HYBRIDOMA
Volume 18, Number 4, 1999
Mary Ann Liebert, Inc.

A Single Chain Fv Specific Against Western Equine Encephalitis Virus

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ABSTRACT

A recombinant single chain Fv (scFv) specific against Western equine encephalitis virus (WEE) was developed and characterized. The scFv was generated from 11D2 hybridoma producing anti-WEE antibody reactive to E1 component of viral envelope glycoprotein. V_L and V_H gene segments of 11D2 scFv were generated and joined together with a $(gly_4ser)_3$ linker by polymerase chain reaction (PCR). The resulting scFv was successfully expressed in *P. pastoris* expression system. Fifteen individual plasmids were tested and six of them were shown to drive scFv expression. DNA sequence analysis from three productive plasmids showed that they all carried the same V_L and V_H gene segments with a few base differences. Comparison of 11D2 scFv DNA sequence to the Kabat database showed that V_H of 11D2 antibody belonged to subgroup IIID and subfamily XIV, while V_L domain did not belong to any known subgroup or subfamily. Western blot analysis of 11D2 scFv using anti-*c-myc* antibody for detection showed different band pattern among clones derived from different plasmids. This was thought to be due to the different glycosylation where amino acid substitution occurred. Successful purification of 11D2 scFv could be done by immobilized metal affinity chromatography with an unoptimized yield of 700 μg/L. Functional studies showed that 11D2 scFv could bind to its respective WEE antigen as demonstrated by Western blot analysis and enzyme-linked immunosorbent assay (ELISA). The binding affinity of 11D2 scFv is reasonably good compared to the parental 11D2 bivalent monoclonal antibody (MAb). Thus, 11D2 scFv and its derivatives have a potential use as immunotherapeutic and immunodiagnostic agents of WEE infections.

INTRODUCTION

WESTERN EQUINE ENCEPHALITIS (WEE) virus is a mosquito-transmitted alphavirus that causes human and equine diseases in the Western hemisphere. Illnesses caused by WEE virus range from mild fevers with headache to aseptic meningitis and encephalitis. Virions of WEE are spherical, enveloped particles They contain the single-stranded positive-sense RNA encapsulated by nucleocapsid protein arranged in an icosahedral configuration. The envelope derived from host cell membrane carries two viral encoded glycoproteins, E1 and E2, which have molecular weight of approximately 50 kDa The trimeric heterodimers of E1 and E2 form spikes that function in attachment to the host cells In addition to envelope glycoproteins, WEE virus also has a nonglycosylated capsid protein that has a molecular weight of 30–34 kDa.[1,2]

Passive immunization using antiviral antibodies has been shown to be beneficial in terms of prophylaxis and treatment of alphavirus infections [3–6] Immune serum passively transferred into mice before or after infection of virulent NSV strain of Sindbis virus protected the animals from fatal disease.[3] Neutralizing and non-neutralizing antibodies to the E1 and E2 surface glycoproteins could confer this protection.[7–9] A more recent study suggested that the antibody worked by binding to viral antigen on infected cell membrane which resulted in improving K^+ flux, host protein synthesis and sustaining the ability to respond to type I interferon.[3] Successful antibody treatment was also shown in other alphaviruses such as Semliki

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Forest virus[5] and Venezuelan equine encephalomyelitis virus (VEE) [4] In WEE virus infection, the use of monoclonal antibodies (MAbs) and their reductants for immunotherapy and immunodiagnosis has not been widely explored.

MAbs specific against WEE virus are available at Defense Research Establishment Suffield (DRES), Ralston, Alberta, Canada to be used for immunodiagnosis as well as for developing an immunotherapeutic model of WEE virus infection. To study the efficacy of WEE treatment using various forms of antibody, a scFv specific against E1 glycoprotein of WEE virus was generated and characterized. In this paper, we report the construction of 11D2 scFv by the joining of variable domains of light and heavy chain gene segments derived from 11D2 hybridoma with a 15 amino acid $(gly_4ser)_3$ linker The scFv was successfully produced from a yeast expression system The molecular nature and binding properties of this scFv were characterized The 11D2 scFv and its derivatives have potential use in immunotherapy as well as in immunodiagnosis of WEE infections

MATERIALS AND METHODS

Cells

Anti-WEE producing hybridoma, 11D2, was generated for DRES by Sci Lab, Inc. (Redcliff, Alberta) through the Hybridoma Facility at Southern Alberta Cancer Research Center, University of Calgary, Alberta, Canada. (Anti-VEE producing hybridoma, 5B4D6, is a generous gift of Dr J T. Roehrig [10]) Anti-*c-myc* producing-hybridoma, 9E10, was obtained from ATCC (Manassas, VA) All cell lines were maintained in RPMI-1640 (GIBCO BRL, Burlington, ON) supplemented with 2 mM L-glutamine (GIBCO BRL) and 10% fetal bovine serum (FBS) (GIBCO BRL) at 37°C, 5% CO_2. Culture supernatant containing monoclonal antibodies were collecetd when the cell concentration reached 1×10^6 cells/mL

Construction of 11D2 scFv plasmid

Strategies for scFv gene construction and the primer sequences are summarized in Fig. 1. V_L and V_H gene segments were generated from 11D2 hybridoma by RT-PCR. Briefly, total RNA was prepared from 11D2 hybridoma using TRIZOL reagent (GIBCO BRL) according to the manufacturer's instructions First-strand cDNA was generated by mixing 2 μg of total RNA with 100 pmoles specific primers (P_L for light chain and P_H for heavy chain) and 200 units SuperscriptII (RNase H-Reverse Trancriptase) (GIBCO BRL) in a total volume of 20 μL containing 50 mM Tris pH 8 3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0 5 mM dNTPS, and 1 unit/μL RNAsin (Promega, Madison, WI) The reaction was incubated at 42°C for 1 h Generation of V_L or V_H were done by mixing 1 μL of cDNA reaction mix, 0 5 μM each primer, 200 μM dNTPs, and 1 unit Vent DNA polymerase (New England Biolabs, Beverly, MA) in 20 mM Tris pH 8 8, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0 1% Triton X-100 and PCR was set at 94°C/55°C/72°C, 45 s/45 s/45 s for 25 cycles. P_1 and P_2 primers were used for the amplification of V_L while P_3 and P_4 were used for the amplification of V_H The V_L (340 bp) and V_H (375 bp) PCR products were gel purified and eluted using QIAEX II gel extraction kit (Qiagen, Chatsworth, CA) Assembly of scFv was performed by mixing equimolar amount of V_L gene segment, linker oligonucleotides (P_5) and V_H gene segment and PCR was set at 94°C/55°C/72°C, 45 s/45 s/45 s for 10 cycles A small aliquot of this mixture (1–2 μL) was transferred to a new PCR mixture containing P_1 and P_4 primers and amplification continued at 94°C/60°C/72°C, 45 s/45 s/45 s for 25 more cycles. The product was analyzed on agarose gel electrophoresis and a 0 8 kb scFv band excised The purified scFv DNA fragment was further digested with *Eco*RI and *Not*I and ligated into *Eco*RI-*Not*I digested pPICZα vector (Invitrogen, San Diego, CA) Transformation of ligation mix was done in TOP10F' strain of *E. coli* (Invitrogen) and plated on LB agar (1% tryptone, 0 5% NaCl and 0 5% yeast extract) containing 25 μg/mL Zeocin (Invitrogen)

DNA sequence analysis

Double-stranded DNA sequencing was done by the sequencing facility, Biological Sciences Department, University of Alberta using Thermo Sequenase II dye terminator cycle sequencing kit (Amersham Pharmacia Biotech, Baie d'Urf'e', QC) and run on Applied Biosystems 373 DNA sequencer. Two sequencing primers were used. alpha factor (5' TAC TAT TGC CAG CAT TGC TGC 3') and 3' AOX1 (5' GCA AAT GGC ATT CTG ACA TCC 3') Alpha factor primer binds to the signal sequence upstream of scFv insert and 3'AOX1 primer is anti-sense that binds to the vector at the transcription termination site

Comparison of DNA sequence to Kabat database was performed through the internet at http://immuno bme.nwu.edu

Generation of recombinants in P. pastoris

Plasmid pPICZα containing 11D2 scFv was linearized by *Ssi*I (GIBCO BRL) digestion and ethanol precipitated. Electrocompetent cells were prepared by growing X-33 strain of *P pastoris* (Invitrogen) until OD_{600} reached 1 5 Cells were washed several times with ice-cold water and then washed once with ice-cold 1 M sorbitol The cell pellet was resuspended in ice cold 1 M sorbitol (0 006× original volume) and was used as electrocompetent cells Linearized plasmid DNA (5–10 μg) was mixed with 80 μL of competent cells in a 0 2 cm Gene Pulser/*E. coli* pulser cuvette (BioRad, Hercules, CA) and placed on ice for 5 min Electroporation was done using EC100 electroporator (E-C Apparatus Corporation, Holbrook, NY) set at 1800 V One milliliter of ice-cold 1 M sorbitol was immediately added and the suspension was transfered to a 15-mL tube After incubating at 30°C without shaking for 1h, aliquots of cell suspension were plated on YPDS agar (1% yeast extract, 2% peptone, 2% dextrose, and 1M sorbitol) supplemented with 100 μg/mL Zeocin and incubated at 30°C for 2–3 days until colonies become visible

Expression and secretion of scFv

P pastoris containing scFv plasmid was grown in BMGY media (1% yeast extract, 2% peptone, 100 mM phosphate buffer pH 6 0, 1 34% yeast nitrogen base, 0 4 mg/L biotin, 1% glycerol) at 30°C, 250 rpm until OD_{600} reached 5 0–6 0 The media was changed to BMMY (1% yeast extract, 2% peptone, 100

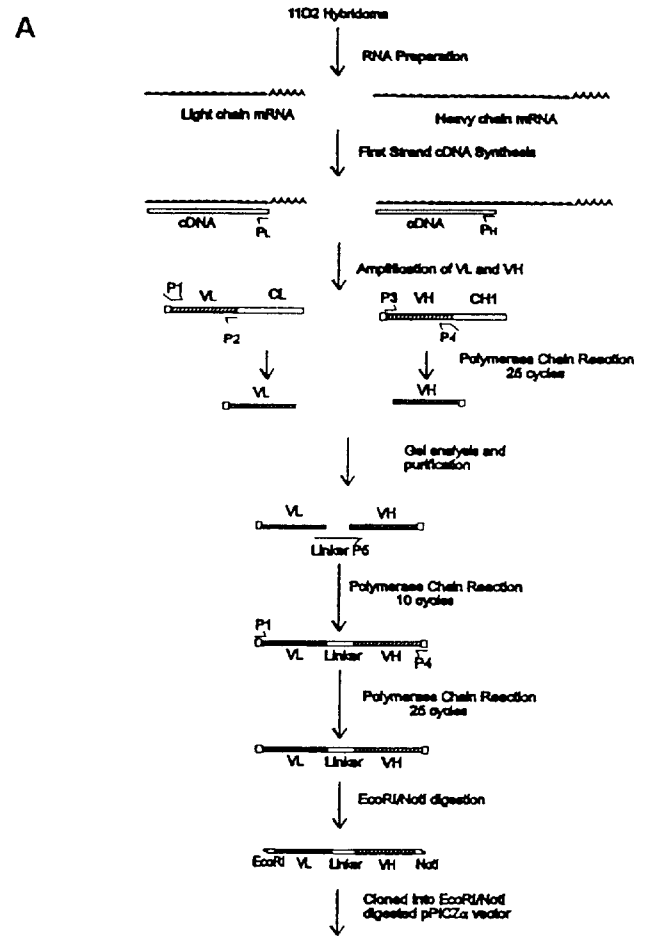

FIG. 1. Schematic representation of 11D2 scFv gene construction V_L and V_H gene segments derived from 11D2 hybridoma was joined together with a $(gly_4ser)_3$ linker (A). An expression cassette was generated by cloning 11D2 scFv encoding sequence into pPICZα (B). The oligonucleotide sequences used for construction of scFv are summarized in (C)

mM phosphate buffer pH 6 0, 1 34% yeast nitrogen base, 0 4 mg/L biotin, 0.5% methanol) and OD_{600} adjusted to 1.0 Culture was further shaken for 2 more days and supernatant was collected for product analysis

Screening of scFv producing clones by dot blot analysis

One microliter of yeast culture supernatant was spotted on nitrocellulose membrane (Hybond ECL, Amersham, Arlington, IL) and air dried The membrane was blocked with 5% skim milk in PBST (0 1% Tween-20 in phosphate buffered saline) for 1 h, washed three times with PBST and reacted to anti *c-myc* antibody (clone 9E10) for 1 h After 3 more washes, blot was reacted to horseradish peroxidase (HRPO) conjugated goat anti-mouse immunoglobulins (Sigma, St. Louis, MO) for 1 h, washed three times and enhanced chemiluminescence (ECL) based detection was performed according to the manufacturer's instructions (Amersham)

Western blot analysis of scFv

SDS-PAGE of scFv was done on 12% polyacrylamide gel using mini-PROTEAN II apparatus (BioRad) Transfer of pro-

teins to nitrocellulose membrane (Hybond ECL, Amersham) was done using mini Trans-blot Apparatus (BioRad) following the manufacturer's instructions. Immunoassay was done as described above for dot blot analysis using anti-*c-myc* antibody and HRPO conjugated goat anti-mouse immunoglobulins.

Purification of scFv by immobilized metal affinity chromatography (IMAC)

Affinity purification of 11D2 scFv was done using His Bind reagent kit (Novagen, Madison, WI) following the manufacturer's instructions Briefly, culture supernatant containing scFv was dialyzed against PBS to remove methanol and adjusted to 5 mM imidazole, 0 8 M NaCl, and 20 mM Tris pH 7 9 The resin was washed with water, charged with 50 mM $NiSO_4$, and adjusted to the same buffer as sample Sample was loaded to the column at a speed of 20 mL/h and washed with buffer containing 60 mM imidazole, 0 5 M NaCl, and 20 mM Tris pH 7 9 scFv was eluted from the column by buffer containing 1 M imidazole, 0.5 M NaCl, and 20 mM Tris pH 7.9 Eluate was desalted and concentrated by Centricon-30 (Amicon, Oakville, ON)

Preparation of WEE virus antigen

WEE strain B11 was kindly provided by Dr G Ludwid (U.S Army Medical Research Institute of Infectious Disease, Frederick, MD) Inactivated virus particles were prepared at DRES and was used as antigen Briefly, Vero cells (ATCC CCL-81) were infected with WEE virus at a multiplicity of infection less than 0 1 and the culture supernatant was harvested when 80–85% cytopathic effect was reached. The virus was precipitated by 7% polyethylene glycol (MW 6000), 2 3% NaCl and centrifuged at 10,000 ×g for 30 min. The pellet was resuspended in PBS and further purified by sucrose gradient centrifugation at 100,000 ×g for 3 5 h at 4°C. The virus band was collected, diluted with Hank's balanced salt solution (HBSS) and centrifuged at 100,000 ×g for 1 h The viral pellet was resuspended in HBSS and inactivated by the addition of 0.5% formalin-buffer saline and incubated for 16–20 h followed by dialysis against several changes of PBS. After safety testing for growth in Vero cell culture, aliquots of inactivated WEE virus were kept at −70°C.

Antigen binding test by Western blot analysis

WEE virus antigen (500 ng/Lane) was electrophoresed on 10% SDS-PAGE and transferred to nitrocellulose membrane as described above The membrane was cut into strips for reacting with various antibodies Immunoassay and detection were as described above in dot blot analysis except for the following changes For testing scFv, the antigen strip was incubated with dialyzed culture supernatant containing scFv for 1 h and washed prior to incubation with anti *c-myc* antibody. In the control experiments, antigen strip was incubated with 11D2 or anti-*c-myc* or 5B4D6 MAb, washed and then incubated with HRPO conjugated goat anti-mouse immunoglobulins

ELISA

ELISA plates (Nunc, Burlington, ON) were coated with 400 ng/well of WEE virus antigen at 4°C for overnight Plates were washed, blocked with 5% skim milk in PBST for 1 h, washed and 100 μL of samples containing scFv or 11D2 MAb were added. After 2 h of incubation, plates were washed, incubated with anti-*c-myc* antibody for 1 h, washed and reacted with HRPO conjugated goat anti-mouse immunoglobulins for 1 h After the final wash, detection was done by the addition of TMB (KPL, Gaithersburg, MD), incubated for 15–30 min and OD_{405} was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader (Vmax kinetic microplate reader, Molecular Devices, Sunnyvale, CA).

RESULTS

Construction and expression of 11D2 scFv

DNA fragment encoding for 11D2 scFv was generated by PCR and cloned into pPICZα vector for expression in *P. pastoris* (Fig 1) The scFv is arranged in V_L–V_H orientation and is joined together with a 15 amino acid $(gly_4ser)_3$ linker The gene transcription is driven by a methanol inducible promoter, P_{AOX1}. The scFv encoding sequence was cloned in frame to alpha factor signal sequence which directs the secretion of the protein The plasmid contains Zeocin resistant gene that can be used for selection of recombinants both in *E coli* and *P. pastoris*. The presence of *c-myc* and histidine tags at the C-terminal of product allow simple detection and affinity purification

Plasmid DNAs, pPICZα containing putative 11D2 scFv sequence, were purified and introduced into X-33 strain of *P. pastoris* by electroporation. Stable transfectants were selected by Zeocin resistance and scFv production was determined two days after methanol induction Several different plasmid DNA constructs were individually tested to select for the productive clones. Three to four different recombinant *P. pastoris* colonies were analyzed from each plasmid to ensure the accuracy of the results. Out of 15 plasmids tested, 6 of them were able to drive expression and secretion of 11D2 scFv as tested by dot blot analysis of culture supernatant using anti *c-myc* antibody for detection (Fig 2A). The ability of these scFvs to bind to WEE antigen was analyzed by ELISA. As shown in Fig. 2B, all *c-myc* positive samples could bind to WEE antigen and gave positive ELISA results. The OD_{405} were comparable in all scFv samples tested, although the ELISA signal was not very strong. Since scFv samples were in methanol containing media, BMMY, we asked if BMMY could suppress reactivity between antigen and antibody A control experiment was set up to measure the activity of 11D2 MAb in the absence or presence of increasing amount of BMMY The results showed that when the amount of BMMY in the sample reached above 90%, ELISA signal was reduced by 60% Whether BMMY inhibited binding of scFv at the same degree as MAb has not been tested Thus, the ELISA results seen in Fig 2B may not reflect the true binding capacity of scFv Rather, it demonstrated the existence of functional scFv producing clones

Sequence analysis of 11D2 scFv

The DNA sequence of 11D2 scFv was analysed from three different productive clones (#5, #20, and #32) and similar results were obtained Representative sequencing data derived from clone #32 is shown in Fig 3 The complementary deter-

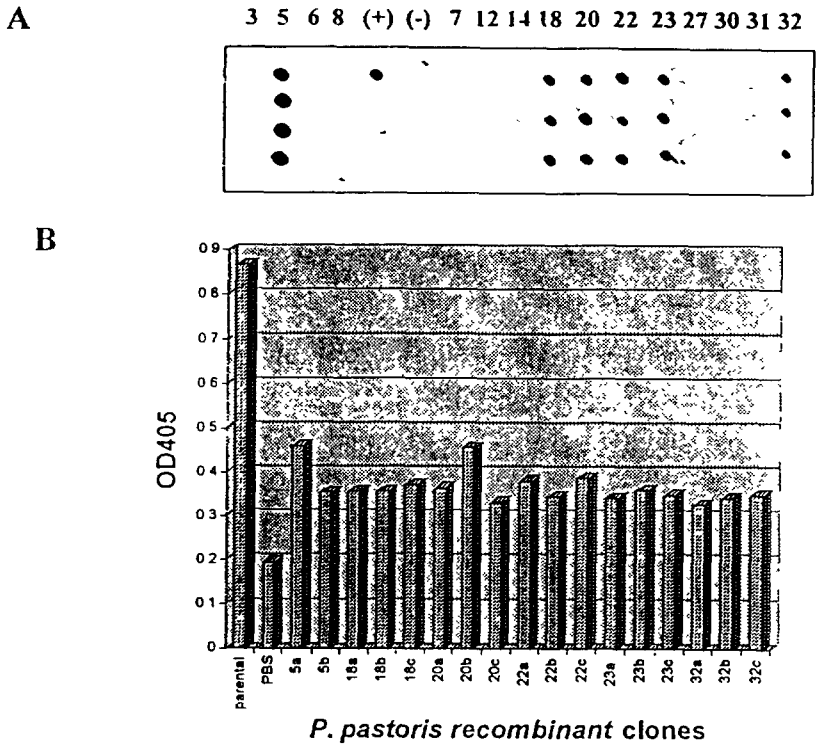

FIG. 2. Analysis of recombinant *P. pastoris* clones derived from various plasmid constructs. The production of 11D2 scFv was analyzed by dot blot analysis (A). Each column represents several yeast isolates derived from the same plasmid The number on top of each column represents the plasmid clone number. A spot of anti-*c-myc* antibody was used as positive control(+) while a recombinant *P. pastoris* transfected with vector only was used as negative control(−). Samples that gave positive results in (A) were tested for WEE antigen binding in ELISA (B).

minant regions (CDR) of V_L and V_H are also indicated. The comparison of 11D2 variable domains to the Kabat database[11] showed that V_H of 11D2 antibody belonged to subgroup IIID and subfamily XIV, while V_L of 11D2 antibody did not belong to any known subgroup or subfamily.

A few base differences were observed among these three clones. A point mutation occurred in $J\kappa$ region of clone #32 (where primer P2 binds), resulting in the change of arginine (R) to glutamine (Q) at residue 107. Also, clone #32 had asparagine (N) at residue 76 while clones #5 and #20 had serine (S). Only a single amino acid difference was found between clones #5 and #20; that is valine (V) at residue 12 of V_H in clone #5 was substituted by methionine (M) in clone #20 (position 135 in Fig 3). All of the amino acid differences were in the framework of variable domains and resulted from a single base difference in the triplet codon. It is thought that these differences may have been introduced during the amplification of DNA by polymerase chain reaction. All the changes did not affect binding of scFv to antigen.

Three of the nonexpressing clones were also sequenced in order to understand the nature of scFv expression. To our surprise, all three of them carried the same V_L gene segment that was distinct from productive 11D2 scFv (sequence not shown), while V_H gene segment remained the same. This different V gene was thought to arise from aberrant message that was coamplified in the primary PCR step (Fig 1) The interference of correct scFv assembly by aberrant messages has also been shown by others.[12]

Characterization and purification of 11D2 scFv

The 11D2 scFvs produced from clones #5, #20, and #32 were analyzed by Western blot analysis using anti-*c-myc* antibody for detection. The results are shown in Fig 4 It is quite interesting that the pattern of positive bands of clones #5 and #20 were different from that of clones #32 Unlike clone #32 that gave a typical 30-kDa protein product, clones #5 and #20 showed multiple bands that were larger than 30 kDa It is not known why but it is thought that different glycosylation of scFv might play a role especially at the positions where amino acid substitution occurred. Although in general, variable domains of immunoglobulins are nonglycosylated in B cells, 11D2 scFv may be different because they are produced by yeast cells that control of post-translational modification may be different from mammalian cells. More experiments will need to be done to prove this hypothesis.

The 11D2 scFv could be successfully purified using immobilized metal affinity chromatography (IMAC) The histidine tag at the C-terminal of scFv could bind to the nickel charged column Once the unbound proteins were washed away, the scFv could be eluted with imidazole For the simplicity in identifying the scFv at 30 kDa on SDS-PAGE, 11D2 scFv produced

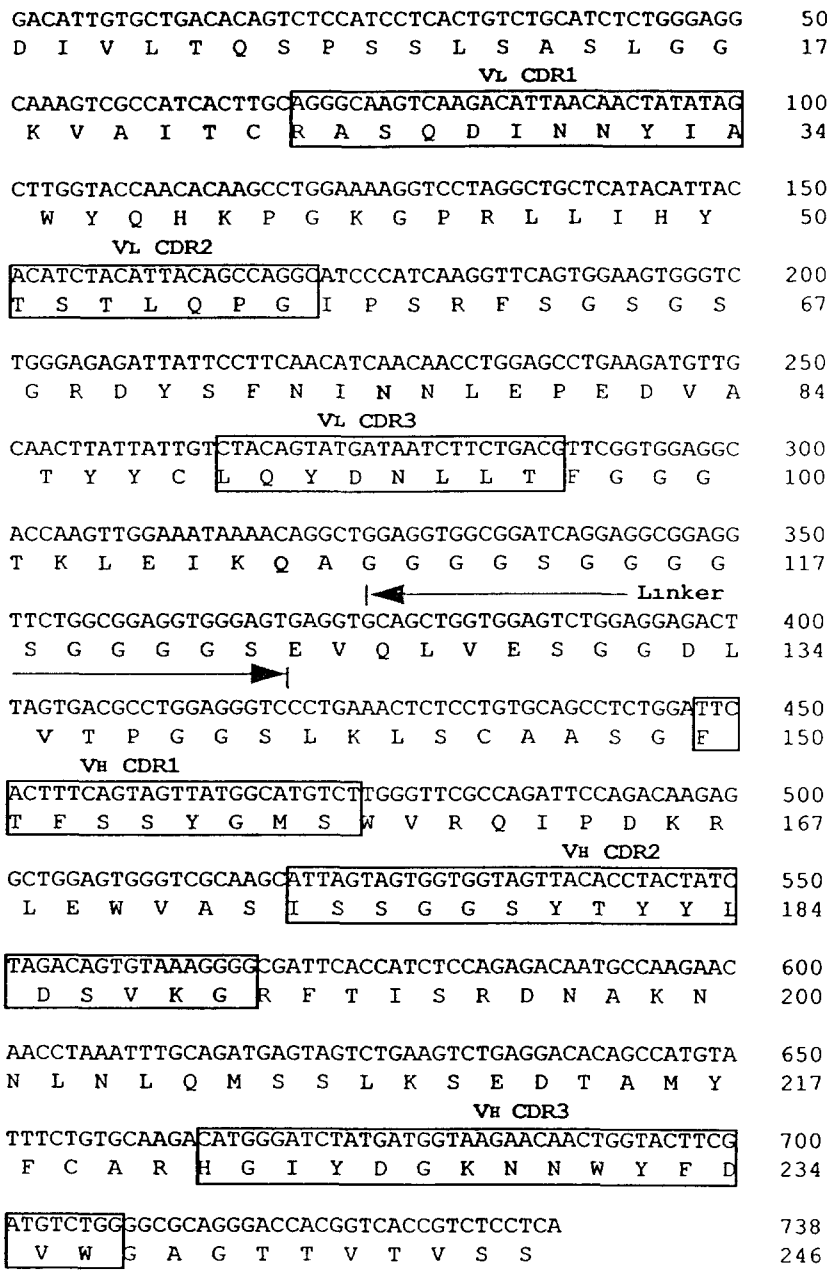

FIG. 3. DNA sequences of 11D2 scFv (clone #32) and its deduced amino acid sequence. The complementary determinant regions were boxed and the linker sequence was shown The number on the right column represented the positions of nucleotide and amino acid of 11D2 scFv Amino acid residues that were different among clones #5, #20, and #32 were shown in bold.

from clone #32 was used as starting material. This particular clone is also designated as P110 As shown in Fig 5, the purified protein has the expected molecular weight of 30 kDa However, they appeared to be more than one band at the very similar molecular weight This was not noticed in the Western blot analysis, probably because the signal of these bands overlapped. Again, it is not known why but we thought that it might reflect the similar post-translational modification among all these 11D2 scFv producing clones

The production yield of 11D2 scFv from P110 in a 250 mL shake flask was about 1 5 mg/L The presence of methanol in the induction medium inhibited the binding of histidine tag to nickel charged column and therefore must be removed by dialysis prior to affinity purification The purified 11D2 scFv was quantitated by comparing band intensity on Coomassie brillant blue stained SDS-PAGE and by dot blot analysis The estimated yield of purified product was 700 μg/L

Binding properties of 11D2 scFv

In addition to ELISA (Fig 2B), the binding of 11D2 scFv to WEE antigen was also demonstrated by Western blot analy-

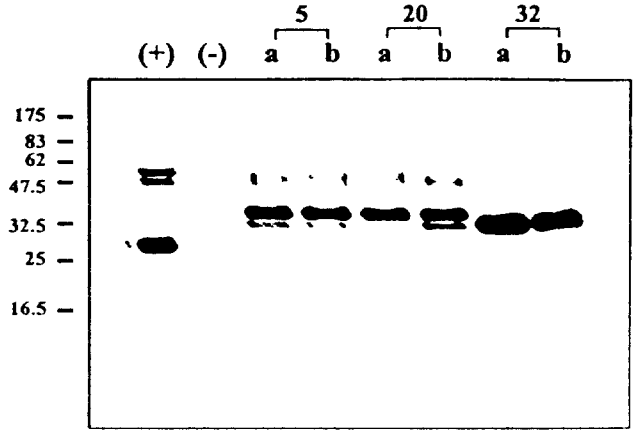

FIG. 4. Western blot analysis of 11D2 scFv produced from different recombinant *P pastoris* clones Two representative clones (a and b) derived from plasmids #5, #20, and #32 were detected by anti-*c-myc* antibody Positive control anti-c-myc antibody, negative control: recombinant *P pastoris* transfected with vector only Molecular weight marker is shown on the left

sis. Antigen was electrophoresed, transferred to the membrane and probed with 11D2 scFv Similar to its parental MAb, 11D2 scFv could bind to E1 glycoprotein of WEE virus and gave positive band at around 55 kDa (Fig 6)

The reduced binding affinity of scFv compared to the parental MAb is generally a concern in antibody engineering To determine the reactivity of 11D2 scFv with WEE antigen compared to the parental MAb, an ELISA was conducted in which various doses of scFv or MAb were titrated against a fixed concentration of WEE antigen coated on the microplate solid phase. As shown in Fig. 7, 11D2 scFv retained good antigen-binding capacity. When calculated at equimolar amount, ELISA signal produced by scFv was approximately half of that of MAb. Although the binding affinity of scFv may not be directly compared to the MAb due to the difference in valency and binding avidity, the results shown here suggested that 11D2

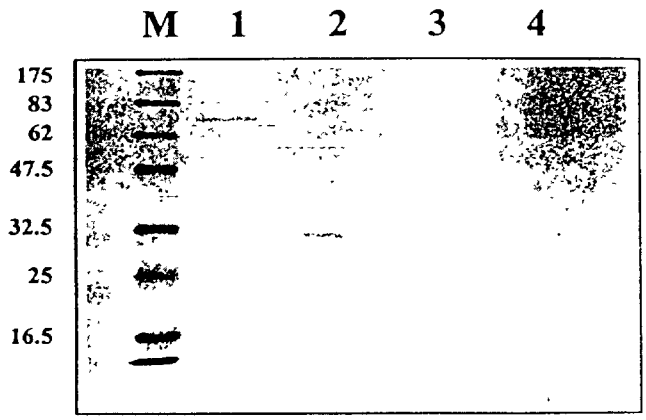

FIG. 5. Purification of 11D2 scFv (clone #32a, P110) by IMAC Coomassie brilliant blue stained SDS-PAGE showed the purified product at 30 kDa M molecular weight marker, lane 1 unpurified sample, lanes 2–3 eluted fractions after IMAC

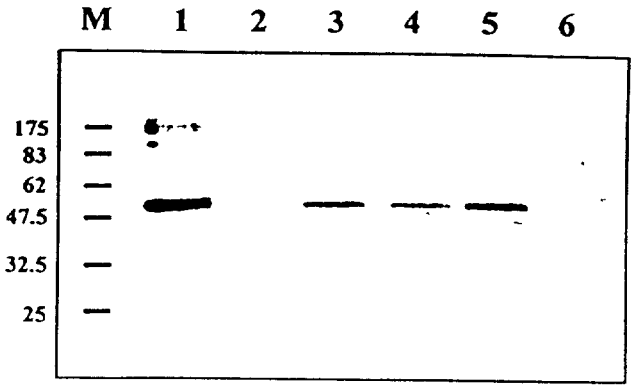

FIG. 6. Binding of 11D2 scFv to WEE antigen was shown by Western blot analysis. WEE antigen was electrophoresed on 10% SDS-PAGE, transblotted and reacted to 11D2 monoclonal antibodies (lane 1) or 11D2 scFv (clone #5, lane 3, clone #20, lane 4, clone #32, lane 5) Antigen strips reacted to anti-*c-myc* antibody (lane 2) or 5B4D6 monoclonal antibody (lane 6) were used as negative controls

scFv retains a reasonably good binding affinity given that the monovalent scFv should be ideally compared with Fab 11D2

DISCUSSION

The role of antibody in the treatment of viral infection has been successfully demonstrated in animal models,[3] thus, it has potential use in human Administration of mouse MAb into hu-

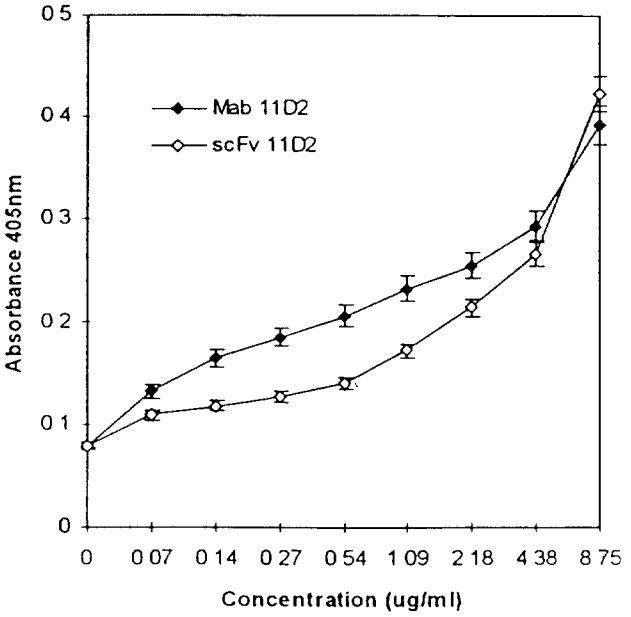

FIG. 7. ELISA of 11D2 scFv compared to parental monoclonal antibody WEE antigen coated plate was reacted to various concentration of antibodies followed by anti-c-myc HRPO-goat anti-mouse immunoglobulins and signal deteceid by the addition of TMB Each data point represents mean and standard deviation of triplicated samples

man, however, creates undesirable side effects such as human anti-mouse antibody (HAMA) response.[13] The use of a smaller size scFv has some advantages over MAb due to the reduced side effects and improved tissue penetration.[14] Single chain Fv antibody fragment can also be customized to suit the downstream applications for immunotherapy and immunodiagnosis by developing bifunctional fusion proteins.

In the present study, 11D2 scFv specific against WEE virus was developed for future study of antibody treatment in animal model of WEE infection and for development of an assay for WEE antigen detection. The 11D2 scFv specific against E1 glycoprotein of WEE virus has been successfully engineered and expressed in *P pastoris* Binding analysis of 11D2 scFv showed that it retained binding property of the parental MAb. In our study, we have decided to generate our scFv from a yeast expression system rather than from a more laborious and time-consuming phage display technology We predicted that the functional messenger RNA in hybridoma should be present in abundant and screening of large number of recombinant clones may not be required. Also, we have made our scFv in the V_L/V_H rather than in the V_H/V_L arrangement because it has been shown that some scFv might not express in the latter orientation.[15] Production of scFv in *P pastoris* is also simple, inexpensive and expected to give a high yield.

Despite the advantages of scFv in *in vivo* application, the binding affinity of this genetically engineered molecule is compromised Although the 11D2 scFv shows lower binding in ELISA compared to parental MAb, further work with Fab is needed to explore if this is due to the bivalency and avidity. Alternatively, to improve or enhance binding affinity of scFv, site directed mutagenesis may be performed and the strong binders may be selected.[16,17] Similar approach has also been used to obtain the high producer clones [18] In the system we described here, selection for high producers may also be done by screening for a small percentage of clones that carry multiple insertion of gene expression cassettes by using high dose of Zeocin. Furthermore, scFv production in yeast can be significantly increased when the culture was set up in the fermenter where optimal growth parameters can be tightly controlled.

The successful production of scFv against WEE virus opens a new avenue to the immunological studies of WEE infections The derivatives of scFv against WEE may be now generated, such as bifunctional recombinant antibodies that would allow their usages as therapeutic and diagnostic agents A bivalent monospecific scFv could be produced in form of diabody[19,20] or by joining of two scFv with a linker[21] if bivalent antibody is required [22] A bispecific single-chain antibody specific against immune cells and viral infected cells can be made for immunotargeting.[23] The DNA vaccine made of scFv intrabody can also be explored.[24,25] For immunodiagnosis, a scFv fused with biotin mimic tag could be produced for *in vitro* ultrasensitive ELISA detection of WEE antigen, an assay that is superior to the use of conventional immunological reagents. Finally, the knowledge gained in these studies can also be applied to the studies of other closely related infections such as VEE and Eastern equine encephalitis (EEE).

ACKNOWLEDGMENTS

This work was supported by the DND-NSERC. M.R S thanks MRC and Biomira, Inc for the University-Industry Chair support.

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Received for publication April 19, 1999 Accepted for publication June 9, 1999.

#519234
CA022368