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Development and Characterization of a Novel Fusion Protein Composed of a Human IgG1 Heavy Chain Constant Region and a Single-Chain Fragment Variable Antibody against Venezuelan Equine Encephalitis Virus

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Murine monoclonal antibody 1A4A1 has been shown to recognize a conserved neutralizing epitope of envelope glycoprotein E2 of Venezuelan equine encephalitis virus. It is a potential candidate for development of a second generation antibody for both immunodiagnosis and immunotherapy. In order to minimize the immunogenicity of murine antibodies and to confer human immune effector functions on murine antibodies, a recombinant gene fusion was constructed. It encoded a human IgG1 heavy chain constant region and a single-chain fragment variable antibody of 1A4A1. After expression in bacteria as inclusion bodies, the recombinant antibody was purified and refolded *in vitro*. The recombinant soluble antibody was demonstrated to retain high antigen-binding affinity to Venezuelan equine encephalitis virus and to possess some human IgG crystallizable fragment domain functions, such as recognition by protein G and human complement C1q binding. On non-reducing and reducing gel electrophoresis analysis of proteolytic fragments of the recombinant antbody, disulfide bond formation was found in the hinge region of the antibody. From these data, it was concluded that the recombinant antibody was capable of antigen recognition, and retained several functional activities. This work forms the basis for characterization of the recombinant antibody as to efficacy *in vivo*.

Key words: antibody engineering, characterization, chimeric antibody, Venezuelan equine encephalitis virus.

Abbreviations. Ab, antibody; ABTS, 2,2′-azino-di-(3-ethyl-benzthiazoline-sulfonic acid) diammonium salt, ADCC, Ab-dependent cellular cytotoxicity, CH, heavy chain constant region, ELISA, enzyme-linked immunosorbent assay, Fab, antigen-binding fragment, $(Fab')_2$, fragment composed of two Fab units and a hinge region, Fc, crystallizable fragment, HRP, horseradish peroxidase; IMAC, immobilized metal affinity chromatography, IPTG, isopropyl β-D-thiogalactopyranoside, MAb, monoclonal antibody, NTA, nitrilotriacetic acid, PBS, phosphate-buffered saline, PBST, PBS containing 0 1% Tween-20, ScFv, single-chain fragment variable, VEE, Venezuelan equine encephalitis virus, VL, light chain variable region, VH, heavy chain variable region, WEE, Western equine encephalitis virus

Venezuelan equine encephalitis virus (VEE), a member of the alphavirus genus of the family *Togaviridae*, is an important pathogen for epidemics in humans, and epizootics in rodents, horses, donkeys, and mules in the Americas (*1*) VEE causes a spectrum of human diseases ranging from inapparent infection to acute encephalitis (*2*). Neurological disease appears in four to 14% of cases (*3*). The incidence of human infection during equine epizootics could be up to 30% (*4*). VEE is a potential biological warfare agent of concern, due to its high infectivity rate or aerosol transmissibility However, there are no antiviral drugs available that are effective against VEE Although live-attenuated and inactivated vaccines against VEE have been developed, these products are far from satisfactory. Approximately 20% of live-attenuated TC-83 vaccine recipients fail to develop neutralizing antibodies (Abs), while another 20% exhibit reactogenicity (*5*). A formaldehyde-inactivated vaccine, C-84, is well tolerated, but requires multiple immunization and periodic booster, and fails to provide protection against aerosol challenge in some rodent models (*6*)

VEE complexes comprise a group of antigenically related, but distinct viruses divided into six subtypes (*7*) VEE virions are composed of an icosahedral nucleocapsid, which is surrounded by a lipid envelope containing two structural glycoproteins, E1 and E2 (*8*) Epitopes on E1 and E2 are the targets of neutralizing Abs Studies have shown that the viral neutralizing epitopes are mainly located on the E2 protein, and that the $E2^C$ epitope appears to be the hub of the neutralization

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epitopes (*9*). Monoclonal antibody (MAb) 1A1A4 is specific for $E2^C$. This MAb has been shown to be efficient in protecting animals from a lethal peripheral challenge with virulent VEE (*9*).

Murine MAbs, however, have serious disadvantages as therapeutic agents in humans (*10*, *11*). They induce human anti-mouse Ab responses Re-treatment may result in rapid clearance of the murine MAbs and anaphylaxis Limitations to the use of murine MAbs for clinical applications led to the development of single-chain fragment variable (scFv) Abs (*12*). ScFv Abs have several advantages compared with the mouse parental MAbs from which they are generated They generally retain the same specificity and similar affinity to antigens (*13*), and exhibit decreased immunogenicity as compared with the parental mouse MAbs (*14*) Furthermore, scFv Abs can be produced economically and in a short time in bacteria or yeast (*15*, *16*), and can be manipulated by genetic engineering to form novel proteins by fusion with other molecules, such as metal-binding proteins (*17*), cytokines (*18*), toxins (*19*), and T cell receptors (*20*).

Our laboratory has cloned and characterized several scFv Abs against genus alphavirus, VEE or Western equine encephalitis virus (WEE) (21–23) An anti-VEE A116 scFv Ab was cloned from 1A4A1 MAb (*24*). However, *in vitro* binding assays demonstrated that A116 scFv Ab had low binding affinity to VEE, as compared to the parental MAb. Sequence analysis of A116 revealed that three bases were missing in conserved framework-1 of the light chain variable region (VL) PCR-based site directed mutagenesis was used to reintroduce the three missing bases, resulting in a repaired A116 scFv Ab, designated mA116 scFv Ab (Alvi *et al* , submitted). This repaired scFv showed affinity to VEE comparable to that of the parental 1A4A1 MAb.

Antiviral immunity is complex, with several factors being involved, such as the Ab response, cell-mediated immunity, and induction of interferon. The Ab response to viruses includes not only neutralization of infectivity for susceptible host cells, but also host effector functions such as complement-mediated lysis of infected host cells and opsonization. The host effector functions of Abs are attributable to the crystallizable fragment (Fc) regions (*10*) ScFv Abs lack a Fc region, and thus lack effector functions.

In this study, we have genetically joined a truncated human IgG1 heavy chain constant region (CH) to anti-VEE mA116 scFv Ab, resulting in clone mA116huFc Ab. The goal of this work was to confer some of the human-associated effector functions on the scFv Ab without increasing the immunogenicity in humans. The mA116huFc Ab gene was expressed in *E. coli*, and the resulting product purified refolded *in vitro*, and characterized The results revealed that mA116huFc Ab retained antigen-binding affinity to VEE and possessed some human Ig G1 Fc domain functions, such as recognition by protein G and human C1q binding

MATERIALS AND METHODS

Construction of pRSmA116huFc—The mA116 scFv Ab gene was originally derived from the A116 scFv Ab gene, which was subsequently replaced by site-directed

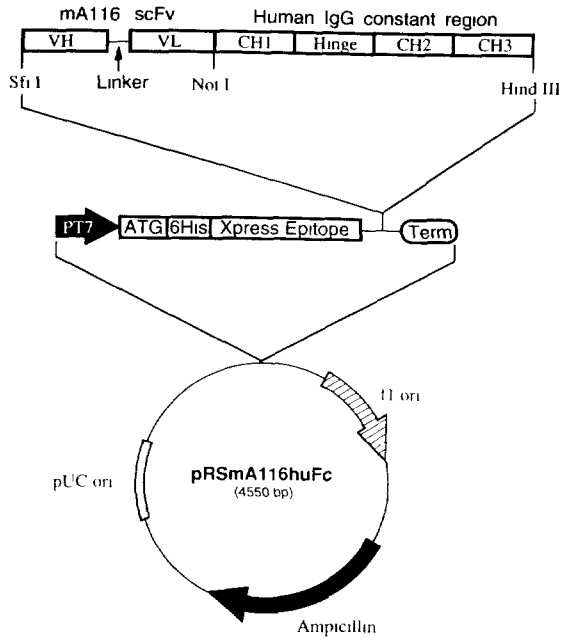

Fig 1 **pRSmA116huFc construct.** The mA116 scFv Ab gene was cloned into the *Sfi*I and *Not*I sites of pRS10B5huFc to replace the 10B5 scFv Ab gene as described under "MATERIALS AND METHODS"

mutagenesis (Alvi *et al* , submitted). The mA116 scFv Ab DNA fragment was separated from vector pCANTAB 5E by restriction enzyme digestion with Sfi I/Not I, followed by gel electrophoresis and purification with a gel extraction kit (Qiagen, Mississauga, ON) The fragment was subcloned into the *Sfi*I/*Not*I site of the pRS10B5huFc expression vector (*22*), replacing the anti-WEE 10B5 scFv Ab gene The fragment was inserted in-frame with the sequence encoding 36 kDa human IgG1 CH, just before the CH1 domain A start codon, followed by a fragment containing a 6 His tag and X-press epitope, was added at the 5′ end for ease of purification and detection. The construct, pRSmA116huFc, was shown in Fig 1. The recombinant plasmid DNA was transferred into *E coli* BL-21 (DE3) pLys S competent cells containing a genomic source of T7 RNA polymerase under *lacUV5* promoter control (Invitrogen, Carlsbad, CA). The recombinant plasmid, containing the mA116 scFv Ab gene insert, was confirmed by restriction digestion and DNA sequencing Six primers for DNA sequencing were made with an Oligo 1,000 DNA synthesizer (Beckman Instruments, Fullerton, CA), and were as follows: SC-1 (5′-CATCATCATCATCATCAT-3′), SC-9 (5′-CAGCAGCCAACTCAGCTT-3′), SC-13 (5′-ACTCCTGACATCCTGTCG-3′), HL-2 (5′-TCTAACGGGACCAGAGACAAC-3′), HL-3 (5′-GGTAAGTTCAGGGACAGG-3′), and HU-4 (5′-TGCAAGGCCAGTCAGGATGTG-3′) The sequencing reactions were performed with a Big Dye Terminator™ cycle sequencing kit (PE Biosystems, Mississauga, ON). The reaction products were purified on Centri-Sep™ columns (Princeton Separations, Adelphia, NJ), and then applied to an ABI 310 genetic analyzer system (Applied Biosystem, Fullerton, CA). Sequences were assembled and analyzed using Lasegene DNA software (DNA Star, Madison, WI)

Expression, Purification, and Refolding of the Recombinant mA116huFc Ab—The recombinant protein expressed in *E. coli* as inclusion bodies was solubilized, purified, and refolded, as previously described, with minor modifications (*22*). Briefly, after induction for 3 h with isopropyl β-D-thiogalactopyranoside (IPTG), *E coli* transformants harboring the pRSmA116huFc expression plasmid were harvested by centrifugation Each cell pellet was resuspended in 5 mM borate sodium, pH 9 3, and 4 M urea, and then a cell lysate was prepared by sonication (three cycles of 10 s, amplitude, 10 μm, 15 s cooling on ice), using a MSE Soniprep 150-probe sonicator (Wolf Laboratories, Pocklington, UK) The sonicates were centrifuged (13,000 ×*g* for 10 min), and the pellets were resuspended in 5 mM borate sodium, pH 9 3, 8 mM urea, and 100 mM sodium chloride. Purification of the recombinant protein was performed on Talon™ metal affinity resin (Clontech, Palo Alto, CA). A solution comprising 5 mM borate sodium, pH 9 3, 8 M urea, and 100 mM sodium chloride was used as the wash buffer. Bound fractions were eluted with 10–250 mM imidazole, and then 1 M arginine (final concentration) was added as a cosolvent, to facilitate the correct folding of the protein molecules. The recombinant protein (~100 μg/ml) was refolded via removal of 8 M urea by dialysis against 5 mM borate sodium, pH 9 3, and 1 M arginine at 4°C with air oxidation for 70 h; the cosolvent was then removed by dialysis against 5 mM borate sodium, pH 9 3, at 4°C for 24 h The purity was checked by SDS-PAGE and Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Mississauga, ON) staining after concentration by ultrafiltration using a Centricon™ YM-30 (Millipore Corp , Bedford, MA).

SDS-PAGE and Western Blot Analysis—Protein samples were electrophoresed on 10% SDS-PAGE gels, using a Mini-PROTEAN II apparatus (Bio-Rad Laboratories) The bands were visualized by Coomassie Blue staining

The separated proteins were also transferred to Immunobilon-P membranes (Millipore Corp) by use of a Western blot semi-dry transfer apparatus (Bio-Rad Laboratories) with Towbin buffer (25 mM Tris-HCl, pH 8 3, 192 mM glycine, and 20% methanol) Unreacted sites were blocked with blocking buffer [3% non-fat skim milk in phosphate-buffered saline (PBS)] Blots were washed three times for 5 min with PBS containing 0 1% Tween-20 (PBST) Some blots were then incubated for 1 h at room temperature with a 1 5,000 dilution of mouse anti-Xpress Ab (Invitrogen). These blots were then washed three times for 5 min with PBST and incubated for 1 h at room temperature with a 1 3,000 dilution of horseradish peroxidase (HRP)–conjugated goat anti-mouse Ig (Caltag Laboratories, Burlingame, CA) in PBST. Other blots were incubated directly with either a 1 2,000 dilution of HRP-conjugated donkey anti-human Ig (Jackson ImmunoResearch Laboratories, West Grove, PA) or a 1:1,000 dilution of HRP-conjugated Ni-nitrilotriacetic acid (NTA) (Qiagen) at room temperature for 1 h. After three washes for 5 min with PBST, and 2 washes for 2 min with deionized water, specific binding was detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Baie d'Urfe', QC)

Inter-Chain Disulfide Bond Formation Assay—MA116-huFc Ab, 1A4A1 MAb, and mA116 scFv Ab were electrophoresed on 10% SDS-PAGE gels under reducing (5% 2-mercaptoethanol) or non-reducing conditions. The bands were visualized by Coomassie Blue staining. In addition, mA116-huFc Ab was digested with papain and pepsin (Sigma, Oakville, ON), respectively, by the method of Andrew and Titus (*25*) Proteolytic fragments of the Ab were analyzed by the above-described SDS-PAGE method

Enzyme-Linked Immunosorbent Assay (ELISA)—The reactivity of mA116huFc Ab to VEE antigen was determined by ELISA. Nunc Maxisorp™ flat-bottomed 96-well plates (Invitrogen, Burlington, ON) were coated overnight at 4°C with live-attenuated, whole VEE (strain TC-83) at a fixed concentration of 10 μg/ml or various concentrations, 0 01–100 μg/ml, in carbonate bicarbonate buffer, pH 9 6, containing 0.02% sodium azide. The plates were washed five times with PBST and then blocked twice in 2% BSA for 1 h at 37°C After 5 washes with PBST, the plates were incubated for 1 h at 37 °C with various concentrations, 0 01–100 μg/ml, or a fixed concentration of 10 μg/ml of a purified Ab (mA116huFc Ab, 1A4A1 MAb, or mA116 scFv Ab) diluted in PBST Following five washes with PBST, the plates were incubated for 1 h at 37°C with HRP-conjugated Ab diluted 1:3,000 in PBST [HRP-donkey anti-human Ig for detection of mA116huFc Ab, HRP-goat anti-mouse Ig for 1A4A1 MAb, or HRP-anti-E tag (Amersham Pharmacia Biotech) for mA116 scFv Ab]. Finally, the plates were washed five times with PBST and developed for 30 min at room temperature with a substrate consisting of 2,2′-azino-di-(3-ethyl-benzthiazoline-sulfonic acid) diammonium salt (ABTS) and hydrogen peroxidate (Kirkegaard and Perry Laboratories, Gathersburg, MD). The reactions were examined as to the absorbance at 405 nm with a microplate autoreader (Molecular Devices, Sunnyvale, CA).

Protein G Binding Assay—The assay for Protein G-binding of the purified recombinant mA116huFc Ab was modified based on the immunoprecipitation protocol (*26*). MA116huFc Ab, 1A4A1 MAb, or mA116 scFv Ab, was incubated with washed protein G agarose (Invitrogen) in radioimmunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 0.1% SDS, and 1% Triton X-100) After 1 h incubation with rocking at room temperature, the agarose was collected by centrifugation at 13,000 ×*g* for 1 min at room temperature. The pellet was washed three times with radioimmunoprecipitation buffer and then centrifuged at 13,000 ×*g* for 1 min. The protein G binding complexes were resuspended in laemmli sample buffer containing 5% 2-mercaptoethanol and then heated in boiling water for 10 min After centrifugation, the supernatants were run on a 10% SDS-PAGE gel, followed by Coomassie Blue staining.

Human Complement C1q Binding Assay—The capacity of purified recombinant mA116huFc Ab to bind to human complement C1q was assayed using a circulating immune complex-C1q test kit (Quidel Corp , San Diego, CA) in accordance with the manufacturer's instructions In brief, eight-well strips coated with human C1q protein were rehydrated with wash buffer (0 05% Tween-20 and 0 01% thimerosal in PBS). The purified recombinant mA116huFc Ab, mA116 scFv Ab, a human IgG1 (positive control) (Sigma), or a human IgG4 (negative control) (Sigma) was serially diluted from 15 nM with the wash buffer, and then incubated with the strips for 1 h at room temperature. After five washes with the wash buffer,

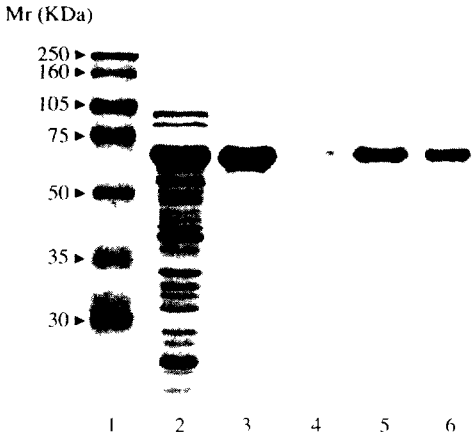

Fig 2 **SDS-PAGE analysis of samples obtained on the purification of mA116huFc Ab.** Samples were resolved on a 10% polyacrylamide gel and then stained with Coomassie Blue Lane 1, molecular weight markers, 2, bacterial lysate, 3 solubilized protein fraction, 4, column flow-through fraction, 5, 10 and 20 mM imidazole eluates, 6, final protein preparation

HRP-conjugated goat anti-human Ab was added to each well and the strips were incubated at room temperature for 30 min, after which they were washed five times with the wash buffer and then incubated with an ABTS substrate solution for 30 min. To stop the enzymatic reaction, a stop solution containing 250 mM oxalic acid was added to the wells, and then the absorbance was measured at 405 nm with the microplate autoreader

RESULTS

Construction, Expression, and Purification—The pRS10B5huFc gene construct, in which the anti-WEE virus 10B5 scFv Ab gene was linked with a truncated human IgG1 CH (*22*), and the anti-VEE mA116 scFv Ab gene in pCANTAB5E were used as source materials to create a mA116huFc Ab gene construct. The 10B5 scFv Ab gene of pRS10B5huFc was replaced by the mA116 scFv Ab gene The resulting plasmid, designated as pRSmA116huFc, contained the mA116 scFv Ab gene, arranged in a heavy chain variable region (VH)-VL orientation via a $(Gly_4Ser)_3$ linker. This was fused to a truncated human IgG1 CH under the control of the T7 promoter (Fig. 1) In addition, there was a sequence with a molecular weight of 6 kDa, including a 6His tag for immobilized metal affinity chromatography (IMAC) purification and a Xpress epitope for detection with anti-Xpress Ab, fused upstream of the VH. The mA116 scFv Ab gene was 723 bp in length, encoding 241 residues with a molecular weight of 26 kDa. The first 66 bases of the CH1 domain were missing from the human IgG1 CH, as the parental IgG1 vector was originally designed for cloning CH domains from antigen-binding fragments (Fabs) The encoded CH protein comprised 322 residues with a molecular weight of 36 kDa The theoretical molecular weight of the whole fusion protein, including the 6His tag and X-press epitope, was about 68 kDa.

The mA116huFc Ab was expressed in *E. coli* BL-21 cells and purified by IMAC. SDS-PAGE demonstrated

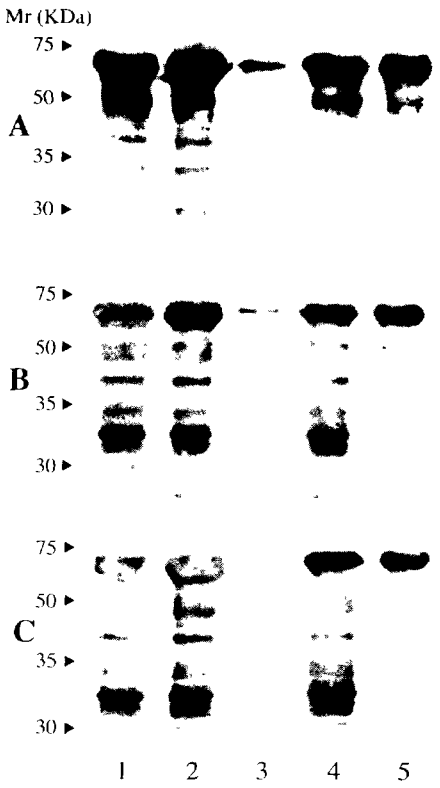

Fig 3 **Western blotting analysis of samples obtained on pu fication of mA116huFc Ab.** Samples were resolved by SD PAGE, transferred to Immunobilon-P membranes, and then prob with (A) HRP-conjugated anti-human Ig, (B) HRP-conjugated N NTA, or (C) anti-Xpress Ab, followed by HRP-conjugated an mouse Ig Lane 1, bacterial lysate, 2, solubilized protein fraction, column flow-through fraction, 4, 10 and 20 mM imidazole eluates, final protein preparation

that there was a large amount of protein in the bacteri lysate of molecular weight ~70 kDa, corresponding to th predicted size (68 kDa) of mA116huFc Ab (Fig. 2, lane 2 After centrifugation of the lysate, and dissolution of th pellet with a denaturing agent, many of the protei were removed from the lysate (Fig. 2, lane 3). The solub lized protein fraction was incubated with metal affini resin, and then loaded on to an empty column. After tho ough washing, the bound fractions were eluted with a imidazole gradient (10 to 250 mM) in elution buffer. Th 10 and 20 mM imidazole eluates each gave a major ban at ~70 kDa accompanied by some weak bands (Fig lane 5), whereas for the eluates with imidazole concen trations of 50 mM and greater, only the 70 kDa band wa observed (Fig 2, lane 6). With this elution protocol, th expressed protein could be purified to >90% The yield purified recombinant mA116huFc Ab was about 30 m per liter of culture medium

Biochemical Characterization—To confirm the presen of intact, expressed mA116huFc Ab, a series of Weste blotting experiments was performed, in which the 70 kD protein was detected with HRP-conjugated anti-huma Ig, HRP-conjugated-Ni-NTA, and anti-Xpress epitop followed by HRP-conjugated-anti-mouse Ig, respectivel As shown in Fig 3, the 70 kDa protein was recognized

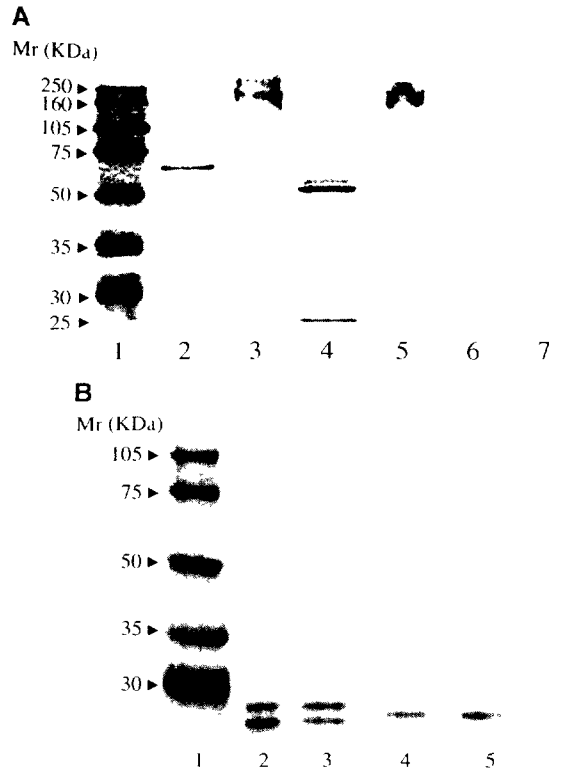

Fig 4 **Disulfide bond formation assay.** A Abs were resolved by SDS-PAGE under reducing and non-reducing conditions, and then stained with Commassie Blue Molecular weight markers (lane 1), mA116huFc Ab under reducing (lane 2) and non-reducing (lane 3) conditions, 1A4A1 MAb under reducing (lane 4) and non-reducing (lane 5) conditions, and mA116 scFv Ab under reducing (lane 6) and non-reducing (lane 7) conditions B SDS-PAGE analysis of proteolytic fragments of mA116huFc Ab digested with papain and pepsin, respectively Molecular weight markers (lane 1), proteolytic fragments of mA116huFc Ab with papain under non-reducing (lane 2) and reducing (lane 3) conditions, and proteolytic fragments of mA116huFc Ab with pepsin under non-reducing (lane 4) and reducing (lane 5) conditions

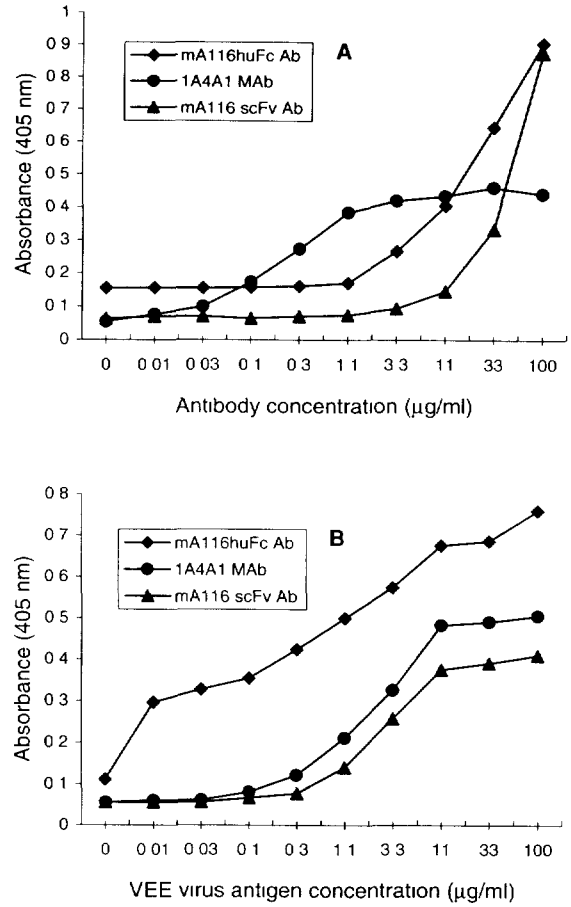

Fig 5 **VEE antigen binding assaying by ELISA.** A Various concentrations of Abs were added to a 96-well plate coated with 10 μg/ml of VEE B 10 μg/ml of Abs was added to a 96-well plate coated with various concentrations of VEE. Binding was detected with HRP-conjugated anti-human Ig, anti-mouse Ig, and anti-E-tag Ab, followed by an ABTS solution Each point represents the mean of four determinations

Western blots by all three probes The HRP-conjugated Ni-NTA and anti-Xpress Ab also detected a 32 kDa fragment in the crude fractions, however, this fragment was not present in the purified fraction (Fig. 3, B and C, lane 5).

The hinge region of an Ab is responsible for disulfide bond formation between two identical Ab heavy chains Since mA116huFc Ab contained an intact hinge region, inter-chain disulfide bond formation was examined by comparing the Ab protein under reducing and non-reducing conditions on SDS-PAGE (Fig. 4A). Under reducing conditions (addition of 5% 2-mercaptoethanol), 1A4A1 MAb appeared as two bands, corresponding to molecular weights of 50 and 25 kDa, representing heavy and light chains, respectively (Fig 4A, lane 4) Under non-reducing conditions, 1A4A1 MAb appeared as a high molecule weight aggregate (Fig 4A, lane 5) Under reducing conditions, mA116huFc Ab migrated as one band corresponding to a molecular weight of ~70 kDa and, under non-reducing conditions, as a high molecule weight aggregate (Fig 4A, lanes 2 and 3) As expected, mA116 scFv Ab migrated as only one band corresponding to a molecular weight of ~30 kDa under both reducing and non-reducing conditions (Fig. 4A, lanes 6 and 7).

In order to confirm the inter-chain disulfide bond formation in the hinge region of mA116huFc Ab, the Ab was digested with papain and pepsin, respectively, and then subjected to SDS-PAGE under non-reducing and reducing conditions. As we know, papain can specifically cleave on the amino-terminal side of a inter-chain disulfide bridge in the hinge region of an IgG molecule, yielding a monovalent Fab, the remaining Fc fragment (around 27 kDa) and some other smaller fragments (*25*) Pepsin cleaves on the carboxyl-terminal side of the same bridge, generating a bivalent fragment composed of two Fab units plus the hinge region [$(Fab')_2$], and some small Fc fragments (*25*). As shown on Fig. 4B, papain-digested mA116huFc Ab appeared as a band corresponding to 40 kDa, corresponding in size to monovalent mA116scFv plus a CH1 domain, accompanied by several small fragments under both non-reducing and reducing conditions (lanes 2 and 3). Pepsin-digested mA116huFc Ab gave a major band corresponding to a molecular weight of ~90

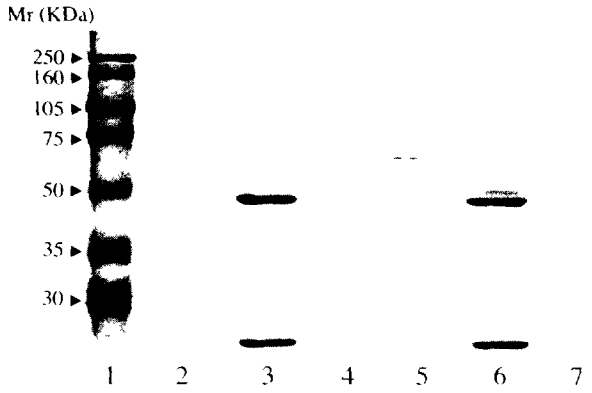

Fig 6 **Protein G binding assay.** Abs were incubated with protein G agarose and then precipitated by centrifugation The pellets were subjected to SDS-PAGE with Coomassie Blue staining Lane 1, molecular weight markers, 2, mA116huFc Ab only, 3, 1A4A1 MAb only, 4, mA116 scFv Ab only, 5, mA116huFc Ab precipitated with protein G, 6, 1A4A1 MAb precipitated with protein G, 7, mA116 scFv Ab precipitated with protein G

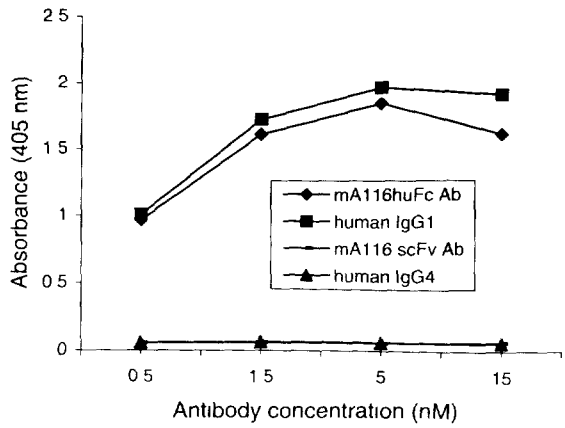

Fig 7 **C1q binding assay.** Various concentrations of Abs were added to strips coated with human C1q protein Binding was detected with HRP-conjugated anti-human IgG, followed by an ABTS solution Each point represents the mean of four determinations

kDa, corresponding in size to bivalent mA116 scFv plus a CH1 domain and hinge region, and a faint band of ~45 kDa, accompanied by several small fragments under non-reducing conditions (lane 4) But under reducing conditions, only a band corresponding to ~45 kDa was observed besides several small fragments (lane 5)

Binding Properties to VEE Antigen—Initially, mA116 huFc Ab was confirmed to bind to E2 protein of VEE, consistent with the specificity of the parental 1A4A1 MAb, on Western blotting (data not shown). The immunoreactivity of mA116huFc Ab toward VEE antigen was further examined by ELISA. When the plates were coated with a fixed concentration of inactivated VEE (10 μg/ml), mA116huFc Ab bound to VEE in a dose-dependent manner, similar to the binding to VEE of the parental 1A4A1 MAb and mA116 scFv Ab (Fig 5A). An additional ELISA test was performed in which a concentration gradient of VEE was titrated against a fixed concentration of Abs (10 μg/ml). A similar dose-response relationship was observed (Fig. 5B)

Protein G and C1q Binding—Although 22 residues were missing from the CH1 domain of mA116huFc Ab, the CH2 and CH3 domains were intact The CH2 and CH3 domains are believed to be associated with essential features of CH of the Abs, such as binding to proteins G and A, and retention of binding sites essential for effector functions. To determine the affinity of binding of mA116 huFc Ab to protein G, mA116huFc Ab was incubated with protein G agarose and then, after thorough washing and centrifugation, analyzed by SDS-PAGE As showed in Fig. 6, mA116huFc Ab, like parental 1A4A1 MAb, efficiently bound to protein G agarose, while mA116 scFv Ab did not To determine whether mA116huFc Ab could initiate complement activation, one of the important effector functions, an ELISA was performed, in which human C1q was coated on the strips. MA116huFc Ab like a human IgG1 could efficiently bind to human C1q in a dose-dependent manner, while mA116 scFv Ab and a human IgG4 did not bind to human C1q (Fig. 7)

DISCUSSION

The *E. coli* expression system has been commonly applied for recombinant immunoglobulin fragments since with this system it is possible to produce proteins in large quantities (*27*). However, the expression of heterologous proteins in bacteria has been hampered by the fact that the large amount of protein produced in the reducing environment of the cytoplasm of bacteria leads to the formation of insoluble inclusion bodies, which contain unfolded protein. It is necessary to solubilize and then refold the protein into the structurally correct form before it becomes functionally active. The inclusion bodies can be easily dissolved with denaturants, such as guanidine-HCl or urea, and then can be used for the refolding procedure, which promotes disulphide bond formation and adoption of the appropriate three-dimensional shape (*28*) Disulphide bond formation can be induced by simple air oxidation (*29*) or a mixture of reduced and oxidized thiol groups (*30*) Refolding can be promoted by artificial chaperones (*31*) or a stabilizing co-solvent, such as arginine, which can facilitate the formation of the native structure of a protein during refolding (*32*) However, there is no universal refolding protocol suitable for every antibody or antibody fragment since each of them is unique In our study, we solubilized the inclusion bodies with the denaturant 8 M urea. After purification, we allowed disulphide bond formation through air oxidation and refolded mA116huFc Ab in the presence of arginine.

The results of Western blot analysis confirmed that the refolded recombinant protein was intact, with a molecular weight of ~70 kDa Comparison of the SDS-PAGE electrophoretic patterns of mA116huFc Ab obtained under reducing and non-reducing conditions was conducted As expected, mA116huFc Ab migrated as a high molecular weight aggregate under non-reducing conditions, as did the parental 1A4A1 MAb. Under reducing conditions, mA116huFc Ab migrated as one band corresponding to a molecular weight of ~70 kDa, correspond-

ing in size to the monovalent mA116huFc Ab. These results suggested that there was inter-chain disulfide bond formation in the mA116huFc Ab Proteolytic digestion of mA116huFc Ab with papain and pepsin, and analysis of the fragments by SDS-PAGE under both non-reducing and reducing conditions were performed Papain-digested mA116huFc Ab gave a band corresponding to 40 kDa, matching that of the monovalent scFv plus the CH1 domain under both non-reducing and reducing conditions, which probably excluded the possibility that there was any inter-chain disulfide bond in the region of scFv plus the CH1 domain. On the other hand, a band corresponding to ~90 kDa of pepsin-digested mA116huFc Ab on SDS-PAGE under non-reducing conditions migrated as a band corresponding to ~45 kDa under reducing conditions, suggesting the ~90 kDa protein was a dimer, matching that of bivalent scFv plus the CH1 domain and hinge region. Since there was no inter-chain disulfide formation in scFv plus the CH1 domain, inter-chain disulfide formation should have occurred in the hinge region of mA116huFc Ab.

The *in vitro* binding characteristics of mA116huFc Ab to VEE antigen were assayed by ELISA The mA116huFc Ab exhibited strong binding activity toward VEE, indicating that the folding was appropriate for the formation of antigen-binding sites The parental 1A1A4 MAb and mA116 scFv Ab showed similar binding activity to VEE, however, direct comparison of the binding affinities of the three Abs was not possible by ELISA, since each Ab required a different conjugated secondary Ab.

The results of the protein G binding assay indicated that mA116huFc Ab could bind to protein G, thus suggesting that the constant region of mA116huFc Ab was correctly folded. Protein G specifically interacts with the CH2 and CH3 domains of Ab (*33*). The finding that mA116huFc Ab could bind to protein G suggested that the Fc region must be close in structure to the native profile. We were also interested in determining whether or not the constant region in mA116huFc Ab was folded well enough to bind to complement. The classical pathway of complement activation is initiated by the constant region of an Ab binding to C1q, a constituent of the first component of complement (*34*). The abilities of human IgG subtypes to bind to human C1q vary widely IgG1 can efficiently bind to C1q, but IgG4 cannot (*35*) We found that mA116huFc Ab, like a positive control, human IgG 1, could strongly bind to human C1q in a dose-dependent manner As expected, mA116 scFv Ab (no Fc domains), similar to a negative control, human IgG4, could not bind to human C1q These results suggest that mA116huFc Ab might be active in the recruitment for complement-mediated cell lysis.

All Abs are glycoproteins and are glycosylated at characteristic positions according to their isotype. An IgG molecule has one conserved glycosylation site, at Asn 297, within the CH2 domain of each of its two heavy chains (*36*). Oligosaccharides are thought to stabilize the molecule and to contribute to the tertiary structure of the constant region, which is very important for such effector functions as complement activation, Fc receptor recognition, and ADCC. Although Asn 457 is present in mA116huFc Ab, bacteria are not capable of glycosylating proteins It has been reported that aglycosylation of IgG could change its binding ability to complement from minimal to profound (*36–39*) However, our results indicated that there was little to no effect of aglycosylation on the ability of mA116huFc to bind to complement.

There have been several reports on recombinant fusions of scFv and different parts of the Fc fragment of human IgG. Coloma and Morrison reported CH3-scFv had some associated effector functions, such as a long half-life and Fc receptor binding (*40*) Munoz *et al.* fused a scFv to hinge-CH2-CH3 to confirm complement C3 could efficiently bind to the remaining constant domains as well as the CH1 domain (*41*). Muller *et al.* showed that a scFv fused to the CH1 domain became linked to another scFv fused to CL of human IgG *via* a disulfide bond to form bispecific miniantibodies (*42*) Gilliland *et al* and Shan *et al* fused hinge-CH2-CH3 to a scFv simply for easy purification using staphylococcal protein A (*43*). We fused CH1-hinge-CH2-CH3 to anti-VEE scFv in order to confer on the scFv some of the human-associated effector functions without increasing the immunogenicity in humans This fusion Ab was shown to be a disulfide-linked homodimer, which demonstrated retention of antigen-binding affinity to VEE antigen. In addition, this Ab was shown to possess some human Ig G1 Fc domain functions, such as protein G and human C1q binding From these findings, it was concluded that this Ab was in a native, functionally active form This work will form the basis for further studies to characterize mA116huFc Ab for efficacy studies *in vivo*

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