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Pharmacokinetics and Brain Uptake of a Genetically Engineered Bi-functional Fusion Antibody Targeting the Mouse Transferrin Receptor

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Abstract

Monoclonal antibodies (MAb) are potential new therapeutics for brain diseases. However, MAb's do not cross the blood-brain barrier (BBB). The present work describes the genetic engineering of a fusion protein comprised of a therapeutic single chain Fv (ScFv) antibody and a mouse/rat chimeric MAb against the mouse transferrin receptor (TfR). The TfRMAb acts as a molecular Trojan horse to ferry the therapeutic ScFv across the BBB in vivo in the mouse. The ScFv is fused to the carboxyl terminus of the heavy chain of the chimeric TfRMAb, and this fusion protein is designated cTfRMAb-ScFv. Chinese hamster ovary cells were permanently transfected, and a high secreting cell line in serum free medium was cloned. The cTfRMAb-ScFv fusion protein was purified to homogeneity on gels and Western blotting with protein G affinity chromatography. The cTfRMAb-ScFv fusion protein was bi-functional and bound both the target antigen, as determined by ELISA, and the mouse TfR, and as determined with a radio-receptor assay. The cTfRMAb-ScFv fusion protein was radio-iodinated with the Bolton-Hunter reagent, and a pharmacokinetics study in mice showed the fusion protein was rapidly cleared from blood with a median residence time of 175 ± 32 min. The fusion protein was avidly taken up by brain with a % injected dose (ID)/g of 3.5 ± 0.7, as compared to an MAb with no receptor specificity, which was 0.06 ± 0.01 %ID/g. These studies demonstrate that therapeutic MAb's may be re-engineered as fusion proteins with BBB molecular Trojan horses for targeted delivery across the BBB in vivo.

Keywords

blood-brain barrier; drug targeting; transferrin receptor; monoclonal antibody

Introduction

Monoclonal antibodies (MAb) are potential large molecule drugs for the brain. However, MAb's do not cross the blood-brain barrier (BBB). Recombinant proteins such as MAb drugs can be re-engineered to cross the BBB as fusion proteins with a BBB molecular Trojan horse[1]. The latter is an endogenous peptide or peptidomimetic MAb against an endogenous BBB receptor-mediated transport (RMT) system, such as the insulin receptor or the transferrin receptor (TfR). The most potent BBB Trojan horse is a MAb against the human insulin receptor (HIR)2. However, the HIRMAb is not active in rodents, and there is

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no known MAb against the mouse insulin receptor that can be used as a BBB molecular Trojan horse. The BBB TfR is also an RMT system3, and rat MAb's against the mouse TfR have been previously shown to undergo rapid transport across the BBB in the mouse4. However, in order to engineer fusion proteins between a therapeutic MAb and a TfRMAb, it is necessary to produce a recombinant form of the molecular Trojan horse. Prior work describes the genetic engineering and validation of a recombinant mouse/rat chimeric TfRMAb, designated cTfRMAb5. Fusion genes encoding both the heavy chain (HC) and the light chain (LC) were engineered which allowed for expression of a chimeric MAb, where the variable region of the heavy chain (VH) of a rat TfRMAb was fused to the constant region of mouse IgG1. In parallel, the variable region of the light chain (VL) of the rat TfRMAb was fused to the constant region of the mouse kappa LC. Therefore, the amino acid sequence of the cTfRMAb is >80% of mouse origin, although the VH and VL were derived from a rat IgG. The presence of the mouse constant region will minimize immunogenicity from chronic administration of the cTfRMAb fusion proteins in mouse models of brain disease.

In the present study a new recombinant fusion protein of the cTfRMAb was engineered, wherein a therapeutic single chain Fv (ScFv) antibody was fused to the carboxyl terminus of the HC of the cTfRMAb. The structure of the fusion protein, which is designated cTfRMAb-ScFv, is shown in Figure 1. The model ScFv used in this study was engineered previously following cloning of the genes encoding the VH and VL of a MAb against the Aβ amyloid peptide of Alzheimer's disease (AD)[6]. Anti-Aβ antibodies (AAA) cause disaggregation of the amyloid plaque of AD, and are potential new therapies for AD7⁻9. It is likely that AAA's must penetrate the BBB to cause plaque disaggregation, since AAAs that bind Aβ plaque, as opposed to soluble Aβ, are the most potent at reversal of the AD amyloidosis in transgenic mouse models10. The purpose of the present study was to engineer, express, and validate the cTfRMAb-ScFv. In vitro studies assess the bi-functional binding properties of the fusion protein using assays that quantitate binding to both the murine TfR and the human Aβ peptide. In vivo investigations were performed with cTfRMAb-ScFv fusion protein radiolabeled with the [^{125}I]-Bolton-Hunter reagent, and the pharmacokinetics (PK) of plasma clearance, and a high level of brain uptake in the mouse are described.

Methods and Materials

Production of CHO line

A tandem vector (TV) was engineered in which the expression cassettes encoding this fusion gene of the ScFv and the HC of the cTfRMAb, as well as the cTfRMAb light chain (LC), and the murine dihydrofolate reductase (DHFR), are all contained on a single strand of DNA similar to a TV described previously[11]. The cTfRMAb LC expression cassette containing the cytomegalovirus (CMV) promoter, the cTfRMAb LC open reading frame and the bovine growth hormone (BGH) polyadenylation sequence was released from the pCD-LC vector described previously5 with NruI and AfeI and inserted into the NruI site of the cTfRMAb HC expression vector located on the 5'-flanking region of the HC CMV promoter. The DHFR expression cassette was later inserted at the AfeI site located on the 3'-flanking region of the cTfRMAb HC expression cassette as previously described11 to form the a tandem vector encoding the cTfRMAb. The cDNA encoding the anti-Aβ ScFv was amplified by PCR as described previously6, and subcloned at the 3'-end of the cTfRMAb HC to form the pcTfRMAb-ScFv tandem vector shown in Figure 2. The TV was linearized and DG44 CHO cells were electroporated, followed by selection in hypoxanthine-thymine deficient medium and amplification with graded increases in methotrexate (MTX) up to 80 nM in serum free medium (SFM). The CHO line was dilutionally cloned at 1 cell/well, and high producing clones were selected by measurement of medium mouse IgG concentrations by enzyme-linked immunosorbent assay (ELISA). The CHO line was stable through

multiple generations, and produced medium IgG levels of 5–10 mg/L in shake flasks at a cell density of 1–2 million cells/mL.

Protein purification

The CHO cells were propagated in 1 L bottles, until 2.4L of conditioned serum free medium was collected. The medium was supplemented with 0.1% Tween-80, ultra-filtered with a 0.2 um Sartopore-2 sterile-filter unit (Sartorius Stedim Biotech, Goettingen, Germany), and applied to a 25 mL protein G Sepharose 4 Fast Flow (GE Life Sciences, Chicago, IL) column equilibrated in 25 mM Tris/25 mM NaCl/5 mM EDTA/pH=7.1/0.1% Tween-80. Following application of the sample, the column was washed with 25 mM Tris/1 M NaCl/5 mM EDTA/pH=7.1/0.1% Tween-80, and the fusion protein was eluted with 0.1 M glycine/ pH=2.8/0.2% Tween-80. The acid eluate was pooled and neutralized to pH=6.5 with 1M Tris base, and concentrated with an Ultra-15 microconcentrator (Millipore, Bedford, MA) and stored sterile-filtered at 4C.

SDS-PAGE and Western blotting

The homogeneity of the cTfRMAb-ScFv fusion protein was evaluated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions using a 4–12% Bis-Tris gel and the Nu-Page system (Invitrogen, Carlsbad, CA). Western blot analysis was performed with a goat anti-mouse IgG (H+L) antibody (Bethyl Labs, Montgomery, TX).

$A\beta^{1-40}$ binding ELISA

The binding of the cTfRMAb-ScFv fusion protein to the Aβ peptide was determined by ELISA. Recombinant streptavidin (SA) was obtained from Sigma Chemical Co. (St. Louis, MO), and plated in 96 well plates overnight at 4C in 100 uL/well of 0.1 M NaHCO3/pH=8.3 (400 ng/well). The SA was removed by aspiration, and the wells were blocked with 0.25% casein (Sigma) in 0.01 M Tris/0.15 M NaCl/pH=7.4 (TBS) for 30 min at room temperature (RT). The wells were washed with TBS/0.05% Tween-20 (TBST) and 100 uL/well of N-biotinyl $A\beta^{1-40}$ peptide (Invitrogen, Carlsbad, CA) was applied (1000 ng/well) for 60 min at RT. Following washing in TBST, 100 uL/well of 30–3000 ng/mL of either the cTfRMAb-ScFv fusion protein or mouse IgG1κ (Sigma) was incubated for 90 min at RT. A conjugate of alkaline phosphatase (AP) and a goat anti-mouse (GAM) kappa light chain antibody (Bethyl Labs) was applied in a volume of 100 uL (100 ng)/well, followed by a 45 min incubation at RT. The wells were washed with TBST and the binding was measured colorimetrically with p-nitrophenylphosphate (Sigma).

Radio-labeling of protein

The 8D3 MAb, which was used for the mouse TfR radio-receptor assay, and the cTfRMAb-ScFv fusion protein, which was injected into mice for a pharmacokinetics analysis, were radiolabeled with [^{125}I]-Bolton-Hunter reagent, which was purchased from American Radiolabeled Chemicals (St. Louis, MO). The labeled protein was purified with a 1×28 cm Sephadex G-25 gel filtration column. The elution buffer for the 8D3 MAb was 0.01 M NaH2PO4/0.15 M NaCl/pH=7.4/0.1% Tween-20, and the elution buffer for the cTfRMAb-ScFv fusion protein was 0.05 M sodium acetate/0.15 M NaCl/pH=6.5/0.18% Tween-80. The 8D3 MAb was labeled to a specific activity of 2.8 uCi/ug and a trichloroacetic acid (TCA) precipitability of >98%. The TCA precipitability of the labeled 8D3 MAb remained >95% for a month after labeling while stored at −70C in 0.01 M NaH2PO4/0.15 M NaCl/ pH=7.4/0.1% Tween-20 (PBST). The cTfRMAb-ScFv fusion protein was labeled to a specific activity of 0.4 uCi/ug and a TCA precipitability of 96%. The labeled cTfRMAb-ScFv fusion protein was administered to mice within 24 hrs of radio-labeling.

Mouse transferrin receptor radio-receptor assay

Binding of the cTfRMAb-ScFv fusion protein to the mouse TfR was measured with a radio-receptor assay (RRA) described previously[5]. The assay employs [^{125}I]-8D3 MAb as the ligand, and the source of the mouse TfR is the mouse 3T3 fibroblast, which were plated on BD BioCoat 24-well dishes (BD Biosciences, Mississauga, Ontario, Canada). Incubations were performed in 500 uL volumes for 3 hours at 4C in the presence of 1.2 nM [^{125}I]-8D3 MAb, and various concentrations of either unlabeled 8D3 MAb, or unlabeled cTfRMAb-ScFv fusion protein. After washing the plates, cell radioactivity was determined with a Perkin-Elmer liquid scintillation counter and the protein was determined with the bicinchoninic acid (BCA) assay (Pierce Chemical Co., Rockford, IL). Binding was expressed as fractional bound per mg protein, by total ligand (pmol) bound per mg protein, and by volume of distribution (VD), uL/mg protein, which is computed from the ratio of the total ligand bound per mg protein divided by the ligand concentration (S), nM, in the medium. The half-saturation constant, KD, in nM, the maximal binding, Bmax, in pmol/mg protein, and the non-saturable binding (NSB), in uL/mg protein, for the binding of the 8D3 MAb was determined by non-linear regression analysis as described previously[5] with the AR program of the BMDP2007 Statistical software (Statistical Solutions, Dublin, Ireland). The half-saturation constant, KI, of cTfRMAb-ScFv fusion protein inhibition of 8D3 MAb binding to the TfR was determined by non-linear regression analysis as described previously5.

Pharmacokinetics and brain uptake in the mouse

Adult male BALB/c mice, weighing 25–27 grams, were obtained from Charles River (Hollister, CA). All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Mice were anesthetized with intra-peritoneal (IP) ketamine and xylazine, and injected intravenously (IV) with 0.1 mL (1.5 uCi) of [^{125}I]-cTfRMAb-ScFv fusion protein in the tail vein, and small aliquots of blood were collected from the femoral vein at 0.25, 2, 5, 15, 30, and 60 min after injection. In separate studies, the [^{125}I]-cTfRMAb-ScFv fusion protein was administered by IP injection. At 60 min after injection, the mice were euthanized and brain and peripheral organs (liver, spleen, lung, heart, and kidney) were removed and weighed. Plasma and tissue samples were analyzed for ^{125}I radioactivity with a gamma counter (Wizard 1470, Perkin Elmer). Organ uptake data was expressed as a volume of distribution (VD), which is the ratio of the 60 min organ radioactivity (DPM/gram) divided by the 60 min plasma radioactivity (DPM/uL), or as % of injected dose (ID)/gram tissue.

The plasma radioactivity, DPM/mL, was converted to % injected dose (ID)/mL, and the %ID/mL was fit to a bi-exponential equation,

$$\%\mathrm{ID/mL} = A1e^{-k1t} + A2e^{-k2t}$$

The intercepts (A1, A2) and the slopes (k1, k2) were used to compute the pharmacokinetics (PK) parameters, including the median residence time (MRT), the central volume of distribution (Vc), the steady state volume of distribution (Vss), the area under the plasma concentration curve (AUC), and the systemic clearance (CL). Non-linear regression analysis used the AR subroutine of the BMDP Statistical Software. Data were weighted by 1/(%ID/mL)^2. PK and organ uptake parameters for the cTfRMAb-ScFv fusion protein were compared to parameters reported previously in the mouse for (a) the 8D3 rat MAb against the mouse TfR, (b) the OX26 mouse MAb against the rat TfR[4], and (c) the genetically engineered cTfRMAb5.

The organ clearance (μL/min/g), also called the BBB permeability-surface area (PS) product, is computed from the terminal organ uptake (%ID/g) and the 60 min plasma AUC (%IDmin/mL) as follows:

$$\text{PS product} = [(\%\text{ID}/g)/\text{AUC}]^{*}1000$$

Results

A tandem vector was engineered, which contained the expression cassettes for the heavy chain fusion gene, the light chain gene, and the DHFR gene on a single strand of DNA. The 3 expression cassettes spanned 6,820 nucleotides (nt). The light chain was comprised of 234 amino acids (AA), which included a 20 AA signal peptide, a 108 AA variable region of the light chain (VL) of the cTfRMAb, and a 106 AA mouse kappa light chain constant region. The predicted molecular weight of the light chain is 23,554 Da with a predicted isoelectric point (pI) of 5.73.The fusion protein of the cTfRMAb heavy chain and the anti-Aβ ScFv was comprised of 707 AA, which included a 19 AA signal peptide. The predicted molecular weight of the heavy chain, without glycosylation, is 75,738 Da with a predicted pI of 7.03. The domains of the fusion heavy chain include a 118 AA variable region of the heavy chain (VH) of the cTfRMAb, a 324 AA mouse IgG1 constant region, a 2 AA linker (Ser-Ser), a 114 AA VH of the anti-Aβ ScFv, a 17 AA linker, and a 113 AA VL of the anti-Aβ ScFv. Single predicted N-linked glycosylation sequences were present in the second complementarity determining region of the anti-Aβ ScFv VH, and in the mouse IgG1 C-region.

The CHO-derived cTfRMAb-ScFv fusion protein was homogeneous on reducing SDS-PAGE with a ~85 kDa heavy chain (HC) and a ~28 kDa light chain (LC) (Figure 3). The fusion protein was also homogenous on non-reducing SDS-PAGE (not shown), and the protein migrated as a single band of approximately 210 kDa. Western blot analysis showed the size of the HC of the cTfRMAb-ScFv fusion protein was about 25–30 kDa larger than the HC of the cTfRMAb, whereas the size of the LC for the cTfRMAb-ScFv fusion protein and the cTfRMAb was identical (Figure 4).

The 8D3 MAb was radio-iodinated and used as the ligand for the mouse TfR RRA with mouse 3T3 fibroblasts, and a self-inhibition curve was saturable and characterized by a KD of 1.1 ± 0.4 nM, a Bmax of 0.34 ± 0.05 pmol/mg protein (Figure 5A), and a NSB of 40 ± 2 uL/mg protein. The KI of inhibition of binding of the ^{125}I-8D3 MAb by unlabeled cTfRMAb-ScFv fusion protein was 1.6 ± 0.4 nM (Figure 5B), which was comparable to the KD of 8D3 binding to the mouse TfR. The design of the ELISA used to measure binding of the cTfRMAb-ScFv fusion protein to the Aβ amyloid peptide is shown in Figure 6A. The cTfRMAb-ScFv, but not mouse IgG1, bound to the Aβ peptide in a dose-dependent mechanism (Figure 6B).

The cTfRMAb-ScFv fusion protein was radiolabeled with the ^{125}I-Bolton-Hunter reagent, and injected into adult BALB/c mice via intravenous (IV) administration. The clearance of the [^{125}I]-cTfRMAb-ScFv fusion protein from plasma after IV administration is plotted in Figure 7. The plasma radioactivity decay curve was fit to a bi-exponential equation to yield the pharmacokinetics (PK) parameters shown in Table 1, which are compared to the PK parameters for the parent [^{125}I]-cTfRMAb reported previously[5].

The uptake of the [^{125}I]-cTfRMAb-ScFv fusion protein by brain and peripheral organs was expressed as a volume of distribution (VD) at 1 hour after IV injection, and these data are shown in Table 2, in comparison with the 60 min VD values for the OX26 MAb, the 8D3

MAb, and the cTfRMAb in the mouse reported previously[4,5]. The brain uptake of the antibodies was also expressed as a %ID/g brain (Figure 8). The BBB permeability-surface area (PS) product of the cTfRMAb-ScFv fusion protein is equal to the ratio of the 60 min %ID/g (Figure 8) and the 60 min plasma AUC (Table 1), and is 2.0 uL/min/g.

The [^{125}I]-cTfRMAb-ScFv fusion protein was also administered by IP injection, and the plasma concentration of the fusion protein is plotted in Figure 7. The 60 min plasma AUC following IP administration of the fusion protein, computed with the trapezoidal rule, 436 %IDmin/mL, is 25% of the corresponding 60 min plasma AUC for the fusion protein following IV administration (Table 1). The brain uptake of the fusion protein at 60 min following IP injection was 0.41 ± 0.03 % ID/g, which is 12% of the brain uptake after IV injection (Figure 8). The plasma radioactivity that was precipitable with TCA was comparable after IP and IV administration. The % TCA precipitable radioactivity was 95 ± 1% and 96 ± 1% at 0.25 min, and 82 ± 1% and 77 ± 6% at 60 min, after IV and IP injection, respectively.

Discussion

The results of this study are consistent with the following conclusions. First, a cTfRMAb-ScFv fusion protein has been engineered, which is derived from the VH and VL of the rat 8D3 MAb, the C-region from mouse IgG1 heavy chain and mouse kappa light chain, and the VH and VL of a mouse MAb against the Aβ peptide of AD (Figure 1). Second, CHO cells have been permanently transfected with a tandem vector (Figure 2), the cloned CHO line secretes the cTfRMAb-ScFv fusion protein in serum free medium, and the cTfRMAb-ScFv fusion protein is homogenous on SDS-PAGE (Figure 3) and Western blotting (Figure 4). Third, the cTfRMAb-ScFv fusion protein is bi-functional, and binds both the mouse TfR (Figure 5) and the Aβ peptide of AD (Figure 6). Fourth, the pharmacokinetics (PK) of cTfRMAb-ScFv fusion protein clearance have been measured following IV and IP injection (Figure 7), and the PK parameters of fusion protein clearance from blood are comparable to the PK parameters for the cTfRMAb (Table 1). Fifth, the brain uptake of the cTfRMAb-ScFv fusion protein is high, >3% ID/g (Figure 8), and is comparable to the brain uptake of the 8D3 MAb or the cTfRMAb, but much greater than the brain uptake of a MAb that has no specificity for a BBB receptor, such as the OX26 MAb against the rat TfR (Table 2).

The delivery of MAb-based therapeutics to the brain could be applied to the development of new drugs for AD and other disorders. MAb therapeutics have been delivered to the brain by cationization[12], BBB disruption[13], or trans-nasal administration[14]. In the present approach, the MAb therapeutic is re-engineered as an IgG-ScFv fusion protein, whereby the anti-Aβ ScFv is fused to a chimeric MAb against the mouse TfR (Figure 1). The expression of the TfRMAb-ScFv fusion protein was enabled by the genetic engineering of a tandem vector encoding the hetero-tetrameric cTfRMAb-ScFv fusion protein (Figure 2). This tandem vector was engineered following the cloning of 6 separate genes, including the VH and VL of the 8D3 MAb against the mouse TfR, the VH and VL of a mouse MAb against the Aβ peptide of AD, the mouse IgG1 HC C-region, and the mouse kappa LC C-region[5,6,11]. Although the parent TfRMAb, the 8D3 MAb, is a rat IgG, the amino acid sequence of the cTfRMAb-ScFv fusion protein is >80% mouse origin[5]. Therefore, the fusion protein can be administered chronically to AD transgenic mice with minimal immune reaction. IgG fusion proteins may be minimally immunogenic, as recent work shows the constant region of IgG contains amino acid sequences called Tregitopes, which induce immune tolerance[15]. Recent work shows that the chronic administration of HIRMAb fusion proteins to primates results in minimal immune reactions[16]. Nevertheless, future chronic administration of the fusion protein will be required to fully ascertain the extent of immune reactions in mice. In

addition, future studies can determine the distribution of the fusion protein in brain at time points beyond 60 min after administration.

The cTfRMAb-ScFv fusion protein is a tri-functional fusion protein (Figure 1). The 'head' of the molecule binds the mouse TfR, to trigger receptor-mediated influx from blood to brain across the BBB on the endogenous TfR. The 'tail' of the molecule binds/disaggregates Aβ amyloid plaque. The 'mid-section' of the fusion protein, at the CH2–CH3 region, binds the neonatal Fc receptor (FcRn), which is expressed at the BBB[17], and binding to the BBB FcRn triggers the receptor-mediated efflux of the IgG-ScFv fusion protein from brain to blood across the BBB6. The BBB FcRn is a reverse transcytosis system that operates only uni-directionally in the brain to blood direction18. These 3 functionalities have been documented previously for the HIRMAb-ScFv fusion protein with in vivo studies in the Rhesus monkey, the rat, and the AD transgenic mouse6. The anti-Aβ ScFv part of the HIRMAb-ScFv fusion protein has the same binding affinity for the Aβ peptide as the original murine anti-Aβ MAb, and binds and disaggregates Aβ amyloid fibrils both in vitro and in vivo[6]. However, the HIRMAb-ScFv does not recognize the murine insulin receptor, and cannot penetrate the BBB of the mouse following peripheral administration. The species specificity of the antibodies against the BBB insulin or transferrin receptor is illustrated by the very low brain uptake in the mouse of the murine OX26 MAb against the rat TfR (Figure 8).

The cTfRMAb-ScFv fusion protein binds the mouse TfR with high affinity (Figure 5), and this correlates with a high degree of brain uptake in the mouse in vivo (Figure 8). The BBB PS product for the cTfRMAb-ScFv fusion protein is 2.0 uL/min/g, which is comparable to the previously reported BBB PS product for the 8D3 MAb in the mouse, 3.3 uL/min/g[4]. The ratio of the PS products for the rat 8D3 MAb, and the cTfRMAb-ScFv fusion protein, parallels the ratio of the KD values of antibody binding to the mouse TfR (Figure 5). The brain uptake (%ID/g) of the cTfRMAb-ScFv fusion protein is slightly higher than the brain uptake of the 8D3 MAb (Figure 8), owing to the higher plasma AUC for the cTfRMAb-ScFv fusion protein (Table 1), as compared to the 8D3 MAb[4].

The high brain uptake of the cTfRMAb-ScFv fusion protein is to be contrasted with the very low brain uptake of a MAb that has no specificity for a BBB receptor transport system. The OX26 MAb is a murine antibody against the rat TfR that does not recognize the mouse TfR[4]. The brain VD of the OX26, 11 uL/g (Table 2), is equal to the cerebral blood volume[4], which indicates the OX26 MAb does not cross the mouse BBB. The VD for the OX26 MAb in peripheral organs in the mouse represents the plasma volume in those organs (Table 2). The ratio of the organ VD for the cTfRMAb-ScFv fusion protein divided by the organ VD for the OX26 MAb is 14, 4, 2, and 2 for brain, liver, kidney, and heart, respectively (Table 2). This analysis shows that the cTfRMAb-ScFv fusion protein is preferentially targeted to brain, relative to peripheral organs in the mouse.

The brain uptake of the OX26 MAb in the mouse is representative of the brain uptake of an antibody that has no specificity for a BBB receptor. An anti-Aβ MAb has no affinity for a BBB transporter, and prior work as shown an anti-Aβ MAb has the same property as the OX26 MAb in the mouse, in that the brain VD is no different from the cerebral blood volume[6]. Such antibodies do not penetrate the BBB, and cannot access the amyloid plaque in brain behind the BBB, in the absence of BB disruption. In contrast, the cTfRMAb-ScFv fusion protein rapidly penetrates the BBB in the mouse, and 3.5% of the injected dose is taken up per gram brain (Figure 8). At a systemic dose of 1 mg/kg of the cTfRMAb-ScFv fusion protein, the brain concentration of the cTfRMAb-ScFv fusion protein is projected to be 4 pmol/gram. Previous studies show that brain concentrations of this magnitude are

sufficient to cause disaggregation and clearance of amyloid plaque in AD transgenic mouse brain[6].

In summary, the present studies describe the genetic engineering of an antibody fusion protein that has been specifically engineered to cross the mouse BBB, as well as bind the Aβ amyloid plaque of AD. The BBB-penetrating properties of the cTfRMAb-ScFv fusion protein enable the antibody to access the amyloid plaque in brain behind the BBB. In addition, the antibody is rapidly cleared from blood with a MRT of 3 hours (Table 1), and this rapid removal from blood will prevent the build-up of high concentrations of the Aβ-binding antibody in plasma. Future treatment studies of AD transgenic mice are required to fully evaluate the therapeutic and toxicologic effects of chronic administration of the cTfRMAb-ScFv fusion protein.

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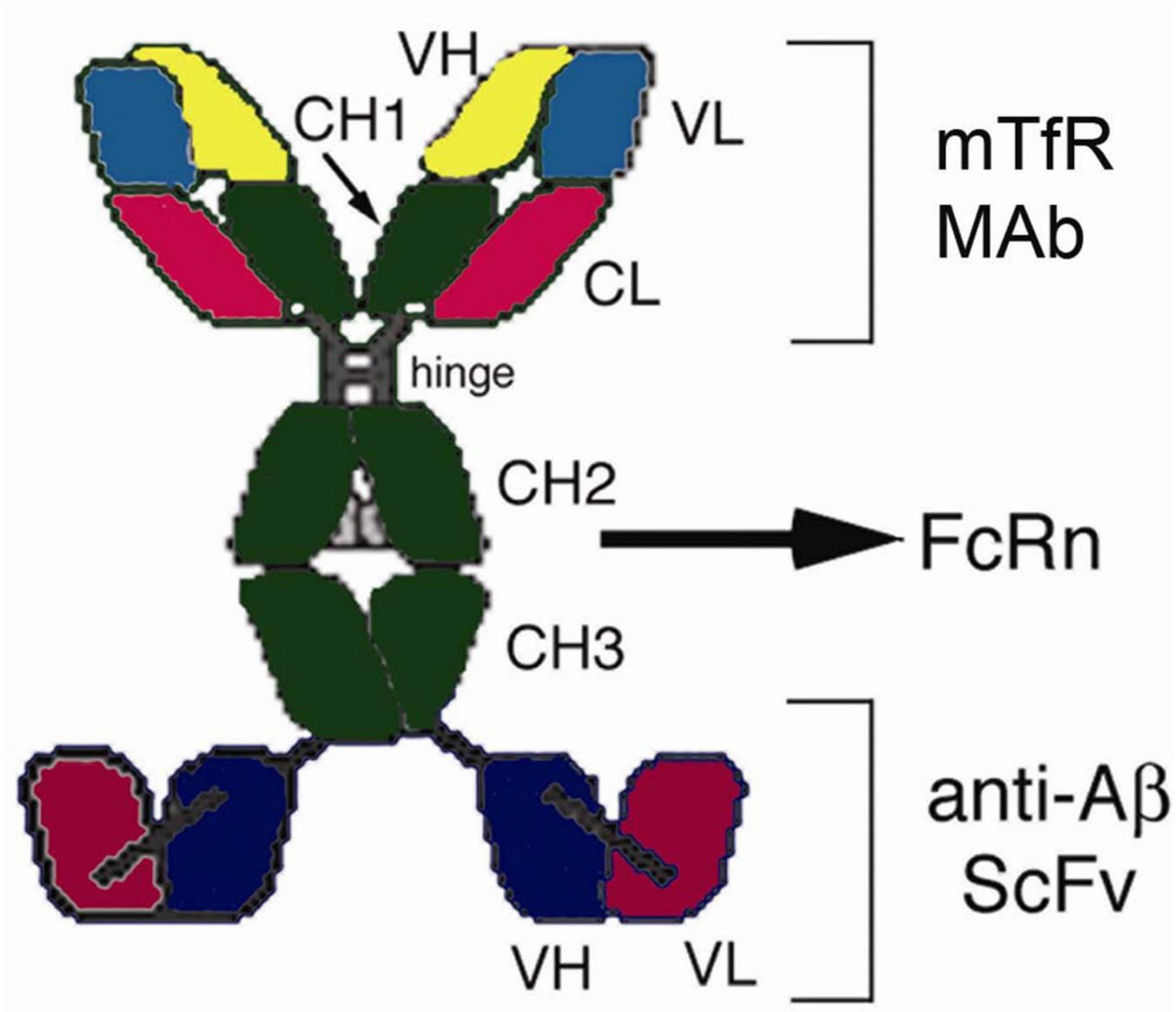

Figure 1.
The cTfRMAb-ScFv fusion protein is formed by fusion of the variable region of the heavy chain (VH) of the rat 8D3 MAb against the mouse transferrin receptor (mTfR) (yellow) to the amino terminus of mouse IgG1 constant-region (green), and fusion of a single chain Fv (ScFv) antibody against the Aβ amyloid peptide to the carboxyl terminus of the heavy chain C-region. The light chain is comprised of the variable region of the light chain (VL) of the rat 8D3 MAb (light blue) and the mouse kappa light chain C-region (CL) (dark red). The heavy chain constant-region is comprised of 4 domains: CH1, hinge, CH2, and CH3. The CH2–CH3 interface is the binding site for the neonatal Fc receptor (FcRn). The ScFv is comprised of the VH (dark blue) and the VL (light red) derived from the anti-Aβ MAb.

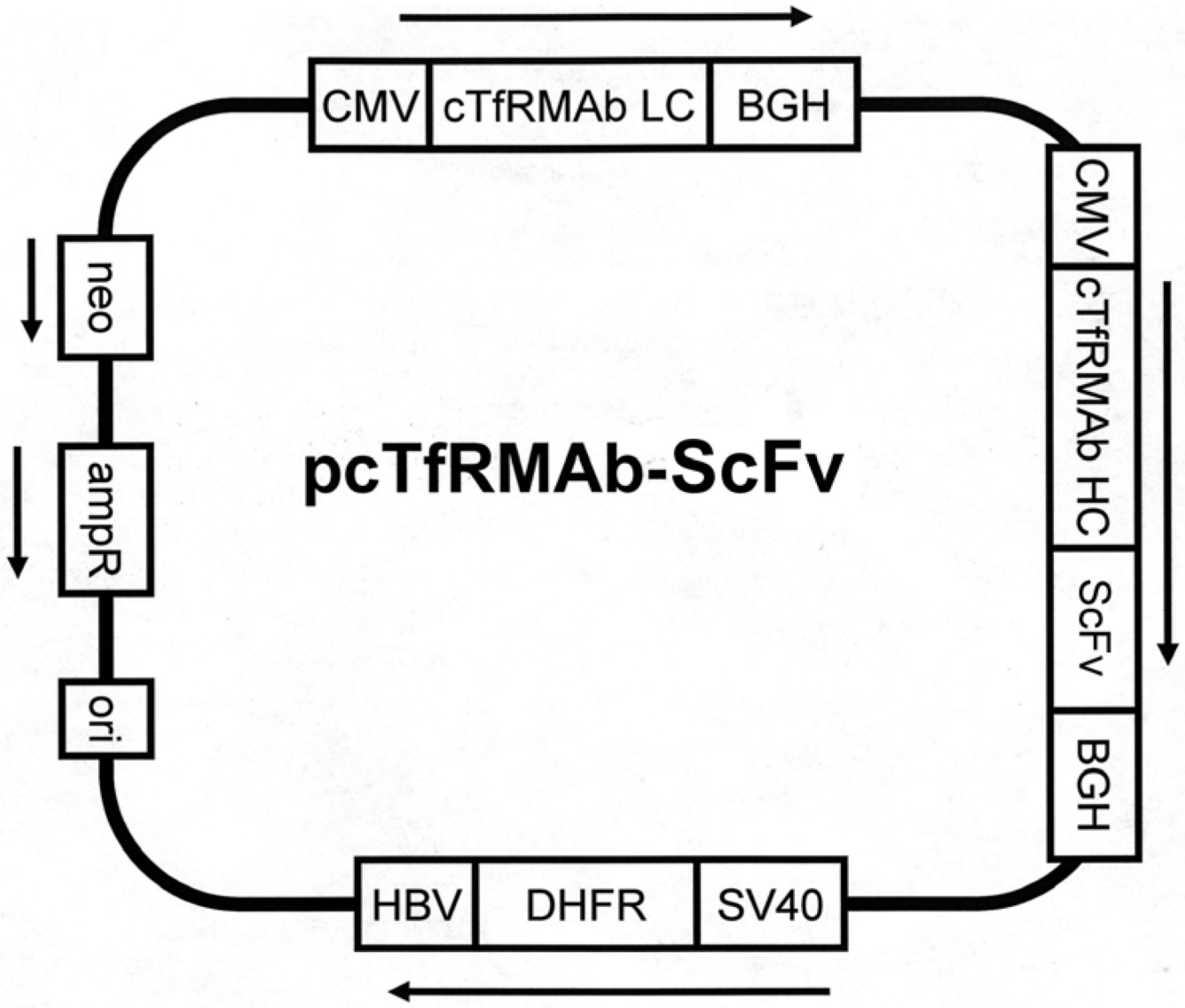

Figure 2.

The tandem vector expressing the cTfRMAb-ScFv fusion protein is comprised of separate expression cassettes on a single strand for the LC of the cTfRMAb, the HC gene formed by fusion of the cDNA encoding the ScFv to the 3' end of the cDNA encoding the HC of the cTfRMAb, and murine dihydrofolate reductase (DHFR), to allow for amplification of cell lines with methotrexate treatment. The HC and LC genes are 5'-flanked by the cytomegalovirus (CMV) promoter, and 3'-flanked by the bovine growth hormone (BGH) polyA sequence, and the DHFR gene is 5'-flanked by the SV40 promoter and the hepatitis B virus (HBV) polyA sequence. The plasmid also contains genes for neomycin (neo) resistance and ampicillin resistance (ampR).

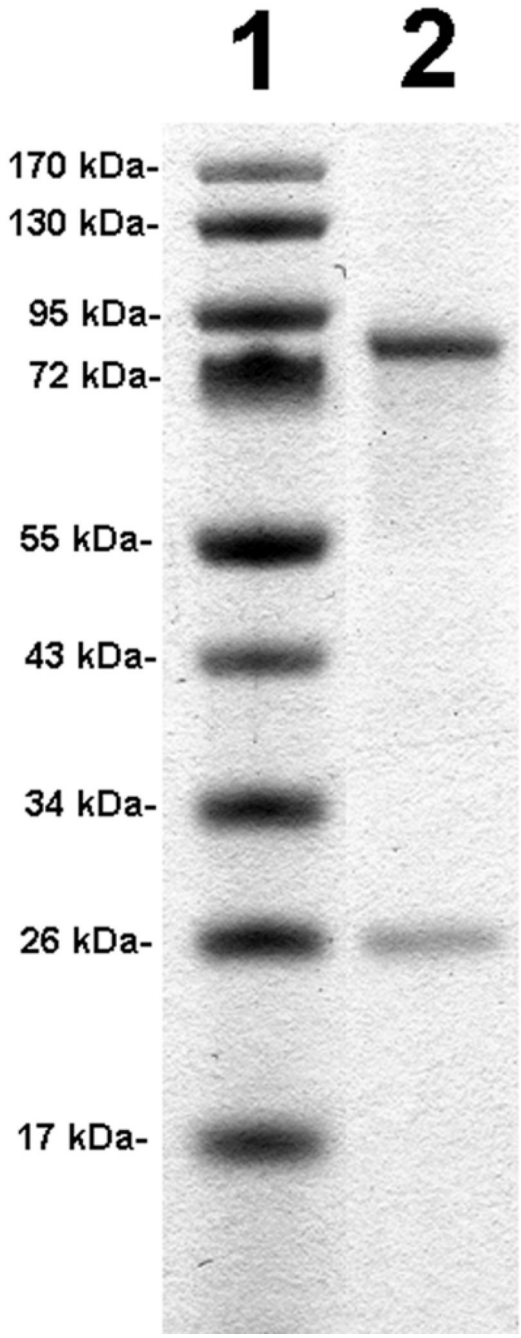

Figure 3.
Reducing SDS-PAGE of molecular weight standards (lane 1) and the cTfRMAb-ScFv fusion protein (lane 2). The size of the heavy chain is about 85 kDa and the size of the light chain is about 26 kDa.

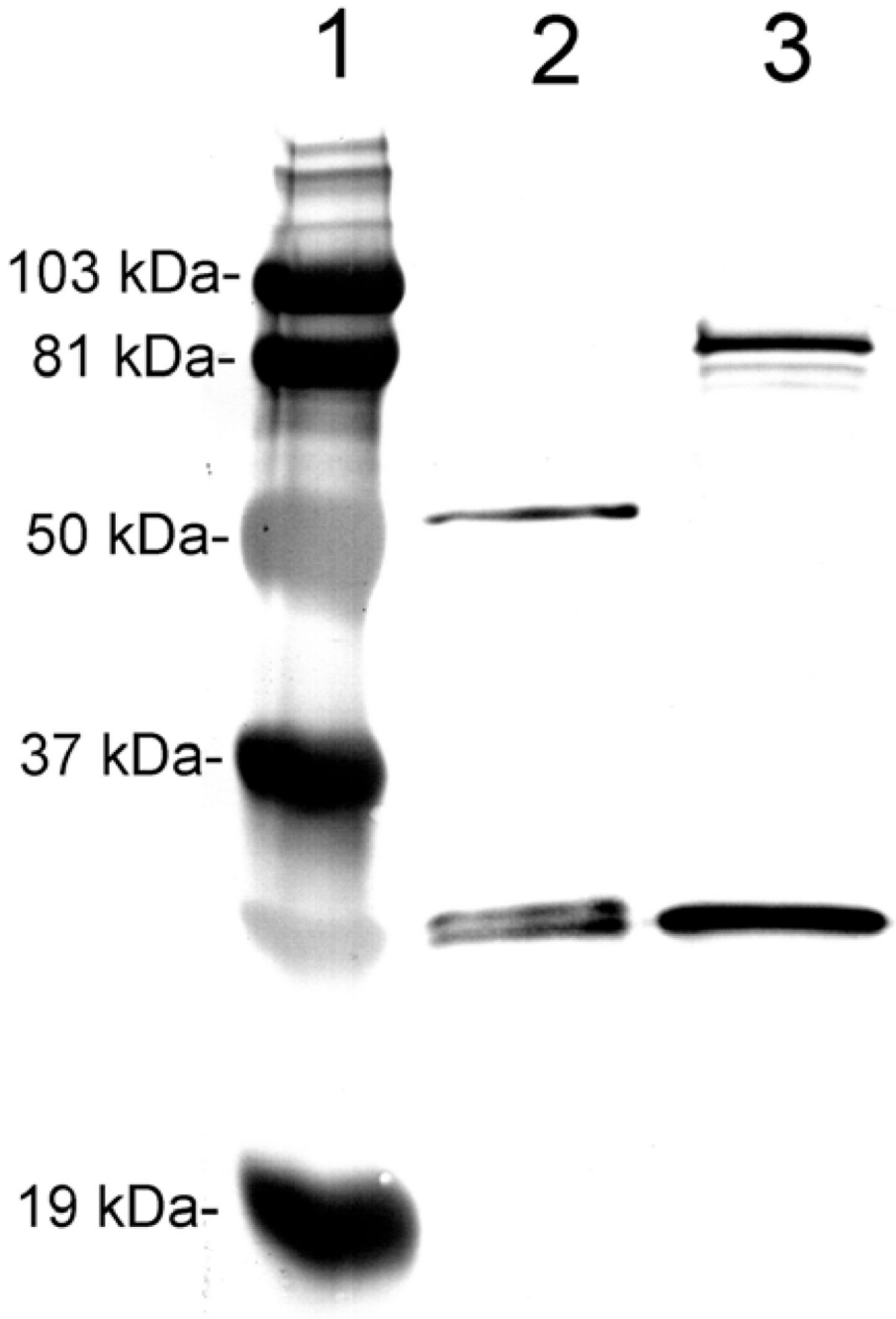

Figure 4.
Western blotting with a primary antibody against mouse IgG and biotinylated molecular weight standards (lane 1), the cTfRMAb (lane 2), and the cTfRMAb-ScFv fusion protein (lane 3). Both the cTfRMAb and the cTfRMAb-ScFv fusion protein share the same light chain. The size of the heavy chain of the cTfRMAb-ScFv fusion protein is about 28 kDa larger than the size heavy chain of the cTfRMAb owing to fusion of the ScFv.

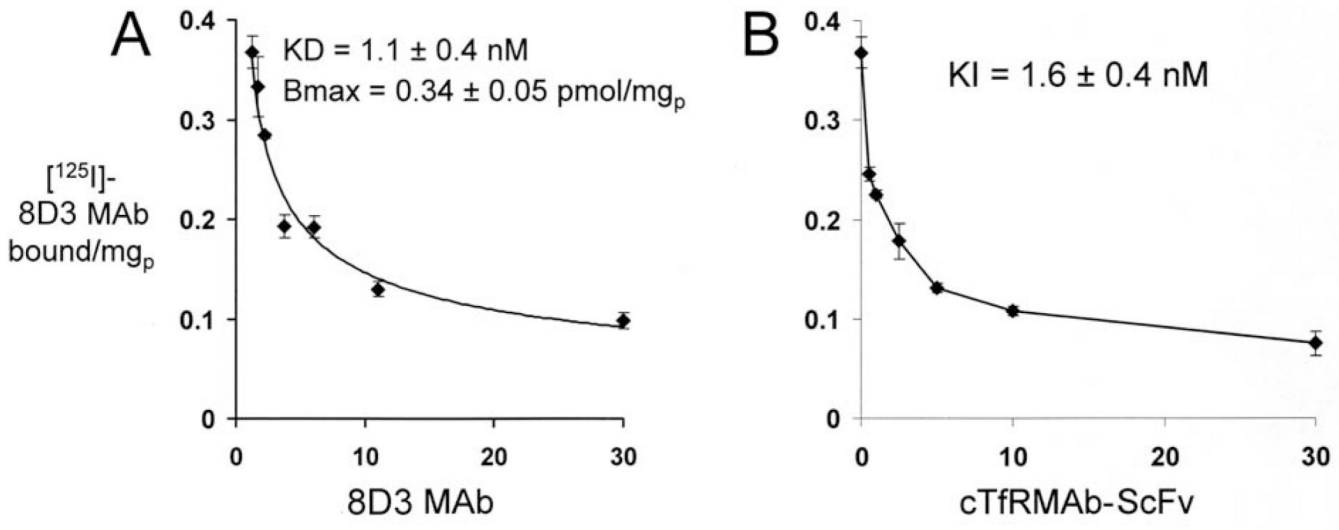

Figure 5.

Radio-receptor assay of the mouse TfR uses mouse fibroblasts as the source of the mouse TfR and [^{125}I]-8D3 as the binding ligand. Binding is displaced by unlabeled 8D3 MAb (A) or the cTfRMAb-ScFv fusion protein at concentrations up to 30 nM (B). The KD of 8D3 self-inhibition and the KI of chimeric TfRMAb cross-inhibition were computed by non-linear regression analysis.

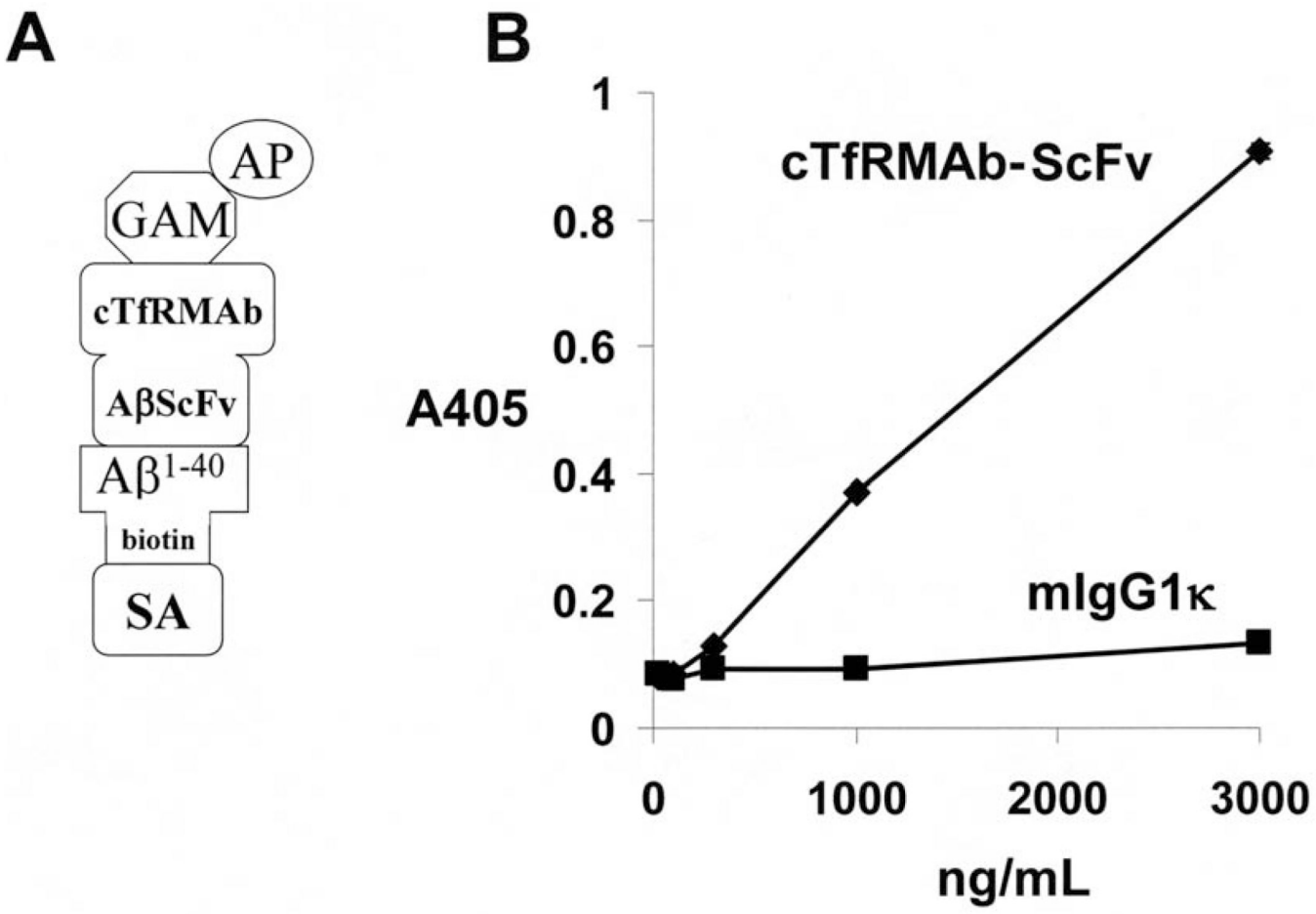

Figure 6.
(A) The ELISA used to measure cTfRMAb-ScFv binding to the $A\beta^{1–40}$ employs a complex of streptavidin (SA) and N-biotinyl $A\beta^{1–40}$ as the capture reagent and a goat anti-mouse (GAM) conjugate of alkaline phosphatase (AP) as the detector reagent. (B) Binding of the cTfRMAb-ScFv fusion protein to the $A\beta^{1–40}$ is linear, whereas there is no binding to the $A\beta^{1–40}$ of mouse (m) IgG1κ.

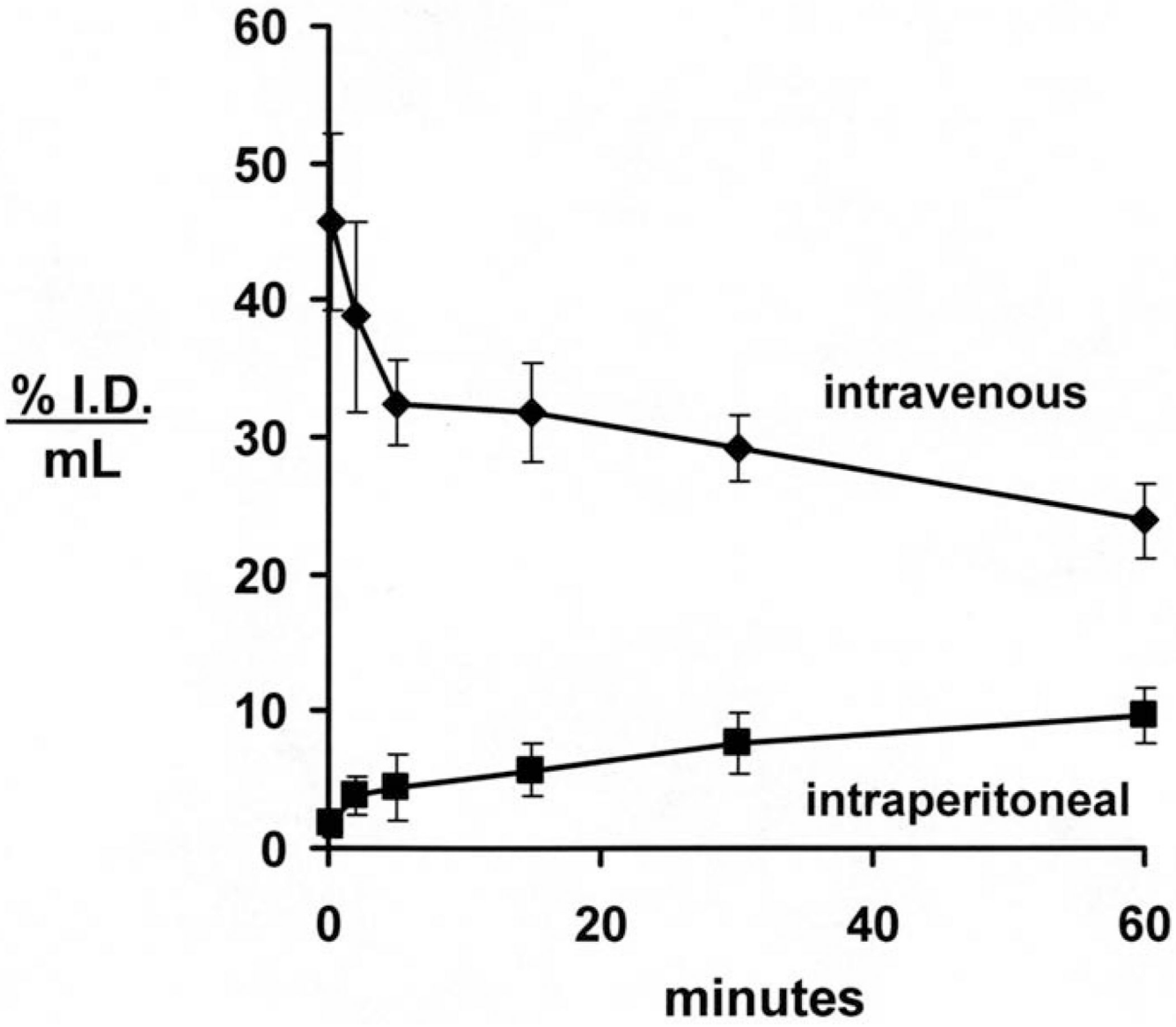

Figure 7.
Plasma concentration, expressed as % of injected dose (I.D.)/mL, of the [^{125}I]-cTfRMAb-ScFv fusion protein after either intravenous or intra-peritoneal injection in the mouse. Data are mean ± S.E. (n=3 mice/point).

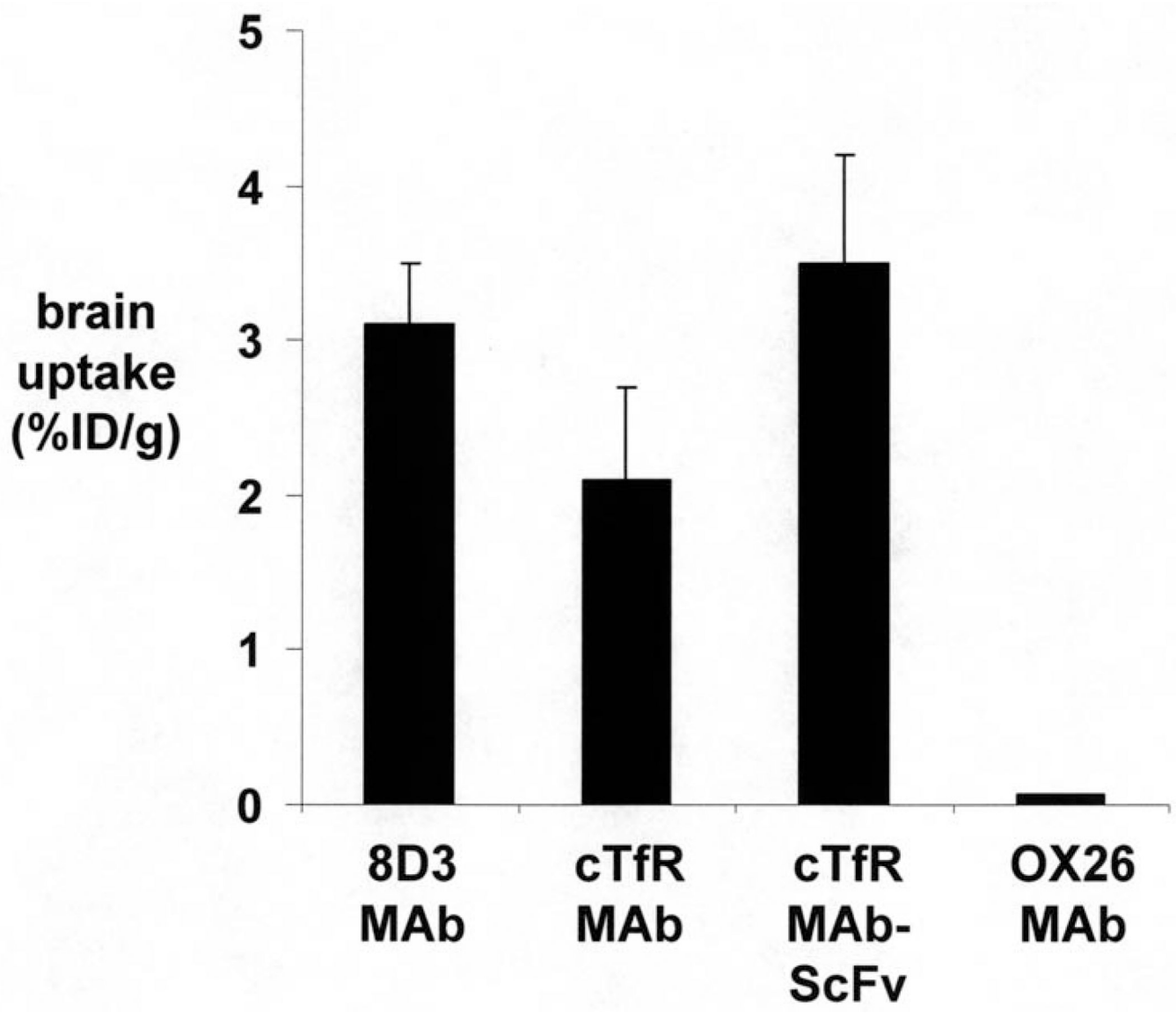

Figure 8.

Brain uptake, expressed as % of injected dose (ID)/gram brain, for the cTfRMAb-ScFv fusion protein, in comparison with the brain uptake values for the rat 8D3 MAb against the mouse TfR, the mouse OX26 MAb against the rat TfR, and the cTfRMAb reported previously4›5. Data are mean ± S.E. (n=3 mice).

Table 1

Pharmacokinetic parameters of cTfRMAb and cTfRMAb-ScFv in the mouse

Parameter	Units	cTfRMAb	cTfRMAb-ScFv
A^1	%ID/mL	18.4 ± 4.2	13.9 ± 2.4
A^2	%ID/mL	33.9 ± 2.1	34.0 ± 1.4
k^1	min^{-1}	0.71 ± 0.37	0.58 ± 0.24
k^2	min^{-1}	0.0048 ± 0.0016	0.0057 ± 0.0010
MRT	min	208 ± 68	175 ± 32
Vc	mL/kg	64 ± 5	81 ± 3
Vss	mL/kg	97 ± 6	114 ± 4
AUC(60 min)	%ID·min/mL	1794 ± 60	1752 ± 32
AUCss	%ID·min/mL	7098 ± 2010	5996 ± 926
Cl	mL/min/kg	0.47 ± 0.13	0.65 ± 0.08

A^1, A^2, k^1, and k^2 are the intercepts and slopes, respectively, of the bi-exponential clearance curves describing antibody removal from plasma. MRT=mean residence time; Vc=plasma volume; Vss=steady state volume of distribution; AUC(60 min)=area under the curve for the first 60 min; AUCss=steady state AUC; Cl=clearance from plasma. Parameters for the cTfRMAb were reported previously[5].

Table 2

Organ volume of distribution (VD) of TfR antibodies in the mouse

Organ	OX26 MAb	8D3 MAb	cTfRMAb	cTfRMAb-ScFv
brain	11± 1	102± 8	81 ± 22	156 ± 46
spleen	n.m.	n.m.	n.m.	712 ± 111
liver	162 ± 19	576± 110	552 ± 16	654 ± 93
kidney	136± 8	213± 7	443 ± 30	309 ± 61
lung	n.m.	n.m.	n.m.	270 ± 33
heart	87± 8	131± 5	175 ± 2	158± 34

VD data for the OX26 MAb, the 8D3 MAb, and for the cTfRMAb have been previously reported [4, 5]. n.m.=not measured.