

Human Monoclonal Antibodies to Sialyl-Lewis^a (CA19.9) with Potent CDC, ADCC, and Antitumor Activity

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Abstract

Purpose: The carbohydrate antigen sialyl-Lewis^a (sLe^a), also known as CA19.9, is widely expressed on epithelial tumors of the gastrointestinal tract and breast and on small-cell lung cancers. Since overexpression of sLe^a appears to be a key event in invasion and metastasis of many tumors and results in susceptibility to antibody-mediated lysis, sLe^a is an attractive molecular target for tumor therapy.

Experimental Design: We generated and characterized fully human monoclonal antibodies (mAb) from blood lymphocytes from individuals immunized with a sLe^a-KLH vaccine.

Results: Several mAbs were selected based on ELISA and FACS including two mAbs with high affinity for sLe^a (5B1 and 7E3, binding affinities 0.14 and 0.04 nmol/L, respectively) and further characterized. Both antibodies were specific for Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAcβ as determined by glycan array analysis. Complement-dependent cytotoxicity against DMS-79 cells was higher (EC₅₀ 0.1 μg/mL vs. 1.7 μg/mL) for r7E3 (IgM) than for r5B1 (IgG1). In addition, r5B1 antibodies showed high level of antibody-dependent cell-mediated cytotoxicity activity on DMS-79 cells with human NK cells or peripheral blood mononuclear cells. To evaluate *in vivo* efficacy, the antibodies were tested in a xenograft model with Colo205 tumor cells engrafted into SCID (severe combined immunodeficient mice) mice. Treatment during the first 21 days with four doses of r5B1 (100 μg per dose) doubled the median survival time to 207 days, and three of five animals survived with six doses.

Conclusion: On the basis of the potential of sLe^a as a target for immune attack and their affinity, specificity, and effector functions, 5B1 and 7E3 may have clinical utility. *Clin Cancer Res*; 17(5); 1024-32. ©2011 AACR.

Introduction

Many tumor-restricted monoclonal antibodies (mAb) resulting from immunization of mice with human cancer cells were shown to be directed against carbohydrate antigens expressed at the cell surface as glycolipids or glycoproteins (1, 2), but carbohydrate chemistry has been rather challenging and the clinical development of such antibodies has been slow for various reasons.

Sialyl-Lewis^a (sLe^a), also known as CA 19.9, is widely expressed on tumors of the gastrointestinal tract and is used as a tumor marker in pancreatic and colon cancer (3, 4). sLe^a is a known ligand for endothelial leukocyte adhesion molecule, and expression of sLe^a was found to have an impact on metastatic potential (5, 6) and to correlate with

increased metastatic potential in human colon cancer (7, 8) and pancreatic adenocarcinoma (9). In a study of 43 breast cancer patients with infiltrating ductal carcinoma it was found in about 79% of samples (10), and there was higher expression in patients with greater node involvement. Interestingly, sLe^a is predominantly expressed in cancers, while the natural counterpart, disialyl-Lewis^a, is found in nonmalignant epithelial cells (4). sLe^a is expressed primarily as a glycolipid, and glycolipids are proven targets for immune attack against cancer cells (1-3). Thus, passive administration of antibodies could potentially eradicate free tumor cells and early metastases in a minimal disease setting and have a significant impact on cancer recurrence.

Here we report the discovery and initial characterization of two fully human antibodies that were generated from blood lymphocytes from individuals immunized with sLe^a-KLH vaccine (11). Two promising candidate antibodies with high affinity for sLe^a (5B1 and 7E3) were expressed as recombinant antibodies and further characterized *in vitro*. Both antibodies were very potent in complement-dependent cytotoxicity (CDC) assays, and, furthermore, the 5B1 IgG1 antibody was also highly active in our antibody-dependent cytotoxicity assays. To evaluate the *in vivo* efficacy, the 5B1 antibodies were tested in a xenograft model of Colo205 tumor cells engrafted into SCID (severe combined immunodeficient) mice. Treatment with 5B1 antibodies

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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Translational Relevance

Carbohydrates expressed on the tumor cell surface are ideal targets for passive immunotherapy. This article describes the discovery of potent fully human monoclonal antibodies (mAb) established from blood samples of patients that were immunized with sialyl-Lewis^a (sLe^a-KLH) conjugate vaccine. The translational relevance is 2-fold: First, the approach serves to validate the antibody response that is elicited by the vaccine at the cellular level, and ultimately this information could be used to improve the vaccine itself. Second, the most potent antibodies that are generated by only a few patients in a clinical trial can be preserved and could ultimately be developed as therapeutics for the target cancer population. The high affinity of mAb 5B1 and potent effector functions support this translational potential.

cured 40% to 60% of the mice depending on dose, while 5 of 5 untreated animals died within 155 days.

Material and Methods

Materials, cells, and antibodies

DMS-79 (12), SW626, EL4, HT29, BxPC3, SK-MEL28, and P3×63Ag8.653 cell lines were purchased from American Type Culture Collection (ATCC). Colo205-luc cells (Bioware ultra) were obtained from Caliper Life Sciences. The murine control mAb 121SLE (IgM) was purchased from GeneTex. sLe^a tetrasaccharide (Cat # S2279) was purchased from Sigma-Aldrich. sLe^a-HSA (human serum albumin) conjugate (Cat # 07-011), monovalent biotinylated sLe^a (sLe^a-sp-biotin; Cat # 02-044), polyvalent biotinylated sLe^a-PAA (Cat # 01-044), biotin-labeled Le^a-PAA (Cat # 01-035), and sLe^x-PAA-biotin (Cat # 01-045) were purchased from GlycoTech. In the polyvalent presentation, the tetrasaccharide is incorporated into a polyacrylamide matrix (PAA), thereby creating a 30-kDa multivalent polymer with approximately every fifth amide group of the polymer chain N-substituted with biotin in a 4:1 ratio and approximately 20% carbohydrate content. Other HSA or BSA glycoconjugates used in this study were prepared in-house using sLe^a pentenyl glycoside as described (11). GD3, fucosyl-GM1, GM2, and GM3 were purchased from Matreya, and GD2 was purchased from Advanced ImmunoChemical.

Generation of anti-sLe^a mAb-producing hybridomas

Blood samples were obtained from 3 patients in an ongoing trial with sLe^a-KLH conjugate vaccine in patients with breast cancer initiated at MSKCC under an MSKCC- and FDA-approved IRB protocol and IND. Blood specimens were selected from 2 patients after 3 or 4 vaccinations, which showed antibody titers of 1/160 and 1/320, respectively, against sLe^a. These sera (and murine mAb 19.9) react well with sLe^a-positive cell lines in FACS assays and mediate potent CDC (11). Peripheral blood mononuclear cells (PBMC) were isolated from approximately 80

to 90 mL of blood by gradient centrifugation on Histopaque-1077 (Sigma-Aldrich).

PBMCs were cultured in RPMI-1640 medium supplemented with L-glutamine, nonessential amino acids, sodium pyruvate, vitamin, penicillin/ streptomycin, 10% FBS (Omega Scientific), 10 ng/mL IL-21 (Biosource), and 1 µg/mL anti-CD40 mAb (G28-5 hybridoma supernatant; ATCC). Cells were fused by electrofusion to P3×63Ag8.653 myeloma cells.

sLe^a ELISA

For the sLe^a ELISA, plates were coated either with 1 µg/mL of sLe^a-HSA conjugate, monovalent biotinylated sLe^a, or with polyvalent biotinylated sLe^a-PAA captured on NeutrAvidin-coated plates. Uncoated wells (PBS) and HSA-coated wells were used as controls. Bound antibodies were initially detected with horseradish peroxidase (HRP)-labeled goat anti-human IgA + G + M (Jackson ImmunoResearch), and positive wells were subsequently probed with IgG-Fc- or IgM-specific secondary antibodies to determine isotypes.

Carbohydrate specificity analysis

Cross-reactivity against the closely related antigens, Le^a and sLe^x, was evaluated by surface plasmon resonance (SPR) and confirmed by ELISA using biotin-labeled Le^a-PAA and biotin-sLe^x-PAA. Binding to gangliosides GD2, GD3, fucosyl-GM1, GM2, and GM3 was tested by ELISA. A competition ELISA was used to evaluate the specificity of the mAbs against several other related carbohydrate moieties. In brief, 2 µg/mL sLe^a-HSA conjugate was coated onto plates followed by blocking with 3% BSA in PBS. Next, 30 µL of different carbohydrate moieties (40 µg/mL in PBS prepared from 1 mg/mL stock solutions) either unconjugated or conjugated to HSA or BSA was mixed separately with 30 µL of test antibody and incubated at room temperature in a sample plate. After 30 minutes 50 µL of the mixture was transferred to the coated assay plate and incubated for 1 hour, followed by incubation with HRP-labeled goat anti-human IgA + G + M, washing and colorimetric detection of bound antibody using a Versamax spectrofluorometer (all steps were carried out at room temperature). The tested carbohydrate moieties included globo H, Lewis Y, Lewis X, sialyl-Thomson-nouveaux (sTn), clustered sTn, Thomson Friedenreich (TF), Tighe Le^b/Le^y mucin, porcine submaxillary mucin (PSM), and sLe^a tetrasaccharide and sLe^a-HSA conjugate. To determine the fine specificity of the antibodies, glycan array analysis was done by the Consortium for Functional Glycomics Core H group. 5B1 and 7E3 antibodies were tested at 10 µg/mL using version 4.1 of the printed array consisting of 465 glycans in replicates of 6.

Immunoglobulin cDNA cloning and recombinant antibody expression

Variable region of human mAb heavy and light chain cDNA was recovered by RT-PCR from the individual hybridoma cell line and subcloned into IgG1 or IgM heavy chain or IgK or IgL light chain expression vector as described

before (13). Ig heavy chain or light chain expression vector was double-digested with Not I and Sal I, and then both fragments were ligated to form a dual-gene expression vector. CHO cells in 6-well plates were transfected with the dual-gene expression vector using Lipofectamine 2000 (Invitrogen). After 24 hours, transfected cells were transferred to a 10-cm dish with selection medium [DMEM supplemented with 10% dialyzed FBS (Invitrogen), 50 $\mu\text{mol/L}$ L-methionine sulfoximine (MSX), GS supplement (Sigma-Aldrich), and penicillin/streptomycin (Omega Scientific)]. Two weeks later MSX-resistant transfectants were isolated and expanded. High anti-sLe^a antibody-producing clones were selected by measuring the antibody levels in supernatants in a sLe^a-specific ELISA assay and expanded for large-scale mAb production.

Human mAb purification

Antibodies were purified using the Äkta Explorer (GE Healthcare) system running Unicorn 5.0 software. In brief, stable clones of 5B1 or 7E3 were grown in serum-free culture medium in a Wave bioreactor, and the harvested supernatant was clarified by centrifugation and filtration and stored refrigerated until used. Human IgG antibodies were purified on appropriate-sized protein A columns using 10 mmol/L PBS and 150 mmol/L NaCl running buffer. Human IgM antibodies were purified on a hydroxyapatite column, and IgM was eluted with a gradient of 500 mmol/L phosphate. The antibody concentrations were determined by OD₂₈₀ using an $E^{1\%}$ of 1.4 and 1.18 for IgG and IgM, respectively, to calculate the concentration. The purity of each preparation was evaluated by SDS-PAGE analysis (1–5 μg per lane) under reducing conditions, and the purity was more than 90% based on the sum of heavy and light chains.

Flow cytometry

sLe^a-positive or -negative tumor cell lines (0.5×10^6 cells per condition) were washed in PBS/2% FBS (PBSF). Test or control human mAb was then added (1–2 $\mu\text{g/mL}$ in complete medium) and incubated on ice for 30 minutes (14, 15). After washing in PBSF, the cells were incubated with Alexa-488 anti-human IgG-Fc γ or anti-human IgM- μ (Invitrogen) for 30 minutes on ice. Cells were washed twice in PBSF and analyzed by flow cytometry using the Guava Personal Cell Analysis-96 (PCA-96) System (Millipore). Colo205-luc cells were incubated with 2 $\mu\text{g/mL}$ of primary antibody, followed by staining with secondary antibodies from SouthernBiotech, and analyzed on a Becton Dickinson FACS Advantage IV instrument using FlowJo 7.2.4 software.

Affinity determination

Affinity constants were determined using the principle of SPR with a Biacore 3000 (GE Healthcare). Biotin-labeled univalent sLe^a (Cat # 02-044) or polyvalent sLe^a-PAA-biotin (Cat # 01-044) were coupled to separate flow cells of an SPA biosensor chip according to the manufacturer's instructions. A flow cell blocked with HSA and culture medium containing free biotin was used as a reference cell. The binding kinetic parameters were determined from

several known concentrations of antibody diluted in HBS-EP buffer (10 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 3.4 mmol/L EDTA, 0.005% surfactant P20) using the sLe^a-PAA-biotin-coated flow cell. The curve-fitting software provided by the Biacore instrument was used to generate estimates of the association and dissociation rates from which affinities are calculated.

CDC assay

sLe^a antigen-positive and -negative cell lines were used for a 90-minute cytotoxicity assay (Guava PCA-96 Cell-Toxicity kit; Millipore; Cat # 4500-0200) using human complement (Quidel; Cat # A113) and purified human mAbs at various dilutions (0.1–25 $\mu\text{g/mL}$) or with positive control mAbs as previously described (15–18). In brief, 2.5×10^6 target cells were painted with carboxyfluorescein diacetate succinymyl ester (CFSE) to yield green/yellow fluorescent target cells. The painted cells ($1 \times 10^5/50 \mu\text{L}$ sample) were incubated for 40 minutes with 100 μL of antibodies on ice. Next, 50 μL of human complement diluted 1:2 in complete medium (RPMI-1640, 10% FCS) or medium alone was added to triplicate samples and incubated for 90 minutes at 37°C. Thus, the final complement dilution in the assay was 1:8. Cells that were killed during this incubation time were labeled by adding the membrane impermeable dye 7-amino-actinomycin D (7-AAD), and samples were analyzed by dual-color immunofluorescence utilizing the Guava CellToxicity software module. Control samples that received NP40 were used to determine maximal killing and samples receiving complement alone served as baseline. The percentage of killed cells was determined by appropriate gating and calculated according to the following formula: % killed = [(% sample – % complement alone)/(% NP40 – % complement alone)] \times 100.

Antibody-dependent cell-mediated cytotoxicity assay

PBMC effector cells were isolated from blood samples obtained under an MSKCC IRB-approved protocol by Ficoll-Hypaque density centrifugation. The target cells were incubated at 5×10^6 cells/mL in complete growth media with 15 μL of 0.1% calcein-AM solution (Sigma-Aldrich) for 30 minutes at 37°C, in the presence of 5% CO₂. The cells were washed twice with 15 mL of PBS-0.02% EDTA and resuspended in 1 mL complete growth medium. Fifty microliters (10,000 cells) of labeled target cells was plated into a 96-well plate in the presence or absence of antibodies at the concentrations described in Figure 3, and incubated with 50 μL of freshly isolated peripheral blood mononuclear cells (effector cells, at 100:1 E/T ratio) accordingly. After 2 hours of incubation, the plate was centrifuged at $300 \times g$ for 10 minutes, and 75 μL of supernatant was transferred into a new flat-bottomed 96-well plate. The fluorescence in the supernatant was measured at 485-nm excitation and 535-nm emission in Fluoroskan Ascent (Thermo Scientific). Spontaneous release was determined from target cells in RPMI-1640 medium with 30% FBS without effector cells and maximum release was determined from target cells in

RPMI-1640 medium with 30% FBS and 6% Triton X-100 without effector cells. Percent cytotoxicity was calculated as [(counts in sample – spontaneous release)/(maximum counts – spontaneous release)] × 100.

mAb internalization assay

Internalization of 5B1 antibody was evaluated by measuring the cytotoxic activity of r5B1 and Hum-ZAP secondary conjugate (Advanced Targeting Systems) complex against sLe^a expressing BxPC3 cells, which were plated into a 96-well plate (2,000 cells/ 90 μL/well) and incubated overnight in duplicates. Various concentrations of 5B1 antibody were incubated with Hum-ZAP secondary conjugates at RT according to the manufacturer's instruction. Next, 10 μL/well of r5B1 and Hum-ZAP complex was added to the cells and incubated for 3 days. Twenty-five microliters of Thiazolyl Blue Tetrazolium Bromide (Sigma-Aldrich) solution (5 mg/mL in PBS) was added to each well and incubated at 37°C. After 2 hours of incubation, 100 μL/well of solubilization solution (20% SDS/50% *N,N*-dimethylformamide) was added to each well and incubated for another 16 hours at 37°C. The OD was measured at 570/690 nm, and values obtained with medium alone were used for plate background subtraction. Eight parallel cultures without antibody were used to normalize the sample values (sample/mean untreated × 100).

Xenograft transplantation model

Female CB17 SCID mice (5–8 weeks old) were purchased from Taconic. Colo205-luc cells (0.5×10^6) in 0.1 mL complete growth media were injected via the tail vein on day 0 using a BD insulin syringe with 28G needle (Becton Dickinson & Co). One hundred micrograms of mAb 5B1 was injected intraperitoneally on days 1, 7, 14, and 21 (experiment 1) or on days 1, 4, 7, 10, 14, and 21 (experiment 2). Mice were monitored for tumor development. All procedures were done under a protocol approved by the Memorial Sloan Kettering Cancer Center Institutional Animal Care and Use Committee. Kaplan–Meier survival curves were generated using GraphPad Prism 5.1 (GraphPad Software) and analyzed using the Mantel–Haenszel log-rank test.

Results

Identification of human monoclonal antibodies by ELISA and generation of recombinant antibodies

Blood samples from 3 vaccinated patients were used for hybridoma generation efforts and many positive wells were

detected in the antigen-specific ELISA assays. Extensive screening was used to eliminate antibodies that showed inferior or nonspecific binding. Eight human antibody-expressing hybridoma cells (1 IgM and 7 IgG) with strong reactivity against sLe^a were initially selected, expanded, and subcloned for further characterization. Two antibodies (9H1 and 9H3) showed strong binding to sLe^a–HSA conjugates but not to sLe^a–PAA-coated plates. Three antibodies (5B1, 5H11, and 7E3) showed strong binding to monovalent and polyvalent sLe^a and sLe^a–HSA conjugates (Supplementary Fig. 1).

The heavy and light chain variable regions from 4 selected antibodies were recovered by RT-PCR and cloned into our full-length IgG1 or IgM expression vectors. Molecular sequence analysis using IMGT/V-Quest (19) revealed that the 3 selected IgG antibodies 5B1 (IgG/λ), 9H3 (IgG/λ), and 5H11 (IgG/λ) were derived from the same VH family and all used lambda light chains. These IgG1 antibodies showed different CDR sequences with 16, 5, or 3 mutations deviating from the germ line, respectively (Supplementary Table 1). The IgM antibody (7E3) utilizes the kappa light chain and has 6 heavy chain mutations. The increased mutations in 5B1 are indicative of affinity maturation. Recombinant antibodies were produced in CHO cell lines in a wave bioreactor system and purified using protein A or hydroxyapatite chromatography for IgG and IgM, respectively. The purified recombinant antibodies retained the properties of the original hybridoma-derived antibodies with respect to ELISA binding and specificity.

Analysis of tumor cell binding

Cell surface binding is crucial for cytotoxic activity and was therefore tested next. Flow cytometry showed strong binding of 5B1, 9H3, 5H11, and 7E3 recombinant antibodies to DMS-79 cells, a small-cell lung cancer suspension cell line (Fig. 1A). Binding of r5B1 and r7E3 was also confirmed on HT29 colon cancer cells (B), BxPC3 pancreatic cancer cells (Fig. 1C), SW626 ovarian cancer cells (Fig. 1D), and Colo205-luc colon cancer cells (Fig. 1F). These antibodies failed to bind to sLe^a-negative (SLE121-negative) SK-MEL28 melanoma cells (Fig. 1E) or EL4 mouse lymphoma cells (data not shown).

Affinity measurements

The relative affinity/avidity of the binding to sLe^a was probed by SPR using a streptavidin-coated biosensor chip to capture biotinylated sLe^a-PPA. As shown in Table 1, r5B1

Table 1. Determination of kinetic parameters of anti-sLe^a antibodies by SPR

mAb	Affinity, nmol/L	K_d , mol/L	K_a , 1/mol/L	Association k_a , 1/mol/L s)	Dissociation k_d , 1/s	Isotype
r5B1	0.14	1.4×10^{-10}	7.0×10^9	1.1×10^6	1.6×10^{-4}	IgG1/λ
r7E3	0.04	3.6×10^{-11}	2.8×10^{10}	8.8×10^5	3.2×10^{-5}	IgM/κ
121SLE	0.35	3.5×10^{-10}	2.8×10^9	2.7×10^6	9.4×10^{-4}	m-IgM

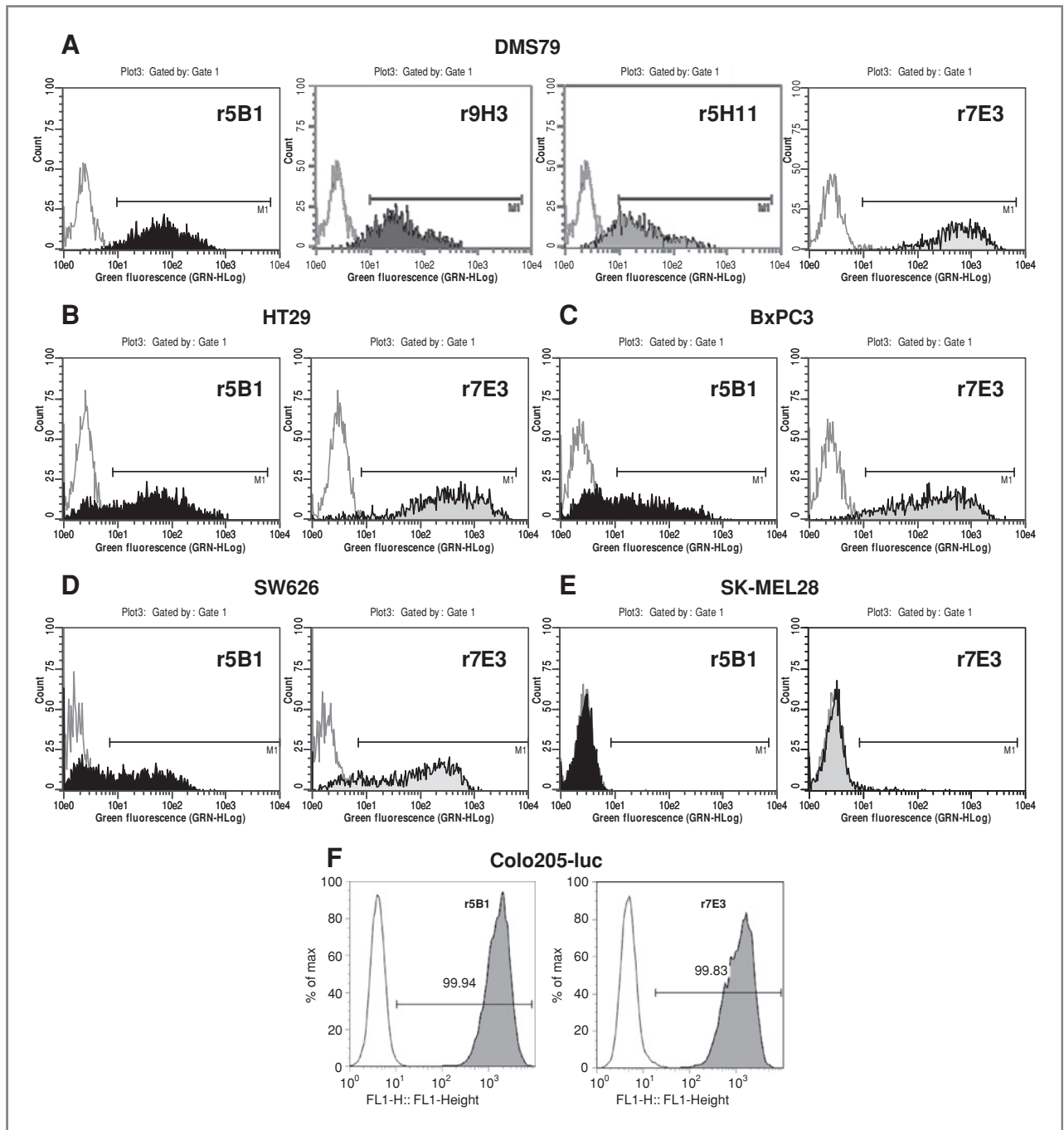


Figure 1. Binding of human anti-sLe^a antibodies to tumor cells analyzed by flow cytometry. DMS-79 cells were stained with recombinant 5B1, 9H3, 5H11, and 7E3 (A). Staining of HT29 (B), P_xPC3 (C), SW626 (D), SK-MEL28 cells (E), and Colo205-luc cells (F) with 1–2 μ g/mL of r5B1 or 7E3 plus IgG or IgM-specific secondary antibody as described in Materials and Methods.

and r7E3 bind rapidly to sLe^a-PPA and show a significantly slower off-rate compared with 121SLE, a commercially available murine IgM anti-sLe^a antibody that was used for comparison. The affinity of 5B1 was measured at 0.14 nmol/L, and the apparent affinity/avidity of 7E3 was approximately 4 times higher (Table 1). Determination of 9H3 affinity was hampered since 9H3 antibodies

(native and recombinant) failed to bind to the sLe^a-PAA-coated biosensor chip.

Specificity analysis

Preliminary assays to probe carbohydrate specificity showed that 5B1, 9H3, and 7E3 did not bind to the closely related sLe^x, Le^a, or Le^y antigens or the gangliosides GD2,

GD3, fucosyl-GM1, GM2, and GM3 as measured by ELISA or SPR. The binding of 5B1 to sLe^a-PAA was also inhibited by sLe^a tetrasaccharide in a dose-dependent manner in a Biacore concentration analysis series (data not shown). These results are consistent with previous observations that sera with high anti-sLe^a antibody titers were found to be specific for sLe^a, that is, not reactive with gangliosides GM2, GD2, GD3, fucosyl GM1, or the neutral glycolipids globo H and Le^y by ELISA (11). In a competition assay with 9 distinct related carbohydrate moieties in various presentations (e.g., as ceramide, or conjugated to BSA or HSA), only sLe^a tetrasaccharide and sLe^a-HSA conjugate were able to inhibit binding to sLe^a-HSA conjugate (data not shown).

To examine the carbohydrate specificity in further detail, 5B1 and 7E3 antibodies were also tested by glycan array analysis done by the Consortium for Functional Glycomics Core H group. Both antibodies were tested at 10 $\mu\text{g}/\text{mL}$ on printed arrays consisting of 465 glycans in 6 replicates. The results confirmed the high specificity of both antibodies with selective recognition of the sLe^a tetrasaccharide, Neu5Ac α 2-3Gal β 1-3(Fuc α 1-4)GlcNAc β and virtual absence of binding to closely related antigens that were present in the array, including sLe^x, Le^a, Le^x, and Le^y. The results are summarized in Supplementary Table 2, and the raw data file is available at http://www.functionalglycomics.org/glycomics/HServlet?operation=view&sideMenu=no&psId=primscreen_3421.

CDC activity

To evaluate the functional activity of 5B1 and 7E3, we tested the cytotoxic activity with DMS-79 cells in the presence of human serum as a source of complement. Both antibodies showed in some assays close to 100% killing activity at 10 $\mu\text{g}/\text{mL}$, while a control antibody with different specificity (1B7, anti-GD2 IgG1 mAb) had no effect at the same concentrations (data not shown). The CDC activity is concentration dependent, and 7E3 was significantly more active than 5B1 in this assay (Fig. 2), which is expected since IgM antibodies are known to be more effective in complement-mediated cytotoxicity assays. The EC₅₀ (50% cytotoxicity) was 1.7 $\mu\text{g}/\text{mL}$ for 5B1 and 0.1 $\mu\text{g}/\text{mL}$ for 7E3, which translates to roughly 85-fold higher potency for 7E3 on a molar basis.

ADCC activity

While 7E3 is significantly more potent in the CDC assay, IgG antibodies are known to have antibody-dependent cell-mediated cytotoxicity (ADCC) activity, which is thought to be important for tumor killing *in vivo*. High levels of cytotoxicity were measured using 5B1 antibody with human PBMC and DMS-79 target cells at various E:T ratios (Fig. 3A). Similar levels of cytotoxicity were observed at lower E:T ratios with primary NK cells (data not shown). A dose-response experiment with PBMC from 2 donors measured at an E:T ratio of 100:1 showed similar efficacy, and more than 85% cytotoxicity was reached at concentrations 0.5 $\mu\text{g}/\text{mL}$ or more of 5B1 (Fig. 3B). The cytotoxicity mediated by 5B1 requires Fc γ RIII receptors since it can be

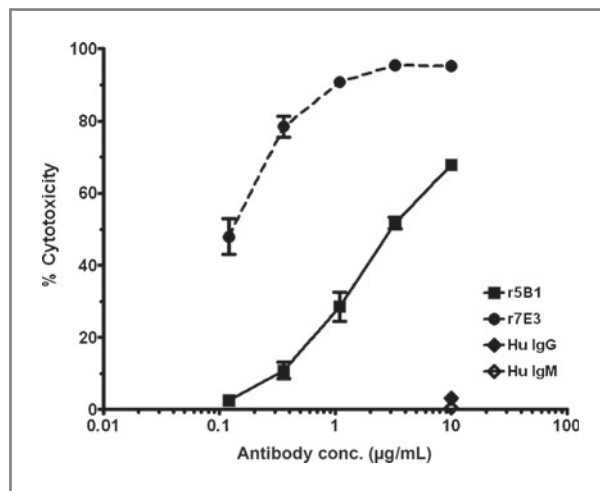


Figure 2. CDC activity of r5B1 and r7E3 antibodies in the presence of human complement. Cytotoxicity of r5B1 (■) and r7E3 (●) at various concentrations against DMS-79 tumor cells in presence of human complement. Nonspecific human IgG (◆) and IgM (◇) tested at 10 $\mu\text{g}/\text{mL}$ were inactive.

blocked with 3G8 anti-CD16 antibodies (data not shown). The ADCC activity achieved with 1 $\mu\text{g}/\text{mL}$ of 5B1 antibodies was superior to the activity observed with antibodies to GM2, fucosyl-GM1, globo H, or polysialic acid. As expected, 7E3 and murine 121SLE (both are IgM) were inactive in this assay (data not shown).

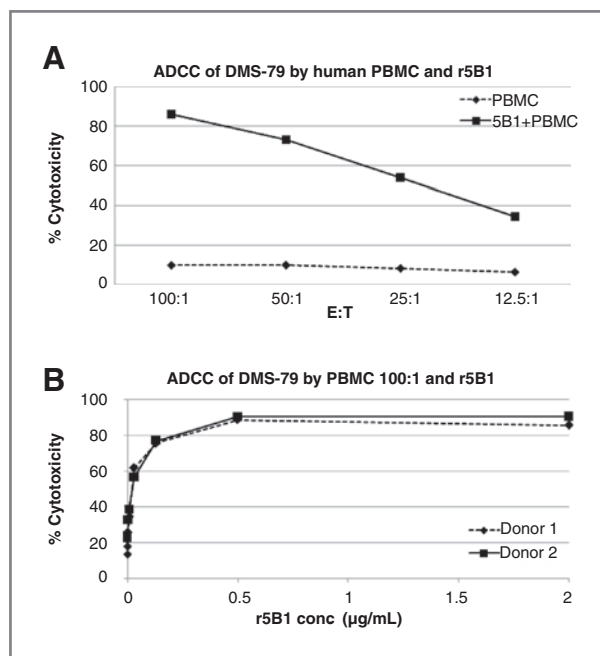


Figure 3. ADCC activity of r5B1 antibodies. A, r5B1-mediated ADCC with human PBMC against DMS-79 cells. PBMC were tested at E:T ratios from 100:1 to 12.5:1 with DMS-79 tumor cells in the presence or absence of 2 $\mu\text{g}/\text{mL}$ r5B1. B, ADCC of r5B1 at various concentrations with PBMCs from 2 donors at an E:T ratio of 1:100 with DMS-79 tumor cells in the presence of the indicated concentrations of r5B1.

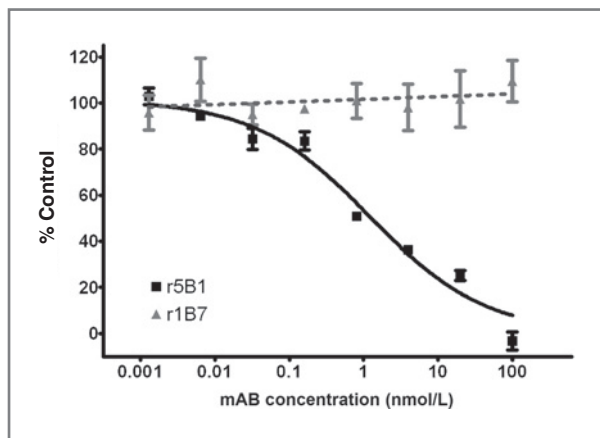


Figure 4. Internalization of sLe^a into BxPC3 cells. BxPC3 pancreatic tumor cells were grown in the presence of r5B1 (anti-sLe^a) or r1B7 (anti-GD2) antibodies complexed with Hum-ZAP, a saporin-conjugated anti-human IgG. After 3 days, the viability of the cells was measured using an MTT assay and the sample values were normalized to the values of untreated cultures.

5B1 internalization assay

Antibody conjugates directed at antigen "closely related to" Lewis Y were previously shown to be rapidly internalized and very effective in animal models (20, 21). To examine whether sLe^a is internalized, we incubated the pancreatic cell line, BxPC3 with 5B1, and then added Hum-ZAP, an anti-human IgG conjugated to the ribosome-inactivation protein saporin (22). Cells that internalize the saporin-containing complex die, while noninternalized saporin leaves the cells unharmed. As shown in Figure 4, BxPC3 cells are effectively killed in the presence of increasing doses of 5B1 while the presence of an isotype-matched IgG1 antibody directed against GD2, which is not expressed on these cells, does not kill the cells.

Activity in xenograft animal model for metastasis

To evaluate the activity of 5B1 *in vivo*, the antibodies were tested in a xenograft model using Colo205-luc tumor cells in SCID mice. Five mice per group were injected with 0.5×10^6 cells into the tail vein on day 0, and successful injection of the cells was verified by imaging the animals using the IVIS 200 *in vivo* imaging system (Caliper Life Sciences). One day later, animals were treated with 5B1 antibodies given intraperitoneal or PBS mock injection. In experiment 1, 100 μ g of 5B1 was given on days 1, 7, 14, and 21 (400 μ g total dose), and in experiment 2 the animals received 100 μ g 5B1 on days 1, 4, 7, 10, 14, and 21 (600 μ g total dose). The average median survival of untreated animals was 102 days in the 2 experiments, and all untreated animals died within 155 days (Fig. 5). Treatment of animals improved survival significantly: the median survival was doubled to 207 days in the group that received 4 doses of 5B1 and 2 of 5 animals survived until termination of the experiment after 301 days (log-rank test, $P = 0.0499$; HR = 3.46). The proportion of survivors further increased to 3 of 5 mice when 6 doses were administered (log-rank test, $P =$

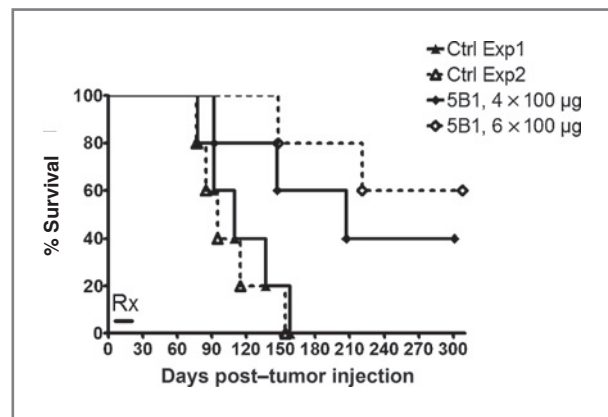


Figure 5. Activity in a xenograft model using Colo205-luc cells. SCID mice (5 per group) received 0.5 million Colo205-luc cells by tail vein injection on day 0. Animals received 100 μ g r5B1 by intraperitoneal injection on days 1, 7, 14, and 21 (experiment 1, Exp1) or on days 1, 4, 7, 10, 14, and 21 (experiment 2, Exp2) for a total dose of 600 μ g. Control (Ctrl) animals received PBS mock injections.

0.0064; HR = 6.375). The second study was terminated after 308 days, and the surviving animals failed to reveal Colo205-luc tumors at the highest sensitivity of the imaging system (data not shown). These data demonstrate a significant survival benefit with 5B1 treatment. PK studies will be required to establish exposure levels and to further refine the dosing schedule.

Discussion

Altered carbohydrate expression is a hallmark of tumor cells that could be an ideal target for active or passive immunotherapy. Unfortunately, the complexity of carbohydrate chemistry and biology has hampered the development of effective therapeutics targeting carbohydrate antigens. We have recently demonstrated that our sLe^a-KLH conjugate vaccine could induce high titers of both IgG and IgM antibodies against sLe^a in mice and humans without cross-reactivity to other similar blood group carbohydrate antigens (11).

To further characterize the humoral immune response to the vaccine, we generated human mAbs from blood samples of vaccinated patients. A number of antibodies were recovered that specifically bind sLe^a in an ELISA assay and on the surface of tumor cell lines. Molecular cloning of some of the antibodies showed evidence of affinity maturation as indicated by the number of CDR mutations deviating from the germ line in the cDNA sequence. Two high-affinity antibodies (5B1 and 7E3) were further characterized *in vitro*, and cell surface binding was demonstrated for CA19.9-positive colon cancer (HT29 and Colo205), ovarian cancer (SW626), small-cell lung cancer (DMS-79), and pancreatic cancer (PxC3) cell lines but not for the CA19.9-negative melanoma cell line (SK-MEL28). Both antibodies failed to bind to sialyl-Lewis^x, Lewis^a, and other related carbohydrates when tested by ELISA and SPR. In addition, binding analysis

on a glycan array with 465 distinct carbohydrates revealed that both antibodies have exquisite specificity for Neu5Ac α 2-3Gal β 1-3(Fuc α 1-4)GlcNAc β . The high specificity of 7E3 measured by glycan array analysis is remarkable since IgM antibodies tend to have lower affinities and show less specificity than affinity-matured IgG antibodies. Both antibodies were very potent in CDC assays, albeit the 7E3 IgM antibody is considerably more potent in this assay, which was expected for a high-affinity/avidity IgM antibody. The 5B1 IgG1 antibody has the added benefit of being highly active in ADCC assays, which is thought to be a major activity contributing to tumor regression. The results obtained so far show that both antibodies have significantly improved affinity/avidity and effector function profile compared with SLE121, a mouse mAb with specificity for sLe^a.

To evaluate the *in vivo* efficacy, 5B1 antibodies were tested in a xenograft model of Colo205 tumor cells engrafted into SCID mice. Treatment with 5B1 antibodies rendered 40% to 60% of the mice disease free for more than 300 days depending on dose, while 100% (5/5) untreated animals died within 155 days under these experimental conditions. Since all animals were injected with Colo205-luc cells that carry the luciferase gene, we could verify that all animals received similar amounts of tumor cells on day 0. The surviving animals failed to show tumors at the highest sensitivity of the imaging system at the end of the study. There could be several reasons why 2 animals died despite 5B1 treatment: Variation of the response could be due to natural variations in the animals' pharmacokinetic (e.g., lower trough levels, faster elimination), variances in effector cell function, differences in complement activation, and any combination of these. Another possibility could be selection of escape mutants in the host that lack or have reduced levels of sLe^a. If escape mutants were generated, one would expect that the percentage of surviving animals would remain similar, independent of the r5B1 dose. We are poised to address this issue in ongoing experiments by evaluating higher doses and, if applicable, by further examining residual tumors. Further animal studies are needed to determine pharmacokinetic parameters and also to evaluate and compare the efficacy of 7E3.

sLe^a is expressed as a glycolipid as well as a glycoprotein on leukosialin (CD43), and the latter plays a dominant role in adhesion to E-selectin, which is thought to be important for hematogenous metastasis (4, 23). A number of mouse antibodies against sLe^a have been discovered and some

(e.g., CA19.9) are routinely used in diagnostic assays, while many others lack specificity and potency. Most of those antibodies were obtained following immunization with cancer cells, and subsequent characterization revealed the antigen's identity. In contrast, the antibodies described here are derived from humans following vaccination with a well-defined carbohydrate (sLe^a) antigen. The results demonstrate that functional, high-affinity antibodies with exquisite specificity and cytotoxic potency can be recovered, and the resulting recombinant antibodies are suitable for clinical testing without the need to initiate time-consuming humanization efforts. We have not observed any toxicity in mice so far, but further studies will be required to evaluate human tissue cross-reactivity, potential cell aggregation in Lewis^a-positive individuals, and hemolytic potential.

Since sLe^a is widely and selectively expressed on human cancers, while disialyl-Lewis^a and other related blood group antigens are predominantly expressed on normal cells, the antibodies described here might have clinical utility and further studies are warranted to explore their activity in various animal models. To our knowledge, there are currently no other fully human anti-sLe^a antibodies in development.

Disclosure of Potential Conflicts of Interest

R. Sawada, S-M. Sun, and W.W. Scholz are full-time employees of MabVax. G. Ragupathi and P.O. Livingston are paid consultants and shareholders of MabVax.

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