

# Construction, Production, and Characterization of Humanized Anti-Lewis Y Monoclonal Antibody 3S193 for Targeted Immunotherapy of Solid Tumors

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## ABSTRACT

The Lewis Y (Le<sup>y</sup>) antigen is a blood group-related antigen that is expressed in a high proportion of epithelial cancers (including breast, colon, ovary, and lung cancer) and is an attractive target for monoclonal antibody-directed therapy. The murine monoclonal 3S193 (IgG3) was generated in BALB/c mice by immunization with Le<sup>y</sup>-expressing cells of the MCF-7 breast carcinoma cell-line. The murine 3S193 showed high specificity for Le<sup>y</sup> in ELISA tests with synthetic Le<sup>y</sup> and Le<sup>y</sup>-containing glycoproteins and glycolipids and also reacted strongly in rosetting assays and cytotoxic tests with Le<sup>y</sup>-expressing cells. We generated a humanized form of the murine 3S193 antibody by linking cDNA sequences encoding the variable region of murine 3S193 with frameworks of the human KOL heavy chain and REI  $\kappa$  chain. The genes for the humanized 3S193 monoclonal antibody IgG1 were transfected into mouse myeloma NS0 cells and cloned for the establishment of high antibody-producing colonies. Humanized 3S193 antibody was subsequently produced through *in vitro* culture and under good manufacturing practice conditions using hollow-fiber bioreactors. The purified humanized 3S193 (hu3S193) was subsequently characterized and validated for use in preliminary immunotherapy investigations. hu3S193 reacted specifically with Le<sup>y</sup> antigen, with similar avidity to the murine form. hu3S193 demonstrated potent immune effector function, with higher antibody-dependent cell-mediated cytotoxicity than its murine counterpart and potent complement-dependent cytotoxicity (ED<sub>50</sub>, 1.0  $\mu$ g/ml). The *in vivo* immunotherapeutic potential of hu3S193 was assessed in a human breast xenograft model using MCF-7, Le<sup>y</sup>-positive cells. Six *i.v.* doses of up to 1 mg of hu3S193 were administered to animals bearing established tumors (120–130 mm<sup>3</sup>) with no significant effect on tumor growth. In contrast, in an MCF-7 xenograft preventive model, a 1-mg hu3S193 dosage schedule was able to significantly slow tumor growth compared with placebo and isotype-matched control IgG1 antibody. hu3S193 has promise for immunotherapy of Le<sup>y</sup>-positive tumors and is currently entering Phase I clinical trials.

## INTRODUCTION

The Le<sup>y2</sup> antigen is a type 2 blood group-related difucosylated oligosaccharide with the chemical structure Fuc $\alpha$ 1 $\rightarrow$ 2Gal $\beta$ 1 $\rightarrow$ 3[Fuc $\alpha$ 1 $\rightarrow$ 4]GlcNAc $\beta$ 1 $\rightarrow$ R (1–7). The Le<sup>y</sup> antigen has been shown to be expressed by 60–90% of human carcinomas of epithelial cell origin, including breast, pancreas, ovary, colon, gastric, and lung cancer (1–5, 8), but does not appear to be expressed on tumors of

neuroectodermal or mesodermal origin (1, 5). Immunohistochemical analysis has demonstrated some expression of Le<sup>y</sup> antigen in normal tissues, particularly in mucosa of the esophagus, stomach, large and small intestine, some exocrine cells of the pancreas, some epithelial cells in the gallbladder, ciliated epithelium of the trachea and bronchus, and type II pneumocytes (5, 7, 8). The restriction of Le<sup>y</sup> expression on normal tissues to epithelial surfaces, as determined by immunohistochemical methods, suggests this antigen normally may be relatively inaccessible to administered antibody (5). Accordingly, the high frequency of Le<sup>y</sup>-expressing tumors, its high density, and altered expression on the surface of tumor cells and relatively homogenous expression in primary and metastatic lesions have led to its selection as an antigenic target for solid tumor immunotherapy (2, 5, 6, 8, 9).

A range of Le<sup>y</sup> antibodies have been identified, but a consistent problem with Le<sup>y</sup> antibodies has been a degree of cross-reactivity with Le<sup>x</sup> and H-type 2 structures and agglutination with human RBCs (6, 10), emphasizing the importance of understanding the target antigen expression and antibody specificity. A small number of Phase I clinical trials with mouse or chimeric anti-Le<sup>y</sup> antibodies have been conducted to date. In a Phase I study of murine BR55-2 in 12 patients with breast cancer at doses up to 80 mg/m<sup>2</sup> for 5 consecutive days, one minor response was observed, transient reduction in skin disease was seen in three patients, and human antimurine antibody prevented repeated treatment cycles (11). The murine anti-Le<sup>y</sup> mAb ABL-364 has been studied in patients with non-small cell lung cancer, at dose of 50 or 100 mg for 6 days over a 12-day period but with no responses observed (12). A chimeric BR-96-doxorubicin construct has been evaluated in a range of patients with advanced cancers including breast cancer at doses up to 700 mg/m<sup>2</sup> (weekly for 3 weeks; Ref. 13). Upper gastrointestinal toxicity was seen in doses of >200 mg/m<sup>2</sup>, and this toxicity was reduced with steroid premedication at higher BR-96-doxorubicin doses. The treatment of micrometastatic disease in bone marrow has also been reported in a study involving 19 patients with breast cancer infused with murine mAb ABL-364 at a dose of 100 mg for 6 days (over 12 days). Minor nausea and vomiting were reported, and a reduction in cytokeratin-positive cells was found in bone marrow in a majority of patients (14). Incorporating the anti-Le<sup>y</sup> monoclonal antibody B3, the LMB-1 immunotoxin (recombinant *Pseudomonas* exotoxin) directed against Le<sup>y</sup> has also been studied, with responses in 5 of 38 patients studied (15).

We have previously reported the development of the murine anti-Le<sup>y</sup> mAb 3S193 (6). To develop an anti-Le<sup>y</sup> antibody with minimal immunogenicity for use in clinical trials, we describe the construction and production and subsequent *in vitro* and *in vivo* characterization of a humanized CDR-grafted construct of 3S193 (hu3S193).

## MATERIALS AND METHODS

The murine monoclonal 3S193 (IgG3) was generated in BALB/c mice by immunization with Le<sup>y</sup>-expressing cells from the MCF-7 breast carcinoma cell

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<sup>2</sup> The abbreviations used are: Le<sup>y</sup>, Lewis Y; mAb, monoclonal antibody; m3S193, murine 3S193; hu3S193, humanized 3S193; MCF-7, Le<sup>y</sup>-positive human breast carcinoma cell line; huA33, humanized A33 mAb; CDC, complement-dependent cytotoxicity; ADCC, antibody-dependent cellular cytotoxicity; GMP, good manufacturing practice; CDR, complementarity binding region; SEC, size exclusion chromatography; IEF, isoelectric focusing; HAHA, human anti-human antibodies.

line. The specificity analysis of m3S193 indicated high specificity for Le<sup>y</sup> in ELISA tests with synthetic Le<sup>y</sup> and Le<sup>y</sup>-containing glycoproteins and glycolipids. In addition, the antibody reacted strongly in rosetting assays and cytotoxic tests with Le<sup>y</sup>-expressing cells. (6).

### Expression of the hu3S193 Antibody

We have briefly described previously the humanization of mu3S193 (6). In an attempt to create an antibody suitable for application in immunotherapy, the antibody m3S193 (IgG3) was humanized using the variable region frameworks of the KOL heavy chain and REI  $\kappa$  chain. Several IgG1 humanized antibodies containing different combinations of the heavy and light chains were produced, and detailed analyses were performed to determine the preferred construct. These antibodies were tested for binding to synthetic Le<sup>y</sup> oligosaccharide-keyhole limpet hemocyanin (Chem-Biomed, Edmonton, Alberta, Canada) and to intact MCF-7 cells by ELISA. The preferred construct was determined, and the genes for the humanized 3S193 monoclonal antibody IgG1 were transfected into mouse myeloma NS0 cells (European Collection of Animal Cell Cultures number 85110503) and cloned for the establishment of high-antibody-producing colonies.

### Production of hu3S193

**Hybridoma Culture.** hu3S193/NS0 was initially cultivated in RPMI 1640 (Life Technologies, Gaithersburg, MD) plus additives (0.6  $\mu$ g/ml insulin, 1.0  $\mu$ g/ml hydrocortisone,  $10^{-5}$  M  $\alpha$ -thioglycerol, 50  $\mu$ g/ml penicillin, and 50  $\mu$ g/ml streptomycin), supplemented with 0.1 M hypoxanthine, 0.016 M thymidine, and 10% fetal bovine serum. Small-scale production of antibody for evaluation of culture conditions and initial characterization was performed under these conditions. The culture was then weaned to serum-free conditions and maintained at 37°C, 5% CO<sub>2</sub> in HyQ-CCM1 medium (HyClone, Logan, UT) supplemented with 0.2% Ex-Cyte VLE, a bovine growth enhancement supplement (Bayer, Pittsburg, PA).

**Large-Scale Production.** Large-scale production of humanized 3S193 IgG1 was achieved by three production runs using an AcuSyst Maximizer 1000 instrument equipped with two 1.2-m<sup>2</sup> cellulose acetate hollow-fiber bioreactors (Cellex Biosciences, Minneapolis, MN). Large-scale production was performed in the Biological Production Facility of the Ludwig Institute for Cancer Research.

Production runs were commenced from frozen ampoules of the hu3S193 NS0 transfectoma with seeding of  $2 \times 10^5$  cells/ml as a stationary culture in T175 tissue culture flasks (Nunclon; Nunc, Roskilde, Denmark). The flasks were incubated at 37°C and 5% CO<sub>2</sub> in HyQ-CCM1 medium (HyClone), containing bovine transferrin (56.0  $\mu$ g/ml) and bovine albumin (1.1 g/ml), supplemented with 0.2% Ex-Cyte VLE (Bayer). The preculture was maintained until a suitable number of cells were obtained to inoculate roller bottles. The roller bottle cultures were further incubated until a suitable number of cells ( $>1.0 \times 10^8$ ) were available for the inoculation of the bioreactor. The culture was prepared for inoculation and pumped into two 1.2-m<sup>2</sup> cellulose acetate hollow-fiber cartridges per production run, according to the manufacturer's specification.

The process temperature was kept at 37°C. Daily samples of culture medium, tested for glucose and lactate concentration, as well as pH and dissolved oxygen, determined the perfusion rate of the system. Whenever possible, the intracellular compartment glucose concentration was kept at  $>0.8$  g/liter, lactate at  $<1.8$  g/liter, and the dissolved oxygen concentration at  $>80$  mm Hg. The continuously perfused cultures in the serum-free conditions described above were harvested daily, monitored, and maintained for up to 55 days.

Antibody-containing medium was harvested continuously into depyrogenated, sterile glass bottles once the culture showed a consistent metabolic pattern. The collected supernatant was centrifuged to clarify the medium from cells and debris and then stored at  $-20^\circ\text{C}$ . Harvest samples were tested by ELISA to monitor the antibody production rate of the run. Biosafety testing was performed on the master and working cell banks as well as on production lots.

### Purification of hu3S193

Pooled bioreactor production runs were clarified by sterile filtration and then subjected to a four-step purification process under GMP conditions,

involving anion exchange, protein A affinity, cation exchange, and SEC. Final SEC fractions containing antibody were pooled and then concentrated using a Minitan concentrator (30-kDa cutoff membranes). The final, concentrated antibody solution was sterile filtered and stored at 2–8°C. After determination of final antibody concentration, the final product was aliquoted, placed in vials, and then stored at  $-80^\circ\text{C}$ .

Antibody content at all steps after the anion exchange resin was estimated by absorbance at 280 nm, using the calculated absorbance value of 1.36 absorbance units/mg of protein. In addition to monitoring the protein profile of the column eluant, the chromatographic purification was monitored by SDS-PAGE and IEF gel analyses. Proteins were visualized by colloidal Coomassie blue or silver staining analyses.

### Protein Characterization of Purified hu3S193

In addition to standard SDS-PAGE and IEF gel electrophoresis analyses, the large-scale hu3S193 product was analyzed by SEC under nonreducing conditions. Separation was achieved on a Phenomenex Biosep SEC S3000 column (300  $\times$  7.8 mm), fitted with an S3000 (35  $\times$  7.8 mm) guard column, on a Hewlett Packard 1100 series chromatograph, at a flow rate of 1.0 ml/min, eluting with 0.01 M sodium phosphate and 0.15 M NaCl (pH 7.2). Protein was detected by absorbance at 214 nm. A molecular weight calibration curve was generated using standard markers containing blue dextran, ferritin, aldolase, ovalbumin, and chymotrypsinogen (Amersham Pharmacia Biotech, Uppsala, Sweden).

### Biological Characterization of Purified hu3S193

**Scatchard Analysis.** Comparison of hu3S193 from small- and large-scale production runs was performed using a cell-based binding assay to determine the association constant for binding to Le<sup>y</sup>-positive MCF-7 cells as described previously (16).

**Competitive Cell Binding Assay.** Comparison of the antibody-antigen affinity of the humanized and murine forms of 3S193 and another murine anti-Le<sup>y</sup> antibody, BR55-2 (IgG3; Z. Stepleski, Wistar Institute, Philadelphia, PA) was performed. The ability of these antibodies to compete with <sup>125</sup>I-hu3S193 for binding to Le<sup>y</sup>-positive MCF-7 cells was assessed using a published method (17). Increasing amounts of each unlabeled antibody were added to MCF-7 cells in triplicate, followed by 20 ng of <sup>125</sup>I-hu3S193, mixed, and incubated at room temperature for 45 min. Cells were washed three times in RPMI 1640 and centrifuged, and the supernatants and pellets were counted in a gamma counter (Cobra II, model 5002, Auto-gamma; Packard Instruments, Canberra, Australia). IgG1 and IgG3 control antibodies huA33 and R24 (Ludwig Institute for Cancer Research, New York, NY) were assessed in parallel.

**Biosensor Analysis.** Biosensor analyses were performed using a BIAcore 2000 (BIAcore AB, Uppsala, Sweden) on a carboxymethyl-dextran-coated sensor chip (CM5). The chip was derivatized with synthetic Lewis Y tetrasaccharide, coupled to BSA (Alberta Research Council, Edmonton, Alberta, Canada), using standard amine-coupling chemistry (*N*-hydroxysuccinimide and *N*-ethyl-*N'*-dimethylaminopropyl-carbodiimide). Samples of hu3S193 and m3S193 were diluted in HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM di-Na-EDTA, and 0.005% Tween 20), and aliquots (30  $\mu$ l) were injected over the sensor chip surface at a flow rate of 5  $\mu$ l/min. After the injection phase, dissociation was monitored by flowing HBS buffer over the chip surface for 300 s. Bound antibody was eluted, and the chip surface was regenerated between samples by injection of 20  $\mu$ l of 10 mM NaOH. For kinetic analyses of binding, varying concentrations of hu3S193 and m3S193 were injected over the sensor chip surface. Apparent  $K_a$  and  $K_d$  rate constants were calculated by nonlinear least squares regression analysis, using BIA-evaluation version 3.0 software (Pharmacia Biosensor, Uppsala, Sweden).

**Immune Effector Function.** Both CDC and ADCC assays used MCF-7 breast carcinoma cells as target cells. An isotype-matched, but unrelated, antibody huA33 (17) was used as a control, as was a control (Le<sup>y</sup> negative) cell line, SW-1222.

**CDC Assay.** Target cells were plated at a density of  $\sim 100$  cells/well, with varying amounts of hu3S193 (0.001–10  $\mu$ g/ml), in microtiter plates (Nunclon;

<sup>3</sup> K. Clarke, F. T. Lee, M. W. Brechbiel, F. E. Smyth, E. Richards, E. Stockert, L. J. Old, and A. M. Scott, unpublished results.

Nunc). Diluted human complement, prepared from the blood of normal, healthy volunteers, was added to each well. Tests were done in triplicate with medium, antibody, and complement controls. After a 4-h incubation at 37°C, cells were fixed with methanol, and viable cells were visualized with Giemsa stain. The percent cytotoxicity of the antibody was calculated as follows: number of viable cells in well treated with antibody + complement ÷ number of viable cells in untreated well × 100. The percent cytotoxicity was plotted versus concentration of antibody (μg/ml), and the concentration of antibody causing 50% cytotoxicity was determined.

**ADCC Assay.** Target cells were incubated for 18 h with 100 μCi of <sup>51</sup>Cr, washed, and plated at a density of 10,000 cells per well into a 96-well microtiter plate. Effector cells (peripheral blood mononucleocytes) were freshly prepared and added to the target cells to achieve E:T ratios of 50:1. Aliquots of hu3S193 (0.003–10 μg/ml) were added to appropriate wells. After incubation at 37°C for 4 h, samples of cell supernatant were collected and counted for released <sup>51</sup>Cr. Controls included in the assay corrected for spontaneous release (medium alone) and total release (detergent). The percentage of cell lysis (cytotoxicity) was calculated according to the formula: percentage cytotoxicity = (sample counts – spontaneous release) ÷ (total release – spontaneous release) × 100. The percent cytotoxicity was plotted versus concentration of antibody (μg/ml), and the concentration of antibody causing 50% cytotoxicity was determined.

### Therapeutic *in Vivo* Studies with hu3S193

**Treatment of Established MCF-7 Tumors in Mice.** To establish MCF-7 human breast xenografts, mice were supplemented with exogenous estrogen. Mice were s.c. implanted with a 60-day slow-release 0.72-mg oestrogen pellet (Innovative Research of America, Sarasota, FL) between the shoulder blades. Subsequently, the mice received 20 × 10<sup>6</sup> MCF-7 cells s.c. into the left inguinal mammary line. The establishment of tumors was monitored by tumor volume measurement [(length × width<sup>2</sup>)/2], where length was the longest axis and width was the measurement at right angles to length. After 21 days, mice with similar mean tumor volumes were divided into treatment groups of five animals. Animals received five retro-orbital i.v. doses of hu3S193 antibody at 72-h intervals. hu3S193 used for therapeutic studies was produced in initial small-scale production runs. Treatment groups consisted of hu3S193 in PBS, 0.03, 0.1, 0.32, or 1.0 mg. The control group received PBS alone. Tumor growth was measured for a further 20 days after completion of treatment.

**Prevention of MCF-7 Tumor Growth in Mice.** The BALB/c MCF-7 xenograft model described above was used in these studies. However, in this study, mice received the first dose of antibody treatment retro-orbitally at the same time as inoculation of 20 × 10<sup>6</sup> MCF-7 cells and insertion of the estrogen pellet. The groups of five animals subsequently received five additional antibody doses retro-orbitally at 48-h intervals. There were four treatment groups; hu3S193 in PBS at 0.1 or 1.0 mg and control groups receiving 1.0 mg of

isotype-matched, unrelated antibody huA33 or PBS alone. Tumor volume was measured [(length × width<sup>2</sup>)/2] for 56 days from commencement of treatment. Results from groups of mice were assessed for statistical significance by Student's paired *t* test.

## RESULTS

### Cloning of VH and VL cDNA

Humanized antibodies were produced using different combinations of the light and heavy chains. The performance of huVKF was found to be slightly better than that of huVK and within 2-fold of that of the chimeric κ chain. The huVH and huVHT were less efficient at binding than the chimeric heavy chain. The inclusion of Thr-24 slightly improved the performance and reduced the cross-reactivity to Le<sup>y</sup>-related structures.

Four further humanized antibodies were produced. These were huVHA/huVKF, huVHAS/huVKF, and huVHAS/huVKF, in which the heavy chain included murine residues selected from Ala-27, Ser-76, and Tyr-79, and huVHTAS/huVKF, which combines the incorporation of Ala-74 and Ser-76 with that of Thr-24. These antibodies were tested for binding to synthetic Le<sup>y</sup> oligosaccharide-keyhole limpet hemocyanin and to intact MCF-7 cells by ELISA. The results indicated the huVHAS/huVKF antibody offered an improvement in binding over the huVHT/huVK antibody. Comparison of the huVHT, huVHAS, and huVHTAS antibodies suggested that combining the two changes was not beneficial.

huVHAS/huVKF showed a little cross-reactivity to Le<sup>x</sup> and H type 2 antigens in ELISAs, which, although the antibody did not cause hemolysis, meant that huVHT/huVKF (previously described as clone 11 in Ref. 6) was the preferred construct. The amino acid sequences of the light- and heavy-chain regions of the translated protein are shown in Fig. 1.

### Antibody Production

The genes for the humanized 3S193 monoclonal antibody IgG1 were transfected into mouse myeloma NS0 cells and cloned for the establishment of high antibody-producing clones. Initial small-scale production runs were performed for antibody characterization. Selected clones were then cultured and weaned to grow and optimally produce antibody in serum-free conditions.

A master cell bank was established and after passing the requisite

Fig. 1. Amino acid sequences of humanized 3S193 heavy and light chains. The CDR regions are shaded.

### Hu3S193 Heavy Chain (HuVHT)

EVQLVESGGG	VVQPGRSLRL	SCSTSGFTFS	DYYMYWVRQA	PGKGLEWVAY <sub>50</sub>
MSNVGAIIDY	PDTVKGRFTI	SRDNSKNTLF	LQMDSLRPED	TGVYFCARGT <sub>100</sub>
RDGSWFAYWG	QGTPVTVSSA	STKGPSVFPL	APSSKSTSGG	TAALGCLVKD <sub>150</sub>
YFPEPVTVSW	NSGALTSGVH	TFPAVLQSSG	LYSLSSVVTV	PSSSLGTQTY <sub>200</sub>
ICNVNHKPSN	TKVDKKEPK	SCDKTHTCPP	CPAPPELLGGP	SVFLFPPKPK <sub>250</sub>
DTLMISRTPE	VTCVVVDVSH	EDPEVKFNWY	VDGVEVHNAK	TKPREEQYNS <sub>300</sub>
TYRVVSVLTV	LHQDWLNGKE	YKCKVSNKAL	PAPIEKTISK	AKGQPREPQV <sub>350</sub>
YTLPPSRDEL	TKNQVSLTCL	VKGFYPSDIA	VEWESNGQPE	NNYKTTTPVL <sub>400</sub>
DSDGSFFLYS	KLTVDKSRWQ	QGNVVFCSVM	HEALHNHYTQ	KSLSLSPGK

### Hu3S193 Light Chain (HuVKF)

DIQMTQSPSS	LSASVGDRTV	ITCRSSQRIY	HSNGNTYLEW	YQQTGKAPK <sub>50</sub>
LLIYKVSNR	SGVPSRFSGS	GGTDFFTFI	SSLQPEDIAI	YYCFQGSHPV <sub>100</sub>
FTFGQGTKLQ	IITRTVAAPS	FIFPPSDEQL	KSGTASVVCL	LNNFYPREAK <sub>150</sub>
VQWKVDNALQ	SGNSQESVTE	QDSKSTSYSL	SSTLTLSKAD	YEKHKVYACE <sub>200</sub>
VTHQGLSSPV	TKSFNRGEC			

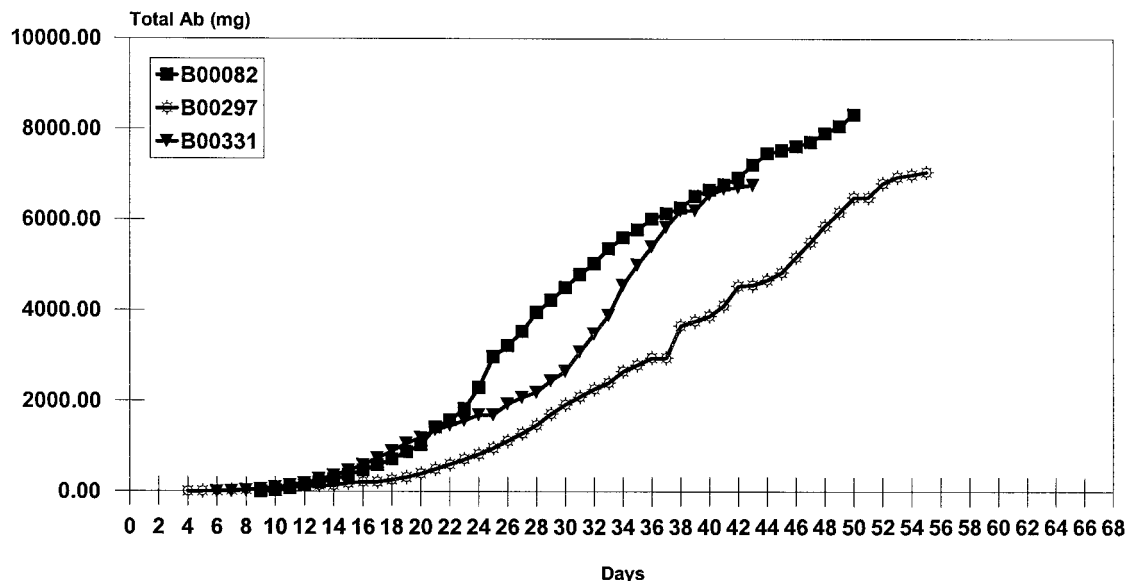


Fig. 2. Large-scale production of 18.8 g of hu3S193 monoclonal antibody. Production rates are presented for three separate Bioreactor production runs. Daily harvests were performed once the culture displayed a consistent metabolic pattern. Run 1 (B00082; ■), 40 harvest days; run 2 (B00297; \*), 51 harvest days; run 3 (B00331; ▼), 37 harvest days.

biosafety testing; a manufacturer's working cell bank was then established. Large-scale production was performed using the working cell bank. Three separate bioreactor production runs were performed to produce the humanized 3S193 large-scale clinical batch. The bioreactors produced hu3S193 at a rate of 114.2–166.5 mg/day, with an average production rate of 144.4 mg/day for the three runs (Fig. 2). The first bioreactor run lasted 50 days. Daily harvests over 40 days yielded 6569 ml of extracapillary medium. The bulk antibody concentration was estimated by ELISA and indicated a total of 8325.6 mg of hu3S193 IgG. The second bioreactor run lasted 55 days (51 harvest days). A total of 5175 ml of extracapillary medium was harvested, which was estimated by ELISA to contain a total of 6279 mg of antibody. The final run was cultured over 43 days. The 37 harvest days produced a total volume of 3907 ml, containing 6554 mg of hu3S193 IgG.

### Antibody Purification

Small-scale production of hu3S193 was purified by protein A and SEC. The large-scale harvested bioreactor cell culture supernatant contained 18.8 g of unpurified hu3S193. This was purified by a four-step column chromatography process, resulting in a 47.5% final yield of 8.93 g of hu3S193 (estimated by  $A_{280}$ ). Minimal levels of leached protein A were observed by ELISA of samples from the post-protein A pool, post-S-Sepharose pool, and post Sephadex 200 pool (2.3 ng/ml). The endotoxin content of the final purified antibody was 0.43 endotoxin units/ml.

### Characterization of hu3S193

**Protein Analysis.** The  $A_{280\text{ nm}}$  trace from SEC analysis of purified hu3S193 showed a single peak with a elution time of 8.62 min, corresponding to a calculated molecular weight of 157,000. No evidence of dimers or other high molecular weight species was observed, in agreement with nonreducing gel analysis. Further analysis by SDS-PAGE of hu3S193, under reducing conditions, indicated the expected molecular masses of 25 and 50 kDa for the hu3S193 light and heavy chains, respectively. No other significant protein bands were demonstrated, attesting to the purity of the preparation. IEF analysis showed a distinct single band for the hu3S193 sample visible with a determined pI of 9.0 (data not shown).

**Scatchard Analysis.** Hu3S193 was analyzed to determine the apparent binding affinity of the purified antibody for its antigen. Samples of the large-scale GMP antibody were found to have an identical apparent binding affinity of  $5.33 \times 10^6/\text{M}$  to small-scale-produced hu3S193. The binding site number per MCF-7 cell was calculated to be  $7 \times 10^6/\text{cell}$ .

**Competitive Cell Binding Assay.** There was a slight loss of apparent binding affinity in hu3S193 compared with m3S193; however, hu3S193 was comparable and slightly superior to another murine anti-Le<sup>y</sup> antibody (BR55-2; Fig. 3).

**Biosensor Determination of Antibody Binding Affinity.** The results of kinetic analysis of binding of hu3S193 are displayed graphically in Fig. 4, and measured parameters are shown in Table 1. The data were fitted globally to a 1:1 Langmuir binding model with compensation for mass transfer. The presence of mass transfer effects was inferred from changes in the shape of the sensorgram at one

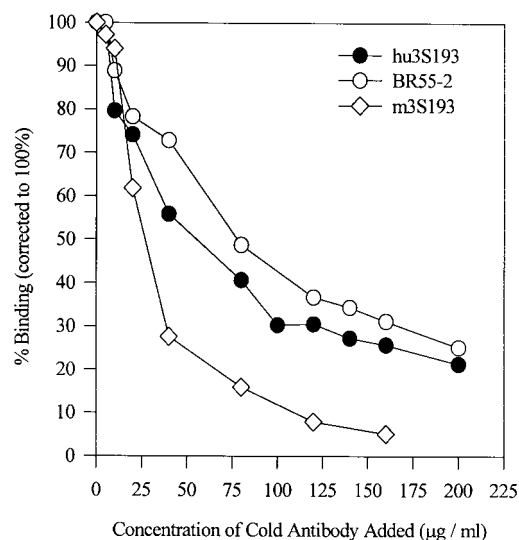


Fig. 3. Competitive cell binding assay comparing affinity of increasing concentrations of hu3S193 (●), m3S193 (◇), and BR55-2 (○) for Le<sup>y</sup>-positive MCF-7 cells in the presence of 20 ng of  $^{125}\text{I}$ -hu3S193. Class-specific controls, huA33 as IgG1 and R24 as IgG3 control, were not inhibited by addition of cold antibody (data not shown).

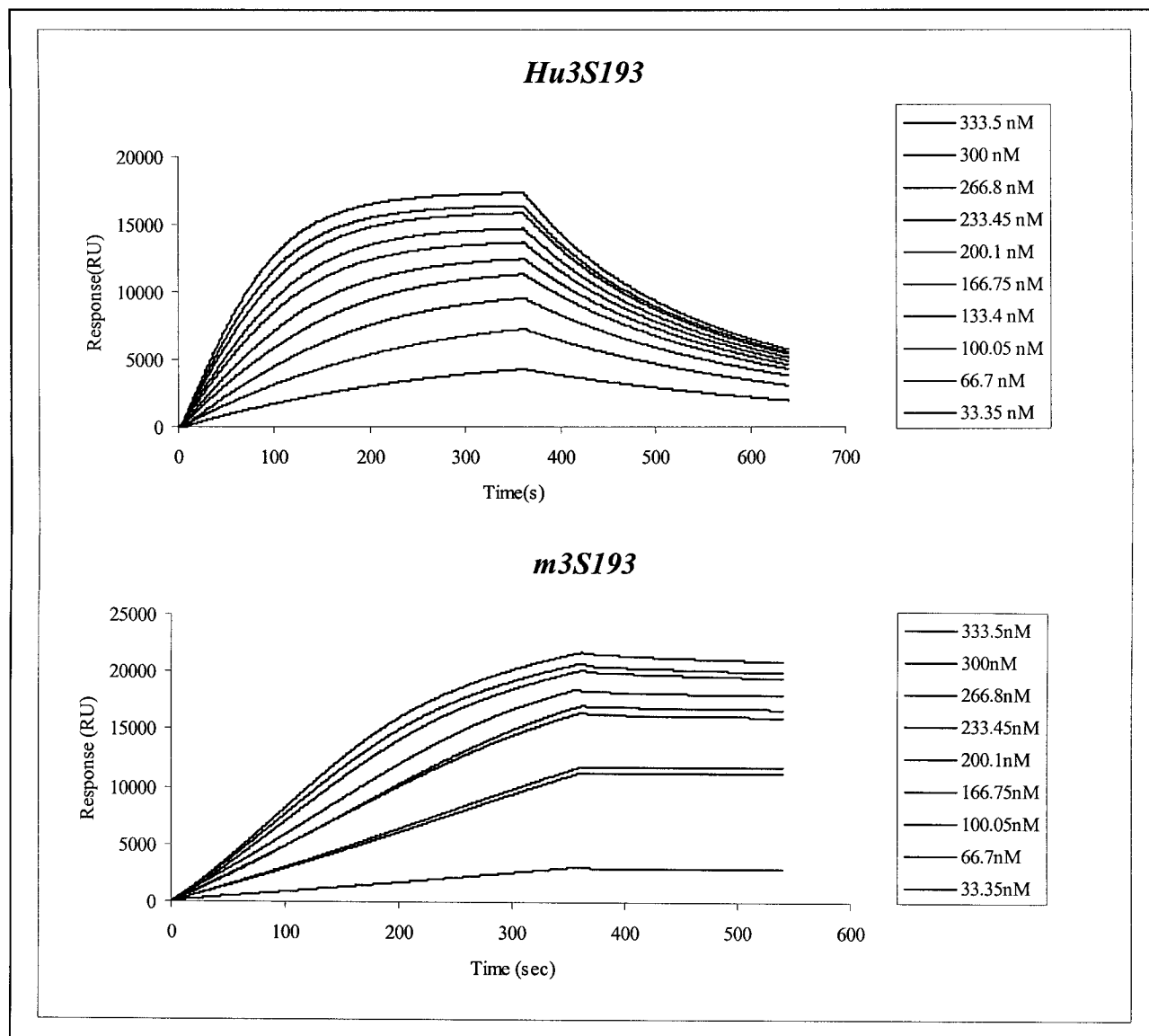


Fig. 4. Sensorgrams from Biosensor analysis of the interaction between anti- $Le^y$  antibodies hu3S193 and m3S193 and immobilized  $Le^y$  tetrasaccharide-BSA conjugate. Varying concentrations of antibody were injected at a flow rate of  $5 \mu\text{l}/\text{min}$  over the tetrasaccharide-BSA conjugate, which had been immobilized onto the chip surface using standard amine-coupling chemistry. Curves were fitted to a 1:1 Langmuir binding model with allowance for mass transfer effects.

analyte concentration but with varying flow rate (data not shown). Data and parameters were compared with those of the equivalent murine antibody. The humanized antibody demonstrated faster on and off rates compared with its murine counterpart, resulting in a reduction in apparent binding affinity. The  $K_a$  of  $4.19 \times 10^6/\text{M}$  for hu3S193 is highly comparable with the specific binding affinity determined by the cell-based Scatchard analysis.

**Immune Effector Function.** The CDC assay demonstrated 50% cytotoxicity at  $1.0 \mu\text{g}/\text{ml}$  for small- and large-scale-produced hu3S193, with negligible cytotoxicity for control antibody (huA33) or

effector cell control. Little difference in CDC was observed comparing humanized and murine versions of 3S193 (Fig. 5A).

The ADCC of hu3S193 on target MCF-7 cells was also evaluated. At an antibody concentration of  $0.32 \mu\text{g}/\text{ml}$ , 50% lysis of target cells was achieved (Fig. 5B). A similar potency was determined for hu3S193 purified from small-scale production runs (data not shown). This cytotoxicity was 3-fold better than that achieved by the murine counterpart. Control antibody huA33 had no evidence of CDC or ADCC activity against MCF-7 cells, and hu3S193 had no cytotoxicity against control SW-1222 cells (data not shown).

#### Therapeutic *in Vivo* Studies MCF-7 Mouse Xenograft Model

**Treatment of Established MCF-7 Tumors in Mice.** Groups of five BALB/c nude mice with established tumors of  $120\text{--}130 \text{ mm}^3$  at day 21 of growth received escalating doses of hu3S193 antibody or placebo. Five doses were administered at 72-h intervals, based on the half-life of murine IgG. The results of the tumor growth are presented in Fig. 6, where day 0 equals the first day of antibody treatment. No

Table 1 Binding parameters for interaction of anti- $Le^y$  antibodies with immobilized  $Le^y$  tetrasaccharide-BSA complex

Parameters were determined using a 1:1 Langmuir binding model with allowance for mass transfer effects.

Antibody	$K_a$ (1/M/s)	$K_d$ (1/s)	$R_{\text{max}}$ (RU)	$K_A$ (1/M)	$K_D$ (M)
hu3S193	$1.03 \times 10^5$	$2.47 \times 10^{-2}$	$1.80 \times 10^4$	$4.19 \times 10^6$	$2.39 \times 10^{-7}$
m3S193	$4.08 \times 10^4$	$2.18 \times 10^{-4}$	$2.19 \times 10^4$	$1.88 \times 10^8$	$5.33 \times 10^{-9}$

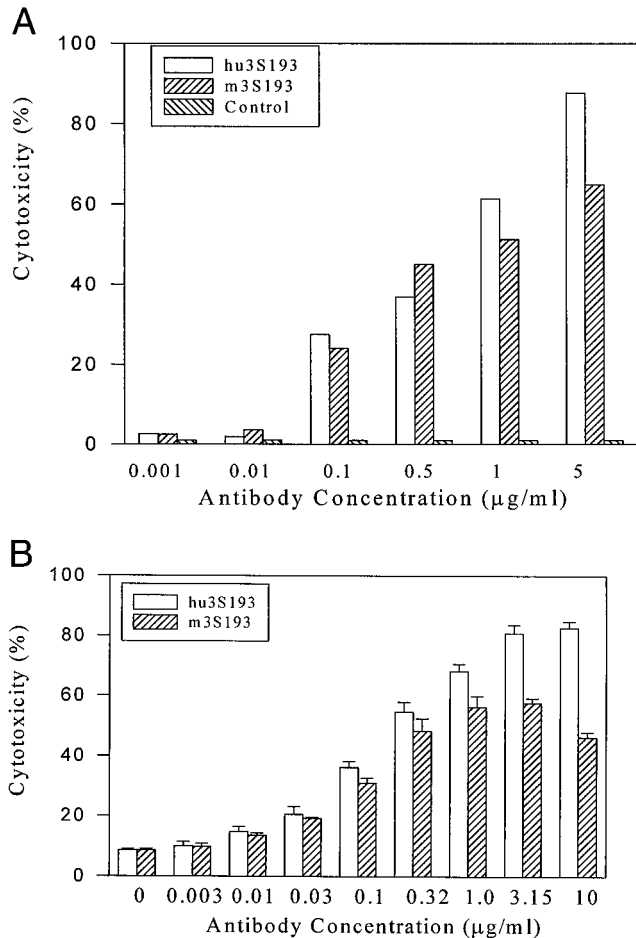


Fig. 5. *A*, comparison of CDC activities: murine and humanized 3S193 antibodies, with control of complement alone (no antibody). *B*, comparison of hu3S193 (□) and m3S193 (▨) ADCC activity over a range of mAb concentrations. % Cytotoxicity is a measure of antibody-mediated cell lysis as measured by release of  $^{51}\text{Cr}$  from labeled target cells.

significant difference was observed between the placebo control group and the antibody treatment groups. Tumor growth continued unabated by the administration of antibody, and animals were culled due to large tumor burdens 35 days from commencement of treatment.

**Prevention of MCF-7 Tumor Growth in Mice.** In this *in vivo* study, four groups of five mice received the first dose of antibody treatment on day 0, at the same time as inoculation of tumor cells. Tumor volume measurements from day 0 of this study are presented in Fig. 7. The concurrent administration of 1 mg of hu3S193 *i.v.* and *s.c.* tumor cell inoculation with four subsequent antibody doses at 48-h intervals was able to significantly slow tumor growth. Statistical analysis was performed with Student's paired *t* test. No difference was observed among 0.1 mg of hu3S193, control huA33 treatment, and placebo treatment. Significant inhibition of tumor growth was observed with treatment with 1 mg of hu3S193 *versus* 1 mg of huA33 at day 56 ( $P = 0.044$ ). Treatment with 1 mg of hu3S193 *versus* placebo showed significant reduction in tumor growth at day 46 ( $P = 0.042$ ), day 50 ( $P = 0.021$ ), and day 56 ( $P = 0.044$ ).

## DISCUSSION

Despite advances in hormonal treatment and chemotherapy in the last 10 years, survival for patients with advanced solid epithelial tumors has not improved significantly, and novel treatment strategies are required. The development of immunological therapies, including monoclonal antibodies that target specific antigens overexpressed on

cancer cells, is a promising area of cancer therapy research. Our research programs have focused on the identification of tumor antigens that are suitable for this approach. We have previously generated a murine anti-Le<sup>y</sup> mAb 3S193 and detailed specificity, affinity, and

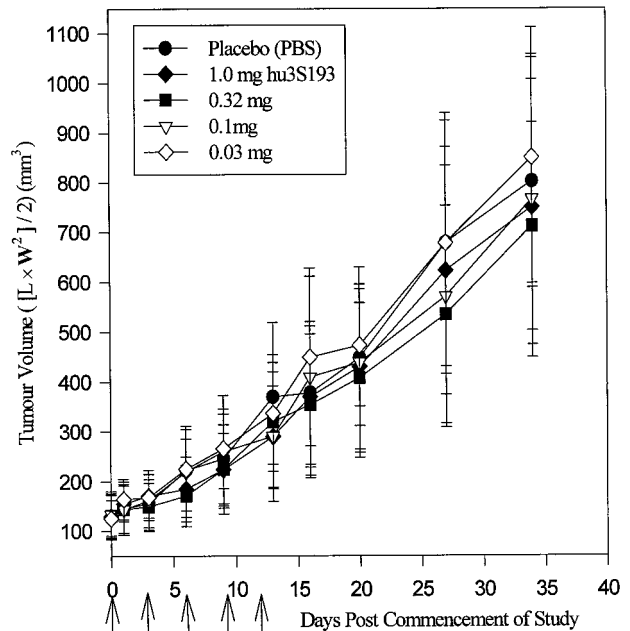


Fig. 6. Therapeutic studies. Mice bearing established MCF-7 xenograft tumors received five doses of hu3S193 at 72-h intervals as indicated by  $\uparrow$ . Day 0 refers to day of first antibody dose. Tumor growth results for the five treatments are presented for the study period: PBS placebo control (●) and four escalating dose levels of hu3S193, 0.03 mg (◇), 0.1 mg (▽), 0.32 mg (■), and 1.0 mg (◆).

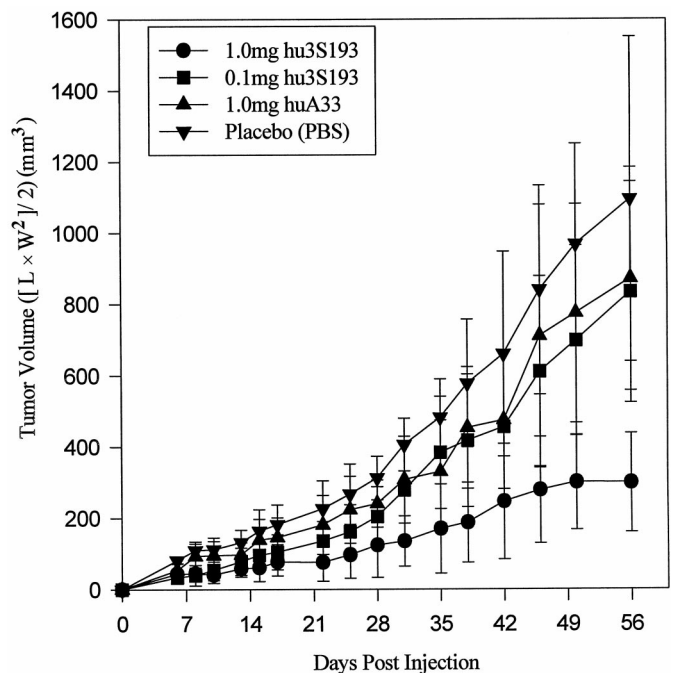


Fig. 7. Prevention studies: dose escalation of hu3S193 in nude mice to prevent establishment of MCF-7 xenografts. Groups of five mice concurrently received the first unlabeled antibody treatment retro-orbitally with the inoculation of  $20 \times 10^6$  MCF-7 cells and the insertion of an estrogen pellet. Treatment with a further five antibody doses administered at 48-h intervals followed. Tumor volumes were recorded three times per week until day 56. Results from hu3S193 dose levels of 0.1 mg (■) and 1.0 mg (●) and controls of unrelated isotype-matched huA33 (▲) and no antibody, PBS placebo (▼) are presented.

cytotoxicity analyses (6). Here we describe the development of a humanized 3S193 antibody for cancer immunotherapy.

The advent of DNA recombinant technology has allowed the synthesis of “reshaped” antibodies. Humanized antibodies combine murine antigen binding (CDR) with human V-region framework determinants. CDR grafting is one method to include human framework regions and aims to make the surface of the antibody seem as fully human as possible but retain the murine antigen binding packaging and interface reactions determining high-affinity binding (18). Although other methods of humanization of murine antibodies have been developed, including veneering of potential B- and T-cell binding sites, and the production of “human” antibodies through recombinant and transgenic techniques (19), there are limited data available on the *in vivo* behavior of these constructs in human studies.

The humanized CDR-grafted construct of 3S193 (hu3S193) was optimized for antigen binding specificity and immune effector function properties. Subsequently the NS0 transfectoma was cloned for stability, high antibody productivity, and clonality.

The retention of antigen binding affinity after humanization is essential to allow optimal binding to tumor *in vivo*. The humanization of 3S193 involved careful analysis of a number of candidate constructs for affinity and specificity for Le<sup>y</sup> in ELISA tests with synthetic Le<sup>y</sup> and Le<sup>y</sup>-containing glycoproteins and glycolipids. The huVHT/huVKF construct (clone 11), containing the murine Thr-24 heavy-chain residue, although not demonstrating as high affinity to Le<sup>y</sup> as other constructs, retained the best specificity with minimal cross-reactivity to other related antigen structures, including Le<sup>x</sup> and H type 2 antigens. Our detailed specificity analysis has been reported previously (6). The minor differences in affinity between constructs, compared with the importance of specificity for Le<sup>y</sup> antigen *in vivo*, resulted in the huVHT/huVKF clone being chosen for subsequent development.

Of particular importance in the humanization strategy was that hu3S193 (IgG1) displays enhanced immune effector function, particularly in mediating cellular cytotoxicity (ADCC), compared with the murine form. Co *et al.* (20) compared murine, chimeric, and humanized versions of the anti-Lewis Y antibody ABL 364 and showed that the humanized antibody had an ADCC (IC<sub>50</sub>, 10 μg/ml on MCF-7 cells) that was superior to the murine form but less effective CDC activity. Pharmacokinetics of the humanized form was prolonged compared with the murine form. Although a direct comparison was not made, hu3S193 is one of the most cytotoxic antibodies yet reported in terms of IC<sub>50</sub> for both CDC (ED<sub>50</sub>, 1 μg/ml), and ADCC (IC<sub>50</sub>, 0.32 μg/ml). This has immediate relevance to its potential for passive immunotherapy of Le<sup>y</sup>-expressing tumors.

The production of hu3S193 under small- and large-scale GMP conditions was achieved without loss of affinity and retention of biological function. The scale up of production under serum-free conditions is often problematic; however, antibody production yields, stability, and consistency of production were attained during GMP bioreactor runs of up to 55 days. Bioreactor production of antibody expressed in mammalian cells under large-scale conditions is well suited to batch runs in the range of 10–100 g, has the advantage of reduced infrastructure requirements of fermenters, and has been used for antibodies approved for clinical use (21). The hu3S193 produced under large-scale conditions was determined to be homogeneous by column and SDS-PAGE analyses. Furthermore, the final product safety tests demonstrated appropriate clearance of DNA, endotoxin, and protein A and sterility. The ability to scale up production of hu3S193 with demonstration of equivalence in affinity and potent immune effector function will allow clinical evaluation of hu3S193 to be optimally performed.

As part of the preclinical development of the hu3S193, *in vitro*

investigations were performed to assess the affinity of the antibody for its antigen. Excellent correlation was found between the apparent binding affinities calculated from the MCF-7 cell-based scatchard analysis and BIAcore analyses of hu3S193 apparent affinity for its Le<sup>y</sup> antigen ( $K_a$ ,  $5.33 \times 10^6$  for cell binding assay compared with BIAcore  $K_A$  of  $4.19 \times 10^6/M$ ). A reduction in avidity was observed with humanization of 3S193; however, this was found to be primarily a result of an increased off rate compared with murine 3S193. The significance of this high off rate *in vivo* is uncertain, because the concentration of antibody surrounding tumor, combined with the high number of binding sites available per cell, would contribute to high antibody localization to tumor cells, as we have demonstrated in an MCF-7 xenograft BALB/c model.<sup>3</sup>

A disparity was observed between the Biosensor calculated avidity of the mu3S193 and hu3S193 and that determined by the cell binding analysis, in which only a 2–3-fold reduction in avidity was apparent after humanization. The differences in apparent binding of mu3S193 may be attributable to the murine antibody having been raised against cultured human tumor lines expressing Le<sup>y</sup> on the cell surface, whereas the Biosensor binding surface was a synthetic Le<sup>y</sup> tetrasaccharide coupled to BSA in a multivalent configuration. The humanization of mu3s193 involved framework alterations, which may also account for the higher BIAcore off rate compared with the murine, without affecting specificity. The exact conformation of the synthetic antigen is unknown, whereas the cell-bound antigen is probably in its native conformation. However, it is not uncommon to observe variations in apparent avidity when using different techniques. This has been noted by ourselves in other antigen systems (22) and by others with the Le<sup>y</sup> antigen system (23).

An *in vivo* therapeutic effect was not observed after i.v. administration of 0.3–1.0 mg of naked hu3S193 to mice with established Le<sup>y</sup>-positive xenografts. The lack of effect is not due to poor tumor localization, because our previous studies have shown excellent tumor targeting of <sup>125</sup>I- and <sup>90</sup>Y-labeled hu3S913 in this model.<sup>3</sup> These experiments determined the mean half-life of hu3S193 to be 43.3 h in this murine model, suggesting that the 72-h dosing schedule was appropriate. The inability of the humanized antibody to have any antitumor effect in this murine model may be due to restricted murine serum complement activity. The potent *in vitro* ADCC of the hu3S193 would suggest that, in conjunction with potent CDC activity in the human, these humoral effects in a clinical setting would augment the therapeutic effects of hu3S193.

In the preventive tumor model, however, significant reduction in tumor growth was observed with the 1-mg hu3S193 dose level. The dosage schedule was selected on the basis of published literature and the known mean half-life of hu3S193 in an MCF-7 BALB/c model.<sup>3</sup> Animals receiving placebo exhibited rapid, unchecked growth of xenograft. Control isotype matched antibody at 1.0-mg doses displayed similar growth curves to 0.1-mg doses of hu3S193. The experiment was not extended past 56 days because of the size of tumors in animals not receiving 1-mg hu3S193 treatment and the fact that the estrogen implants were only able to release estrogen for 60 days, after which the model becomes less reliable. The data suggest that the differences in tumor growth would have become more marked if further observation had been possible. These results demonstrate the potential efficacy of hu3S193 in patients with small-volume disease. The differences between mouse and human models of cancer are well known, but the potent complement-mediated activity of hu3S193, in conjunction with other Fc effector mechanisms (including ADCC), does provide evidence of an attractive therapeutic mechanism of tumor killing.

The high frequency of Le<sup>y</sup>-expressing tumors, the high density and altered expression of Le<sup>y</sup> on the surface of tumor cells, and the

relatively homogenous expression in primary and metastatic lesions have led to its selection as an antigenic target for cancer immunotherapy (2, 5, 6, 8, 9). The expression of Le<sup>y</sup> antigen in normal tissues (including the gastrointestinal tract) is of concern in targeting strategies against this antigen; however, the accessibility of normal tissues to anti-Le<sup>y</sup> antibodies remains unclear. Results of studies with murine and chimeric antibodies against Le<sup>y</sup> suggest gastric toxicity may be relevant in proposed clinical trials; however, significant toxicity has only been observed at protein doses of >200 mg (14). The optimal protein dose for effective targeting of Le<sup>y</sup> tumors (tumor:blood ratios and normal tissue uptake) is yet to be defined with careful biodistribution and biopsy-based trials. In this context, high protein doses of anti-Le<sup>y</sup> antibodies reported to cause toxicities may not be required for effective tumor targeting.

The introduction of humanized antibodies into the clinic allows the immunogenicity of these constructs to be determined. The recombinant human mAb HER2 has had minimal HAHA responses seen in hundreds of patients entered into clinical trials to date, including patients receiving weekly infusions for >1 year (24, 25). Other humanized antibodies, including huCTMO1 and huM195, have been administered to patients with minimal toxicity and no evidence of HAHA (26, 27). In comparison, humanized antibody A33, administered to patients with advanced colorectal carcinoma, has been shown to induce HAHA in a subset of patients after prolonged treatment (28). Thus, although there has been a consistent observation of markedly reduced incidence of immune responses to humanized antibodies in patients, the humanization process does not totally remove the potential for HAHA. The development of a humanized form of 3S193 has been undertaken to minimize the risk of immune recognition and to allow repeated infusions over time. The potent immune effector functions of hu3S193 *in vitro* and *in vivo*, combined with its high specificity for Le<sup>y</sup> antigen, and the humanization strategy used in production suggest that hu3S193 has promise in the treatment of Le<sup>y</sup>-expressing tumors in cancer patients.

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