A recombinant single chain antibody neutralizes coronavirus infectivity but only slightly delays lethal infection of mice

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The variable region genes of a murine anti-coronavirus monoclonal antibody (mAb) were joined by assembly polymerase chain reaction and expressed in *Escherichia coli* in a single chain variable fragment (scFv) configuration. After induction of expression, the expected 32-kDa protein was identified by Western immunoblotting with specific rabbit anti-idiotype antibodies. The scFv fragments were purified from soluble cytoplasmic preparations by affinity chromatography on nickel agarose, which was possible with an N-terminal but not with a C-terminal histidine tag. Purified scFv fragments retained the antigen-binding properties of the parental antibody, could inhibit its binding to viral antigens with apparently higher efficiency than monovalent antigen-binding (Fab) fragments, but neutralized viral infectivity with lower efficiency (about sevenfold at a molar level). To evaluate the usefulness of these smaller and less immunogenic molecules in the treatment of viral diseases, mice were treated with purified recombinant scFv fragments and challenged with a lethal viral dose. A small delay in mortality was observed for the scFv-treated animals. Therefore, even though the scFv could neutralize viral infectivity *in vitro*, the same quantity of fragments that partially protected mice in the form of Fab only slightly delayed virus-induced lethality when injected as scFv fragments, probably because of a much faster *in vivo* clearance: the biologic half-life was estimated to be about 6 min. Since a scFv derived from a highly neutralizing and protective mAb is only marginally effective in the passive protection of mice from lethal viral infection, the use of such reagents for viral immunotherapy will require strategies to overcome stability limitations.

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1 Introduction

Coronaviruses are members of the Coronaviridae virus family that includes important pathogens of the respiratory, gastrointestinal and neurological systems of humans and various animals [1, 2]. Neurotropic strains of the murine coronavirus MHV can induce neurological disorders in rodents that are similar to multiple sclerosis [3], providing an excellent animal model for the study of human nervous system diseases and immune protection mechanisms. Passive protection from MHV infection has been achieved by administration of mAb specific for all four major structural proteins of the virus [4–8]. We have recently shown that $F(ab')_2$ and Fab fragments of mAb 7–10A specific for the viral surface glycoprotein can also neutralize the virus *in vitro* and protect mice *in vivo* [9].

The utilization of mAb in the treatment of important viral diseases is an attractive approach because of their wide specificities and potent biological effects. However, their clinical use has been hampered by their immunogenicity in humans [10]. The development of molecular biology techniques which make it possible to express antibody fragments in bacteria and eukaryotic cells offers the possibility of developing immunological reagents with very high specificity and sensitivity, with even less immunogenicity than antibody fragments obtained by enzymatic digestion [11–13]. To explore the possibility of using antibody fragments expressed in bacteria for the treatment of viral diseases, an scFv was constructed from the sequences of MHV-specific mAb 7–10A and its *in vitro* neutralization and *in vivo* protection properties were evaluated.

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Abbreviations: scFv: Single chain variable fragment **MHV:** Mouse hepatitis virus **PBS-T:** PBS containing 0.1 % (v/v) Tween 20

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2 Results

2.1 Construction, cloning and sequencing of scFv 7–10A

The variable regions of the heavy and light chains of mAb 7–10A were amplified by PCR with V_H- and V_K-specific primers using cDNA synthesized from RNA extracted from 7–10A hybridoma cells (Fig. 1). Assembly of the variable regions of heavy and light chains of mAb 7–10A was done by splicing with overlap extension [24]. A linker molecule $(Gly_4\ Ser)_3$ was used to bridge the two chains together in an scFv configuration [25]. The assembly product of the correct size (750 bp) was gel-purified and cloned into the bacterial expression vector pET-22b. The nucleotide and deduced amino acid sequences of scFv 7–10A were determined (Fig. 2). The V_H region was 92 % homologous (percentage of nucleotide identity) to the rearranged V_H gene of the mouse VGAM 3-2 VDJ region [26] and belongs to subgroup I(A) according to the classification of Kabat et al. [27]. The V_L region of the κ chain was 97 % homologous to the Abelson virus transformed B cell line T24B [28] and belongs to subgroup V.

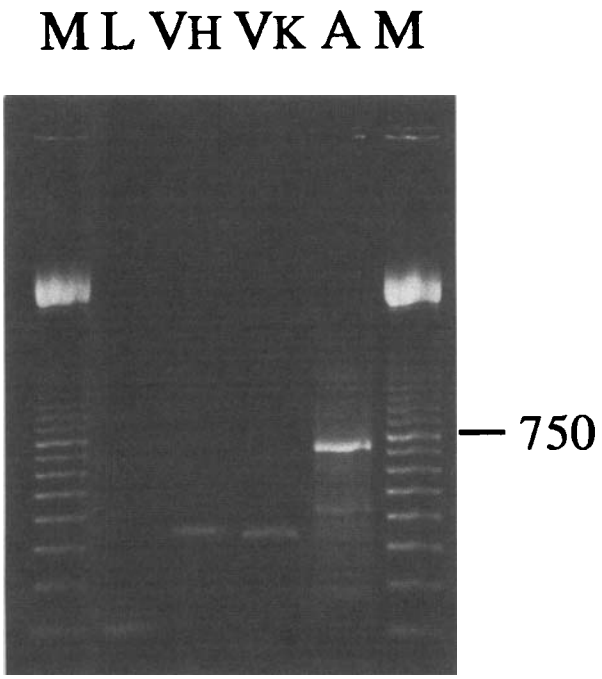

Figure 1. Ethidium bromide-stained agarose gel showing the assembly PCR of the V_H and V_K regions. M: 100-bp DNA ladder (Pharmacia), L: PCR product of the linker molecule, VH: PCR product of the variable region of the heavy chain, VK: PCR product of the variable region of the kappa light chain, A: 750-bp PCR product of the assembly reaction.

2.2 Expression of the scFv 7–10A

Expression of the recombinant scFv was induced with 1 mM IPTG for 18 h at 30 °C and total cellular proteins were analyzed by SDS-PAGE (Fig. 3A). Coomassie blue staining of induced cells revealed a major protein band of 32 kDa corresponding to the predicted size of the scFv and that was undetectable in uninduced cells. Fractionation of soluble and insoluble material revealed that the majority of the recombinant protein was contained in insoluble inclusion bodies (data not shown). The identity of the scFv was verified by Western immunoblotting with polyclonal anti-idiotype antibodies produced against the parental 7–10A antibody [22]. These antibodies reacted with a 32-kDa protein present only in induced cells (Fig. 3B).

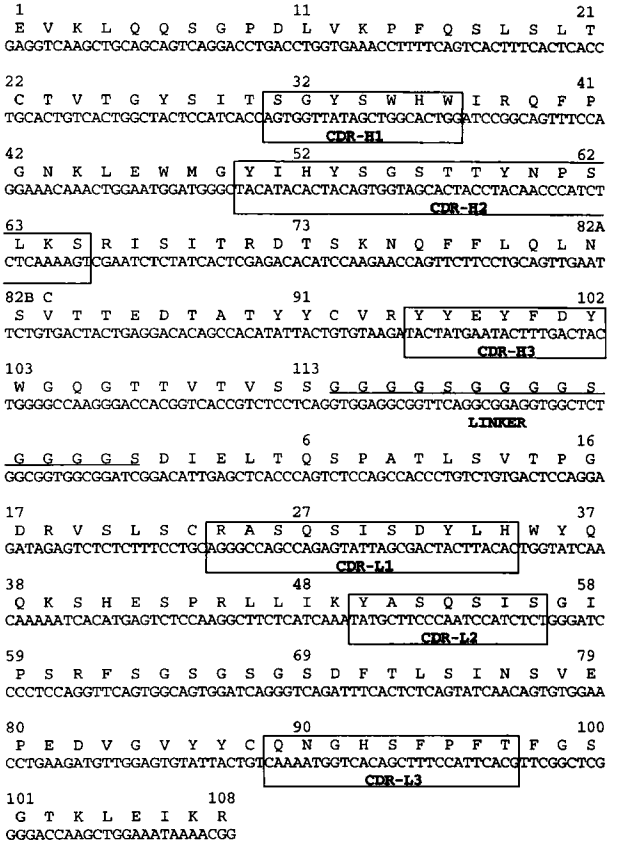

Figure 2. Nucleotide and deduced amino acid sequences of the scFv. Numbers on top of amino acid residues represent the position as attributed by the Kabat et al. database [27]. Boxed residues represent the three heavy (H) and three light (L) chains CDR. Underlined amino acids represent the $(Gly_4Ser)_3$ linker molecule. These sequence data are available from GenBank under accession number U73335.

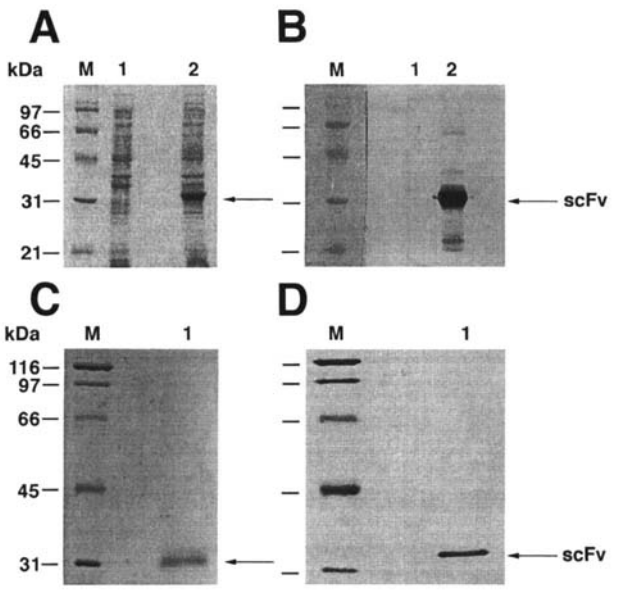

Figure 3. SDS-PAGE and Western blot analysis of purified scFv fragments. Samples of crude cell extracts or purified scFv were separated by SDS-PAGE on 10% acrylamide gels under reducing conditions and stained with Coomassie blue or electrotransferred onto nitrocellulose. (A) Coomassie blue stained gel of uninduced (lane 1) or induced cell extract (lane 2). (B) Western immunoblot of uninduced (lane 1) or induced cell extract (lane 2). (C) Coomassie blue-stained gel of affinity-purified scFv (lane 1). (D) Western immunoblot of affinity-purified scFv (lane 1). M: Molecular weight standards (Bio-Rad).

2.3 Purification of the scFv 7–10A

Attempts to affinity purify the recombinant scFv expressed in the pET-22b vector by Ni-NTA agarose column chromatography under either non-denaturing or denaturing conditions failed. It is possible that the C-terminal histidine tag was so embedded in the protein core even under denaturing conditions that it was inaccessible to the Ni^{2+} cations. We subcloned the scFv into the pET-16b vector which expresses the histidine tag at the N-terminal end of the protein. Although most of the recombinant scFv was also produced in insoluble inclusion bodies, enough soluble protein was present in cytoplasmic extracts to be purified on the Ni-NTA agarose column, with a yield of about 0.2 mg/l of bacterial culture. Adsorbed proteins were eluted with 60 mM imidazole and the fractions were analyzed by SDS-PAGE and Western immunoblotting. Coomassie blue staining of the eluted fractions revealed a unique band of 32 kDa (Fig. 3C), which was also revealed in Western immunoblotting with the anti-7–10A anti-idiotype antibodies (Fig. 3D).

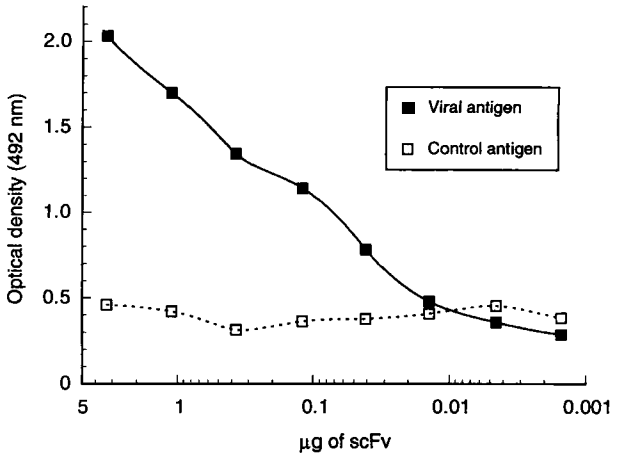

Figure 4. Reactivity of the scFv fragments for MHV antigen. Microtiter plates were coated with 500 ng/well of viral antigen preparations (■) or uninfected cell lysates (□). The binding of threefold dilutions of purified scFv fragments was detected using 7–10A-specific anti-idiotype antibodies and horseradish peroxidase-labeled goat anti-rabbit IgG antibodies.

2.4 Binding of the scFv 7–10A to viral antigen

To verify whether the purified scFv fragments had retained the antigenic specificity of the bivalent parental immunoglobulin, their binding to viral antigen prepara-

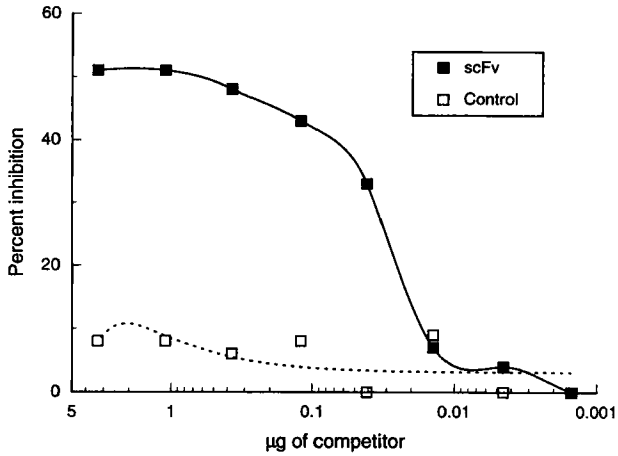

Figure 5. Inhibition of parental antibody binding to MHV antigen by purified scFv fragments. Microtiter plates were coated with 500 ng/well of viral antigen preparations. Serial threefold dilutions of purified scFv fragments (■) or control antibody (□) were added to the plates and the binding of a fixed concentration of the parental antibody was detected using Fc-specific horseradish peroxidase-labeled anti-mouse antibodies.

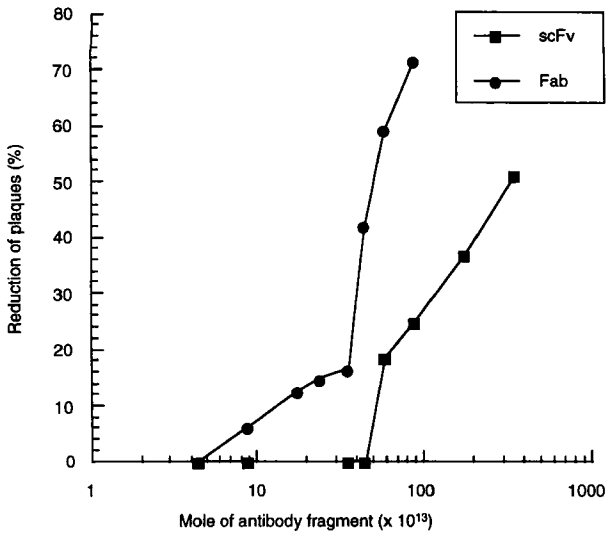

Figure 6. Comparative MHV-A59 neutralization profiles by scFv and Fab fragments. Serial dilutions of scFv or Fab fragments were incubated for 1 h at 37 °C with 50 PFU of MHV-A59 and residual infectious virus measured by plaque assay to evaluate the reduction of plaques. Neutralization of 50 % of input virus required about 50 × 10^{-13} moles of Fab fragments and 350 × 10^{-13} moles of scFv fragments.

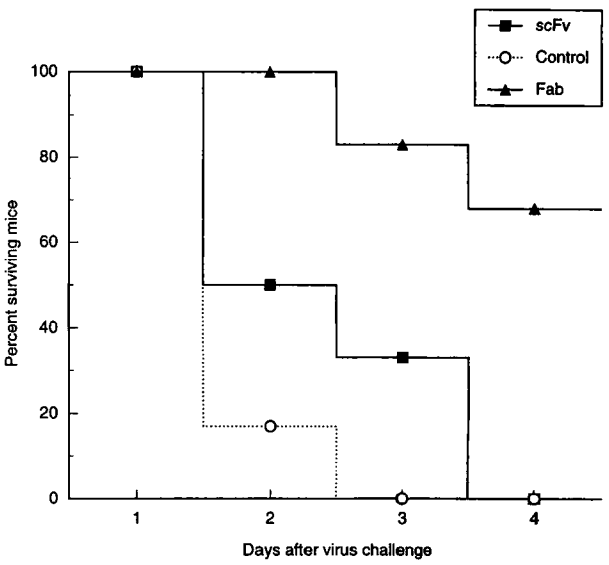

Figure 7. Protection of mice from MHV-A59 infection by passive transfer of antibody fragments. Three groups of six BALB/c mice received either a 500-μg injection of purified scFv (■) or Fab (▲) fragments in PBS or an equivalent volume of PBS (□) and were challenged 30 min later with 10 LD_{50} of MHV-A59.

tions was tested by ELISA using the specific anti-7–10A anti-idiotype antibodies for detection (Fig. 4). The scFv could indeed bind in a concentration-dependent manner to viral proteins present in infected cell lysates whereas no specific interaction with preparations from uninfected cells was observed. In order to determine the relative affinity of the scFv fragment for antigen, its ability to inhibit the binding of the bivalent natural antibody was examined (Fig. 5). Fifty percent inhibition of 7–10A binding was achieved with 0.6 mg of purified scFv fragments. In contrast, we have previously shown that the same amount of purified Fab fragments inhibited less then 20 % of the intact antibody binding [9].

2.5 Neutralization of virus infectivity

The neutralization capacity of the recombinant scFv fragment was evaluated and compared to that of the Fab fragment by incubating 50 PFU of virus with dilutions of purified fragments and determining the residual viral infectivity on murine fibroblast cells (Fig. 6). The neutralizing titer of scFv fragments (350 × 10^{-13} mole) was about sevenfold lower than that of Fab fragments (50 × 10^{-13} mole).

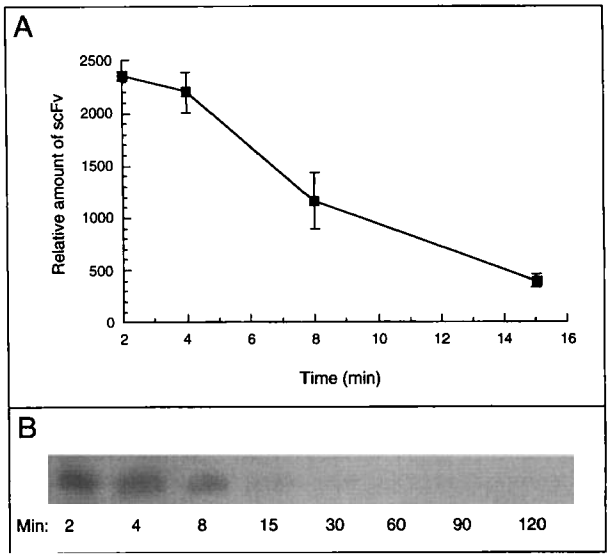

Figure 8. Biological half-life of scFv 7–10A. Two BALB/c mice received a 15-μg injection of purified radioiodinated scFv fragments. At the times indicated, small plasma samples were obtained and circulating levels of scFv fragments were quantitated by densitometric analysis (panel A) of autoradiograms of SDS-PAGE gels (panel B). The estimated half-life was about 6 min.

2.6 Protection

BALB/c mice were treated with 500 μg of purified scFv or Fab fragments and were challenged 30 min later with 10 LD_{50} of infectious MHV. No mice survived the viral infection but a small delay in the mortality of the animals treated with the scFv was observed, which contrasted with the protection of about 70 % of animals treated with Fab fragments (Fig. 7).

2.7 Biological half-life of scFv 7–10A

Given the relatively efficient *in vitro* virus-binding properties of scFv compared to Fab fragments, we evaluated whether the limited *in vivo* protective capacity of the scFv fragments was due to faster clearance. Indeed, we estimated the half-life of scFv 7–10A to be only about 6 min (Fig. 8).

3 Discussion

Murine antibodies that neutralize virus infectivity and have the capacity of protecting against viral infection are attractive candidates as potential immunotherapeutic agents. However, their large scale use has been hampered by allergic immune reactions in humans [29] and the difficulty and costs of producing large quantities of antibodies. Recombinant antibody fragments present several advantages over conventional monoclonal antibodies: they are less immunogenic in humans and can be produced in large amounts and at lower costs. These advantages have encouraged the development of a number of genetically engineered virus-specific antibody fragments with neutralizing properties [30–33]. As a model for the utilization of recombinant antibodies in the treatment of viral diseases, we tested whether the same antibody engineering technology could be employed for the production of a scFv that could protect from virus infection in a convenient animal model.

We report the construction and expression of a scFv rescued from a hybridoma line that secretes anti-coronavirus IgG2a mAb which can neutralize virus infection *in vitro* and protect mice against a normally lethal dose of virus. We show that the location of the histidine tag, either at the C- or N-terminal end of the recombinant protein, may have a major importance for purification by affinity chromatography on a nickel agarose column. Indeed, we have observed that the scFv fragment produced in this study could only be purified when the histidine tag was expressed at the N-terminal end. In contrast, Lake et al. [34] have reported the purification on a nickel agarose column of an anti-insulin scFv with a C-terminal histidine tag. This demonstrates that the conformation of the particular scFv will determine whether the expression of a C- or N-terminal histidine tag will be accessible to the Ni^{2+} cations and will allow purification by metal chromatography.

The scFv described in the present report showed biological properties similar to Fab fragments obtained by papain digestion. In fact, they exhibited much better inhibition of parental antibody binding to viral antigen than Fab fragments, which is consistent with a higher affinity. Indeed, 50 % inhibition of binding of the parental antibody to viral antigen was achieved with only 0.6 μg of scFv whereas 10 μg of Fab fragments only inhibited 42 % of binding [9]. However, this did not correlate with a better neutralization activity of the scFv, with molar titers about sevenfold lower than these of Fab fragments. Even with an apparent higher affinity than Fab fragments, the scFv was less effective in the passive protection of animals against lethal viral infection. This was most likely due to a shorter half-life, which we measured to be about 55-fold shorter (6 min) than that of Fab fragments. The rapid *in vivo* blood clearance of scFv is well documented, with reported half-lives ranging from 2.4 to 32 min [35–39], as compared to 5.5 h for Fab fragments of mAb 7–10A [9]. Although large quantities of scFv did not achieve complete protection of a proportion of treated mice, as could Fab fragments, the short delay in mortality suggests that the scFv fragments did have a limited protective effect on viral infection. It is likely that repeated injections of even larger doses of scFv fragments or a less overwhelming viral challenge would reproduce the passive protection achieved with Fab fragments [9].

The very fast blood clearance of scFv fragments represents an advantage for some clinical uses such as tumor immunotargeting for diagnosis or treatment of cancer but represents a major limitation for their utilization in viral immunotherapy. However, some reports have suggested that the *in vivo* stability of these small antibody fragments can be significantly prolonged, for example by disulfide stabilization [38, 40] or the identification and introduction of stabilizing mutations [41].

Importantly, the results presented in the current study with a murine coronavirus have very recently been confirmed in another animal model, vesicular stomatitis virus [39]. These authors also concluded that a short half-life of the antibody fragments hampered passive protection of mice against lethal infection and showed that protection required pre-incubation of the challenge virus with antibody fragments. This confirms that monovalent antibody fragments may be able to passively protect against viral infections and emphasize the need to engineer more stable molecules before clinical uses can be envisaged.

4 Materials and methods

4.1 Animals

Male or female, 6- to 7-week-old, MHV-seronegative BALB/c mice (Charles River, St-Constant, Canada) were used in the protection experiments.

4.2 Virus and cells

The neurotropic A59 strain of MHV (MHV-A59) was obtained from the American Type Culture Collection (Rockville, MD), plaque-purified twice, and passaged on DBT cells as described previously [14].

4.3 Construction and expression of scFv 7–10A

Total cellular RNA was isolated from 7–10A hybridoma cells as described previously [15]. Ten micrograms of total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Pharmacia Canada Inc., Baie-d'Urfé, Canada) using the CH_1 primer MOCG12FOR (5'-CTCAATTTTCTTGTCCACCTTGGTGC-3') and the C_K primer CKFOR (5'-CTCATTCCTGTTGAAGCTCTTGAC-3'). These primer sequences were obtained from Dr. Greg Winter (Medical Research Council, Cambridge, Great Britain). Amplification of variable regions were carried out by PCR of cDNA using Taq DNA polymerase (BIO/CAN Scientific, Mississauga, Ontario, Canada) for 30 cycles (94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min). Primers VH1FOR-2 (5'-TGAGGAGACGGTGACCGTGGTCCCTTGGCCCC-3') [16] and VH1BACK (5'-AGGTSMARCTGCAGSAGTCWGG-3': S = C or G; M = A or C; R = A or G; W = A or T) [17] were used to amplify V_H and primers VK4FOR (an equimolar mix of JK1: 5'-CCGTTTGATTTCCAGCTTGGTGCC-3', JK2: 5'-CCGTTTTATTTCCAGCTTGGTCCC-3', JK4: 5'-CCGTTTTATTTCCAACTTTGTCCC-3' and JK5: 5'-CCGTTTCAGCTCCAGCTTGGTCCC-3') and VK2BACK (5'-GACATTGAGCTCACCCAGTCTCCA-3') [18] to amplify V_K. The linker DNA was similarly amplified from the plasmid pSW2scD1.3 [19] using primers MO-LINK-BACK and MO-LINK-FOR (complementary to VH1FOR-2 and VK2BACK respectively). Gel purified V_H and V_K amplicons (100 ng each) were mixed with 20 ng of the linker DNA fragment encoding the peptide $(Gly_4Ser)_3$ in a 50-μl reaction mixture without primers and cycled 7 times (94 °C for 1 min, 72 °C for 2.5 min) with Vent DNA polymerase (New England Biolabs, Ltd., Mississauga, Canada) to randomly join the fragments, then amplified for 23 cycles (94 °C for 1 min, 60 °C for 2 min and 72 °C for 2 min) using 25 pmol each of VH1BACK and VK4FOR primers to which NcoI and NotI restriction sites where appended, respectively. The amplification product was digested with NcoI and NotI for cloning into the pET-22b vector (Novagen, Inc., Madison, WI) containing a C-terminal histidine tag. The scFv product was also cloned into the pET-16b vector (Novagen) for the expression of an N-terminal histidine tag. The scFv 7–10A insert contained in the pET-22b plasmid was amplified using primers VH1BNDE (5'-GGAATTCCATATGGCCGAGGTCAAGCTGC-3') and JKSAL (5'-ACGCGTCGACCCGTTTTATTTCCAGCTTGG-3') and the PCR product was digested with NdeI and SalI and ligated into the NdeI- and SalI-digested pET-16b vector. The ligation products were used to transform the XL1-blue strain of *E. coli* (Stratagene, La Jolla, CA). Positive clones were subcloned into *E. coli* strain BL21 (DE3) (Novagen) for expression. Transformed BL21 (DE3) cells were grown at 37 °C in LB containing 100 μg/ml ampicillin (Boehringer Mannheim Canada, Laval, Canada) until the OD at 600 nm reached 0.6, at which time 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added (Clonetech Laboratories Inc., Palo Alto, CA). Following induction, the cultures were grown for an additional 18 h at 30 °C. Induced cells were centrifuged for 20 min at 10 000 × g and resuspended in 1/25 volume of 500 mM NaCl and 20 mM Tris-HCl, pH 7.9. Lysozyme (Boehringer Mannheim) was added to a concentration of 100 μg/ml and the suspension was incubated at 30 °C for 30 min. To shear the DNA, the suspension was sonicated on ice for 1 min or until the solution lost viscosity using a Braun-Sonic 2000 sonicator. The lysate was centrifuged at 29 000 × g for 30 min and the supernatant filtered through a 0.45-μm Sterifil-D membrane (Millipore Canada, Nepean, Canada) for column chromatography.

4.4 Nucleotide sequencing of scFv 7–10A

The PCR assembly product of scFv 7–10A was cloned into the pCRII TA cloning vector (Invitrogen Corporation, San Diego, CA). Nucleotide sequencing was performed on both strands of two PCR products by the dideoxynucleotide chain terminating method [20] using T7 DNA polymerase (Pharmacia) and [α-^{35}S] dATP (ICN Pharmaceuticals Canada Ltd., Montréal, Canada) according to the manufacturer's instructions (Pharmacia).

4.5 Purification of scFv 7–10A

A soluble cytoplasmic extract was used for the purification on a Ni-NTA agarose column (Qiagen Inc., Chatsworth, CA). The column was washed with ten volumes of binding buffer (500 mM NaCl and 20 mM Tris-HCl, pH 7.9) and loaded with the prepared cell extract at a flow rate of about 10 column volumes per hour. After loading, the column was washed with binding buffer until the OD at 280 nm reached the base line level, after which time the bound proteins were eluted with binding buffer containing 60 mM imidazole (Sigma-Aldrich, Canada, Ltd., Mississauga, Canada). Elution fractions were analyzed by SDS-PAGE and Western immunoblotting. Fractions containing the purified scFv were pooled and dialyzed against PBS for the biological assays.

4.6 SDS-PAGE analysis and Western immunoblotting

Induced cell extract preparations and elution fractions were separated by SDS-PAGE [21] and stained with Coomassie blue for direct visualization or electrotransferred onto Hybond-C Extra nitrocellulose membranes (Amersham Searle Corp., Oakville, Canada) for 1 h at 100 V. Membranes were blocked for 1 h with PBS-T and incubated for 90 min with a 2 μg/ml solution of purified anti-7–10A rabbit anti-idiotype antibodies [22] in PBS-T. Membranes were washed five times with PBS-T and incubated for 60 min with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (diluted 1/1,000; Kirkegaard & Perry Laboratory, Gaithersburg, MD). Membranes were again washed five times with PBS-T and incubated with hydrogen peroxide (Sigma) and 4-chloro-1-naphtol (Bio-Rad Laboratories Ltd.).

4.7 ELISA

All incubations were performed at room temperature (about 25 °C). Each well of 96-well microtiter plates was coated with 500 ng of viral antigen prepared from MHV-A59-infected cells, as described previously [23]. After overnight incubation, the remaining binding sites in the wells were blocked with PBS containing 10 % (v/v) FCS and 0.2 % (v/v) Tween-20 for 90 min. Serial threefold dilutions of purified scFv fragments were added to the wells and incubated for 90 min. The wells were washed five times with PBS-T and a 2 μg/ml solution of purified rabbit anti-7–10A anti-idiotype antibodies in blocking solution was added and incubated for 90 min. The wells were washed as described above and peroxidase-labeled goat anti-rabbit IgG antibodies (Kirkegaard & Perry Laboratories, Inc) were then added and the plates incubated for another 90 min. The plates were washed five times with PBS-T and the bound peroxidase revealed by incubation with *o*-phenylenediamine (Sigma) and hydrogen peroxide. The reaction was stopped with 1 N HCl and the absorbance read at 492 nm using an SLT EAR 400 AT plate reader.

4.8 Inhibition of binding of mAb 7–10A

The wells of 96-well microtiter plates were coated with viral antigen as described above and incubated overnight at room temperature. Serial threefold dilutions of purified scFv fragments were added to the wells and incubated for 90 min, after which the wells were emptied and purified mAb 7–10A added without any previous washing. After incubation for 60 min, Fc-specific peroxidase-labeled goat anti-mouse IgG (ICN) was added and the plates incubated for 90 min. All subsequent steps were performed as described above. The amount of purified mAb 7–10A added in this test was determined in a binding ELISA in which an absorbance value at 492 nm of about 1.0 was achieved in the absence of inhibition by antibody fragments.

4.9 Virus-neutralization assay

Duplicate serial dilutions of scFv or Fab fragments were incubated with approximately fifty PFU of MHV-A59 for 1 h at 37 °C. The mixtures were transferred onto 12-well plates containing confluent monolayers of DBT cells. After an adsorption period of 1 h at 37 °C, the virus-scFv mixtures were removed and cells were overlaid with 1.5 % (w/v) agarose in Earle's minimum essential medium/Hank's M199 (1:1, v/v) (Gibco Canada, Burlington, Canada) supplemented with 5 % (v/v) FCS. Plates were incubated for 48 h at 37 °C in a humidified atmosphere containing 5 % (v/v) CO_2, after which the cells were fixed with formaldehyde and stained with crystal violet. Viral neutralization titers are expressed as the amount of antibody fragments that could neutralize 50 % of input viral infectivity.

4.10 Passive immunizations

MHV-seronegative 6-week-old BALB/c mice (Charles River) were injected intraperitoneally with 500 μg of antibody fragments 30 min prior to challenge with 5×10^5 PFU (10 LD_{50}) of MHV-A59 injected intracerebrally.

4.11 Biological half-life of scFv 7–10A

The purification of scFv 7–10A was as described above except that it was performed in batch, using 50-ml tubes. Two milliliters of Ni-NTA agarose resin were washed three times with 30 ml of binding buffer and mixed with 15 ml of bacterial cell extract for 60 min at 4 °C on a rocker platform. The resin was washed twice with binding buffer and three times with wash buffer (binding buffer containing 30 mM imidazole). The scFv 7–10A were then eluted from the resin with 5 ml of elution buffer (binding buffer containing 1 M imidazole). Purified scFv fragments were analyzed by SDS-PAGE and Western immunoblotting as described above, concentrated with Aquacide II (Calbiochem-Novabiochem Corporation, La Jolla, CA) and dialyzed against PBS.

Radioiodination of the purified scFv 7–10A was performed with the Iodo-Beads® radioiodination reagent (Pierce, Rockford, IL). Three beads were washed in 0.1 M phosphate buffer, pH 7.2, dried on filter paper and resuspended in 0.1 ml of the same buffer, to which 5 mCi $Na^{125}I$ (ICN) were added. After 5 min incubation, 1 mg of purified scFv 7–10A was added and iodination was allowed to proceed for 14 min at room temperature. The reaction was stopped by removing the beads. Radioiodinated scFv 7–10A were separated from free iodine by Sephadex G-25 chromatography (Pharmacia) and their purity reverified by SDS-PAGE followed by autoradiography of the dried gel using a Kodak X-Omat AR X-ray film.

To measure biological half-life *in vivo*, two BALB/c mice (Charles River) were injected intravenously with 15 μg of radioiodinated scFv 7–10A. After 2, 4, 8, 15, 30, 60, 90 and 120 min, 60 μl of blood was collected from the retro-orbital plexus into heparinized capillary tubes. Plasma samples were analyzed by SDS-PAGE and autoradiography and the 32-kDa scFv bands were quantitated by videodensitometry using an AlphaImager™ 2000 Documentation and Analysis System with AlphaImager™ 3.24i software (Applied Innotech, San Leandro, CA). The validity of this densitometric procedure was ascertained using classical laser densitometry (Bio-Rad), with identical results.

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