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Evaluation of human carcinoembryonic-antigen (CEA)-transduced and non-transduced murine tumors as potential targets for anti-CEA therapies

Patricia Horan Hand, Paul F. Robbins, Michael L. Salgaller, Diane J. Poole, Jeffrey Schlom

Laboratory of Tumor Immunology and Biology, National Cancer Institute, The National Institutes of Health, Bethesda, MD 20892, USA

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Abstract. The MC-38 C57BL/6 mouse colon adenocarcinoma cell line has been transduced with a retroviral construct containing cDNA encoding the human carcinoembryonic antigen (CEA) gene [Robbins PF, Kantor JA, Salgaller M, Horan Hand P, Fernsten PD, Schlom J (1991) Cancer Res 51: 3657]. Two clones, MC-38-cea1 and MC-38-cea2, expressed high levels of CEA on their cell surface. A third CEA-expressing cell line, MCA-102-cea3, was similarly derived by transduction of the MCA-102 C57BL/6 mouse fibrosarcoma cell line and is described here. In this study, the three CEA-transduced murine tumor cell lines (MC-38-cea1, MC-38-cea2, MCA-102-cea3) were evaluated for their tumorigenic potential, as well as their ability to serve as in vivo model systems for active and passive immunotherapy studies. Parameters that were investigated include tumor growth rate, the antibody response of the host to CEA, and the CEA content of the tumors. The MC-38-cea2 model appeared to be the most appropriate for immunotherapy studies. Biodistribution studies, using an ^{125}I-labeled anti-CEA mAb, demonstrated efficient tumor targeting of MC-38-cea2 tumors in C57BL/6 and athymic mice.

Key words: Carcinoembryonic antigen – Immunotherapy – CEA-transduced tumor cells

Introduction

Several human tumor-associated antigens (TAA) have been identified and represent potential targets for active immunotherapy. To date, human TAA such as carcinoembryonic antigen (CEA) [9], TAG-72 [13], 19-9 [16], DF-3 [17] and OC-125 [2], or close analogues of these antigens, have not been detected in murine tumors. Since human tumors can not grow in immunologically intact mice, it has been difficult to assess the ability of human TAA to serve as targets for active immunotherapy in an animal model. Recently, however, the genes encoding a number of TAA have been cloned [3, 26, 31]. This has opened up the possibility to carrying out immunotherapy studies in animal model systems using recombinant gene products.

One requirement for the study of active immunotherapy is the development of an in vivo model in an immunologically intact animal. Such a model system would also be advantageous for the study of passive immunotherapy, i.e., mAb-directed antigen-specific therapy. One approach to active immunotherapy is the use of a recombinant vaccinia virus. Estin et al. [7] have reported active immunotherapy studies directed against the p97 human melanoma antigen. Immunization with a p97 vaccinia virus recombinant was found to protect mice from challenge with syngeneic K1735 mouse melanoma cells transfected with the human p97 gene [7].

Another potentially useful target for therapy is CEA. Expression of CEA has been found in more than 90% of all colon tumors, as well as in significant numbers of pancreatic, gastric, breast, and lung tumors [27]. It has been postulated that CEA is a homoadhesion molecule, and that the expression of CEA may lead to a more malignant cell population [4, 12].

The recent cloning of CEA has shown that it is a member of a multi-gene family which is contained within the immunoglobulin supergene family [23, 32]. The most closely related members of the CEA gene family, non-specific cross-reactive antigen [30] and biliary glycoprotein [1], are widely expressed in normal tissues, as well as in many gastrointestinal, mammary, and lung tumors.

Recently, a CEA–vaccinia-virus recombinant (rV-CEA) has been developed and shown to elicit an immune response in mice [15]. In a separate study, the MC-38 C57BL/6 mouse colon adenocarcinoma cell line has been transduced with a retroviral construct containing cDNA encoding the human CEA gene [25]. Two clones, MC-38-

Correspondence to: P. Horan Hand, Laboratory of Tumor Immunology and Biology, Building 10, Room 8B07, 9000 Rockville Pike, Bethesda, MD 20892, USA

cea1 and MC-38-cea2, expressed levels of CEA on their cell surface comparable to the high CEA levels found on the GEO and CBS human colon carcinoma cell lines [25]. The CEA expressed by the MC-38-cea1 clone had a molecular mass of 180 kDa, similar to that of native CEA. In contrast, a deletion of two of the three repeated CEA domains occurred in the MC-38-cea2 clone, resulting in expression of a 70-kDa CEA gene product [25]. The CEA expressed by the MC-38-cea2 clone contained antigenic determinants detected by eight distinct anti-CEA mAbs [25]. Recent studies using the MC-38-cea2 cell line and the non-transduced MC-38 cell line demonstrated the ability of the rV-CEA construct to inhibit the growth of established MC-38-cea2 tumors and to prevent the transplantation of MC-38-cea2 tumors following immunization with rV-CEA [14]. Subsequently we have developed and report here a third CEA-transduced cell line, MCA-102-cea3, derived from transduction of the MCA-102 C57BL/6 mouse fibrosarcoma cell line with CEA.

The CEA-transduced (MC-38-cea1, MC-38-cea2, MCA-102-cea3) and non-transduced (MC-38, MCA-102) cell lines thus provide matched sets of cells to define the specificity of responses to active immunotherapy and the ability of mAb to target tumors. Furthermore, using immunologically intact C57BL/6 mice as an animal model presents a more cost-effective option than the widely used athymic (nu/nu) mice.

In this report, we have analyzed the biological properties of the CEA-transduced cell lines in immunologically intact C57BL/6 mice, athymic mice and irradiated C57BL/6 mice. Parameters that were investigated include tumor growth rate and espression of CEA in tumors, as well as the anti-CEA antibody response of the host. Furthermore, the ability of a radiolabeled anti-CEA mAb to target CEA-transduced tumor cells in vivo was investigated.

Materials and methods

Cell lines. The MC-38 B/6 mouse colonic adenocarcinoma cell line [8] and MCA-102 C57BL/6 fibrosarcoma cell line were obtained from Dr. Bernard Fox in the laboratory of Dr. Steven Rosenberg (National Cancer Institute, NIH, Bethesda, Md.). These cell lines were established by continuous in vitro passage of MC-38 and MCA-102 tumor cells [18]. MC-38-cea1 and MC-38-cea2 were developed by transduction of the MC-38 B/6 cell line with a retroviral construct containing cDNA encoding the human CEA gene, as described previously [25]. The LS-174T human colon adenocarcinoma cell line [29] was obtained from the American Type Culture Collection, Rockville, Md. The WiDr human colon adenocarcinoma cell line [22] was provided by Dr. P. Noguchi (Center for Biologics Evaluation and Research, NIH, Bethesda, Md.). The A375 melanoma cell line was received from Dr. S. Aaronson, National Cancer Institute, NIH, Bethesda, Md. The ecotropic retroviral packaging cell line, ψ2 [20], was obtained from Dr. R. Bassin, National Cancer Institute, NIH, Bethesda, Md. All cell lines were maintained in the growth medium recommended by their respective source.

Transduction of MCA-102 with CEA. The ψ2 ecotropic retroviral packaging cell line was transfected with a vector containing a CEA cDNA clone, and a clone expressing CEA was isolated as described previously [25]. Supernatant obtained from the ψ2 clone was used to transduce MCA-102 cells, and the transduced cells were cloned by limiting dilution. Isolated clones were examined for expression of cell-surface CEA by flow cytometry (FACScan Becton-Dickinson, Mountain View, Calif.), as previously described [25]. A clone (MCA-102-cea3) expressing high levels of cell-surface CEA was selected for use in the studies reported here. The M_r of the product expressed in this clone was close to that of native CEA.

In vitro culture of tumor cells. Tumor cells were isolated from subcutaneous tumors, as described previously [18]. Briefly, tumors were minced into fragments 1–2 mm in size in Hanks balanced salt solution (HBSS). The fragments isolated from one tumor were then incubated in 40 ml HBSS containing 4 mg type I deoxyribonuclease (Sigma Chemical, St. Louis, Mo.), 40 mg type IV collagenase (Sigma Chemical), and 100 units type V hyaluronidase (Sigma Chemical) for 4 h at room temperature with gentle mixing. Following this incubation, cells were washed three times in HBSS, and transfered to one 75-cm² flask containing 12 ml Dulbecco's modified Eagle's medium with 10% (v/v) fetal bovine serum.

Western blotting. For Western blot analysis, proteins were separated on a 4%–12% gradient sodium dodecyl sulfate/polyacrylamide gel (Novex, Encinido, Calif). Proteins were transferred to a polyvinylidene difluoride membrane (0.45 μm pore size) (Millipore Corporation, Bedford, Mass.) using a semi-dry blotter (Bio-Rad, Richmond, Calif.) in 20% methanol, 0.039 M glycine, 0.048 M TRIS for 1 h at 1 W/30 cm². Following transfer, membranes were incubated for 1 h with mAb COL-1 (1 μg/ml). Bound antibody was detected using ^{125}I-labeled goat anti-(mouse IgG) (Kirkegaard and Perry, Gaithersburg, Md.), as described [21].

Cell-surface expression of CEA. Analysis of CEA expression on the surface of transduced cells was carried out using an immunofluorescence assay, as described previously [10, 25]. The anti-CEA mAb COL-1 (IgG2a) [21] and the negative-control murine myeloma mAb UPC-10 (IgG2a) (Cappel, Organon Teknika Corp., West Chester, Pa.) were used as primary antibodies. Fluorescein-labeled goat anti-mouse IgG + IgM (Kirkegaard and Perry, Gaithersburg, Md.) was used as the secondary antibody. Analysis was performed with a FACScan (Becton-Dickinson, Mountain View, Calif.) equipped with a blue laser excitation of 15 mW at 488 nm.

Tumor growth studies. Female athymic mice (nu/nu) and C57BL/6 mice were obtained from Charles River Inc. (Wilmington, Mass.) at approximately 6 weeks of age. One week later, mice were injected subcutaneously (s.c) in the scapular region with 1×10^6 CEA-transduced or control MC-38 or MCA-102 cells. Selected C57BL/6 mice were exposed to 5 Gy external-beam irradiation using a Gamma Cell-40 (Nordion International Inc., Kanata, Canada) prior to injection of tumor cells. Tumor measurements (mm²) were recorded three times per week and were discontinued at the onset of visual detection of necrosis.

Quantification of CEA. Cell extracts were prepared from finely minced tissues by homogenization for 2–3 min in 10 mM TRIS/HCl (pH 7.2) containing 0.2 mM $CaCl_2$. The homogenate was subjected to pressure homogenization using a cell disruption bomb (Parr Instrument Co., Moline, Ill.) for 5 min at 7 MPa (1000 lb/in²) and then clarified at 1000 *g* for 10 min. The supernatant was sonicated on ice for 1–2 min using 15-s intervals (Branson sonifier, setting 7). The sonicate was centrifuged at 10000 *g* for 10 min, and the supernatant was assayed for CEA using a CEA radioimmunoassay (RIA) kit obtained from Abbott Laboratories Inc. (Chicago, Ill.) or using a CEA enzyme-linked immunosorbent assay (ELISA) kit obtained from Hoffmann-LaRoche (Alameda, Calif.). Protein was determined by the method of Lowry et al. [19].

Immunohistochemical studies. Frozen sections (methanol-fixed) of tumor isografts, allografts and xenografts were assayed for CEA expression using a modification of the method of Thor et al. [28]. Briefly, frozen sections were treated with methanol containing 0.3% H_2O_2 for 10 min at room temperature (to block endogenous peroxidase activity), and then were assayed for CEA expression using the anti-CEA mAb, COL-6 [21]. The mAb COL-6 was conjugated with biotin-*N*-hydroxysuccinimide using the method of Guesdon et al. [11]. After incubation of

Table 1. Characteristics of cell lines used

Parent cell line	Description	Transduced gene product (kDa)	Transduced cell line designation
MC-38	Murine colon carcinoma	180	MC-38-cea1
MC-38	Murine colon carcinoma	70	MC-38-cea2
MCA-102	Murine fibrosarcoma	180	MCA-102-cea3

the sections with 10% normal horse serum, the sections were treated with the biotinylated mAb COL-6 at a concentration of 40 µg/ml (8 µg/section) for 30 min at room temperature. Following a rinse with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), the avidin-dehydrogenase:biotinylated-horseradish-peroxidase-H complex (Vectastain ABC Kit, Vector Laboratories Inc., Burlingame, Calif.) was added (30 min at room temperature). CEA reactivity was visualized using 0.06% diaminobenzidene (Sigma Chemical, St. Louis, Mo.) as chromogen. The sections were then counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and mounted under a coverslip.

ELISA for murine anti-CEA antibody in sera. CEA (50 ng/well) or thyroglobulin (50 ng/well) was used to coat wells of 96-well polyvinyl microtiter plates. The plates were treated with 5% BSA in PBS for 1 h at 37° C, and dilutions of serum samples (50 µl) or mAb COL-1 in PBS containing 1% BSA were added to the wells and incubated for 1 h at 37° C. The wells were then washed with PBS, and peroxidase-labeled goat anti-mouse IgG (Southern Biotechnology, Birmingham, Ala.) was added. The plates were then incubated for 1 h at 37° C. After washing, *o*-phenylenediamine dihydrochloride was added to the wells in the presence of H_2O_2 and plates were read after color development, as described [24].

Radiolabeling of mAb COL-1 with iodine-125. Purified mAb COL-1 was labeled with NA^{125}I using the Iodo-Gen (Pierce Chemical, Rockford, Ill.) method, as previously described [5], resulting in a specific activity of 3.7 µCi/µg. Radiolabeled mAb COL-1 was tested for retention of immunoreactivity using a solid-phase radioimmunoassay (RIA) with protein extracts of human colon carcinoma (LS-174T; CEA-positive) and human melanoma (A375; CEA-negative) cell lines, as previously described [6]. The average immunoreactivity for the radiolabeled mAb versus LS-174T was approximately 28%. Reactivity to A375 was not more than 1%.

Biodistribution studies. Separate groups of athymic mice (nu/nu) and C57BL/6 mice were injected s.c. in the scapular region with 1×10^6 MC-38-cea2 cells, MC-38 cells, or LS-174T cells. Biodistribution studies were performed 6 days later, when the tumors were approximately 0.5 cm in diameter. Tumor-bearing mice (4 per time point) were injected in the tail vein with approximately 2.7 µCi/mouse ^{125}I-COL-1. Mice were killed by exsanguination at 48 h or 96 h after injection of radiolabeled mAb. Blood, tumor and all major organs were collected and wet-weighed with an analytical balance. Radioactivity was measured in a gamma scintillation counter. The percentage of the injected dose per gram (%ID/g) for each organ was determined, and the radiolocalization indices (%ID/g in tumor divided by %ID/g in normal tissue) were calculated.

Statistical analysis. Statistical analysis was done using a two-tailed Student's *t*-test.

Results

In vivo tumor growth characteristics

The three CEA-transduced murine tumor cell lines (MC-38-cea1, MC-38-cea2 and MCA-102-cea3) (Table 1) were evaluated for their potential to serve as in vivo model systems for syngeneic tumors expressing human CEA. Since it was possible that the human CEA gene would be highly immunogenic in mice, one possibility was that the CEA-transduced tumors would not grow following implantation. In the first series of experiments the in vivo growth characteristics of the three CEA-transduced cell lines and their respective non-transduced control cell lines were therefore evaluated. For each cell line, mice were injected s.c. with a dose of 10^6 cells/per animal. Each animal was evaluated for the time of appearance of tumor and the rate of tumor growth.

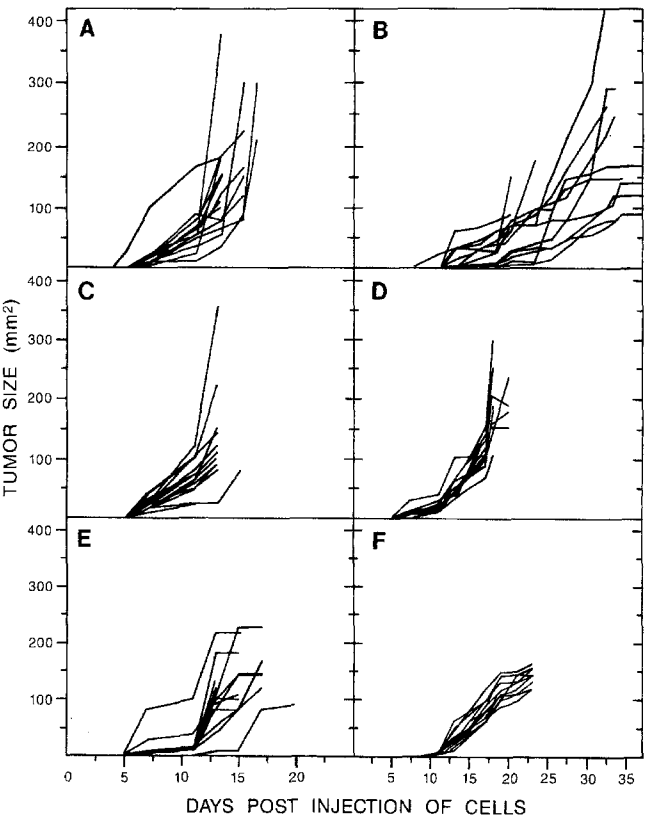

Fig. 1A–F. Growth of MC-38 (non-transduced) and MC-38-cea1 (CEA-transduced) murine adenocarcinoma cells in vivo. MC-38 cells (10^6/mouse) were injected s.c. into 20 C57BL/6 (**A**), 15 athymic (**C**) and 15 irradiated C57BL/6 (**E**) mice. MC-38-cea1 cells (10^6 cells/mouse) were injected s.c. into 20 C57BL/6 (**B**), 15 athymic (**D**) and 15 irradiated C57BL/6 (**F**) mice. Tumor measurements were recorded three times per week

The growth of the non-transduced MC-38 and the CEA-transduced MC-38-cea1 adenocarcinoma cells was initially determined in intact syngeneic C57BL/6 mice. Tumor isografts grew in all mice (20 of 20) that received MC-38 control cells. Tumors were first observed 7 days after the injection of cells for all 20 mice, and, as shown in Fig. 1A, individual tumors grew at approximately the same rate. In contrast, MC-38-cea1 tumors were detected in only 15 of 20 C57BL/6 mice during the 61-day observation period with the time of detection varying from 11 to 34 days after the injection of cells (Fig. 1B, data not shown for tumor detected at 34 days). Furthermore, there was substantial variation in the rate of growth of MC-38-cea1

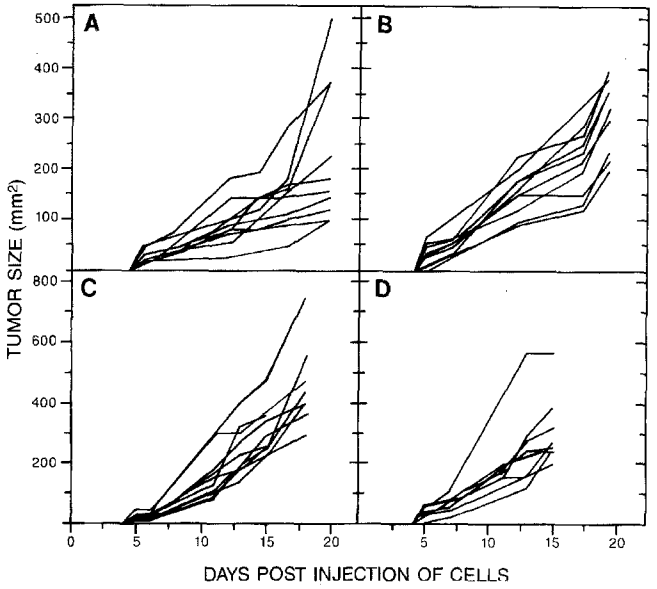

Fig. 2A–D. Growth of MC-38 (non-transduced) and MC-38-cea2 (CEA-transduced) murine adenocarcinoma cells in vivo. C57BL/6 mice were injected with MC-38-cea2 (**A**) or MC-38 (**C**) cells (10^6 cells/mouse). Athymic mice were injected with MC-38-cea2 (**B**) or MC-38 (**D**) cells (10^6 cells/mouse). Tumor measurements were recorded three times per week

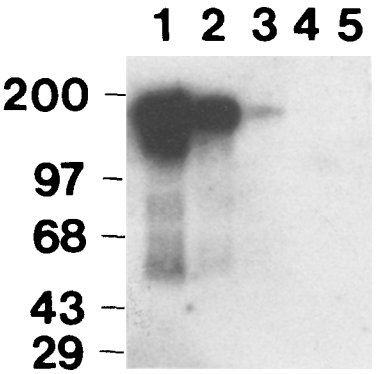

Fig. 3. Western blot analysis of CEA gene products. A Western blot was carried out on protein extracts of MC-38-cea1 (*lane 1*), MCA-102-cea3 (*lane 2*), WiDr (*lane 3*), MC-38 (*lane 4*), and MCA-102 (*lane 5*)

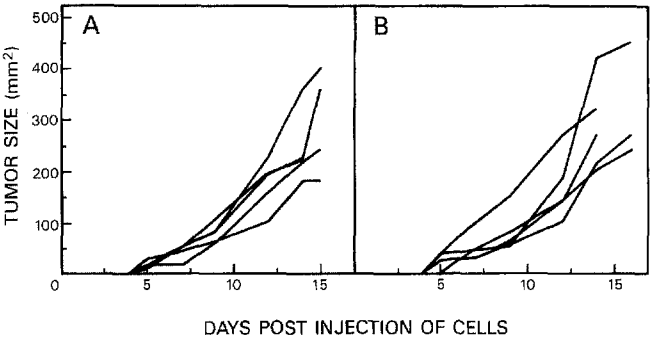

Fig. 4A, B. Growth of MCA-102 non-transduced and MCA-102-cea3 CEA-transduced murine fibrosarcoma cells in vivo. C57BL/6 mice (5/group) were injected s.c. with MCA-102 or MCA-102-cea3 cells (10^6 cells/mouse). Tumor measurements were recorded three times per week

tumors, which appeared to be unrelated to the time of tumor appearance (Fig. 1B).

In an attempt to determine if host factors were responsible for differences in growth rate between the non-transduced and CEA-transduced tumors, MC-38 and MC-38-cea1 cells were then grown in athymic (nu/nu) mice. The growth rate and time of appearance of MC-38 tumors were similar in athymic and C57BL/6 mice (Fig. 1A, C). The growth rate of the MC-38-cea1 cells in athymic mice was similar to that of the MC-38 cells, and distinctly different from the tumor growth characteristics observed in C57BL/6 mice (Fig. 1B, D). Tumors grew in all 15 athymic mice injected with MC-38-cea1 cells, and, in contrast to the variable onset of tumor formation in C57BL/6 mice, all MC-38-cea1 tumors in athymic mice were initially detected 7–11 days after the injection of cells (Fig. 1D).

Experiments were then conducted to determine if sublethal irradiation of C57BL/6 mice would allow MC-38-cea1 tumors to exhibit growth characteristics similar to those observed in athymic mice. C57BL/6 mice were irradiated, as described in Materials and methods, and then injected with MC-38 or MC-38-cea1 cells. As shown in Fig. 1E, MC-38 tumors grew at a similar rate in normal and irradiated C57BL/6 mice (Fig. 1A). Tumors grew in all 15 irradiated mice injected with the non-transduced cells, with initial detection of the vast majority (14 of 15) of tumors at 7 days (Fig. 1E). MC-38-cea1 isografts, which grew in all 15 irradiated mice, had a strikingly similar onset of appearance; 15 of 15 tumors were detected at 11 days (Fig. 1F). The growth rate of the MC-38-cea1 isografts in the irradiated C57BL/6 mice (Fig. 1F) was somewhat slower than that observed in athymic mice (Fig. 1D), but was far more consistent among the individual mice than that observed among non-irradiated C57BL/6 mice (Fig. 1B).

The in vivo growth characteristics of the MC-38-cea2 adenocarcinoma cell line, which expresses the 70-kDa CEA protein product, were then evaluated in C57BL/6 and athymic mice (Fig. 2). Tumors were observed in all mice injected with MC-38 or MC-38-cea2 cells (10 per group). For C57BL/6 mice, the time of tumor onset was identical (5 days) for each mouse injected with either MC-38 or MC-38-cea2 cells. However, the majority of the MC-38-cea2 tumors had a slower growth rate than the control tumors (Fig. 2A, C). In athymic mice, MC-38-cea2 and MC-38 tumors were detected 5–7 days after injection of mice with cells (Fig. 2B, D).

A third CEA-transduced cell line, MCA-102-cea3, was produced by transduction of CEA into the MCA-102 C57BL/6 fibrosarcoma cell line. Western blot analysis was used to determine the size of the CEA gene product expressed by the transduced fibrosarcoma cells. The immunoreactive gene product present in the MCA-102-cea3 cells was approximately 180 kDa (Fig. 3, lane 2), similar to the 180-kDa CEA observed in the WiDr human colon adenocarcinoma cell line (Fig. 3, lane 3).

The non-transduced and CEA-transduced MCA-102 fibrosarcoma cells were injected s.c. into syngeneic C57BL/6 mice (5 mice/group), and the growth characteris-

Table 2. Tumor growth rate[a]

Tumors	Growth rate (days)		
	C57BL/6	Athymic	C57BL/6 (irrad.)[b]
Murine adenocarcinoma[c]			
MC-38	10.9 ± 1.3	10.3 ± 2.0	12.1 ± 2.0
MC-38-cea1	22.3 ± 7.8[e]	13.3 ± 1.8	14.6 ± 0.9
MC-38-cea2	9.3 ± 3.4	6.9 ± 2.0	NT
Murine fibrosarcoma[d]			
MCA-102	7.8 ± 0.4	NT	NT
MCA-102-cea3	7.6 ± 0.9	NT	NT

[a] Mice were injected s.c. with 10^6 cells/mouse. Tumor measurements were recorded three times per week. Values represent the mean time ± SD required after inoculation of cells to achieve a tumor size of 50 mm^2

[b] Mice were exposed to 5 Gy of external-beam irradiation prior to injection of tumor cells

[c] Twenty C57BL/6, 15 athymic and 15 irradiated C57BL/6 mice were injected s.c. with MC-38 or MC-38-cea1 cells. Ten C57BL/6 mice and 10 athymic mice were injected s.c. with MC-38-cea2 cells

[d] Five C57BL/6 mice were injected s.c. with MCA-102 cells, and 5 C57BL/6 mice were injected with MCA-102-cea3 cells

[e] Tumors were observed in 15 of the 20 mice. Value represents mean ± SD of 15 tumor-bearing mice, 100% of mice injected in all other groups had measurable tumors

Table 3. Murine anti-CEA antibody levels in sera of mice bearing MC-38-cea1 tumors

Tumor	Animal no.	Anti-CEA level[a]		
		C57BL/6	Athymic	C57BL/6 (X-irrad.)
MC-38-cea1	1	10000	NEG[b]	NEG
	2	6500	NEG	NEG
	3	1100	NEG	NEG
	4	4700	NEG	NEG
	5	1500	NEG	210
	6	12150	NEG	NEG
	7	NT[c]	NEG	NEG
	8	NT	NEG	NEG
	9	NT	NT	NEG
	10	NT	NEG	140
	11	NT	110	1650
	12	NT	NEG	190
	13	NT	NEG	NEG
	14	NT	NEG	NEG
	15	NT	NEG	NEG
MC-38	1	NEG	NT	NT
	2	NEG	NT	NT
	3	NEG	NT	NT

[a] Values represent the dilution of sera to yield an A_{490} in the enzyme-linked immunosorbent assay (ELISA) equivalent to 1 ng anti-CEA mAb COL-1

[b] NEG, A490 ≤ 0.27 at a 1 : 50 dilution of the serum sample

[c] NT, not tested

tics of the resulting isografts were observed. Isografts derived from MCA-102 and MCA-102-cea3 were detected at similar times (5 days for MCA-102; 5–7 days for MCA-102-cea3), and had comparable growth rates (Fig. 4A, B). However, many of the tumors derived from both transduced and non-transduced fibrosarcoma cell lines showed areas of visible necrosis by day 14.

To compare the growth rates of the three CEA-transduced murine tumor cell lines, the time required to develop a 50-mm^2 tumor was determined for each tumor-bearing animal and then averaged for each group of mice. As shown in Table 2, the MC-38 adenocarcinoma cell line grew at a similar rate in C57BL/6 and athymic mice. The growth rate of MC-38 cells in irradiated C57BL/6 mice was somewhat slower than that observed in C57BL/6 ($0.05 \geq P \geq 0.025$) or athymic ($0.025 \geq P \geq 0.01$) mice. In C57BL/6 mice, of the three CEA-transduced cell lines, MC-38-cea1 grew at the slowest rate, ($P < 0.005$). The growth of MC-38-cea2 was more comparable to that of MC-38 ($0.1 \geq P \geq 0.05$) (Table 2). There was no statistically significant difference between the growth rates of the CEA-transduced murine fibrosarcoma cell line, MCA-102-cea3, and its non-transduced control.

Anti-CEA antibody in mouse sera

Experiments were then conducted to determine if anti-CEA antibodies were present in mice bearing CEA-transduced tumors. Sera were collected from C57BL/6, athymic and irradiated C57BL/6 mice bearing MC-38-cea1 tumors approximately 20–30 days after the injection of cells. Sera were tested for the presence of murine anti-CEA antibody using an ELISA, as described in Materials and methods. As shown in Table 3, anti-CEA antibodies were detected in 6 of 6 C57BL/6 mice bearing MC-38-cea1 isografts. In contrast, low levels of anti-CEA antibodies were observed in 1 of 14 athymic mice and 4 of 15 irradiated C57BL/6 mice (Table 3). Anti-CEA antibodies were not detected in C57BL/6 mice bearing MC-38 isografts (Table 3).

Anti-CEA antibody levels were also measured in C57BL/6 and athymic mice bearing MC-38-cea2 tumors. In this experiment, sera were collected from all mice 20 days after cell injection; in a subset of animals, sera were also collected immediately prior to injection of the mice with the MC-38-cea2 or MC-38 cells (day 0) and then at 8 and 13 days. As shown in Table 4, no anti-CEA antibody was observed in pre-immune sera or on day 8 in the C57BL/6 mice bearing MC-38-cea2 tumors, but anti-CEA antibodies were detected on day 13 in 2 of 4 mice. By day 20, sera from 10 of the 10 C57BL/6 mice bearing MC-38-cea2 isografts contained anti-CEA antibodies (Table 4), whereas no anti-CEA antibody was detected in athymic mice bearing MC-38-cea2 tumors at any time tested (data not shown). Furthermore, anti-CEA antibody was not detected in the sera of C57BL/6 (Table 4) or athymic mice (data not shown) bearing MC-38 control tumors.

Anti-CEA antibody was also detected in the sera of C57BL/6 mice bearing MCA-102-cea3 murine fibrosarcoma tumors. At day 13, 3 of 3 mice had positive sera titers for CEA, whereas C57BL/6 mice bearing MCA-102 tumors were negative. It should be pointed out that the immune response to CEA did not prevent the CEA-ex-

Table 4. Murine anti-human CEA antibody levels in C57BL/6 mice bearing MC-38-cea2 tumors

Cell line	Mouse no.	Day	Anti-CEA antibody
MC-38-cea2	1	0	< 50[a] (0)[b]
		8	< 50 (0)
		13	< 50 (0.1)
		20	250
	2	0	< 50 (0)
		8	< 50 (0)
		13	50
		20	>500
	3	0	< 50 (0)
		8	< 50 (0)
		13	300
		20	>500
	4	0	< 50 (0)
		8	< 50 (0)
		13	< 50 (0)
		20	>500
	5	20	>500
	6	20	>500
	7	20	>500
	8	20	>500
	9	20	>500
	10	20	>500
MC-38	1	0, 8, 13, 20	< 50 (0)
	2	0, 8, 13, 20	< 50 (0)
	3	0, 8, 13, 20	< 50 (0)

[a] Values represent the dilution of sera to yield an absorbance in the ELISA assay equivalent to 1 ng anti-CEA mAb COL-1

[b] Value in parentheses represent the A_{490} of the sample at a dilution of 50

Table 5. CEA levels in protein extracts of MC-38-cea1 tumors (derived from athymic mice) and treated with hyperimmune mouse sera[a]

Tumor	Sera		CEA[b] (ng/mg protein extract)
	Antibody vs CEA	Anti-CEA titer	
MC-38-cea1	None	N/A	21.1
	Positive[c]	12150[e]	11.0
	Negative[d]	<50 (0.1)[f]	20.3
MC-38	None	N/A	0.0
	Positive	12150	0.0
	Negative	<50 (0.1)	0.1

[a] Protein extracts of MC-38 or MC-38-cea1 tumors derived from athymic mice were incubated with mouse sera for 30 min at 37° C prior to assay of the protein extract for CEA levels. Sera were used at a 1 : 4 final dilution

[b] CEA levels were quantified using an Abbott CEA radioimmunoassay (RIA) kit

[c] Serum was collected from a C57BL/6 mouse bearing a MC-38-cea1 tumor

[d] Serum was collected from a C57BL/6 mouse bearing a MC-38 tumor

[e] Values represent the dilution of sera required to yield A_{490} in the ELISA equivalent to 1 ng anti-CEA mAb COL-1

[f] Values in parentheses represent the A_{490} of the serum at a dilution of 50

pressing tumors from growing in C57BL/6 mice. Although no attempt has been made to quantify and compare the absolute levels of anti-CEA antibodies elicited by the three CEA-transduced tumor models, these experiments demonstrate that all three CEA-transduced tumors are immunogenic in C57BL/6 mice.

CEA levels in tumors derived from CEA-transduced cells

One potential problem related to the presence of murine anti-CEA antibodies in the sera of some tumor-bearing mice is that it may affect the accurate quantification of CEA in tumors through the formation of antigen-antibody complexes. Studies were conducted to determine if such a situation existed in C57BL/6 mice bearing tumors derived from the CEA-transduced cells. First, the amount of CEA in a protein extract of a MC-38-cea1 tumor derived from an athymic mouse (anti-CEA antibody negative) was determined (Table 5). The same protein extract was then incubated with serum from a C57BL/6 mouse bearing a MC-38-cea1 tumor. This serum was positive for the presence of anti-CEA antibody. As shown in Table 5, the amount of CEA detected in the tumor extract treated with the positive serum was approximately 52% of the amount detected in the untreated extract. Incubation of the MC-38-cea1 tumor extract with CEA-negative serum collected from a C57BL/6 mouse bearing a MC-38 tumor had no effect on the detection of CEA in the extract (Table 5). No effect on the quantification of CEA was also observed following treatment of a CEA-negative MC-38 tumor extract with either the anti-CEA-positive or -negative murine sera (Table 5). These results suggest that the presence of anti-CEA antibodies in vivo might affect the quantification of CEA in tumors derived from CEA-transduced cells.

With the above results in mind, the level of CEA expression was evaluated in extracts of MC-38-cea1 tumors derived from C57BL/6, athymic, and irradiated C57BL/6 mice. Since C57BL/6 mice bearing MC-38-cea1 tumors may contain anti-CEA antibodies, accurate quantification of CEA in these tumors was not possible; however, Table 6 shows that CEA was present in these tumors. CEA levels were also evaluated in tumors from athymic and irradiated C57BL/6 mice, where antibody levels were negative or slightly above background (Table 3). CEA amounts varied among the MC-38-cea1 tumors from 11.9 ng to 102.1 ng CEA/mg protein in athymic mice and from 11.4 ng to 77 ng CEA/mg protein in irradiated C57BL/6 mice, whereas the amount of CEA in the MC-38-cea1 cell line was 230 ng CEA/mg (Table 6 [24]). Differences in CEA levels of MC-38-cea1 tumors grown in (a) C57BL/6 compared to athymic mice and (b) C57BL/6 compared to C57BL/6 X-irradiated mice were statistically significant ($0.05 \geq P \geq 0.025$). No statistically significant difference was observed in CEA levels of MC-38-cea1 tumors in athymic compared to C57BL/6 X-irradiated mice ($P > 0.25$). For comparison, CEA levels in colon carcinoma biopsy specimens from 5 patients were 5, 140, 200, 201, and 227 ng CEA/mg protein. No CEA was detected in the non-transduced MC-38 tumors or in two normal spleen samples from non-carcinoma patients.

MC-38-cea1 tumors were also evaluated by immunohistochemical analyses for CEA expression. Frozen sec-

Table 6. CEA levels in MC-38-cea1 tumors

Tumor	Animal no.	CEA (ng/mg protein)		
		C57BL/6	Athymic	C57BL/6 (X-irrad.)
MC-38-cea1	1	9.9[a]	17.6	26.1
	2	26.8	16.1	22.9
	3	14.6	102.1	25.5
	4	2.5	28.3	NT
	5	6.1	41.2	NT
	6	NT	11.9	18.4
	7	NT	14.1	27.1
	8	NT	12.8	29.1
	9	NT	13.3	77.0
	10	NT	31.6	NT
	11	NT	21.0	NT
	12	NT	31.0	NT
	13	NT	41.9	11.4
	14	NT	26.9	19.5
	15	NT	87.8	23.4
Av ± SD[b]		11.9 ± 9.42 ($0.05 \leq P \leq 0.10$)[c]	33.17 ± 27.06 ($0.01 \leq P < 0.025$)[c]	28.0 ± 17.95 ($0.01 \leq P \leq 0.025$)[c]
MC-38	1	0.0	0.2	0.4
	2	0.1	0.3	0.3
	3	0.2	0.3	0.3
Av ± SD		0.1 ± 0.1	0.27 ± 0.06	0.33 ± 0.06

[a] Values represent CEA levels detected using an Abbott RIA kit
[b] Av ± SD average ± standard deviation
[c] P value for difference between Av ± SD of MC-38-cea1 and MC-38 in the same mouse strain

tions of MC-38-cea1 tumors were stained with the anti-CEA mAb COL-6, and the percentage of positive tumor cells was noted. The percentage of positive tumor cells varied between each group of mice and among the individual mice within each group (Fig. 5). MC-38-cea1 tumors from C57BL/6 mice expressed the least amount of CEA; from 0 to 20% of the tumor cells were positive (Fig. 5A). MC-38-cea1 tumors from athymic mice expressed the highest amount of positive tumor cells (30%–70%), while those from irradiated C57BL/6 mice had an intermediate level (20%–50%) (Fig. 5B, C). No MC-38 tumor cells stained positive for CEA (Fig. 5D). These results are consistent with those observed above using an RIA for CEA quantification.

An attempt was also made to determine CEA levels in MC-38-cea2 and MCA-102-cea3 tumors. Previous results indicated that CEA could not be detected in the MC-38-cea2 cell line using a CEA RIA kit (Abbott Laboratories Inc.), but could be detected using an ELISA kit (Hoffmann-LaRoche), which reacts with different epitopes on CEA [25]. Expression of CEA in extracts of MC-38-cea2 tumors grown in C57BL/6 or athymic mice was below the level of detection of the ELISA (<2.5 ng/mg), whereas 57 ng CEA/mg was detected in extracts of the MC-38-cea2 cell line [25]. The possible reasons for this result will be discussed below.

Using the RIA, MCA-102-cea3 fibrosarcoma tumor extracts derived from C57BL/6 mice were shown to contain 1.3–30.4 ng CEA/mg protein while the MCA-102-cea3 cell line contained 380 ng CEA/mg protein (Table 7) [25]. The tumors that were harvested 7 days after inoculation of cells into the mice appeared to express higher CEA levels than those removed at later times ($0.1 \geq P \geq 0.05$) (Table 7). However it is possible that antibody present at later times masked the presence of the antigen. CEA was not detected in non-transduced MCA-102 tumor extracts at any time tested. Taken as a whole, the results on tumor growth and CEA expression observed in this study with the MC-38-cea1, MC-38-cea2 and MCA-102-cea3 tumors demonstrate that the expression of CEA, at the levels detected here, does not overtly influence the tumorigenicity of colon carcinoma cells grown subcutaneously.

Expression of CEA in tumor cells following in vitro culture

The apparent low levels of CEA expression in transduced tumors may have resulted from the binding of antibodies to antigen in the tumors. Modulation of CEA in vivo, as well as proteolysis of the expressed gene product in mice may also have contributed to the low levels of CEA found in the transduced tumors. In addition, rare variants of the original transduced clone, which lack or express only low levels of CEA, may have outgrown the predominant cell population, which expresses high levels of CEA in mice. In order to address this issue, an MC-38-cea2 tumor was removed from C57BL/6 mice 21 days after transplantation, and individual cells were isolated by enzymatic digestion of the tumor. Following in vitro culture for 13 days, cells were analyzed for cell-surface expression of CEA (Fig. 6). The majority of cells cultured from this tumor express CEA (78%), which is comparable to the expression of CEA

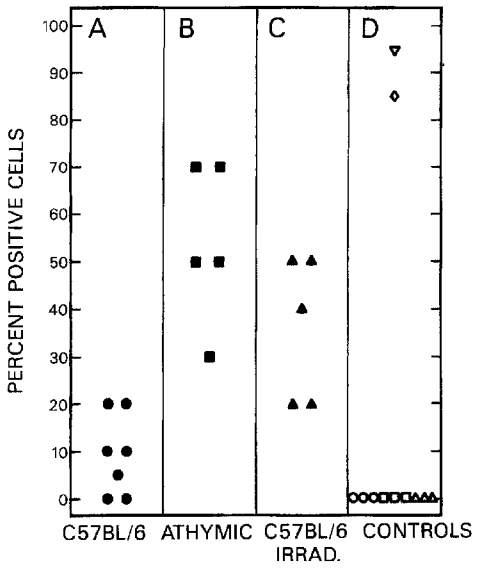

Fig. 5A, B. Reactivity of biotinylated mAb COL-6 to frozen sections (methanol-fixed) of MC-38-cea1 tumors. Each tumor section was reacted with 8 μg biotinylated mAb COL-6 using the immunoperoxidase method. MC-38-cea1 tumors derived from (**A**) C57BL/6 mice (●), (**B**) athymic mice (■), and (**C**) irradiated C57BL/6 mice (▲). **D** Control tumors: MC-38 tumors derived from C57BL/6 (○), athymic (□), and irradiated C57BL/6 (△) mice; LS-174T human colon adenocarcinoma xenograft from athymic mouse (▽); human colon adenocarcinoma (◇). Each symbol represents a separate tumor. The percentage positive cells denotes the number of tumor cells reactive with biotinylated mAb COL-6 divided by the total number of tumor cells and multiplied by 100

Table 7. Tumor CEA levels in C57BL/6 mice bearing MCA-102-cea3 tumors

Tumor	Animal no.	Day[a]	CEA[b] (ng/mg protein extract)
MCA-102-cea3	1	7	30.4
	2	7	19.5
	3	20	1.3
	4	20	1.4
	5	13	6.8
	6	13	2.1
	7	13	3.7
	8	14	2.6
	9	14	3.6
			7.93 ± 10.16[c]
MCA-102	1	7	0.2
	2	17	0.0
	3	14	0.0
	4	14	0.0
	5	17	0.0
			0.04 ± 0.09[c]

[a] Days after inoculation of mice with tumor cells
[b] Value represent CEA detected using an Abbott RIA kit
[c] Average ± standard deviation. Difference between average ± standard deviation for MCA-102-cea3 and MCA-102 was statistically significant ($0.01 \leq P \leq 0.025$)

(90%) in control MC-38-cea2 tissue-culture cells that had never been transplanted. The mean fluorescence intensity of the cultured tumor cells, expressed in arbitrary units (57 units), was, however, lower than the level found in control tissue-culture cells (114 units). Similar results were found with four additional MC-38-cea2 tumors, as well as with three MC-38-cea1 and two MCA-102-cea3 tumors (data not shown).

Biodistribution of anti-CEA mAb COL-1

Experiments were then initiated to determine if radiolabeled anti-CEA mAb could detect CEA on the surface of the transduced cells in vivo, and if these CEA-transduced tumors could be used as a model for tumor targeting of mAb to CEA. For this experiment, mAb COL-1 was radiolabeled and injected i.v. into athymic and C57BL/6

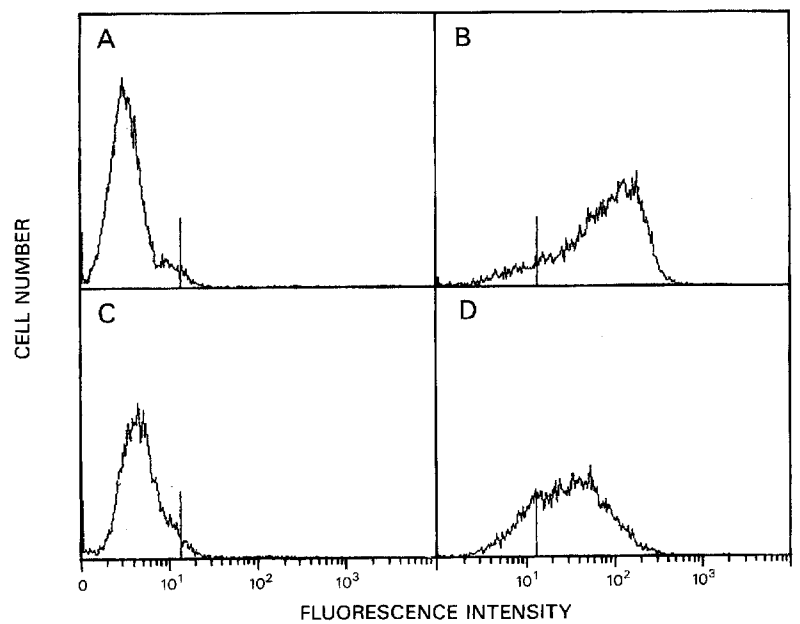

Fig. 6 A – D. Expression of CEA in cells isolated from a MC-38-cea2 tumor. The expression of CEA was examined by staining cells with either mAb UPC-10 (**A, C**) or mAb COL-1 (**B, D**). **A, B** Flow cytometry profiles of the MC-38-cea2 tissue culture line; **C, D** profiles of the cells isolated from a MC-38-cea2 tumor, as described in Materials and methods

Table 8. Radiolocalization of ^{125}I-mAb COL-1 in mice bearing murine or human tumors[a]

Location	^{125}I-mAb (%injected dose/g)				
	Athymic (nu/nu) mice			C57BL/6 mice	
	MC-38[b]	MC-38-cea2[c]	LS-174T[d]	MC-38	MC-38-cea2
Tumor	2.9±0.6	14.4±5.7	5.8±1.7	3.7±0.7	11.4±2.3
Blood	4.8±0.8	2.5±0.3	3.6±0.5	7.3±0.5	6.3±1.3
Liver	2.2±0.4	1.0±0.2	1.4±0.3	2.3±0.1	2.0±0.2
Spleen	2.3±0.6	1.3±0.6	1.2±0.2	2.0±0.3	1.6±0.3
Kidneys	1.5±0.4	0.9±0.1	0.9±0.1	2.1±0.2	1.9±0.3
Lungs	2.1±0.3	2.4±0.3	1.5±0.3	2.9±0.4	2.3±0.4

[a] Athymic and C57BL/6 mice (4 per group) bearing murine or human colon adenocarcinoma xenografts were injected with approximately 2.7 μCi ^{125}I-labeled mAb COL-1 (3.7 μCi/μg) and sacrificed at 48 h
[b] MC-38: murine colon adenocarcinoma xenograft [8]
[c] MC-38-cea2: MC-38 transduced with a retroviral construct containing cDNA encoding the human CEA gene [25]
[d] LS-174T: human colon adenocarcinoma xenograft [29]

Table 9. Radiolocalization of ^{125}I-mAb COL-1 in mice bearing murine or human tumors[a]

Location	Tumor: normal tissue ratios[b]				
	Athymic (nu/nu) mice			C57BL/6 mice	
	MC-38[c]	MC-38-cea2[d]	LS-174T[e]	MC-38	MC-38-cea2
Blood	0.6±0.2	6.0±3.2	1.6±0.5	0.5±0.1	1.9±0.6
Liver	1.3±0.2	13.7±4.4	4.0±0.6	1.6±0.2	5.8±1.4
Spleen	1.3±0.2	12.2±6.6	4.9±1.1	1.8±0.2	7.2±2.0
Kidneys	2.1±0.6	15.8±6.0	6.7±1.9	1.8±0.3	6.3±2.0
Lungs	1.4±0.2	6.2±1.4	3.8±1.0	1.3±0.2	5.2±1.5

[a] Athymic and C57BL/6 mice (4 per group) bearing murine or human colon adenocarcinoma xenografts were injected with approximately 2.7 μCi ^{125}I-labeled mAb COL-1 (3.7 μCi/μg) and sacrificed at 48 h
[b] Tumor: normal tissue ratio is the %ID/g of the tumor divided by the %ID/g of the normal tissues
[c] MC-38: murine colon adenocarcinoma xenograft [8]
[d] MC-38-cea2: MC-38 transduced with a retroviral construct containing cDNA encoding the human CEA gene [25]
[e] LS-174T: human colon adenocarcinoma xenograft [29]

mice bearing MC-38-cea2 tumors. As a specificity control for localization of anti-CEA antibodies, athymic and C57BL/6 mice bearing MC-38 tumors were used. Athymic mice bearing xenografts of the LS-174T human colon adenocarcinoma cell line served as a positive control. Tumors (MC-38, MC-38-cea2 and LS-147T) were approximately 0.5 cm in diameter at the time of injection of ^{125}I-COL-1. The LS-174T cell line has previously been shown to be a high expressor of CEA as compared to most human colon carcinomas. LS-174T xenografts contain approximately 240 ng CEA/mg tumor extract, as determined by CEA RIA (data not shown). Mice were sacrificed 48 h and 96 h after injection with ^{125}I-COL-1, and the percentage injected dose (ID) of the radiolabeled mAb/g in tumors and normal tissues was determined.

In athymic mice, mAb tumor localization was observed in the CEA-transduced MC-38-cea2 tumors as well as the positive control LS-174T xenografts expressing high levels of CEA (Table 8). In contrast, no tumor localization was seen in the non-transduced MC-38 cells (Table 8). The tumor:tissue ratios (%ID/g in tumor divided by the %ID/g in normal tissue) at 48 h demonstrated, for example, a tumor:liver ratio of 13.7 for the CEA-transduced cells ($0.0062 \geq P \geq 0.005$ in comparison with MC-38 tumor:liver ratio), and a 4.0 tumor:liver ratio for the LS-174T human colon carcinoma cells ($P < 0.005$ in comparison with MC-38 tumor:liver ratio) (Table 9). Similar results were observed at the 96-h time point.

mAb tumor localization was also observed in CEA-transduced MC-38-cea2 tumors grown in C57BL/6 mice (Table 8). As observed for the athymic mice, no tumor localization was seen in the non-transduced MC-38 tumors grown in C57BL/6 mice. For example, tumor:liver ratios at 48 h were 5.8 and 1.6 ($P<0.005$) for the MC-38-cea2 and MC-38 tumors, respectively (Table 9). No conclusions could be reached at 96 h concerning the %ID/g values of MC-38-cea2 tumors in C57BL/6 mice, since the mean ± standard deviation for the four tumors was 3.3±2.8. These studies thus demonstrate the ability of an anti-CEA mAb to localize a CEA-transduced tumor in an allograft or isograft setting.

Discussion

One purpose of these studies was to analyze three model systems for potential use in anti-CEA immunotherapy studies (either for passive immunotherapy using anti-CEA mAb, or active immunotherapy using anti-CEA vaccines such as rV-CEA). It appears that MC-38-cea2 is the most

appropriate cell line for these studies. When C57BL/6 mice were inoculated with MC-38-cea2 cells, 100% of the mice developed tumors, whereas only 75% of mice inoculated with MC-38-cea1 cells developed tumors. In addition, the in vivo growth rate of MC-38-cea2 tumors is more similar to that of the parental MC-38 tumors than is the growth rate of MC-38-cea1 tumors. In vivo growth of MCA-102-cea3 cells was comparable to that of the parental MCA-102 cell line; however, there were visible areas of necrosis at substantially earlier times (i.e. approximately 15 days after transplant) than observed with MC-38-cea1 and MC-38-cea2 tumors. Furthermore, MC-38 is a colon adenocarcinoma and may be more relevant to human CEA studies than the MCA-102 fibrosarcoma.

Therefore, because of its in vivo growth characteristics and tumor of origin, the MC-38-cea2 cell line was subsequently analyzed as a target for a radiolabeled anti-CEA mAb and shown to be as efficient or more efficient as a target in C57BL/6 or athymic mice than the LS-174T human colon adenocarcinoma xenograft was in athymic mice. The LS-174T cell line, as well as tumors derived from this cell line have previously been shown to be high expressors of CEA [10].

Some results that can not be fully explained at this time were obtained when the CEA-transduced cells were transplanted in vivo. First, the MC-38-cea1 cells demonstrated a reduced growth rate in immunologically intact C57BL/6 mice compared to athymic mice (Table 2), suggesting the influence of an anti-CEA immune response on the growth of this tumor in immunologically competent C57BL/6 mice. Indeed, this was supported by the appearance of anti-CEA antibodies in sera of C57BL/6 mice and not in athymic mice following tumor transplant (Table 3). One would then anticipate that there might be a selection of CEA-negative cells in the tumor mass. This is indicated by the data generated using the immunoperoxidase method, which demonstrated a lower percentage of MC-38-cea1 tumor cells was positive for expression of CEA from C57BL/6 versus athymic mice. Levels of CEA in extracts of MC-38-cea1, MC-38-cea2, and MCA-102-cea3 tumors grown in normal C57BL/6 mice were significantly lower than the level of CEA in extracts of these cells grown in vitro. While these latter results may be explained by the masking of CEA by anti-CEA antibodies in sera, the level of CEA expression in extracts of MC-38-cea1 tumors grown in irradiated C57BL/6 mice and athymic mice, where no anti-CEA antibodies were detected, was also significantly lower than that of the in vitro passaged line. When cells isolated from the in vivo passaged tumors were cultured in vitro, the cell-surface expression of CEA was similar to that of cells passaged continuously in vitro (Fig. 6), although there was a shift to a lower level of staining in the in vivo passaged cells. Variants that express differing levels of CEA may have arisen during the expansion of this clone in vitro, and those expressing lower levels of antigen may have expanded more quickly during in vivo growth of the tumor. Nevertheless, this shift is not significant enough to account for the dramatic loss of antigen expression seen during the in vivo growth of this tumor. The reasons for this apparent modulation of CEA expression in vivo are not known at this time.

It should be noted that the growth rates of the MC-38-cea2 transduced and non-transduced cells, unlike those of MC-38-cea1, were similar in athymic and C57BL/6 mice. Moreover, preliminary studies have indicated that the MC-38-cea2 line expresses CEA at a level that allows it to act as a target for active immunotherapy responses following administration of a recombinant vaccinia virus expressing CEA [14]. That is, mice vaccinated with the recombinant vaccine were resistant to tumor challenge of CEA-transduced MC-38-cea2 cells, and not MC-38 cells. Moreover, in mice bearing MC-38-cea2 tumors that had been transplanted 7 days previously, treatment with the recombinant vaccine inhibited or eliminated tumor growth. No effect of the rV-CEA was seen on similarly established MC-38 cells. Thus, tumors expressing only low levels of CEA can successfully be treated with anti-CEA immune reagents. Finally, the studies reported here have demonstrated that a radiolabeled anti-CEA mAb is able to target MC-38-cea2 tumors efficiently (Tables 8, 9). This demonstrates that tumors that express only low levels of CEA may function as targets for anti-CEA-directed therapies.

As more human tumor-associated genes become identified, and more recombinant vaccines are developed using these genes and become available for use in active immunotherapy, model systems such as the one identified here will become more necessary to define the biological activity and the specificity of the vaccines in question. Finally, the use of a transduced and non-transduced cell line in a tumor model system, the only difference between the two tumors being the inserted tumor gene, will help define the specificity of the immunotherapeutic agent being evaluated.

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