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# Novel Antitransferrin Receptor Antibodies Improve the Blood–Brain Barrier Crossing Efficacy of Immunoliposomes

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ABSTRACT

Surface functionalization with antitransferrin receptor (TfR) mAbs has been suggested as the strategy to enhance the transfer of nanoparticles (NPs) across the blood–brain barrier (BBB) and to carry non-permeant drugs from the blood into the brain. However, the efficiency of BBB crossing is currently too poor to be used *in vivo*. In the present investigation, we compared 6 different murine mAbs specific for different epitopes of the human TfR to identify the best performing one for the functionalization of NPs. For this purpose, we compared the ability of mAbs to cross an *in vitro* BBB model made of human brain capillary endothelial cells (hCMEC/D3). Liposomes functionalized with the best performing mAb (MYBE/4C1) were uptaken, crossed the BBB *in vitro*, and facilitated the BBB *in vitro* passage of doxorubicin, an anticancer drug, 3.9 folds more than liposomes functionalized with a nonspecific IgG, as assessed by confocal microscopy, radiochemical techniques, and fluorescence, and did not modify the cell monolayer structural or functional properties. These results show that MYBE/4C1 antihuman TfR mAb is a powerful resource for the enhancement of BBB crossing of NPs and is therefore potentially useful in the treatment of neurologic diseases and disorders including brain carcinomas.


## Introduction

In spite of decades of research, the specific transport of drugs and imaging agents to the brain remains challenging because of the efficiency of the blood–brain barrier (BBB), a tightly packed layer of endothelial cells that protect the brain from potentially harmful endogenous compounds and xenobiotics.[1] Several strategies bypassing the BBB have been proposed,[2,3] but the potential benefits of such systems must be weighted against their impact on the defensive function of BBB. In this context, targeted delivery achieved by directly coupling a brain-targeting ligand to a drug molecule or by encapsulating drugs into brain-targeted nanoparticles (NPs) has been proposed as an innovative and noninvasive tool for delivering drugs to the brain. NP-mediated brain drug delivery might be achieved by functionalizing the NP surface with BBB targeting agents, thus allowing the NP to cross the BBB by exploiting the physiological mechanisms of transport.[4] Transferrin receptor (TfR), also known as CD71, has received the greatest attention for use in target-mediating NP brain entry, owing to its expression on BBB endothelial cells for the regulation of brain uptake of iron.[5,6]

The purpose of this investigation was to identify, among a panel of antihuman TfR mAbs, the most efficient to promote BBB crossing of nanoliposomes (nano-LIPs)—colloidal vesicles formed by biodegradable and biocompatible phospholipids and sphingolipids frequently used as a delivery system for drugs. For this reason, 6 antihuman TfR mAbs, specific for different epitopes of the human TfR I, namely CB26 (IgG1), CBMIEL-2 (IgG1), MYBE/4G3 (IgG1), MYBE/5F5 (IgG1), MYBE/4C1 (IgG1), and CBMIEL-1 (IgM) were prepared. The criteria for selection were the mAb ability to cross an *in vitro* BBB model made up of human brain capillary endothelial

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cells (hCMEC/D3). The best performing mAb was used to functionalize LIP, and the permeability across the BBB model of the resulting functionalized NP was studied to verify their potential as nanocarriers for applications in the treatment of brain diseases.

## Experimental

### Materials

Bovine brain sphingomyelin (Sm), cholesterol (Chol), and 1,2-distearoyl-sn-glycero-3-phospho-ethanolamine-N-[maleimide (polyethyleneglycol)-2000] were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)sphingosyl-phosphocholine (BODIPY-Sm) was from Molecular Probes (Life Technologies, Carlsbad, CA). *N*-acetyl-cysteine, Sepharose CL-4B, doxorubicin hydrochloride (DOX), 3-(4,5-dimetiltiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), and Triton X-100 were purchased from Sigma-Aldrich. Tritiated sphingomyelin ([$^3$H]-Sm) was from PerkinElmer (Waltham, MA). Amicon Ultra-15 centrifugal 10K filter devices and polycarbonate filters for the extrusion procedure were purchased from Merck Millipore (Billerica, MA). The Thermobarrel Extruder was from Lipex Biomembranes (Vancouver, BC, Canada). Purified rat antimouse RI7217 was from BioLegend (San Diego, CA). Ultrapure and deionized water were obtained from Direct-Q5n system (Millipore, Italy). All other chemicals were reagent grade. All the stock solutions for cell cultures were from Euroclone (Milano, Italy). The Elisa kits for IgM and IgG quantitative detection were from eBioscience (San Diego, CA). Texas Red-X phalloidin and 4′,6-diamidino-2-phenylindole were from Molecular Probes (Life Technologies, Carlsbad, CA).

### Methods

#### Preparation of Antihuman TfR mAbs

A panel of specific murine mAbs was produced through immunization with murine L-fibroblasts transfected with the human TfR-1 (L-CD71$^+$), obtained as described.[7] Female Balb/c mice were injected twice intraperitoneally with $5 \times 10^6$ cells. Five days before somatic fusion with the P3.X63.Ag8/653 myeloma line, mice were injected intravenously with $2 \times 10^6$ cells. Four days after the last injection, the spleen was removed for fusion using conventional techniques.[8] The reactivity of the individual anti-CD71 mAbs was analyzed by cytofluorimetric analysis (see later). Positive clones underwent 2 rounds of cultures after limiting dilution and were then expanded in massive cultures. Individual clones were tested for isotype by means of Outcherlony technique. The mAbs identified were CB26 (IgG1), CB-MIEL-2 (IgG1), MYBE/4G3 (IgG1), MYBE/5F5 (IgG1), CB-MIEL-1 (IgM), and MYBE/4C1 (IgG1). Each mAb was purified using an HPLC technique as described.[9] The purified mAbs were then sterilized by 0.22-μm filtration (Millipore Polyethersulfone Millex-GP Syringe Filter Unit, radiosterilized) and detoxified by Detoxi-Gel Endotoxin Removing Gel (Thermo Scientific). The work for the production of the murine mAbs was performed under the control of a local Committee of Animal Care, after a permission issued by the Istituto Superiore di Sanità (National Institute of Health, Rome).

#### Cytofluorimetric Analysis

L-CD71$^+$ cells ($2.5 \times 10^5$) washed in phosphate-buffered saline (PBS) containing 1% bovine serum albumin and $NaN_3$ were incubated with the panel of anti-CD71 mAbs (1 h, 4°C). Unbound mAb was eliminated by washing twice and incubated 30 min, 4°C with fluorescein isothiocyanate (FITC)-conjugated F(ab')2 goat antimouse IgG+IgM (Jackson Immuno-Research Laboratories, West Grove, PA). The samples were washed, resuspended in PBS, and acquired on an FACScan (Becton-Dickinson, San Jose, CA) using CellQuest Software (Becton-Dickinson). Data WinMDI 2.9 software collected were analyzed using a WinMDI 2.9 software (Scripps Research Institute, La Jolla, CA). IB4 was analyzed with a similar approach using murine NIH-3T3 cells trasfected with human CD38 (NIH-3T3/CD38+). Mock-transfected L-cells were used as negative controls.

#### Preparation of LIP Functionalized With mAbs (MYBE/4C1-LIP, IB4-LIP, and RI7217-LIP)

LIPs were prepared in 10 mM PBS, pH 7.4, using Sm/Chol/1,2-distearoyl-sn-glycero-3-phospho-ethanolamine-N-[maleimide (polyethyleneglycol)-2000 (49.5:49.5:1 molar ratio), by extrusion of the multilamellar vesicles through 100-nm pore-size filters, as previously described.[10] For preparation of fluorescently labeled LIP, 0.5 molar% of total Sm was substituted with BODIPY-Sm. For preparing radiolabeled LIP, 0.001 molar% of [$^3$H]-Sm (100 μCi/mL) was added as a tracer to track lipid distribution by measuring radioactivity. For the covalent coupling to maleimide-containing LIP, MYBE/4C1, IB4, and RI7217 were thiolated, as described.[10] Thiolated mAbs were then incubated with LIP (4 mM) overnight at 25°C at a 1:1000 molar ratio of mAbs/phospholipids. Unbound mAbs were removed by using gel exclusion chromatography (Sepharose 4B-CL column). The amount of mAb bound to LIP was quantified using the Bradford assay.[11] Phospholipids in final samples were quantified by using Stewart's assay.[12] mAb-functionalized LIPs are called IB4-LIP, MYBE/4C1-LIP, and RI7217-LIP.

#### Preparation of DOX-Loaded LIP (MYBE/4C1-DOX-LIP, IB4-DOX-LIP, and RI7217-DOX-LIP)

The procedure described by Sakakibara et al.[13] was followed with only small changes. LIPs composed as previously described were prepared in ammonium sulfate (500 mM, pH 5.5) and extruded through polycarbonate membranes of 100-nm pores. LIPs were then dialyzed against HEPES (10 mM, pH 7.4) and incubated with DOX for 1 h at 65°C. Free DOX was removed by gel filtration (Sepharose 4B-CL column). DOX loading was quantified fluorometrically ($\lambda_{ex} = 495$ nm; $\lambda_{em} = 592$ nm) after vesicle disruption with 0.1% Triton X-100.[14] To obtain DOX concentration in the solution, fluorescence intensities were compared with a previously established calibration curve for DOX in HEPES (10 mM, pH 7.4). Drug loading was calculated using the following equation: drug loading% = DOX concentration in NP solution or NP concentration in the same solution × 100. LIPs were then functionalized with mAbs as described previously.

#### Size and Charge Characterization of LIP

Size, ζ-potential, polydispersity index, and stability were analyzed by dynamic light scattering (DLS), as described previously.[15] The reported data are the mean of at least 5 different measurements.

#### Cell Cultures

Immortalized hCMEC/D3 were provided by the Institut National de la Santé et de la Recherche Médicale, Paris, France) and cultured as previously described.[16]

#### Cellular Uptake of mAb-Functionalized LIP by Human Brain Endothelial Cells

hCMEC/D3 (65,000 cells cm$^{-2}$) were cultured for 2 days on rat type I collagen-coated cover slips (diameter, 22 mm) positioned in culture dishes and then incubated with fluorescent (BODIPY-Sm) IB4-LIP or MYBE/4C1-LIP (30 μg mL$^{-1}$of mAbs) at 37°C. After 2 h, cells were treated as described.[10] Cellular uptake of LIP was