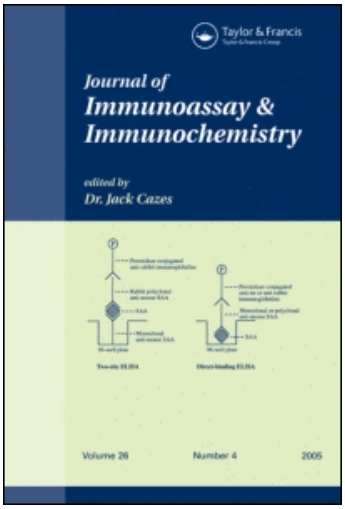

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AN ELISA FOR QUANTIFICATION OF T84.66, A MONOCLONAL ANTI-CEA ANTIBODY, IN MOUSE PLASMA

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□ *T84.66 is a monoclonal antibody with high affinity and specificity for tumor-associated carcinoembryonic antigen (CEA). In this work, we have developed an enzyme linked immunosorbent assay to determine T84.66 concentrations in mouse plasma. The assay was validated with respect to precision and accuracy by evaluating the recovery of T84.66 from mouse plasma. The working range of the assay is 25–200 ng/mL, and the limit of quantification is 2.5 μg/mL. Intra-assay recoveries ranged from 90.6 to 97.4%, and intra-assay precision reported as the percent coefficient of variation (CV%), ranged from 4.58 to 12.6%. Inter-assay recoveries were between 92.6 to 98.1% and the CV% ranged from 4.9–6.5%. The assay was tested for possible interference from soluble CEA. Soluble CEA, at concentrations up to 5 ng/mL, did not influence the recovery of T84.66. The assay was applied to study the pharmacokinetics of T84.66 in athymic Fox^{nu} mice.*

Keywords athymic mice, carcinoembryonic antigen (CEA), enzyme immunoassay, T84.66 anti-CEA antibody

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly occurring cancer in the world. Statistics from the American Cancer Society estimate that there will be nearly 150,000 new cases of colorectal cancer and about 49,000 deaths in 2008.[1] Chemotherapeutic agents such as 5-flurouracil, irinotecan, and capecitabine have been used either alone or in combination to treat this disease. The response rates to these agents have been modest, and the chemotherapeutics are associated with severe side-effects. Newly approved antibody-based therapies such as bevacizumab and cetuximab target specific receptors, namely VEGF (vascular endothelial growth

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factor) and EGFR (epidermal growth factor receptor), and these targeted therapies have demonstrated significant increases in overall survival time when combined with chemotherapy, with very modest untoward effects. These promising results have encouraged further research in the field of targeted cancer therapy.

Carcinoembryonic antigen (CEA) is an attractive target for antibody-based treatments and tumor imaging since it shows limited expression in healthy tissues. CEA expression is increased by several-folds in tumors arising in the gastrointestinal tract, especially in colorectal cancer.[2] T84.66, a mouse monoclonal IgG directed against human CEA, was first produced and characterized by Wagener and coworkers in 1983.[3,4] It is known to bind to CEA with very high affinity ($Ka = 2.6 \times 10^{10}\,L/M$) and specificity, and does not bind to other members of the CEA family, including BGP (biliary glycoprotein) and NCA (nonspecific cross reacting antigen).[5,6]

This laboratory is interested in utilizing T84.66 in an antibody-mediated drug delivery approach to selectively target CEA-expressing adenocarcinomas. Athymic Fox^{nu} mice are generally used as preclinical models to establish xenografts. Understanding the disposition of T84.66 in athymic mice in the presence and absence of tumors is essential for evaluating our targeting strategy.

Radiolabeled anti-CEA antibodies have been extensively used for tumor imaging[7,8] and targeting.[9] However, use of an ELISA assay can enable determination of anti-CEA levels over an extended period with fewer safety concerns, and with avoidance of several other problems associated with use of radiolabeled antibodies (e.g., poor stability of the radiolabel tag, altered antibody binding and disposition with radiolabeling, and difficulties in distinguishing metabolites and intact antibody).

Some groups have reported using ELISA assays to detect the presence of anti-CEA antibodies following immunization with CEA,[10] and others have used ELISA to investigate the anti-CEA auto-antibodies in cancer patients.[11] However, to the authors' knowledge, this work is the first report of a validated ELISA to detect and quantify concentrations of a therapeutic anti-CEA antibody. The assay was successfully applied to study the plasma disposition of T84.66 in control and in athymic Fox^{nu} mice bearing CEA-expressing xenografts.

EXPERIMENTAL

Production and Purification of Anti-CEA Antibody

Hybridoma cells producing the anti-CEA antibody, T84.66, were purchased from the American Type Culture Collection (ATCC # HB-8747,

Manassas, VA). To allow large-scale antibody production, hybridoma cells were grown in 1 L spinner flasks containing serum free media (Hybridoma SFM, Invitrogen, Grand Island, NY). Anti-CEA antibody was purified from culture supernatant by Protein-G chromatography (Amersham Biosciences, Uppsala Sweden) using a Bio-Rad medium-pressure chromatography system (Bio-Rad Laboratories, CA). 20 mM Na_2HPO_4 (pH 7.0, Sigma Chemical) was used as the loading buffer, and the elution buffer was 100 mM glycine (pH 2.8, Sigma Chemical). Eluted antibody was collected in glass tubes containing 1 M Tris buffer (pH 9.0) to neutralize the solution and minimize antibody aggregation.

ELISA Procedure

T84.66 standards were prepared by diluting a stock solution to appropriate concentrations (0, 25, 50, 100, 150, and 200 ng/mL) with phosphate buffered saline (pH 7.4) and mouse plasma (1% v/v). Validation of assay precision and accuracy was performed by determining the recovery of quality control samples (QCs) of T84.66 in mouse plasma, prepared at low, mid, and high concentrations, with respect to the standard curve (25, 100, and 175 ng/mL). Standards were prepared immediately prior to use in the assay, and QCs were prepared in bulk, aliquoted, and stored at 4°C. Standards and QCs were run on each microplate, with samples, and unique standard curves were generated for each microplate.

Recombinant carcinoembryonic antigen (rCEA, Protein Sciences Corporation, CT) was diluted in 20 mM Na_2HPO_4 to a final concentration of 400 ng/mL. Nunc Maxisorp 96 well plates (Nunc model # 62409-002, VWR, Bridgeport, NJ) were incubated over night at 4°C with rCEA (250 µL/well). The plates were washed thrice with PB-Tween (0.05% Tween in 0.02 M Na_2HPO_4, no pH adjustment), followed by two washes with double distilled water. Plates were then incubated with standards and samples in triplicate (200 µL) for 2 h at room temperature. At the end of incubation, the plates were washed as described before. Next, the plates were incubated with 100 µL of goat anti-mouse-Fab alkaline phosphatase conjugate (Sigma, Cat #A1682, St. Louis, MO) for 1 h at room temperature (1:500 dilution of the conjugate with PB-Tween with 30% BSA). The plates were then washed and rinsed, and p-nitro phenyl phosphate solution (Pierce, Rockford, IL, 4 mg/mL in diethanolamine buffer, pH 9.8) was added to each well (250 µL/well). The change in absorbance at 405 nm with time (dA/dt) was monitored with a plate reader (Spectra Max 250, Molecular Devices, Sunnyvale, CA), and standard curves were obtained by fitting the dA/dt vs. T84.66 concentration using the linear equation: $y = mx + c$.

Influence of Soluble Carcinoembryonic Antigen on Assay Recovery

Additional quality control samples in mouse plasma, with T84.66 at concentrations of 25, 100, and 175 ng/mL, were prepared with soluble rCEA (5 ng/mL). The accuracy and precision of T84.66 recovery was then determined, based on the standard curve.

Xenograft Model

The human colorectal cancer cell line LS174 T (ATCC# CL-188, Manassas, VA), known to express large amounts of carcinoembryonic antigen, was used to develop xenografts in athymic Fox^{nu} mice (20–25 g, 5–6 weeks old, Harlan, Indianapolis, IN). Mice were housed in a sterile room and were handled under aseptic conditions. 100 µL of LS174 T cells ($\sim 5 \times 10^6$ cells) were injected subcutaneously into the right flank of the mice. The mice were examined for tumor growth and their body weight was monitored regularly. The tumor size was measured by use of vernier calipers, and tumor volume (mm^3) was calculated by the formula, volume $= (a^2 \text{ x } b)/2$. In this equation, a represents the width and b represents the length of the tumor. All animal experiments were approved by the Institutional Animal Use and Care Committee of the University at Buffalo.

Examining the Presence of Shed CEA

A pilot study was performed to assay for circulating CEA in plasma samples obtained from mice bearing LS174 T xenografts. Briefly, 100 µL of LS174 T cells ($\sim 5 \times 10^6$ cells) were injected s.c into the right flank of mice (n = 5). Blood samples were collected from retro-orbital plexus using Unopette® capillary tubes (100 µL) pre-rinsed with EDTA. Samples were collected every third day, starting from 1 day prior to tumor cell inoculation and up to the point when the tumor volumes reached 2000 mm^3. Blood samples were centrifuged at 13,000 rpm for 3 min. The plasma fraction was separated and stored at −20°C until analyzed by ELISA for CEA. The ELISA for CEA, which was developed in this laboratory, has a limit of quantification of 2 ng/mL in mouse plasma; validation work has demonstrated intra-assay recovery and inter-assay recovery in the range of 93–105%, with CV% ranging from 2.7–12.8% (accepted for publication in The Journal of Immunoassay and Immunochemistry).

Pharmacokinetic Study

T84.66, 25 mg/kg, was administered intravenously through the penile vein to control and tumor-bearing athymic Fox^{nu} mice (tumor volume

$\sim$600 mm^3, n = 3/group). Blood samples (25 µL) were collected from the retro-orbital plexus using calibrated capillary pipettes (Drummond Scientific Company, Cat # 2–000-020) pre-rinsed with EDTA at 1 h, 3 h, 8 h, 1d, 2d, 4d, 7d, 12d, 21d, and 35 days for control athymic mice and at 1 h, 3 h, 8 h, 1d, 2d, 4d, 7d, and 12 days for xenograft bearing mice The blood was centrifuged at 13,000 rpm for 3 min to obtain plasma. Plasma samples were stored at −20°C until analyzed by ELISA. Pharmacokinetic parameters were estimated by non-compartmental pharmacokinetic analysis using WinNonlin, version 5.0 (Pharsight Corporation, Palo Alto, CA).

RESULTS AND DISCUSSION

Assay Validation

The calibration curve was linear over the standard curve range and correlation coefficients typically greater than 0.997 were obtained on fitting to the equation: y = mx+c (Figure 1). Inter-assay and intra-assay precision and accuracy parameters are listed in Table 1. The intra-assay recovery was in the range of 90.6–97.4% and the CV% ranged from 4.58–12.6%. Inter-assay recoveries were in the range of 92.6–98.1% and the percent coefficient of variation ranged from 4.90–6.5%. The working range of the assay was 25–200 ng/mL, which corresponds to a limit of quantitation of

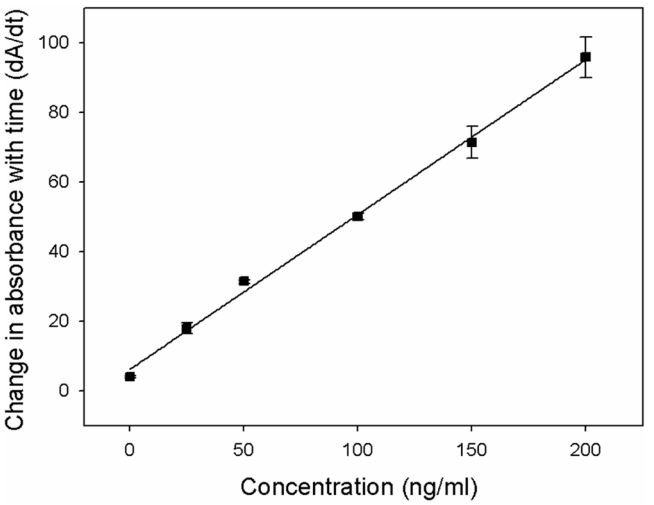

FIGURE 1 Representative standard curve for T84.66, anti-CEA IgG over the range of 25–200 ng/mL. The curve is fitted with a cubic equation, and $r^2 = 0.997$. Error bars represent the standard deviation across the mean of 3 replicates.

TABLE 1 Intra-assay (Within Plate) and Inter-Assay (Between Plates) Precision and Accuracy for T84.66, Anti-CEA IgG Quality Control Samples in Mouse Pasma

Actual Concentration (ng/mL)	Recovered Concentration (ng/mL)	Recovery (%)	CV (%)
Intra-assay Variability (n = 4)			
25	23.9	95.5	12
100	97.4	97.4	5.3
175	159	90.6	4.5
Inter-assay Variability (n = 3)			
25	23.8	95.1	6.5
100	98.1	98.1	4.9
175	162	92.6	6.1

*CV-Coefficient of variation.

2.5 μg/mL (i.e., since the samples are diluted 1:100 to contain a final concentration of 1% mouse plasma).

Influence of Soluble Carcinoembryonic Antigen (CEA) on Recovery of T84.66

Small amounts of spiked rCEA (5 ng/mL) did not influence the recovery of the quality control samples prepared at the low, medium, and high end of the standard curve. Results are reported in Table 2.

Shed Carcinoembryonic Antigen (CEA)

Circulating CEA may have any impact on the recovery of anti-CEA IgG from mouse plasma and, hence, it essential to detect the presence of any antigen shed from the xenograft. Plasma samples obtained from non-tumor bearing Fox^{nu} mice did not contain detectable concentrations of CEA. Plasma samples obtained from the xenograft-bearing mice demonstrated very low levels of circulating CEA (<3 ng/mL in all mice). Based on

TABLE 2 Influence of Spiked rCEA (5 ng/mL) on Recovery of T84.66, anti-CEA IgG from Mouse Plasma Samples (n = 3)

Actual Concentration (ng/mL)	Recovered Concentration (ng/mL)	Recovery (%)	CV (%)
25	21.6	86.5	11.4
100	99.3	99.3	3.90
175	157	89.9	12.5

*CV-Coefficient of variation.

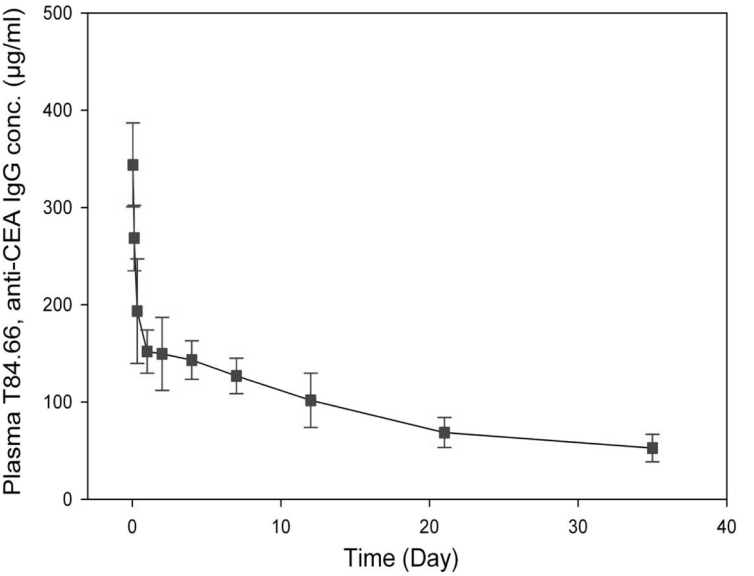

FIGURE 2 Pharmacokinetics of T84.66, monoclonal anti-CEA IgG following a 25 mg/kg dose in control athymic Fox^{nu} mice (no xenograft). Each point represents average plasma concentration obtained from 3 mice. Error bars denote standard deviation about the mean.

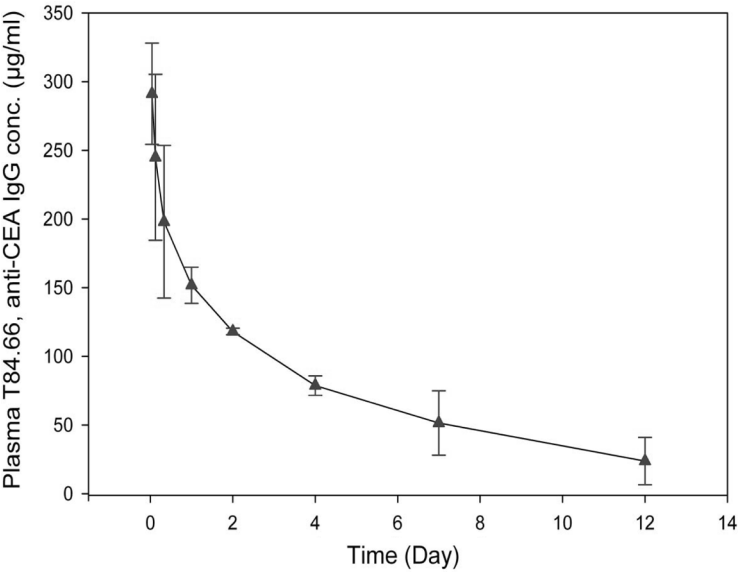

FIGURE 3 Pharmacokinetics of T84.66, monoclonal anti-CEA IgG following a 25 mg/kg dose in athymic Fox^{nu} mice bearing CEA expressing LS174T xenograft. Each point represents average plasma concentration obtained from 3 mice. Error bars denote standard deviation about the mean.

TABLE 3 Pharmacokinetic Parameters for anti-CEA Antibody, T84.66 Following Administration of 25 mg/kg dose to Control and Xenograft Bearing Athymic Fox^{nu} Mice

Parameter*	Control	Tumor Positive
Vss (mL/kg)	153 ± 31	146 ± 44
CL (mL/day/kg)	5.548 ± 0.55	23.3 ± 7.9
Terminal half-life (day)	19.8 ± 4.4	5.99 ± 4.0

*Vss refers to the volume of distribution at steady state.
CL refers to the systemic clearance.

these observations and the performance of our assay in the presence of low amounts of soluble recombinant CEA, we are confident that shed CEA will not interfere with our ability to investigate the pharmacokinetics of T84.66 using the LS174 T tumor model.

Pharmacokinetics of T84.66, Monoclonal anti-CEA IgG

The concentration vs. time profile following intravenous administration of T84.66 in control and xenograft bearing Fox^{nu} mice is shown in Figures 2 and 3, respectively. CL, Vss, and terminal half-life parameters obtained by non-compartmental analysis are reported in Table 3. The pharmacokinetic parameter values in control mice are similar to that of other murine monoclonal antibodies in mice.[12]

CONCLUSIONS

The sensitive ELISA method described in this report permits quick, accurate, and precise quantification of monoclonal anti-CEA IgG in mouse plasma. Previous reports have described ELISA methods to detect endogenous anti-CEA IgG or IgM antibodies in human serum; however, to the authors' knowledge, this is the first report of a validated ELISA method for quantification of an exogenous, monoclonal anti-CEA IgG in mouse plasma. The assay has been successfully applied to investigate T84.66 pharmacokinetics in mice, and the assay will be used to guide the development of novel targeting strategies for treatment of colorectal cancers.

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