Transport Across the Primate Blood-Brain Barrier of a Genetically Engineered Chimeric Monoclonal Antibody to the Human Insulin Receptor

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Purpose. Brain drug targeting may be achieved by conjugating drugs, that normally do not cross the blood-brain barrier (BBB), to brain drug delivery vectors. The murine 83-14 MAb to the human insulin receptor (HIR) is a potential brain drug targeting vector that could be used in humans, if this MAb was genetically engineered to form a chimeric antibody, where most of the immunogenic murine sequences are replaced by human antibody sequence.

Methods. The present studies describe the production of the gene for the chimeric HIRMAb, expression and characterization of the protein, radiolabeling of the chimeric HIRMAb with 111-indium and 125-iodine, and quantitative autoradiography of living primate brain taken 2 hours after intravenous administration of the [^{111}In]chimeric HIRMAb.

Results. The chimeric HIRMAb had identical affinity to the target antigen as the murine HIRMAb based on Western blotting and immunoradiometric assay using partially purified HIR affinity purified from serum free conditioned media produced by a CHO cell line secreting soluble HIR. The [^{125}I]chimeric HIRMAb was avidly bound to isolated human brain capillaries, and this binding was blocked by the murine HIRMAb. The [^{111}In]chimeric HIRMAb was administered intravenously to an anesthetized Rhesus monkey, and the 2 hour brain scan showed robust uptake of the chimeric antibody by the living primate brain.

Conclusions. A genetically engineered chimeric HIRMAb has been produced, and the chimeric antibody has identical reactivity to the human and primate BBB HIR as the original murine antibody. This chimeric HIRMAb may be used in humans for drug targeting through the BBB of neurodiagnostic or neurotherapeutic drugs that normally do not cross the BBB.

KEY WORDS: blood-brain barrier; BBB; drug targeting; drug delivery; human insulin receptor.

INTRODUCTION

Drug targeting to the central nervous system (CNS) is the limiting factor in CNS drug development because >98% of all drugs do not cross the brain capillary endothelial wall, which makes up the blood-brain barrier (BBB) in vivo (1). One strategy for drug targeting to the brain is to use endogenous BBB transport systems. Chimeric peptides are formed when a drug, that is normally not transported through the BBB, is conjugated to a peptide or peptidomimetic monoclonal antibody (MAb) that undergoes receptor-mediated transcytosis through the BBB in vivo on one of the endogenous peptide receptor systems localized within the brain capillary endothelial plasma membranes (2). Circulating peptides such as insulin, insulin-like growth factors (IGF), transferrin, or leptin undergo receptor-mediated transport through the BBB in vivo (3–9). These peptides could be used as brain drug delivery vectors. However, the administration of insulin conjugates would cause hypoglycemia owing to activation of insulin receptors in peripheral tissues. The 83-14 murine MAb is a 150 kDa insulin peptidomimetic MAb that binds an exofacial epitope on the α-subunit of the human insulin receptor (HIR) (10), and this MAb (i) is bound with high affinity to isolated human brain capillaries, used as an in vitro model of human BBB transport, and (ii) undergoes receptor-mediated transcytosis through the BBB in vivo in Old World primates such as the Rhesus monkey (11). The 83-14 MAb to the human insulin receptor is the most potent brain drug delivery vector known to date and has a BBB permeability-surface area (PS) product in the primate that is 9-fold greater than murine MAbs to the human transferrin receptor (TfR) (12). Approximately 4% of the injected dose (ID) of the HIRMAb is delivered to the primate brain in vivo (11), and this level of brain delivery is comparable to the brain uptake of many neuroactive small molecules (12).

The chimeric peptide technology has been used to deliver both neurodiagnostic (12,13) and neurotherapeutic molecules (14,15) across the rodent or primate BBB, and could be used for BBB drug targeting in humans, should the peptidomimetic MAb transport vector be genetically engineered to remove the immunodominant murine sequences that would produce immune reactions in humans. Human/mouse chimeric MAbs are genetically engineered antibodies wherein approximately 85% of the sequence is human and 15% is murine (16). The murine sequences encoding the variable region of the heavy chain (V_H) or the variable region of the light chain (V_L) may be spliced into gene fragments containing the constant regions of the heavy chain (HC) and the light chain (LC) of human immunoglobulin (17). Following transfection of mammalian cell lines, the chimeric MAb may be expressed and affinity purified. The present studies describe the genetic engineering, expression, and purification of a chimeric MAb to the HIR. These studies characterize the affinity of the chimeric HIRMAb for the human insulin receptor, describe the labeling of the chimeric HIRMAb with 125-iodine or 111-indium, and demonstrate brain imaging with the [^{111}In]-chimeric HIRMAb in the living primate.

METHODS

Materials

The murine 83-14 HIRMAb was purified with protein G affinity chromatography from concentrated serum free media conditioned by the hybridoma, as described previously (11);

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125-iodine, 111-indium, and Reflection x-ray film were purchased from Dupont-New England Nuclear (Boston, MA). Diethylenetriamine pentaacetic acid (DTPA) dianhydride was obtained from Aldrich Chemical Company (Milwaukee, WI). Kodak Biomax MS-1 x-ray film and all chemical reagents were obtained from Sigma Chemical Co. (St. Louis). A 32-year old male Rhesus monkey (8.5 kg) was obtained from the Manheimmer Foundation (Homestead, FL). Immobilon-P filters (0.45 micron) were purchased from Millipore Corp. (Bedford, MA). The ABC avidin-peroxidase immunostaining kit, biotinylated horse anti-mouse IgG, and biotinylated goat anti-human IgG were obtained from Vector Laboratories (Burlingame, CA). IM-9 cells (CCL159) were obtained from the American Type Culture Collection (ATCC, Rockville, MD)

Genetic Engineering

The 83-14 hybridoma cell line was kindly provided by Dr. Kenneth Siddle (Cambridge, UK). Total RNA was isolated from 5×10^5 hybridoma cells using the single step guanidinium/phenol method (18). First strand cDNA was synthesized and used as template in a polymerase chain reaction (PCR) to amplify the variable regions as described previously (19). A first PCR was performed using degenerate leader and constant region primers specific for the heavy and the light chain. The PCR products were cloned into the PCR2 T-A plasmid (Invitrogen) and the complete rearranged variable region sequence determined. Then the amplified heavy and light chain variable regions were modified with leader and J region primers containing appropriate restriction sites to allow cloning into expression vectors. The expression vectors containing human Gamma I and human Kappa constant regions have been widely used to produce chimeric antibodies of different specificity (19). The light chain variable region was cloned into the expression vector pAG4622 (19) as a EcoR V-Sal I fragment generating pAG4691. Due to the presence of a EcoR V site within the framework I region of the heavy region, this was cloned into the expression vector pAH4604 as a Ssp I-Nhe I fragment giving rise to pAH4695 (Fig. 1).

The non producer hybridoma cell line NSO 1 was used as recipient and was kept in IMDM medium containing 5% bovine calf serum until transfection. Eight μg of maxi prep DNA from each expression vector (pAG4691 and pAH4695) was linearized by digestion with Bspc 1 (Stratagene, Pvu I isoschizomer) and 1×10^7 NSO 1 cells were co-transfected by electroporation with standard parameters. The transfected cells were plated into five 96 well plates at a concentration of 10^4 cells/well in IMDM with 10% calf serum. After 48 hrs, selection was started with a final concentration of 10 mM histidinol. After two weeks, transfectants were screened by ELISA to test for the secretion of both chains using plates coated with anti-human-IgG1 and developed with anti-human kappa conjugated to alkaline phosphatase. High producers secreting both H and L chains were expanded for further analysis and subcloned. To determine proper assembly and size and to visualize the antibodies secreted, 1×10^6 transfectants were biosynthetically labeled overnight with 25 μCi ^{35}S methionine; cytoplasmic and secreted antibodies were immunoprecipitated with rabbit anti-human Ig and protein A and the immunoprecipitates were fractionated on SDS-PAGE 5% acrylamide 0.1-% sodium phosphate buffered gels were used to confirm proper assembly of heavy (H) and light (L) chains and 12% acrylamide Tris-Glycine gels were used to analyze antibodies reduced by treatment with 0.15 M 2-mercaptoethanol to confirm the size of the unassembled H and L chains. The gels were stained, dried and exposed to film.

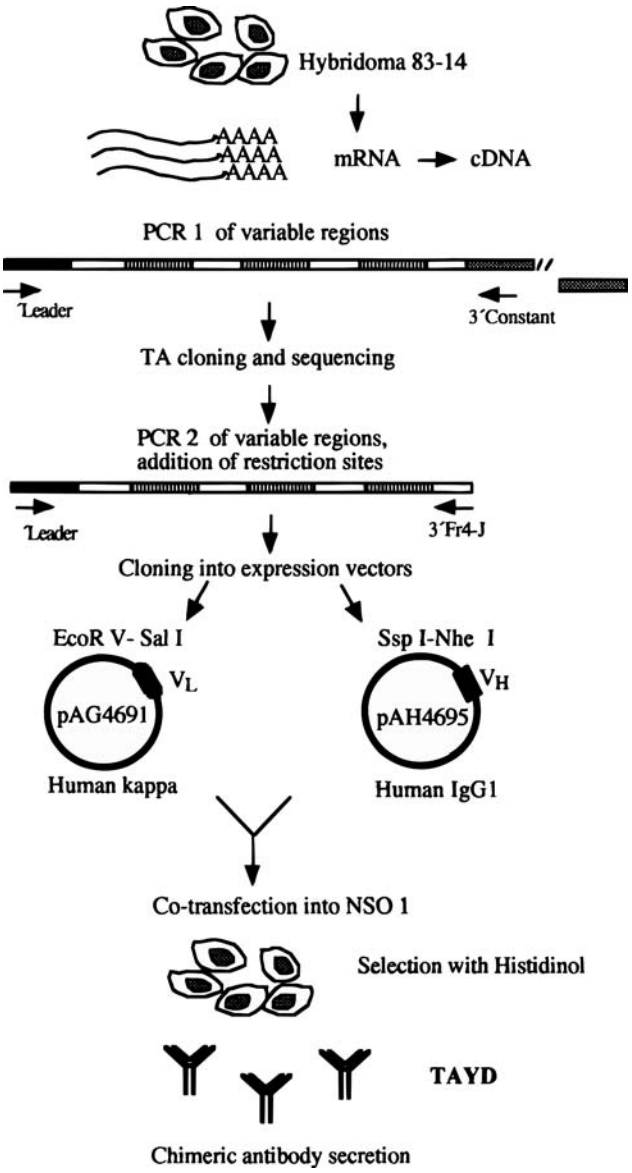

Fig. 1. The cloning strategy used to produce a mouse-human chimeric antibody specific for the human insulin receptor is shown. The expression vectors, pAG4622 and pAH4604, are used for IgG light chain and heavy chain cloning, respectively, and allow for direct cloning of PCR generated variable regions; these plasmids contain human IgG1 or human Kappa constant regions, respectively, as well as an immunoglobulin promotor and enhancer (19). The resulting expression vectors pAG4691 and pAH4695 where used to co-transfect NSO-1 cells giving rise to TAYD.

The selected transfectant producing the chimeric HIRMAb was designated TAYD, and was expanded to 2 liter roller bottles and grown at 37°C in IMDM supplemented with 1% alpha calf serum (Hyclone Labs, Logan, UT) and 100x Glutamax for 2–4 weeks or until high density was reached (~1×10^7 cells/

ml). Supernatants were harvested by centrifugation, filtered and stored at 4°C after addition of sodium azide to 0.02%, EDTA to 1mM, sodium phosphate pH 6.5 to 10 mM, NaCl to 0.45 M and the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) to 2 mM. The supernatants were chromatographed for three times at 4°C over 2 ml of a rabbit anti-human kappa column, and 1 ml fractions eluted into 150 μl 1 M Tris pH 8.0; elution was with 1 ml 0.1 M citric acid pH 4.5, 4 ml 0.1 M glycine pH 2.5 and 3 ml 0.1 M glycine pH 2.0. The antibody eluted at pH = 2.5, and the purity was checked by PAGE. The eluted antibodies were concentrated using Amicon microcentrators and dialyzed against PBS with 0.02% azide. The protein content was quantified by the BCA assay and the sample was either stored at 4°C or snap frozen at −70°C.

To confirm the preservation of the specificity of the chimeric protein, purified antibody was incubated with the human lymphoblastoid cell line IM-9 which expresses the insulin receptor on its surface and specific binding detected by fluorescence activated cell sorting (FACS). IM-9 cells were maintained in 5% calf serum until used. Cells were washed 3 times in cold assay buffer (PBS with 1% FCS) prior to the assay, and resuspended in the same buffer to a final concentration of 5×10^6 cells/ml. All incubations were on ice; 100 μl of cells were incubated for 1 hour with 1 μg of purified recombinant TAYD or antibody with irrelevant specificity. After 3 washes, 100 μl of a biotinylated anti-human IgG diluted1:200 in assay buffer were added for 30 min and after washing, 100 μl of the 15 μg/ml streptavidin-phycoerythrin (Caltag Labs, San Francisco, CA) diluted 1:500 were added and incubated for 30 min. The stained cells were finally fixed with 200 μl 1% paraformaldehyde, transferred to 5 ml Falcon FACS tubes and covered with foil; 1×10^5 cells were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 15 mW, 488 nm air-cooled argon-ion laser. Initial gating was done using forward- and side- scatter to eliminate dead cells and cell debris from subsequent measurements. For each sample, 10,000 events were collected, and data was analyzed using LYSIS II software and histograms plotted. These studies showed a significant shift when TAYD was bound to IM-9 cells while no shift was observed when an anti-dansyl antibody was used as a control, indicating that the specificity of the TAYD chimeric MAb to the HIR is maintained.

Purification of Soluble Human Insulin Receptor

A CHO cell line that was stably transfected with a gene encoding soluble human insulin receptor with a partially truncated beta subunit (20), was kindly provided by Dr. Colin Ward of the CSIRO Division of Molecular Science (Parkville, Victoria, Australia), and was grown in glutamine free minimal essential media (MEM) with 10% dialyzed fetal calf serum, and 100 μM of methionine sulfoxime (MSX) until >5 L of serum free conditioned medium was obtained. This was reduced to a final volume of 200 mL with an Amicon concentrator (Model RA2000) using an S1Y10 membrane. The solution was further reduced to a volume of 40 mL with a Centricon + 80-Biomax 100 microconcentrator (Amicon Corp.). This was then applied to a 5 mL column of Sepharose 6MB conjugated wheat germ agglutinin (WGA) obtained from Sigma Chemical Company (St. Louis, MO), and the soluble HIR was eluted with 20 mL of 0.3 M N-acetylglucosamine (NAGA) in 0.01 M HEPES/ pH = 7.4, as described previously (3). The A_{280} peak was pooled and the volume was reduced to 1.0 mL with a Centricon + 80-Biomax 100 microconcentrator and then dialyzed overnight at 4°C against 10 L of PBS with a 12,000 Dalton molecular weight cutoff. The final protein content was measured with the bicinchoninic acid (BCA) assay from Pierce Chemical Company (Rockford, IL). A typical yield of NAGA eluate from the WGA column was 235 μg of protein per liter of unconcentrated serum free media.

Western Blotting

The binding of the murine and chimeric HIRMAb to the HIR purified from CHO cell media was measured with Western blotting; 35 μg of NAGA eluate was applied per lane of 7.5% mini polyacrylamide gels followed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing (5% β-mercaptoethanol) conditions. Biotinylated molecular weight standards were also applied to the gel. Following SDS-PAGE, the gel was blotted to Immobilon-P filters with 15% methanol at 100 volts for 60 minutes. The filters were reacted with either 3 μg/mL of murine HIRMAb or chimeric HIRMAb, mouse IgG2a isotype control, or human IgG. The secondary antibody used for either the murine HIRMAb or the mouse IgG2a was a biotinylated horse anti-mouse antibody; the secondary antibody used for either the chimeric HIRMAb or the human IgG was a biotinylated goat anti-human IgG antibody, and both secondary antibodies were used at 3 μg/mL concentration.

Antibody Radiolabeling

The murine or chimeric HIRMAb was radioiodinated to a specific activity of 21 or 6 μCi/μg, respectively, and trichloroacetic acid (TCA) precipitability of >98%, with 125-iodine and chloramine T followed by Sephadex G-25 gel filtration chromatography using methods described previously (11). For labeling with 111-indium, the chimeric HIRMAb was conjugated with DTPA-dianhydride, as described previously (21). The molar ratio of DTPA-dianhydride to chimeric HIRMAb was 15:1. The DTPA-chimeric HIRMAb was radiolabeled with 111-indium by mixing 26 μg (172 pmol) of DTPA-chimeric HIRMAb in ABS buffer with 2.5 mCi (225 pmol) of 111-indium followed by G25 gel filtration chromatography (0.7×28 cm column) in PBS buffer (0.01 M NaH_2PO_4, 0.15 M NaCl, pH = 7.4). The void volume of a column containing the [^{111}In-DTPA] chimeric HIRMAb was pooled and used within 24 hours for pharmacokinetics and QAR brain scanning in anesthetized Rhesus monkeys.

Immunoradiometric Assay (IRMA)

The affinity of the chimeric HIRMAb for the HIR was determined by IRMA using methods described previously (21). NUNC Maxisorb 96 well ELISA plates were plated with 16 μg per well of NAGA eluate of the CHO cell soluble HIR extracellular domain dissolved in 0.1 M $NaHCO_3$ (pH = 8.3). Each well (100 μL) contained 0.04 μCi of [^{125}I] murine HIRMAb (0.02 μg/mL) and 0.01–10 μg/mL of either unlabeled murine HIRMAb, chimeric HIRMAb, or DTPA conjugated chimeric HIRMAb in PBST buffer, where PBST = PBS plus 0.05% Tween 20. Following incubation for 2.5 hours at room

temperature, the wells were washed three times with a 180 μL per well of cold PBSHT buffer and the removable wells were counted for [^{125}I] radioactivity.

Binding fractions of the labeled murine HIRMAb were expressed as the ratio of bound to total counts, and dissociation constants (K_D) for antibody binding to the soluble HIR were estimated by unweighted least squares fits of the competition curves using the nonlinear regression program BMDP3R (22). Predicted bound fractions were computed by a Newton-Raphson technique for a 1-site model with separate K_D for each of the three HIRMAb species but a common binding capacity and non-specific binding (23). An additional scaling parameter was estimated to account for differences in yield of soluble HIR when separate competition studies were fit simultaneously. Estimated K_D's and their ratios are summarized as regression estimates ± asymptotic standard errors.

Human Brain Capillary Radioreceptor Assay

The binding of the [^{125}I] chimeric HIRMAb to human brain capillaries was determined by incubating isolated human brain capillaries in 0.45 mL of Ringer-HEPES buffer (RHB) containing 0.1% human serum albumin (HSA) and 0.2 μCi/mL of [^{125}I] chimeric HIRMAb (3). The human brain capillaries were isolated with a mechanical homogenization technique from human autopsy brain as described previously (3). Prior studies showed isotype control antibodies cause no inhibition of the human brain capillary binding of the HIRMAb (11). The microvessels are patent (24), so that both luminal and abluminal endothelial membranes are simultaneously exposed in the binding experiment.

Pharmacokinetics and Quantitative Autoradiography

The pharmacokinetics and brain uptake of the [^{111}In] chimeric HIRMAb was measured in an 8.5 kg 32-year old male Rhesus monkey, as described previously (11,12). The intravenous injection solution was 0.8 mL of PBS containing 400 μCi of [^{111}In-DTPA] chimeric HIRMAb and 1 mL of arterial blood was sampled at 0.5, 1, 2.5, 5, 15, 30, 60, and 120 minutes after injection. The animal was euthanized with 100 mg/kg of sodium pentobarbital intravenously followed by decapitation for rapid removal of brain. Small aliquots (approximately 1.0 g) were removed from the occipital lobes for capillary depletion analysis as described previously (11). The remaining brain was sagittally sectioned and divided into 5 coronal slabs, which were rapidly frozen for film autoradiography on 30 micron frozen sections using Biomax MS film, as described previously (12). The x-ray film was scanned with a Hewlett-Packard Scanjet IIcx/T flatbed scanner and transferred to Adobe Photoshop on a Power Macintosh 7100/66 microcomputer, followed by colorization with NIH image software (12).

The pharmacokinetic parameters were estimated by fitting the plasma radioactivity data to a biexponential equation (25), by derivative-free nonlinear regression analysis (BMDP program AR, Biomedical computer P-series developed at the UCLA Health Science Computing Facility) (22). Parameters were estimated with omission of the 0.5 min data and fitting unweighted plasma data. The inclusion of the 0.5 min data point did not result in a significant change in the parameters, but caused a 100-fold increase in the residual sums of squares. The area under the plasma concentration-time curve (AUC), the steady state volume of distribution (V_{ss}), the total plasma clearance (Cl), and the mean residence time (MRT) were calculated as described previously (11,12). The BBB PS product was calculated at 120 minutes as described previously (11), using a brain plasma volume of 30 μL/g, which has been measured previously (11).

Serum Chromatography Analysis

The stability of the 111-indium labeled chimeric HIRMAb in the primate in vivo was determined by injecting the 120 minute serum on to a Superose 12 HR 10/30 gel filtration fast protein liquid chromatography (FPLC) column with elution at 0.5 mL/min in PBST and collection of 0.5 mL fractions. Controls included the 111-indium labeled chimeric HIRMAb mixed in either PBS or in control Rhesus monkey serum.

RESULTS

The reactivity of the murine HIRMAb and the chimeric HIRMAb with the α-subunit of the HIR was assessed by Western blotting as shown in Fig. 2, where the murine HIRMAb is designated as 83-14 and the chimeric HIRMAb is designated as TAYD. Both antibodies reacted with a 130 kDa band, the size characteristic of the α-subunit of the HIR (3). The murine 83-14 HIRMAb was detected with a horse anti-mouse secondary antibody, whereas the chimeric TAYD HIRMAb was

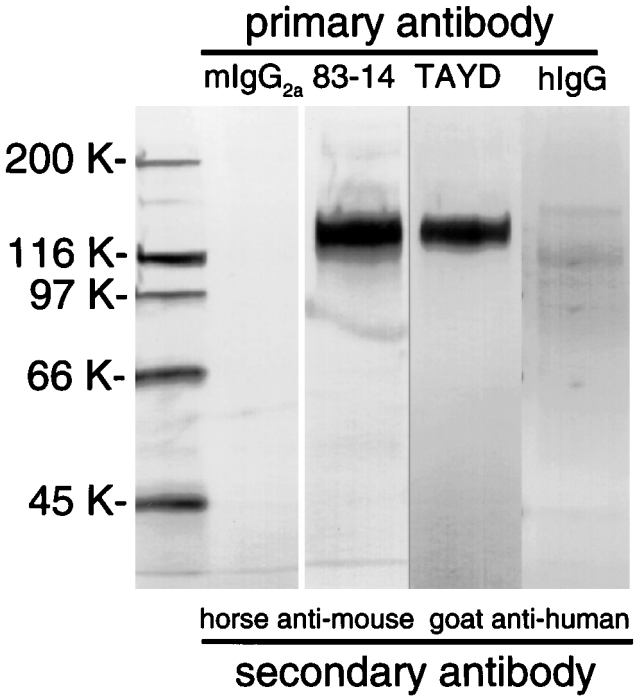

Fig. 2. Western blot showing reactivity of mouse IgG2a isotype control, murine 83-14 HIRMAb, chimeric TAYD HIRMAb, or human IgG as the primary antibody. The filters were blotted following SDS-PAGE of soluble human insulin receptor produced from a transfected CHO cell line. The secondary antibody used for the mouse IgG2a or the 83-14 murine HIRMAb was a horse anti-mouse IgG. The secondary antibody used for the chimeric TAYD HIRMAb or the human IgG was a goat anti-human IgG.

detected with a goat anti-human secondary antibody (Fig. 2). The murine 83-14 HIRMAb and the chimeric TAYD HIRMAb also gave equal reactivity in the IRMA (Fig. 3). Nonlinear regression analysis of this data yielded the K_D of MAb binding to the CHO cell secreted HIR, 0.13 ± 0.06 nM. This K_D was not significantly different from K_D for chimeric HIRMAb, or the DTPA conjugated chimeric HIRMAb, as the ratio of the K_D for the chimeric/murine MAbs, 1.54 ± 0.40, or the DTPA-chimeric/murine MAbs, 0.89 ± 0.41, respectively, were not significantly different from unity.

The TAYD chimeric HIRMAb was radiolabeled with 125-iodine and added to isolated human brain capillaries, which are shown in Fig. 4A. The binding increased with time and was nearly completely suppressed by the addition of 10 μg/mL of murine 83-14 HIRMAb (Fig. 4B).

The [^{111}In-DTPA] chimeric HIRMAb was injected intravenously into the anesthetized Rhesus monkey and the decrease in plasma radioactivity over 2 hours after injection is shown in Fig. 5. These data were subjected to pharmacokinetic analysis (Methods) and the pharmacokinetic parameters are listed in Table 1 in comparison with previously reported pharmacokinetic parameters for the 83-14 murine HIRMAb. The chimeric HIRMAb was metabolically stable and there was no measurable production of low molecular weight radiolabeled metabolites in the serum as shown by the gel filtration FPLC profile in Fig. 6.

The 2 hour brain V_D, BBB PS product, and %ID/100 g brain are shown in Table 1 for the [^{111}In]chimeric HIRMAb,

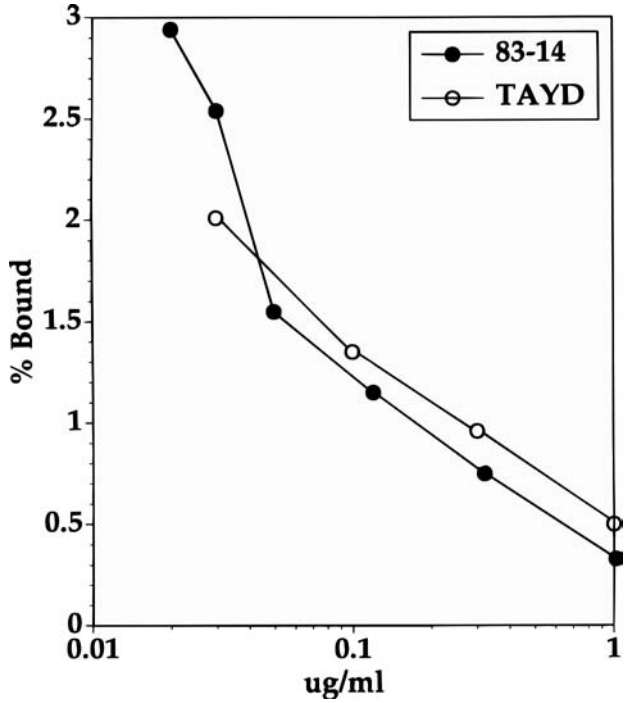

Fig. 3. Immunoradiometric assay (IRMA) was performed by binding [^{125}I] murine 83-14 HIRMAb (0.02 μg/mL) to wells plated with soluble HIR produced from transfected CHO cells. The binding of the radiolabeled murine HIRMAb to the plate was competitively inhibited by increasing concentrations of unlabeled murine 83-14 HIRMAb (closed circles) or chimeric TAYD HIRMAb (open circles). The abscissa is total antibody binding of the [^{125}I] murine 83-14 HIRMAb.

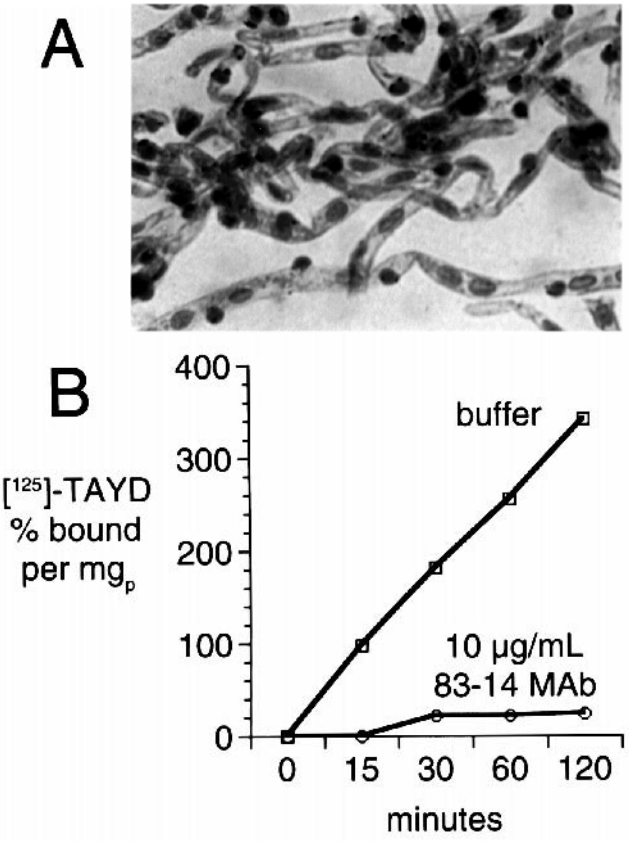

Fig. 4. (A) Light micrograph of isolated human brain capillaries showing the capillary structures are free of the adjoining brain tissue. (B) The binding to the human brain capillaries of [^{125}I] TAYD chimeric HIRMAb is plotted versus the time of incubation and is expressed as % bound per mg of capillary protein. Each tube contained approximately 0.05 mgp, indicating about 20% of the total antibody was bound per tube. The binding increased with time in the presence of buffer, but was nearly completely suppressed by the addition of 10 μg/mL murine 83-14 HIRMAb.

and these data are compared to previously reported data for the [^{125}I]murine HIRMAb (11). The capillary depletion analysis generates the ratio of brain V_D that is in the post-vascular supernatant relative to the brain V_D in the capillary pellet; a ratio of 1.0 indicates 50% of the antibody that is taken up from blood has transcytosed through the BBB to enter the brain interstitium and brain cells (11). The data in Table 1 indicate the degree of transcytosis of the chimeric HIRMAb at 2 hours is comparable to the murine HIRMAb at 3 hours.

The 2 hour brain scan, as determined by QAR (Methods), is shown in Fig. 7. These data show uniform uptake of the [^{111}In]-chimeric HIRMAb in brain with a preferential uptake in gray matter tracks relative to the white matter tracks of brain.

DISCUSSION

These studies describe the production of a chimeric HIRMAb and are consistent with the following conclusions. First, the murine and chimeric HIRMAb have equal reactivity with the HIR produced from CHO cells based on both Western blotting (Fig. 2) and IRMA analysis (Fig. 3). Second, there is avid binding of the [^{125}I] chimeric HIRMAb to isolated human

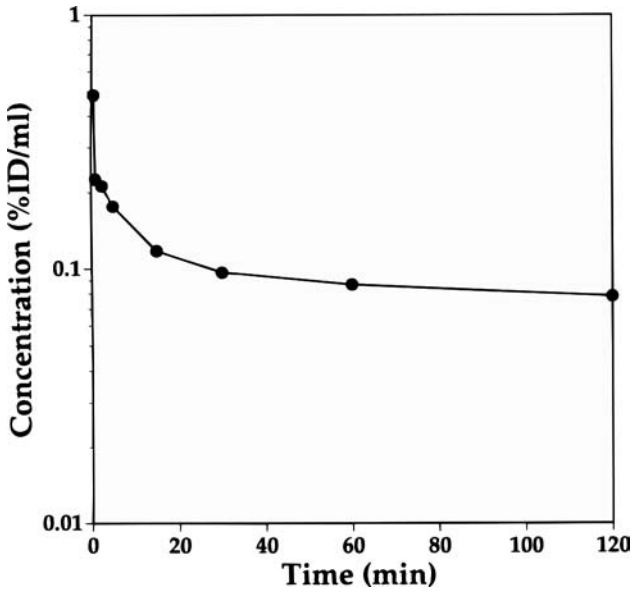

Fig. 5. The percent injected dose (ID) per mL plasma of [^{111}In-DTPA] chimeric HIRMAb is plotted versus time after single intravenous injection of the isotope in the anesthetized Rhesus monkey.

brain capillaries (Fig. 4) and this degree of binding is comparable to that reported previously for the murine HIRMAb (11). Third, the pharmacokinetic parameters of plasma clearance of the [^{111}In-DTPA] chimeric HIRMAb are comparable to the previously reported pharmacokinetic parameters for the [^{125}I] murine HIRMAb (Fig. 5, Table 1). Fourth, there is avid uptake by the primate brain in vivo of the [^{111} In-DTPA] chimeric HIRMAb (Fig. 7), and this reflects the brain uptake of the actual MAb and not low molecular weight radiolabeled metabolites, based on the metabolic stability of the [^{111}In-DTPA]chimeric HIRMAb as shown by the FPLC analysis (Fig. 6).

Table 1. Pharmacokinetic and Brain Uptake Parameters

Parameter (units)	HIRMAb	
	[^{111}In]-chimeric	[^{125}I]-murine
K_1 (min^{-1})	0.12 ± 0.01	0.27–0.29
K_2 (min^{-1})	0.0018 ± 0.0010	0.060–0.14
$t^1_{1/2}$ (min)	5.8 ± 0.6	1.9–2.4
$t^2_{1/2}$ (min)	380 ± 39	300–672
A_1 (%ID/mL)	0.15 ± 0.01	0.21–0.27
A_2 (%ID/mL)	0.10 ± 0.01	0.027–0.038
$AUCl^{\infty}_0$ (%ID•min/mL)	55 ± 5	12.5–38.1
V_{SS} (mL/kg)	116 ± 11	367–406
C1 (mL/min/kg)	0.22 ± 0.08	0.39–1.00
MRT (hr)	8.9 ± 0.9	6.8–15.9
brain V_D (μL/g)	287 ± 3	1263–1329
BBB PS (μL/min/g)	1.7 ± 0.1	5.3–5.4
%ID/100 g brain	2.0 ± 0.1	2.5–3.8
V_D (sup)/V_D (capillary)	0.78 ± 0.08	0.60–0.80

Note: The chimeric data were measured over a 2 hour period for 1 monkey and the data are mean of 3 triplicate measurements from the same animal (Fig. 5). The murine data were measured over a 3 hour period reported previously (11), and the range for 2 monkeys is shown.

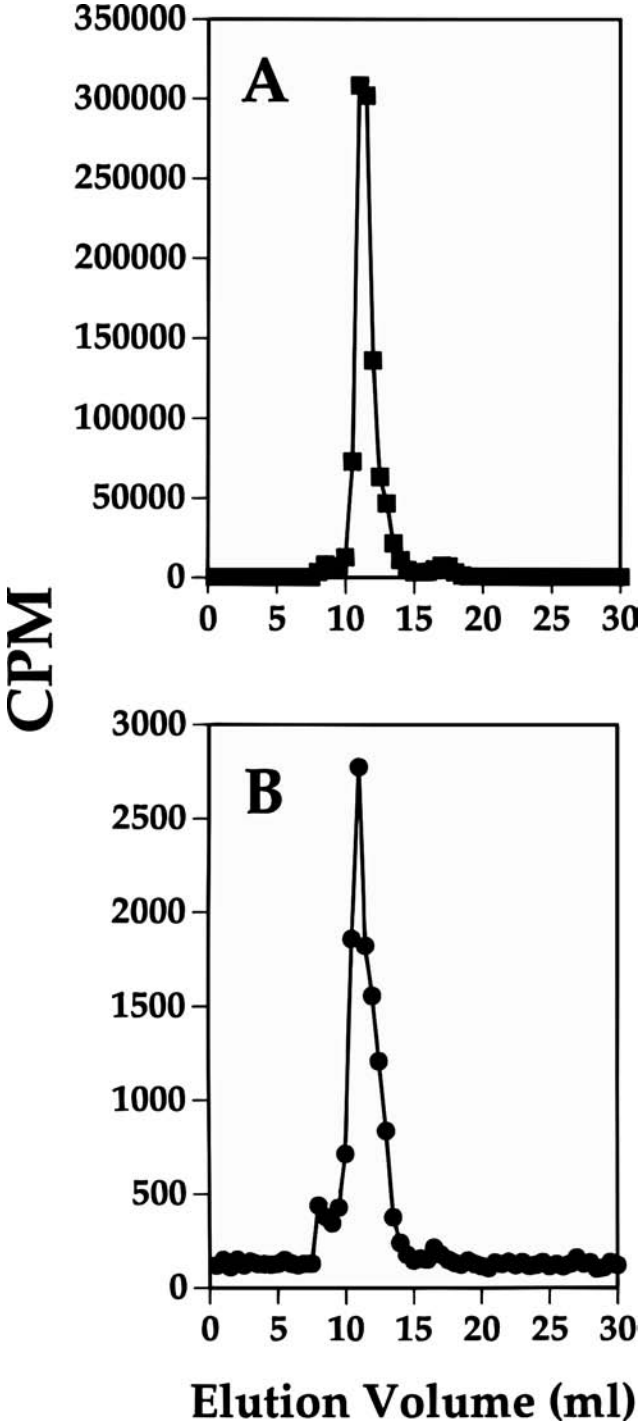

Fig. 6. Elution from a single Superose 12 HR 10/30 FPLC column of either control Rhesus monkey serum pre-mixed with [^{111}In-DPTA] chimeric HIRMAb (A), or monkey serum obtained 120 minutes after intravenous injection of 400 μCi of [^{111}In-DTPA] chimeric HIRMAb (B). The uninjected [^{111}In-DTPA] chimeric HIRMAb added to undiluted monkey serum (A) and migrates at the same elution volume as the chimeric HIRMAb in serum taken 120 minutes after injection (B).

The epitope on the HIR that reacts with the 83-14 murine

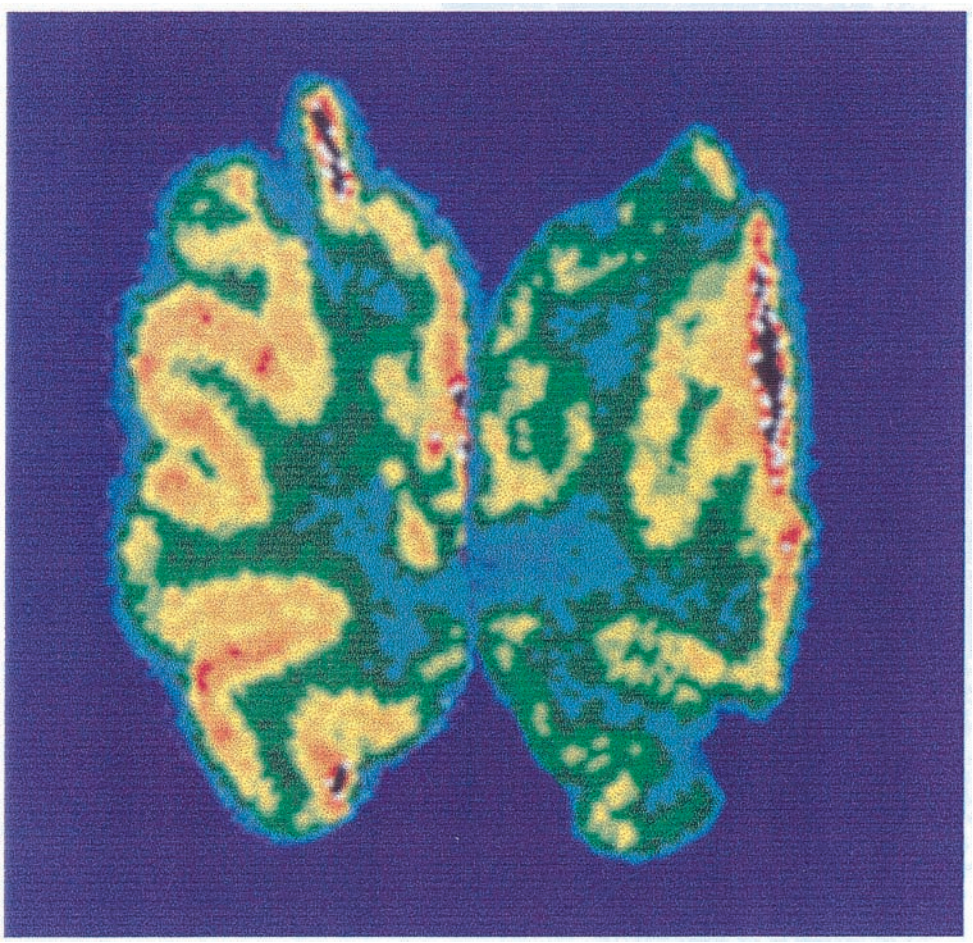

Fig. 7. Film autoradiography of coronal brain sections obtained from a Rhesus monkey 2 hours after the intravenous injection of [^{111}In-DTPA] chimeric HIRMAb. Biomax MS x ray film was exposed for 13 days at 2 70°C with intensifying screens. The respective coronal section through the midbrain for the left and right hemisphere were prepared separately and exposed and scanned separately. The scanned images were then merged in Adobe Photoshop to yield the image of the entire primate brain shown in the figure.

MAb is within amino acids 469±592 of the a-subunit (10). The availability of a CHO cell line secreting soluble extracellular domain of the HIR allowed for assessment of the binding of the chimeric HIRMAb to the target antigen qualitatively by Western analysis and quantitatively by IRMA (Figs. 2, 3). This transfected CHO cell line secretes a soluble HIR wherein the protein is truncated at the transmembrane region of the b-subunit of the HIR (20); the receptor has an intact a-subunit which has a molecular weight of approximately 130 kDa based on Western blotting (Fig. 2). The IRMA allowed for quantitation of the K_D of the binding to the soluble HIR of the either the murine or chimeric antibodies (Fig. 3). The K_D of binding of the murine 83-14 MAb, 0.13 6 0.06 nM (Results), is comparable to the previously reported K_D of [^{125}I] murine 83-14 HIRMAb binding to the membrane bound HIR within human brain capillaries (11); these latter studies were performed using a radioreceptor assay and isolated human brain capillaries similar to that described in Fig. 4. In addition, the IRMA analysis showed that there was no loss in affinity caused by DTPA conjugation of the chimeric HIRMAb (Results). The affinity of the murine or chimeric HIRMAb for the HIR is quite high, approximating $10^{2\,10}$ M, and this high affinity underlies the very avid binding of the HIRMAb to human brain capillaries and the high BBB PS product reported previously (11). For example, the binding to isolated human brain capillaries of either the [^{125}I] chimeric HIRMAb (Fig. 4B) or the [^{125}I] murine 83-14 HIRMAb (11), approximates 400% binding per mg of protein of brain capillaries at 2 hours after incubation. Since the incubations contain approximately 0.05 mg_P of brain capillary derived protein, the actual binding was approximately 20% per incubation tube. The binding of either the murine or chimeric HIRMAb to both the human BBB and the Rhesus monkey BBB is consistent with previously reported data (11), and with the genetic similarity between humans and Old World primates such as the Rhesus monkey (26). In contrast, the murine 83-14 HIRMAb does not react with the HIR in brain capillaries of New World primates such as squirrel monkeys (11).

The pharmacokinetic parameters for the 111-indium labeled chimeric HIRMAb and the 125-iodine labeled murine HIRMAb are compared in Table 1. These results are derived from only 3 monkeys, but the data do show there is a higher clearance of the murine HIRMAb labeled with 125-iodine. This slower clearance of plasma radioactivity in the case of the chimeric HIRMAb labeled with 111-indium may be more reflective of the rates of disposal of the two isotopes, [^{111}In] vs [^{125}I], rather than the chimeric vs murine HIRMAb, per se.

Although the murine 83-14 HIRMAb labeled with 125-iodine was relatively metabolically stable, the plasma TCA precipitability did decrease from 99% to 94% at 2 hours after intravenous injection (11), which is indicative of the formation of low molecular weight metabolites labeled with 125-iodine. As described recently for [^{125}I] EGF and [^{111}In] EGF, the brain uptake of radioactivity is always higher when the peptide is radiolabeled with 125-iodine as opposed to 111-indium (13). This is because of the greater metabolic stability of the MAb labeled with 111-indium. The brain uptake of the 125-iodine metabolites explains the slightly higher BBB PS product for the [^{125}I] murine HIRMAb compared to the PS product for the [^{111}In]-chimeric HIRMAb (Table 1). As shown by the FPLC study in Fig. 6, there is no formation of low molecular weight metabolites labeled with 111-indium in the circulation. Because of the greater metabolic stability of the formulation with the 111-indium radionuclide, this brain imaging study has virtually no "noise" with this isotope (13). Therefore, the brain scan shown in Fig. 7, indicating avid brain uptake of the chimeric HIRMAb, represents actual transport of the chimeric HIRMAb across the BBB and into primate brain. This conclusion was supported by the results of the capillary depletion analysis (Results). Although time course studies were not done in primates, previous studies in rats show the brain uptake of the peptidomimetic MAb peaks at 1–3 hours after administration (27). The rate of export of the 111-indium from the primate brain is not known, but 111-indium is rapidly cleared from rat brain (13), and the $t_{1/2}$ of 125-iodine clearance from primate brain is 16 hours following targeting through the BBB (12).

In summary, these studies describe the production of a chimeric MAb to the human insulin receptor and the retention of affinity of the MAb for the target antigen following the genetic engineering. Chimeric MAbs are now administered to humans and are FDA approved therapeutics (28). The chimeric HIRMAb described in these studies could be administered to humans. Based on past experience, there would be minimal immunologic sequalae from this administration (28). The brain scan in humans using the chimeric HIRMAb should be just as intense as that shown for the living primate (Fig. 7), given the high reactivity of the HIRMAb for human brain capillaries (Fig. 4), which form the human BBB in vivo. Peptidomimetic MAbs such as the HIRMAb or the TfRMAb have been shown in previous studies to carry across the BBB neuropeptides that can be used as neuro-diagnostics (12,13) or neuro-therapeutics (14,15). Conjugation of mono-biotinylated therapeutics to BBB drug targeting vectors is facilitated by the production of single chain antibody/streptavidin fusion proteins (29) or MAB/avidin constructs (30). The vast majority of neuropharmaceuticals that do not cross the BBB can be converted into active CNS pharmaceuticals if these molecules undergo a molecular re-formulation, wherein the pharmaceutical is conjugated to a BBB drug delivery vector. The availability of the chimeric HIRMAb may allow for future drug targeting of neurodiagnostics or neurotherapeutics to the human brain.

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