Mice Transgenic for Human Carcinoembryonic Antigen as a Model for Immunotherapy

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ABSTRACT

Mice transgenic for the human carcinoembryonic antigen (CEA) gene were prepared for use as a preclinical model for immunotherapy. A 32.6-kb fragment containing the complete human CEA gene and flanking sequences was isolated from a genomic cosmid clone and used to produce transgenic C57BL/6 mice. A homozygous line was established that was designated C57BL/6J-TgN(CEA)18FJP. Southern blot analysis showed that this line contained intact copies of the cosm id clone, with approximately 19 integrated copies at one chromosomal location. A mouse-human chimeric anti-CEA monoclonal antibody was used to examine CEA expression by immunohistochemical staining of frozen tissue sections. In the cecum and colon, approximately 20% of the luminal epithelial cells had strong cytoplasmic staining, whereas occasional glands showed intense staining. CEA was also expressed in gastric foveolar cells, whereas small intestine villi had only a few (<1%) positive cells. CEA was not found by immunohistochemistry in other tissues of the digestive tract, nor was it found in a wide range of other tissues or organs. Concordance in results was obtained between immunohistochemistry and analysis of tissue extracts by enzyme immunoassay. The lone exception was the testis, which was positive only by enzyme immunoassay. Expression of human CEA was not observed in tissues derived from transgenic mice. The fecal content of CEA in transgenic mice was approximately 100-fold less than that observed for humans. Circulating CEA was not detected. A CEA-transfected syngeneic murine colon carcinoma cell line, MC-38, was prepared that had stable expression of CEA in vitro and in vivo. The molecular size of CEA produced by CEA-transfected MC-38 cells and by the colon of transgenic mice was similar to that obtained with CEA purified from human colon tumors. Anti-CEA antibody appeared in nontransgenic but not transgenic mice bearing transplanted MC-38 tumors. These findings demonstrate that CEA distribution and its properties in tissues of mice transgenic for the human CEA gene are similar to those observed in human tissues. As in humans, immune responsiveness to CEA, as reflected by antibody formation, was not detectable in transgenic mice bearing CEA-positive tumors. Thus, CEA transgenic mice may serve as a useful model for studying the efficacy and safety of various immunotherapy strategies directed at this tumor self-antigen.

INTRODUCTION

Self-antigens expressed on tumor cells have been implicated as targets for human B- and T-cell responses against autologous cancer (1-5). Several of these antigens are under clinical study, in an effort to specifically boost antitumor reactions. CEA3 is abundantly expressed on a variety of human carcinomas, although its presence in normal tissues is mainly limited to the large intestine (6, 7). In the immediate years after CEA was discovered, numerous reports appeared concerning CEA’s immunogenicity or lack thereof in humans (8-11). Anti-CEA activity was even found in sera of pregnant women and was thought to arise from sensitization by antigen expressed by the fetus (12). However, the conclusion from these early studies was that humans were tolerant to CEA, and when reactivity was detected, this appeared to be associated with such factors as antibodies reacting with blood group determinants on CEA, contaminants in the CEA preparations, and nonspecific reactions. Thus, the immune repertoire for CEA in humans appears truncated because the liberation of antigen during malignant spread does not cause an appreciable change in responsiveness.

The issue of immune responsiveness to CEA has recently been revisited. In vitro immunization of peripheral blood lymphocytes from healthy individuals with an anti-CEA idiotype antibody was found to induce the appearance of anti-CEA antibodies (13). Similar results were obtained when human tumor infiltrating lymphocytes or peripheral blood T cells from colorectal cancer patients were exposed to the anti-idiotype antibody. Human anti-CEA antibodies have also been derived from hybridomas that used draining lymph node cells from colorectal cancer patients as fusion partners (14).

Three recent clinical trials in cancer patients were directed at inducing anti-CEA responses. Foon et al. (15) found that treatment with an anti-CEA idiotype antibody produced both high titer antibodies against CEA and anti-idiotype-specific lymphoproliferative responses. Two other studies have used a recombinant CEA vaccinia vaccine. In one, HLA-restricted anti-CEA CTLs were identified following repeated restimulation of peripheral blood lymphocytes with peptide-pulsed autologous antigen-presenting cells (16). In the other, peripheral blood lymphocytes from two patients showed a significant lymphoproliferative response to CEA (17). Thus, mounting evidence suggests that CEA, as a self-antigen, may be a target for eliciting autologous antitumor reactivity.

A preclinical animal model is desirable for the study of vaccines designed to elicit CEA reactivity, as well as to identify any adverse autoimmune effects that may accompany the latter. However, no true CEA homologue has been identified in mice or rats to date (18). As an alternative, we have prepared mice that are transgenic for the human CEA gene, and we describe herein their preparation, the tissue distribution properties of CEA in these mice, and the development of a syngeneic CEA-expressing tumor system.

MATERIALS AND METHODS

CEA Genomic DNA Isolation. CEA genomic DNA was isolated from the cosmid cosCEA1 (19) provided by Dr. John Shively (City of Hope National Medical Center). cosCEA1 was digested with Aatll (New England Biolabs, Beverly, MA), and the 32.6-kb fragment containing the entire CEA genomic insert along with the flanking cosmid sequences was isolated by sucrose gradient centrifugation, as described (20). Isolated DNA was exchanged with an anti-CEA idiotype antibody. Human anti-CEA antibodies have also been found in a wide range of other tissues or organs. Concordance in results was obtained between immunohistochemistry and analysis of tissue extracts by enzyme immunoassay. The lone exception was the testis, which was positive only by enzyme immunoassay. Expression of human CEA was not observed in tissues derived from transgenic mice. The fecal content of CEA in transgenic mice was approximately 100-fold less than that observed for humans. Circulating CEA was not detected. A CEA-transfected syngeneic murine colon carcinoma cell line, MC-38, was prepared that had stable expression of CEA in vitro and in vivo. The molecular size of CEA produced by CEA-transfected MC-38 cells and by the colon of transgenic mice was similar to that obtained with CEA purified from human colon tumors. Anti-CEA antibody appeared in nontransgenic but not transgenic mice bearing transplanted MC-38 tumors. These findings demonstrate that CEA distribution and its properties in tissues of mice transgenic for the human CEA gene are similar to those observed in human tissues. As in humans, immune responsiveness to CEA, as reflected by antibody formation, was not detectable in transgenic mice bearing CEA-positive tumors. Thus, CEA transgenic mice may serve as a useful model for studying the efficacy and safety of various immunotherapy strategies directed at this tumor self-antigen.

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3 The abbreviations used are: CEA, carcinoembryonic antigen; FISH, fluorescence in situ hybridization; MAb, monoclonal antibody; RT, room temperature; TBS, 0.1 M Tris-HCl (pH 7.5); 0.9% NaCl; PI-PLC, phosphatidylinositol phospholipase C.
0.1 mM Tris (pH 8.0), containing 0.01 mM EDTA, 0.5% Nonidet P-40, and 0.5% Tween 80. Digests were extracted with phenol and precipitated with ethanol prior to PCR. PCR used 50 ng of tail sample DNA and AmpliTaq DNA polymerase (Perkin-Elmer, Branchburg, NJ) in standard reactions using 1.5 mM MgCl₂. The sense strand primer (TCCCCGGACGAGGCCTCGCT-CAC) and antisense strand primer (GCTAGGAGAGCAGACGAG- CATCGG) corresponded to the CEA genomic exon 1 (nucleotides 1806–1830) and CEA intron 1 (nucleotides 2725–2701), respectively (19). PCRs using these primers generated a fragment of 920 bp. The size of the CEA cDNA copy in CEA-transfected cells was determined by PCR using as primers: sense strand, CAGATGGTCATCCTGGTCAGGG; and antisense strand, CTTATAACATGTATACGACGACCCACCAC. Oligonucleotides were synthesized by the City of Hope DNA synthesis facility using a standard. Detection and quantitation of CEA in samples. The T84.1 MAb (500 ng/well), followed by biotin-labeled goat antihuman IgG (Vector Laboratories, Burlingame, CA). Sites of antigen localization were disclosed using peroxidase- streptavidin conjugate (Vector) and diaminobenzidine. The primary anti-CEA MAb was omitted on control sections.

**Western Blotting.** Colons from nontransgenic and transgenic mice were cleared of fecal and maternal DNA then homogenized at 4°C in 0.01 M Tris-HCl buffer (pH 8.0), containing 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide, and 0.025% NaN₃. Following centrifugation at 13,000 × g for 30 min at 4°C, the supernatant was preincubated by incubation for 1 h at 4°C with a 1:1 (v/v) slurry of Sepharose 4B-normal mouse IgG adsorbant (Pharmacia, Piscataway, NJ). The gel was removed from the latter mixture by centrifugation at 200 × g for 5 min, and the precleared supernatant was mixed with 1:1 slurry of Sepharose 4B-T84.66 immunoadsorbant. The immunoadsorbants were prepared by coupling 5 mg of normal mouse IgG or T84.66 MAb per ml of cyanogen bromide-activated Sepharose 4B (Pharmacia). Incubation of the supernatant-immunoadsorbant mixture was carried out overnight at 4°C with continuous agitation. The latter mixture (100 μl) was poured into a small pipette column, the unbound fraction was eluted, and then bound material was removed with 300 μl of SDS-PAGE sample buffer lacking 2-mercaptoethanol.

The bound fractions from the T84.66 immunoadsorbant were applied to a 7.5% SDS-polyacrylamide gel after samples were heated at 100°C for 5 min. After electrophoresis, transfer to nitrocellulose (Hybond; Amersham, Arlington Heights, IL) was carried out at 100 mA/gel for 50 min using a semidry transfer cell (Bio-Rad) and 0.048 mM Tris, 0.039 glycine, and 20% methanol (pH 9.2) buffer. After transfer, the nitrocellulose was blocked overnight at 4°C with 5% nonfat dry milk in TBS. The transfer paper was reacted with T84.66 MAb (0.5 μg/ml in 3% BSA-TBS) for 2 h at RT with agitation, followed by washing in TBS containing 0.1% Tween 20. Peroxidase coupled goat antlmouse IgG (Pierce) in 3% BSA, TBS, and Tween 20 was allowed to react for 1 h at RT, washed, and then developed for 5 min with Pierce SuperSignal Substrate according to the manufacturer’s instructions. The transfer paper was then exposed to Biomax X-ray film (Eastman Kodak Company, Rochester, NY).

**Transfections.** The chemically induced colon adenocarcinoma cell line, MC-38, was obtained from Dr. Steven A. Rosenberg (National Cancer Institute, Bethesda, MD) and was maintained in DMEM high-glucose medium supplemented with nonessential amino acids, 4 mM glutamine, 2 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin (all obtained from Irvine Scientific, Santa Ana, CA), and 10% fetal bovine serum (Gemini, Carlsbad, CA). MC-38 cells were transfected with Lipofectin (Life Technologies, Inc., Gaithersburg, MD) and the CEA cDNA-containing plasmid pHBAP-r-neo provided by Dr. John Shively. Transfected cells were selected with 500 μg/ml G418 (Life Technologies). and CEA-expressing clones were identified first by ELISA, followed by flow cytometry using T84.66 MAb for CEA detection. Flow cytometry analysis was carried out in the City of Hope Flow Cytometry and Sorting facility using a MoFlo (Cytomation, Ft. Collins, CO).

For PI-PLC digestion, cells were suspended in RPMI 1640 (Irvine Scientific) containing 0.1% BSA, 0.02 mM EDTA, and 0.5 units/ml PI-PLC. The PI-PLC obtained from *Bacillus thuringiensis* was provided by Dr. Donald Diamond (Department of Bone Marrow Transplantation, City of Hope National Medical Center). Cells were incubated for 45 min at 37°C with agitation, washed 3 times, and analyzed by flow cytometry.

**Growth of CEA-transfected MC-38 Cells in Vivo.** CEA-expressing MC-38 cells were released from culture flasks with trypsin-EDTA (Irvine Scientific), washed twice in serum-free RPMI 1640, and 2.5 × 10⁶ cells were implanted s.c. Tumor growth was monitored by caliper measurements in two
perpendicular dimensions, and tumor volume was determined according to the formula:

\[
\text{Tumor volume} = \frac{(\text{Short dimension})^2 \times (\text{long dimension})}{2}
\]

The expression of CEA on transfected MC-38 cells growing in vivo was determined by flow cytometry. Tumors were excised and minced, and single-cell suspensions were prepared by digestion of fragments at RT for 4 h with a mixture of 0.1 mg/ml hyaluronidase type IV, 1 mg/ml collagenase type IV, and 0.1 mg/ml DNase Type IV (Sigma) in RPMI 1640 (29). Cells were placed in cell culture and propagated in the absence of G418 for 8–13 days prior to analysis by flow cytometry.

The presence of anti-CEA antibody in the serum of tumor-bearing mice was measured by ELISA. Polystyrene microtiter wells were coated with 200 ng of purified CEA in 0.1 M sodium bicarbonate (pH 9.6) overnight at 4°C. After blocking with 0.5% BSA, doubling dilutions of serum, beginning at 1:100, were added to the wells, and the plates were incubated at 37°C for 1 h. The plates were washed in PBS containing 0.05% Tween 20 and then reacted with alkaline phosphatase-labeled goat antimouse IgG+IgM (Jackson ImmunoResearch) for 1 h at 37°C. The plates were washed, reacted with chromogen, and read in a microplate reader.

RESULTS

Establishment of CEA Transgenic Mouse. A 32.6-kb AatII restriction fragment that contains the entire human CEA genomic region was used to generate transgenic mice. This DNA fragment has been shown to contain the regulatory sequences required for tissue-specific CEA expression (19). Of 19 mice born, 4 were found to be positive for the CEA gene, as determined by PCR analysis of DNA derived from tail samples (data not shown). To determine whether any of the positive mice expressed CEA, we took advantage of the observation that CEA can be detected in human feces (30). Extracts of mouse fecal pellets were assayed for CEA in a two-site MAb ELISA. Three of the four founder mice were found to contain immunoreactive CEA in their feces. The \( A_{405} \) nm for the three CEA-expressing mice ranged between 0.8 and 1.3, as compared to a range of 0.05–0.11 for feces obtained from the nontransgenic littersmates, as well as control nontransgenic mice. Of the three CEA-expressing founder mice, successful breeding was only obtained with one, and this mouse line has been designated C57BL/6J-TgN(CE4Ge)18FJP or line 18, as it will be referred to hereafter. Using the 32.6-kb CEA genomic fragment for FISH analysis of interphase nuclei from peripheral blood, homozygous mice were identified and bred. FISH also demonstrated integration of the CEA gene into one chromosomal site (data not shown).

Southern Blot and Copy Number Determination. Fig. 1 shows the results of Southern analysis using transgenic and normal mouse DNAs. The AatII CEA genomic fragment used to generate the transgenic mice contains the 13.0-, 9.0-, 4.8-, and 2.6-kb fragments. All of the latter fragments were also contained within the transgenic DNA digest, indicating that this line harbors an intact CEA gene. The cosCEAl digestion gives rise to an additional 4.4-kb band, corresponding to CEA-cosmid junction fragments. The two minor bands at 3.7 and 5.1 kb obtained with the transgenic DNA digest are consistent with head-to-tail or head-to-head junction fragments created by multiple insertions. Determination of copy number revealed that line 18 contains 19.2 ± 2.9 (\( n = 3 \)) copies of the CEA gene.

Tissue Distribution of CEA Expression. Immunoperoxidase staining was carried out on acetone-fixed, frozen tissue sections using a mouse/human chimeric anti-CEA MAb, identical to the antibody used by Eades-Perner et al. (31) for demonstration of CEA in their CEA transgenic mice. With the latter MAb, it was possible to use an antihuman secondary antibody to avoid nonspecific background staining arising from endogenous mouse immunoglobulin in tissue sections that would result from the application of antimouse secondary antibodies. In the cecum and large intestine, 10–20% of the luminal epithelial cells had strong cytoplasmic and apical staining, whereas occasional glands showed intense staining. An area from a transgenic colon is depicted in Fig. 2 and shows a region in which the majority of epithelial cells are CEA positive. By contrast, the colon from a nontransgenic mouse did not display any positive staining (Fig. 2). The pattern of CEA localization in the transgenic colon is very similar to CEA staining that we have previously reported for morphologically normal human colon (32). Besides the cecum and colon, CEA staining was also observed in gastric foveolar cells and in a sporadic epithelial cell in the proximal portion of the small intestine. All other tissues (tongue, esophagus, middle and distal portion of the small intestine, liver, spleen, kidney, bladder, lung, thyroid, salivary gland, skin, testis, ovary, uterus, and cervix) were negative for CEA expression, as measured by immunohistochemistry.
The expression of CEA was also measured by ELISA in extracts of the same tissues examined by immunohistochemistry. Tissues from three males and one female were used for this analysis. In the gastrointestinal tract, the highest level of CEA content was found in the colon (25.9 ± 7.5 ng/mg protein), followed by stomach (12.2 ± 5.9) and cecum (5.5 ± 1.6). CEA was also found in the testis (9.1 ± 2.8), which represented the only discrepancy between immunohistochemistry and ELISA results. CEA expression was not observed in the same tissues derived from nontransgenic mice when examined by ELISA.

The CEA content of feces and serum was compared between line 18 mice and normal humans in ELISA. To enhance extraction of CEA from fecal samples, both detergent (1% Triton X-100) and sonication were used. Although CEA was present in transgenic mouse feces, its level was approximately 100-fold less than that measured in feces from normal humans (Table 1). CEA was not detected in the serum of transgenic mice.

### Table 1 CEA content of feces and serum obtained from CEA transgenic mice

<table>
<thead>
<tr>
<th>Source</th>
<th>Feces CEA (ng/mg of total protein)</th>
<th>Serum (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transgenic</td>
<td>11.7 ± 4.0 (n = 4)</td>
<td>&lt;2.5</td>
</tr>
<tr>
<td>Nontransgenic</td>
<td>0</td>
<td>&lt;2.5</td>
</tr>
<tr>
<td>Human</td>
<td></td>
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<tr>
<td>Normal</td>
<td>1570 ± 683 (n = 3)</td>
<td>&lt;2.5&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>a</sup> Literature value (53).
CEA TRANSIENT MICE

Fig. 3. Characterization of CEA and/or CEA gene in colons of transgenic mice or CEA-transfected MC-38 cells. A, analysis by Western blotting using chemiluminescence of CEA in colon of transgenic mice and CEA-expressing MC-38 cells. Colon extracts were adsorbed to an immunoadsorbant containing T84.66 anti-CEA MAb, and then applied nonreduced to a 7.5% acrylamide gel for SDS-PAGE. Lane 1, CEA purified from human colon adenocarcinomas (40 ng); Lane 2, immunoadsorbant bound fraction from colon of nontransgenic mouse (20-μl sample); Lane 3, immunoadsorbant bound fraction from colon of CEA transgenic mouse (20-μl sample); Lane 4, extract of CEA-transfected MC-38 cells, clone C15-4.3; Lane 5, extract of parental MC-38 cells. Blots were stained with T84.66 MAb. B, analysis by PCR of CEA DNA in clone C15-4.3 cells. Lane 1, length standard (mixture of A// HindIII and XhoI74 HaeIII); Lane 2, PCR product of plasmid containing CEA; Lane 3, PCR product of parental MC-38 cells; Lane 4, PCR product of CEA-transfected MC-38 cells.

clearing step with a mouse IgG adsorbant. The gel was eluted with SDS-PAGE sample buffer, and the eluate was analyzed by Western blotting. The same volume of material bound from either nontransgenic or transgenic extracts was applied to the SDS-polyacrylamide gel. As shown in Fig. 3A, a major band in the same size range as that of CEA purified from human colon tumors was observed with colon extracts from transgenic mice. A second intense band of slower migration was also observed in transgenic colon extracts that most likely represented aggregates arising from sample processing (33). Similar band patterns were observed in reduced and nonreduced gels. Bands in the same size range as those obtained with transgenic colon extracts were also observed with extracts from colons of nontransgenic mice (Fig. 3A). However, the bands appearing in colons from the latter mice were much weaker than those observed with colons from transgenic mice. We have repeatedly observed this background staining with nontransgenic colon extracts from different mice, but it is always much weaker than that observed with colons from different transgenic mice. Furthermore, exposure of blots to an irrelevant mouse MAb instead of the anti-CEA MAb also resulted in similar weak bands for colon extracts from both transgenic and nontransgenic mice (data not shown). Although the basis for this nonspecific reactivity is unknown, these results show that the CEA molecule produced by the transgenic mice is of the same molecular size range as CEA derived from human colon adenocarcinomas.

Establishment of Syngeneic Colon Tumor Cell Line Expressing CEA. Transfection of MC-38 colon carcinoma cells with the CEA cDNA-containing plasmid, pH3Apr-1-neo, resulted in the establishment of a clone, C15-4.3, that has maintained CEA expression for over one year in continuous culture in the absence of drug selection. The flow cytometry profile for C15-4.3 cells stained with the anti-CEA MAb T84.66 is depicted in Fig. 4, and it shows that over 90% of the cells are expressing CEA on their surface. CEA expressed on the surface of human colon carcinoma cells is linked to the cell surface by a phosphatidylinositol glycan linkage (34). To determine whether the CEA expressed on the C15-4.3 cell surface had a similar linkage, the cells were treated with PI-PLC, which cleaves phosphatidylinositol glycan-linked proteins from the cell surface (35). The majority of C15-4.3 cells became CEA negative following treatment with PI-PLC (Fig. 4), demonstrating that C15-4.3 cells have CEA attached to their cell surface via a phosphatidylinositol glycan linkage.

Quantitation of the amount of CEA produced by tissue cultured C15-4.3 cells was carried out by ELISA. C15-4.3 cells secreted 27.3 ± 1.0 ng of CEA per 10^6 cells every 24 h, whereas CEA was not detected in supernatants derived from the parental MC-38 cells. Under similar conditions, the human colon adenocarcinoma cell line, LS-174T, secreted 71.4 ± 8.1 ng of CEA.

The molecular size of the CEA produced by C15-4.3 cells was analyzed by Western blotting. Cultured cells (0.75–1.6 × 10^6 cells) were washed with PBS, resuspended in SDS-PAGE sample buffer, and heated at 100°C for 5 min prior to electrophoresis. As shown in Fig. 3A, two bands were observed with C15-4.3 cell extracts, one major band migrating the same as the reference purified human CEA and a second weaker band appearing at M, ~50,000. The faster migrating band was absent in other C15-4.3 cell extracts (data not shown), suggesting that it may have represented a breakdown product. CEA-expressing tumor cells grew at a faster rate in transgenic mice; all of these mice consistently developed progressively growing tumors. One nontransgenic mouse had a tumor that grew transiently and regressed by day 14. On day 28, the sizes of the tumors growing in transgenic and nontransgenic mice were 418 ± 91 and 179 ± 47 mm^3, respectively (P = 0.03). The latter experiment was repeated, and a similar difference in growth rate of C15-4.3 cells was observed between transgenic and nontransgenic mice. To determine whether the C15-4.3 cells maintained expression of CEA while growing in transgenic mice, tumors were removed from 3 mice 31–43 days after implantation. The tumors were enzyme-digested, and the

![Graph](https://example.com/graph.png)

Fig. 4. Expression of CEA on CEA-transfected MC-38 colon carcinoma cells, as measured by flow cytometry. Clone C15-4.3 was stained with T84.66 anti-CEA MAb (——) or control IgG (· · · · · · · · · · · · · ·). Cells were also treated with PI-PLC prior to staining with T84.66 (shaded area). Bound antibody was detected with fluorescein-labeled antimouse IgG.
released cells were placed in tissue culture using medium lacking G418. Flow cytometry was then carried out on the cultured cells 3–8 days later. All three cultures stained positive for CEA (94–99%), a percentage similar to that obtained with C15-4.3 cells maintained in continuous culture (data not shown). However, the density of CEA expression appeared to be reduced because the mean fluorescence intensity for the in vivo passaged cells was 63–73% of that observed for the cells grown in continuous culture. The reduction in the intensity of CEA expression may result from a loss of a subpopulation of highly positive cells in vivo, a slow adaptation of the latter cells to growth in vitro because the assay was carried out 3–8 days after removal of the tumors from the animals or a reduction in expression level by the general cell population. Nonetheless, these results demonstrate that propagation of the C15-4.3 cells in vivo did not result in a complete or rapid loss of CEA expression.

**Anti-CEA Antibody in Transgenic and Nontransgenic Mice with Progressively Growing C15-4.3 Tumors.** When tumor-bearing mice from the previous experiment required euthanasia, blood was collected to test by ELISA for the presence of antibody against CEA. The day after implantation for removal of a blood sample ranged between 28 and 36 and 28 and 59 days for transgenic and nontransgenic mice, respectively. The shorter residence time of tumors in transgenic mice resulted from their faster growth rate in these mice. The day after implantation for removal of a blood sample ranged between 28 and 36 and 28 and 59 days for transgenic and nontransgenic mice, respectively. The shorter residence time of tumors in transgenic mice resulted from their faster growth rate in these mice. The day after implantation for removal of a blood sample ranged between 28 and 36 and 28 and 59 days for transgenic and nontransgenic mice, respectively. 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**DISCUSSION**

A major limitation in immunotherapy studies of human tumor antigens is the general lack of appropriate preclinical models. Clinical studies can be difficult to implement, particularly when a clear understanding of the potential efficacy, limitations, and safety of an immunotherapeutic strategy is not available from relevant animal investigations. Immune-mediated therapies directed at tumor self-antigens such as CEA also deal with a balance between desirable antitumor responses and unwanted autoimmune reactions. However, mice carrying a transgene for a human tumor self-antigen may provide a more acceptable experimental model in which knowledge about the above issues can be enhanced prior to initiating clinical trials. With respect to CEA, two laboratories have previously described the preparation of CEA transgenic mice, differing chiefly in the type of CEA DNA used (31, 36).

Hasegawa et al. (36) were the first to create a CEA transgenic mouse line by using a full-length CEA cDNA fragment containing the SV40 early promoter. Northern and Southern blotting revealed expression of CEA in all tissues examined, including the brain, thymus, lung, spleen, liver, kidney, and colon. The ectopic expression of CEA by the brain, thymus, spleen, liver, and kidney was not unexpected because of the incorporation of the SV40 promoter. However, some cellular specificity of expression was evident because only the epithelial cells in the lung and colon were positive by immunohistochemical staining.

**Fig. 6. Anti-CEA antibody in mice bearing CEA-transfected MC-38 tumors (C15-4.3 clone). Serum obtained from nontransgenic (n = 6; A) or transgenic (n = 5; B) mice was assayed for the presence of anti-CEA antibody by ELISA beginning at 1:100 dilution. □ (A), reaction produced by nontransgenic C57BL/6 mouse serum.**

**Fig. 5. Growth of CEA-transfected MC-38 cells in transgenic mice. Clone C15-4.3 cells (2.5 × 10⁶) were implanted s.c. into transgenic (□; n = 5) or nontransgenic (○; n = 6) mice.**
Schrewe et al. (19) cloned the complete gene for CEA, including flanking regulatory elements, and showed that this genomic clone conveyed cell-type specific expression of CEA. Furthermore, transfection of this cosmids clone into CHO cells resulted in CEA expression, demonstrating that the factors responsible for activation of the CEA gene were conserved between humans and rodents (37). This prompted Eades-Perner et al. (31), followed by ourselves in this study, to prepare transgenic mice with genomic CEA in hopes that the tissue-specific expression observed in humans would be also reflected in transgenic mice. The former laboratory established four independent lines having a C57BL/6 × CB6 background, all of which contained intact copies of the cosmids insert. A restricted pattern of CEA expression was observed in all four lines, as determined by Northern blot and immunohistochemical analysis. However, ectopic appearance of CEA with respect to its expression in adult humans (6) was observed in the esophagus, small intestine, trachea, and lung of all transgenic lines. In the line 18 mouse described here, CEA was only detected by immunohistochemistry in the large intestine, cecum, and stomach, more closely approximating the adult human pattern. Because the same MAb and similar tissue processing procedures were used in our studies as those of Eades-Perner et al. (31), differences in levels of gene expression most likely account for the discordance in tissue expression between the mice derived in the two studies. The levels of CEA in the colons of transgenic mice produced by Eades-Perner et al. (31) were over 50- and 10-fold higher than that of the line 18 mouse and humans, respectively. Similarly, fecal content of CEA in the former mice was 2–4 times higher than that of human feces, whereas line 18 mouse feces contained 100-fold less CEA. The high level of CEA production was not related to copy number among the four lines studied by Eades-Perner et al. (31), nor was it related to the line 18 mouse because the latter had twice the number of copies of the mouse line with the highest copy number in their study. The former transgenic lines also had elevated levels (14–30 ng/ml) of circulating CEA, whereas it was below detection limits in the serum from line 18 mice, as it is in normal human serum. Although differences between transgenic mice and humans in posttranslational regulatory mechanisms or turnover rates may explain the lack of correlation in CEA expression with copy number or CEA mRNA levels (31), our findings with the line 18 mouse suggest that the level of CEA transcription may also be important and is perhaps influenced by site of insertion. Nonetheless, the pattern of CEA expression of the line 18 mouse confirms previous findings (31) that the regulatory mechanisms contributing to tissue-specific CEA expression in humans are conserved in mice.

The CEA produced by the colon of line 18 mice was similar in size to that of CEA purified from human colonic adenocarcinomas, demonstrating that the CEA molecule was processed normally. However, it is likely that differences exist in glycosylation of CEA in mice as compared to humans (38). The CEA produced by the colon epithelium of line 18 mice was predominantly localized to the cells in the apical regions of the crypts, similar to the pattern observed in human colons (32). In contrast, Eades-Perner et al. (31) found that CEA was expressed throughout the lumen of the colonic crypts in their four transgenic lines, again demonstrating heightened CEA expression in these mice.

Depending upon the intended study, the high level of CEA expression by the four lines produced by Eades-Perner et al. (31) may be less desirable than a transgenic mouse with lower antigen expression. As a potential model for antibody targeting studies, high circulating levels of CEA could result in the formation of complexes with an injected antibody, as has been observed clinically with radioantibodies (39). This could compromise the use of the mouse model because serum CEA levels are rare in early-stage colorectal cancer, although 95% of colorectal cancers express CEA (40). Aberrant expression of antigen in ectopic sites could also enhance inappropriate localization of antibody to these locations. Furthermore, as discussed below, vaccine development may be more arduous because of difficulties in breaking tolerance to CEA and may not truly mimic requirements to breach the tolerant state in humans.

Besides potential limitations caused by elevated levels of CEA expressed by the transgenic lines generated by Eades-Perner et al. (31), our interest in producing another CEA transgenic mouse line was motivated by the desire to have a syngeneic model for tumor immunotherapy. For a tumor system, a syngeneic, transplantable colon carcinoma tumor, MC-38 (41), was transplanted with human CEA cDNA. One clone (C15-4.3) contained a full-length copy of the CEA cDNA, expressed a high level of CEA on its surface, and secreted antigen at a rate that was about one-third the level of antigen produced by the LS-174T human colon adenocarcinoma cell line. This clone also expressed antigen that was similar in molecular size to that of CEA purified from human colonic tumors and was attached to the cell surface via a phosphatidylglycerol glycan linkage, which is typical for CEA. The transfected MC-38 cells produced progressively growing tumors following implantation into transgenic mice. Importantly, expression of CEA similar to that of cells under continuous cell culture was maintained throughout tumor growth in vivo.

Other groups have previously described the production of CEA-positive rodent colon carcinomas (42, 43). The CEA-transfected MC-38 cell line, MC-38-cea-2 (43), has been used in several experimental immunotherapy studies (44–48). However, about one-half of the CEA gene was missing in these cells, and this cell line was apparently selected based on its superior growth properties in normal mice, as compared to transfectants harboring the complete CEA gene (49). An underlying anti-CEA immune response appears to be the reason for the retarded growth of the latter cells in normal mice (49). Likewise, we found that the C15-4.3 cells did not grow as well in nontransgenic as did transgenic mice. Pfeigrin et al. (42) described the growth of several CEA-transfected rat colon carcinoma lines. They reported that, in normal rats, the CEA-positive cell lines grew at a slower rate than did nontransfected cells, and some of the highest CEA expressers regressed. Thus, although other approaches exist for the preparation of tumor models in transgenic mice, such as direct chemical induction or mating with mice with a high tumor incidence, the use of CEA-transfected tumor cells, as shown here, appears to provide a suitable alternative. Unlike the use of transfectants bearing a foreign antigen in normal mice, transfectants expressing authentic antigen grow well in transgenic mice.

The growth in vivo of CEA-transfected MC-38 cells was accompanied by the appearance of antibody in nontransgenic but not transgenic mice. Despite the presence of a humoral response, the C15-4.3 produced progressively growing tumors in nontransgenic mice, albeit at a slower rate than in transgenic animals. The role that anti-CEA antibody has in inhibiting tumor growth, as well as contributions of cell-mediated components to an antitumor response in the former mice, are not known. More importantly, the failure of transgenic mice to develop anti-CEA antibody under these conditions shows that the line 18 mice are tolerant to CEA. Ongoing studies are currently evaluating the extent to which tolerance to CEA in the latter mice can be assigned to the B- and/or T-helper cell compartments. Adelstein et al. (50), in experiments carried out in hen egg lysozyme transgenic mice, showed that T cells were tolerant, irrespective of the level of lysozyme expressed. B-cell tolerance did not occur when the antigen concentration in the serum was below 0.1 nM. On the basis of these observations, it appears likely that B-cell tolerance to CEA in line 18...
mice is absent or weak and certainly less than that of the lines
developed by Eades-Perner et al. (31). Because the latter mouse lines
express CEA to much higher levels than that found in either line 18
mice or in humans, it may be more difficult to break tolerance to CEA
in these mice. Nonetheless, the tolerant state of the latter mice presents a
more demanding challenge than do nontransgenic animals for
examing the ability of vaccines to elicit anti-CEA responses that
may also be therapeutically effective. We have shown here that
syngeneic transplantable tumor transfectants expressing high levels
of authentic CEA can be prepared and that these grow well in
transgenic mice. Thus, these CEA transfectants provide a means to
begin testing the antitumor potency of vaccines, whereas other
mouse models are under development. Because line 18 mice also
express antigen in normal tissue locations, as is found in humans,
it is possible to search for the appearance and properties of auto-
immune reactions against these tissues once tolerance to CEA is
circumvented. Finally, the line 18 transgenic mouse model provides an
alternative for antibody targeting studies, one in which experiments can be carried out in an immunocompetent host. The
analysis of immunocomjugates in which their activities at least
partly depend on an intact immune system would be enhanced in
transgenic mice (51, 52).

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