% SDS-PAGE gel. Indicator antibody was HRP-labelled anti-E Tag antibody. ısition of molecular weight markers is indicated on the right hand side of the gel. (B) Screening of expression-positive ScFv otein lysates from *E. coli* TG-1 cells for functional reactivity by ELISA. Wells were coated with 100 uL inactivated VEE at a ıncentration of 4 ug/mL. Fifty uL of each test lysate was added per well. Indicator antibody was HRP-labelled anti-E Tag an- ıody, diluted 1:4000 in antibody dilution buffer. Plates were read 30 min following addition of substrate solution. Data points e the mean of triplicate determinations. ■, antigen; □, no antigen.

sates and lysates from 1A4A1-12 were included as negative ıntrols, while 5B4D6 lysate, another VEE ScFv shown previ- ısly to react with VEE antigen,[20] was used as positive con- ol (Fig. 2B). Only 1A4A1-16 ScFv clone gave positive ELISA sults, as indicated by retention of ScFv products in the pres- ıce of VEE antigen. Wells with VEE antigen specifically re- ıned the expressed ScFv products at absorbance levels at least ıce as high as that observed in wells without VEE antigen, dicating that 1A4A1-16 ScFv antibody was functionally ac- ve. It may also be observed from Fig. 2B that the 1A4A1-16 :Fv clone product, although functional, was less active than e 5B4D6 ScFv positive control lysate. The results of this LISA complemented the expression results shown in Fig. 2A.

xpression of functional soluble ScFv proteins in E. oli HB2151 cells

To demonstrate the functionality of the soluble 30 kDa ScFv oducts when expressed in the absence of the ~70 kDa ScFv- 33 fusion protein, 1A4A1-16 DNA was transformed into a on-suppressor background cell type, *E. coli* HB2151. To dis- nguish between TG-1 and HB2151 transformants, transfor- ants obtained as a result of HB2151 transformations were ferred to as A116, while TG-1 transformants were called A4A1-16.

To establish that A116 clones expressed soluble 30 kDa ScFv otein, 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 expression of 30 kDa soluble ScFv protein (Fig. 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 (Fig. 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

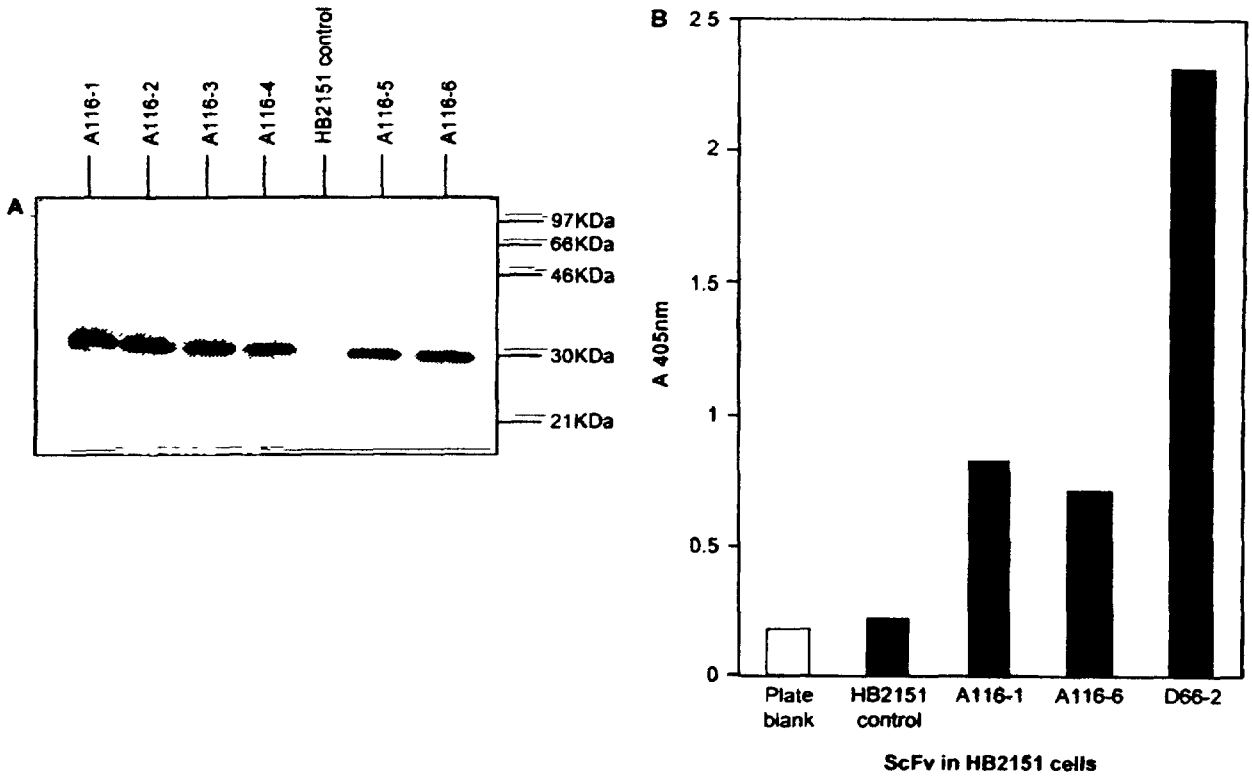


FIG. 3. (A) Screening of A116 ScFv protein in whole cell lysates of *E. coli* HB2151 cells for expression of soluble ScFv antibody by western blotting. Six μL of lysate in a final volume of 24 μL was loaded per well and electrophoresed on 12% SDS-PAGE gels. HRP-labelled anti-E Tag antibody was used at 1:2000 dilution. Position of molecular weight markers is indicated on the right hand side of the gel. (B) 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 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 min 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.

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, a sandwich ELISA was performed (Fig. 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 (Fig. 4B) or from the whole cell lysate (Fig. 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 reengineer 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 $(Gly_4Ser)^3$. 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

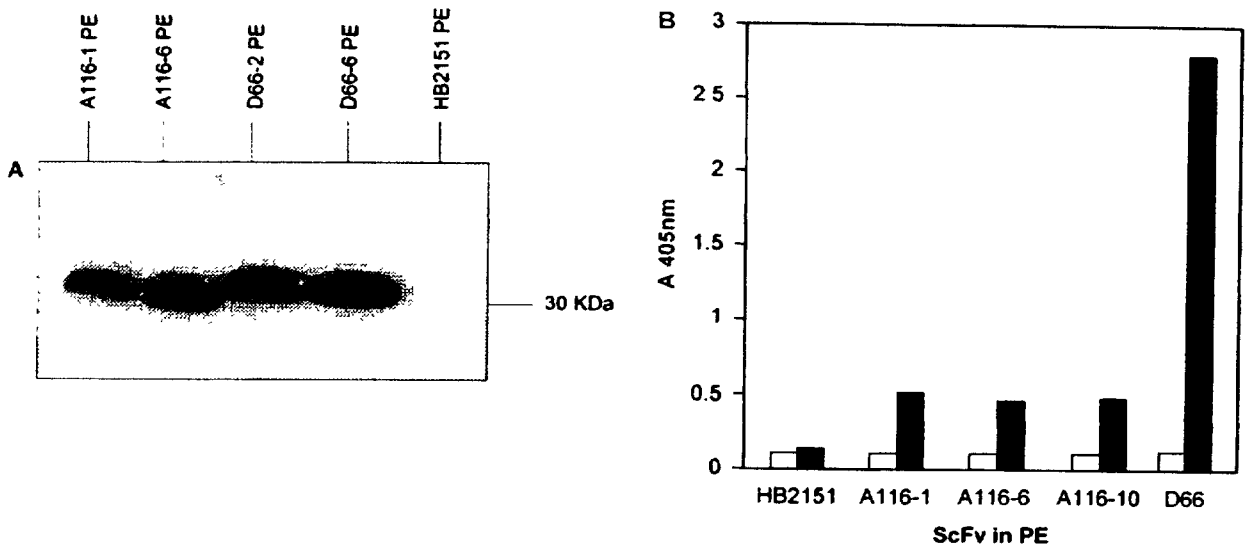


FIG. 4. (A) Western blot of soluble A116 ScFv protein in periplasmic extract (PE) of *E. coli* HB2151 cells. Fifteen ~~microliters~~ of PE in a final volume of 25 μL was loaded per well and electrophoresed on a 12% SDS-PAGE gel. Indicator antibody was HRP-labelled anti-E Tag antibody. Position of the 30 kDa molecular marker is indicated on the right hand side of the gel. (B) Functional analysis of A116 ScFv protein from ~~periplasmic extract (PE)~~ of *E. coli* HB2151 cells by ELISA. Wells were coated with 100 ng inactivated VEE at a concentration of 10 μg/mL. Thirty μL 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 min following addition of the substrate. Data points are the mean of triplicate determinants. ■, antigen; □, no antigen.

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, is shown (Fig. 5A and 5B). 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 (Fig. 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 (Fig. 5B). The protein sequence also diverged in the N-terminal part of the V_L regions of A116: the first framework region of the V_L chain of A116 between the end of the linker and just before the beginning of the CDR1, had mutated.

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 1A 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 (Fig. 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 (Fig. 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 de-

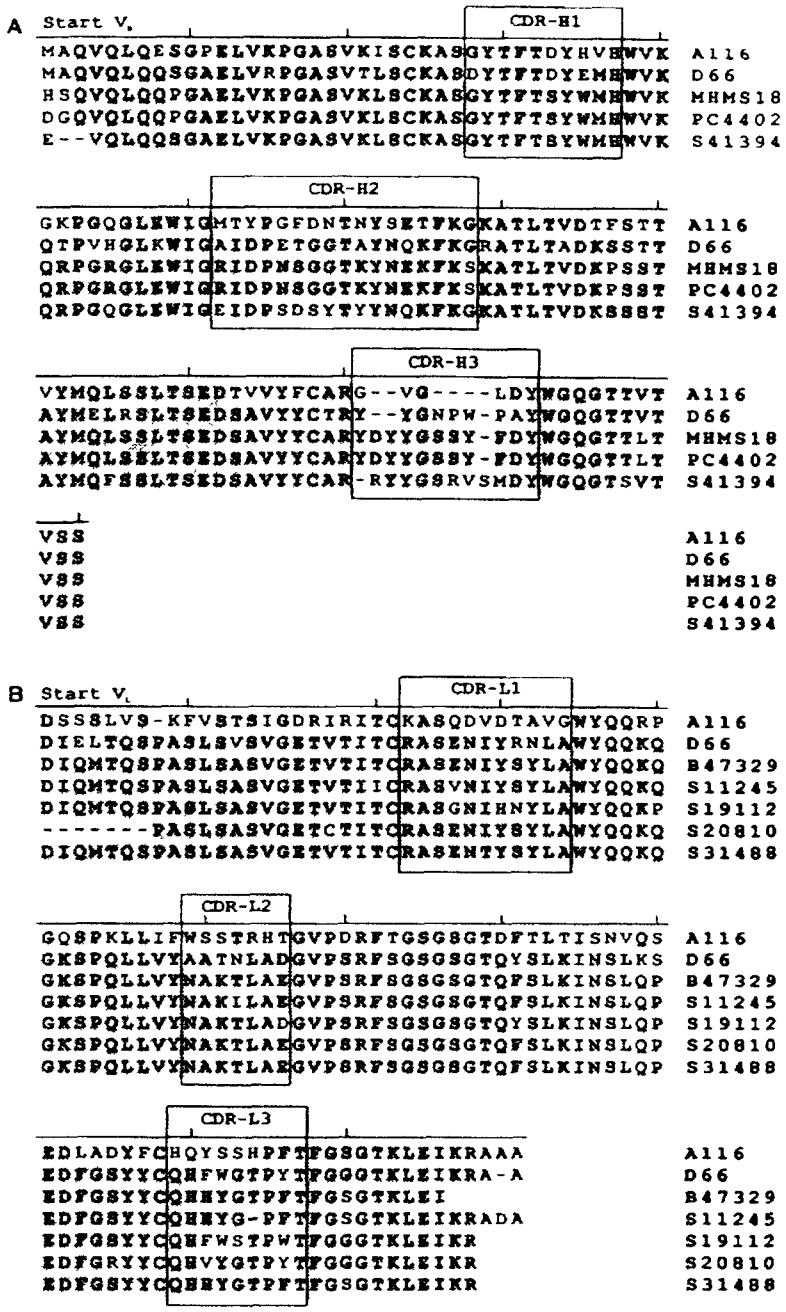


FIG. 5. V_H and V_L amino acid sequence comparisons. The deduced amino acid sequence of A116 V_H and V_L regions from the determined nucleotide sequence of the A116 clone is shown in the top lane. The A116 amino acid sequence is compared to the amino acid sequence of D66 ScFv antibody and to other murine 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. (A) V_H amino acid comparisons. (B) V_L amino acid sequence comparisons. The PIR database accession numbers of the compared sequences are indicated on the right hand side of the sequence array. 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.

duced, 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

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ACATCGAGCTCACTCAGTCTCCAAATTCGT-GTCCACATC  1A4A1-10
ACATCGAGCTCACTCAATCTCCATCCTCCTTATCTGCCTC  1A4A1-12
ACATCGAGCTCACTCAGTCTCCATCCTCCTTATCTGCCTC  1A4A1-17
ACATCGAGCTCACTCAGTCTCCAAATTCGT-GTCCACATC  1A4A1-24
ACATCGAGCTCACTCAGTCTCCAAATTCGT-GTCCACATC  1A4A1-30
AC-TCGAGCTCACTC-GTCTCCAAATTCGT-GTCCACATC  1A4A1-16
  A             A             T
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FIG. 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.

antibodies that are both functionally active and genetically stable.[22,23] In a previous publication, we reported the cloning and expression of D66 ScFv, a functionally active ScFv antibody produced from VEE hybridoma 5B4D6.[20] In this study, we report the cloning and expression of another functionally active anti-VEE ScFv, A116, prepared from VEE hybridoma 1A4A1.

VEE MAb 1A4A1, from which ScFv 1A4A1 was cloned in this study, has been previously well characterized by Roehrig et al.[7] 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 done 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 we adopted a screening protocol 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-labelled 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 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 sandwich 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 us to further investigate the A116 clone from the perspective of its protein sequence. We compared the A116 ScFv amino acid sequence with that of D66 ScFv, as well as with ScFv sequences of murine origin that had previously been shown to be functional and were available in the GCG database. 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 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, we proposed 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, shown to be conserved in all other ScFv antibodies with which it was compared, supports this hypothesis. By comparing the nucleotide sequences of the six 1A4A1 ScFv clones, we were able to locate the three individual nucleotide deletions that could have caused the proposed localized frame shift. 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 this regard, future experiments are planned to reintroduce the

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