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ORIGINAL ARTICLE

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Induction of cytotoxic T cells and their antitumor activity in mice transgenic for carcinoembryonic antigen

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Abstract In order to develop immunotherapy strategies that are based on eliciting immune responsiveness to the self-antigen, human carcinoembryonic antigen (CEA), we examined whether cytotoxic T lymphocyte (CTL) activity against CEA could be elicited in CEA-transgenic and nontransgenic mice. CEA-transgenic [C57BL/6-TGN(CEAGe)18FJP] and nontransgenic mice were primed with CEA-transfected syngeneic fibroblasts in combination with *Corynebacterium parvum*. Spleen cells from immunized mice were cultured with irradiated syngeneic MC-38 colon carcinoma cells transfected with CEA (MC-38.CEA) as stimulators prior to the measurement of CTL activity. Primed nontransgenic spleen cells showed augmented CTL activity against MC-38.CEA cells as compared with control parental MC-38 cells, nontransfected or transfected with vector only. Moreover, primed CEA transgenic spleen cells showed augmented CTL activity against MC-38.CEA cells that was similar to that observed in nontransgenic mice. All CTL clones derived from either transgenic or non-transgenic mice showed cross-reactivity with MC-38 cells expressing the CEA-related antigen, nonspecific cross-reacting antigen, but not biliary glycoprotein. CEA-specific CTL clones were not identified. Adoptive transfer of cloned CTL resulted in inhibition of MC-38.CEA but not MC-38.BGP tumor growth. Tumor cures were elicited in mice treated with a combination of cloned CTL and cyclophosphamide. Histopathological examination of CEA-expressing colons from either immunized mice or recipients of cloned CTL did not reveal any autoimmune reactions. These studies demonstrate that CTL recognizing cross-reactive class I epitopes on the CEA molecule can be induced in transgenic mice. The expression of these epitopes on tumor cells creates effective targets for CTL in vivo without inducing adverse reactions in CEA-expressing normal tissues. Since anti-CEA CTL have been generated in humans, CEA-transgenic mice may be a useful model to study vaccines that are based on CTL effector mechanisms.

Key words Carcinoembryonic antigen · Transgenic mice · Cytotoxic T cell · Tolerance · Adoptive therapy

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Introduction

The emergence of human tumor antigens that have been identified with autologous T cells or antibodies derived from cancer patients has stimulated efforts to boost immune reactivity against these antigens for immunotherapy [2, 18, 22, 38]. Many of these antigens are products of normal genes and can be expressed by normal tissues as well, predominantly in a tissue-specific manner. Examples of these self-antigens that are targets for recognition by T cells include MAGE, BAGE, GAGE, gp100, tyrosinase, and Melan-A/MART-1 [16]. MHC-class-I-restricted peptide epitopes have been defined for all of the latter molecules, and immunization of melanoma patients with a MAGE peptide has been found to elicit melanoma-reactive cytotoxic T lymphocytes (CTL) with peptide specificity [32]. Besides tissue-specific self-

antigens, other self-antigens on human tumor cells that have a wider normal tissue distribution can also stimulate T cell or antibody responses [16].

Tissue-specific self-antigens expressed on human tumor cells against which there is no apparent immune reactivity in cancer patients may also serve as antigen targets for vaccines. The carcinoembryonic antigen (CEA) is a member of the immunoglobulin gene superfamily that is expressed in a high percentage of human colon, breast, and lung carcinomas [9, 12, 37, 43, 49, 51]. Because of its limited normal tissue distribution, radiolabeled antibodies against CEA have been studied experimentally and clinically for immunodiagnosis and therapy [19, 39, 40, 50]. Until recently, the evidence for immune responsiveness in humans to CEA was conflicting [17, 24, 26, 33]. However, clinical studies with anti-idiotype antibodies [14] and in vitro experiments with peripheral blood lymphocytes from both cancer patients and normal individuals [3, 47] have demonstrated that the human T and B cell repertoire can recognize CEA. Thus, CEA may offer a suitable self-antigen target for eliciting immune responsiveness against a variety of cancer types that express this molecule.

Recently, we have produced a mouse line that carries the transgene for human CEA [6]. This CEA-transgenic mouse line expresses CEA in a tissue-specific manner, as observed in humans, where the colon is the main site of CEA production. In contrast to nontransgenic mice, it was further observed that CEA-transgenic mice failed to develop antibodies against this antigen following implantation of a syngeneic tumor transfected with the CEA gene. Thus, it appears that this particular transgenic line may provide a preclinical system in which to effect an immune responsiveness to CEA and to examine the outcome of the induced reactivity in terms of tumor rejection as well as adverse effects on antigen-positive normal tissues. In the present report, we describe studies that evaluate whether CTL reactive with CEA can be elicited in CEA-transgenic mice, and if the induced CTL have antitumor properties in vivo. These experiments were intended to begin defining tolerance to CEA in these mice for comparison to humans and as a setting for future preclinical vaccine studies.

Materials and methods

Mice

The CEA-transgenic line, C57BL/6J-TgN(CEAGe)18FJP [6], was maintained at the Vanderbilt Animal Care Facility. C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, Me.). All animal studies were carried out under the approval of the Institutional Research Animal Care Committee. Housing and care of animals was consistent with the PHS Policy, the Guide for the Care and Use of Laboratory Animals, the Animal Welfare Act and other applicable state and local regulations.

Cell lines and transfectants

The C57BL/6 SV-40-transformed fibroblast cell line (TIB88) was obtained from the American Type Culture Collection (ATCC, Rockville, Md.). It was transfected by lipofection with CEA cDNA subcloned into the mammalian expression vector, pHβA pr-1-neo, according to procedures described elsewhere [6], and is designated fibroblasts.CEA. The chemically induced colon adenocarcinoma cell line, MC-38 [8], was obtained from Dr. Steven A. Rosenberg (National Cancer Institute, Bethesda, Md.). It was transfected with CEA, biliary glycoprotein (BGP), or nonspecific cross-reacting antigen (NCA) cDNA (provided by Dr. John E. Shively, City of Hope, Duarte, Calif.) as described for fibroblasts. The CEA-expressing (clone C15-4.3), BGP-expressing (clone D1), and NCA-expressing (clone 4-4) MC-38 transfectants were designated MC-38.CEA, MC-38.BGP, and MC-38.NCA respectively. MC-38 cells were also transfected with the empty vector to serve as a vector control, designated MC-38.PHB. All cell lines were cultured in Dulbecco's modified Eagle's medium (Irvine scientific, Santa Ana, Calif.) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin-G, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 1 mM non-essential amino acids, and 2 mM glutamine. Transfectants were maintained in culture in the presence of 500 μg/ml of G418 sulfate (Gibco, Grand Isalnd, N.Y.).

Flow cytometry

The cell-surface expression of antigen on transfectants was determined by flow cytometry using CEA-specific and CEA-cross-reactive mAb for antigen detection. Flow-cytometry analysis was carried out by a FACS Calibur (Becton Dickinson, Mountain View, Calif.) as previously described [41].

Immunization with CEA-expressing fibroblasts

Mice received four applications (50 μl) of 1.5×10^6 fibroblasts.CEA cells suspended in 100 μg *C. parvum* (Ribi Immunochem Research Inc., Hamilton, Mont.), injected i.d. in the flank at 3-week intervals.

In vitro stimulation of spleen cells

Spleen cells were harvested and suspended in RPMI-1640 medium (Irvine Scientific) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 2 mM glutamine, 10 mM HEPES, and 50 mM 2-mercaptoethanol (CTL medium). Spleen cells (6×10^6 cells in 2 ml/well) were cultured in 24-well plates (Corning Inc., Corning, N.Y.) at a 20:1 ratio with irradiated (40 Gy) MC-38.CEA cells for 5 days at 37 °C in a humidified 5% CO_2 atmosphere.

Generation of CTL lines and clones

After the initial in vitro stimulation, spleen cells were maintained in 24-well plates in CTL medium containing 5% concanavalin A supernatant (Collaborative Research Inc., Bedford, Mass.). Spleen cells were restimulated with irradiated (50 Gy) MC-38.CEA cells biweekly. Normal mouse spleen cells, cultured for 3 days in the presence of 25 μg/ml lipopolysaccharide (*E. coli* 0111:B4; Difco, Detroit, Mich.), were used after irradiation (20 Gy) as a source of feeder cells at a 3:1 responder:feeder cell ratio. After long-term cultures had been established, the cell lines were cloned by limiting dilution. Clones were maintained in CTL medium containing 5% concanavalin A supernatant and were routinely restimulated biweekly with irradiated MC-38.CEA cells.

Cytotoxic assay

Cytotoxic activity was measured by a 4-h chromium(^{51}Cr)-release assay using the labeled syngeneic tumor cells described above. Target cells (3×10^6– 6×10^6) were labeled with 200 μCi ^{51}Cr

(DuPont, Boston, Mass.) for 1 h at 37 °C. The labeled target cells were washed and resuspended to 1×10^5 cells/ml in CTL medium, and 100 µl was mixed with an equal volume of effector cells at various ratios in triplicate in 96-well U-bottomed tissue-culture plates (Corning Inc.). After incubation for 4 h at 37 °C in 5% CO_2, supernatants were harvested using cotton wicks (Skatron Instruments Inc., Sterling, Va.), and the released radioactivity was determined with a gamma counter (Packard Instrument Co., Downers Grove, Ill.). The percentage specific ^{51}Cr release was calculated as: 100 × (experimental release − spontaneous release)/(total release − spontaneous release). Spontaneous release was determined using target cells without effectors while total release was determined with target cells exposed to 1% Triton X-100. Spontaneous release and the variation between replicates were less than 10%.

Antibody blocking experiments with anti-CD8 (53-6.72) or anti-CD4 (GK1.5) mAb were carried out by pre-incubating effector cells for 60 min at 37 °C. Blocking experiments with anti-H-2 D^b (28-4-8S) or anti-H-2 K^b (AF6 88.5.3) mAb were carried out by pre-incubating labeled target cells for 60 min at 37 °C. All antibodies used in blocking experiments were purified by protein G (Pharmacia, Piscataway, N.J.) chromatography from ascites fluid produced by hybridomas obtained from ATCC.

Adoptive transfer

Cloned T cells were cultured for 7–8 days in the presence of 60 IU/ml interleukin-2 and irradiated MC-38.CEA cells at a 1:1 ratio. CEA-transgenic mice, male or female, were implanted s.c. with 1×10^6 MC-38.CEA or MC-38.BGP cells 2 days prior to injection of cloned CTL. Mice were injected once i.v. with $(30\text{–}45) \times 10^6$ cloned CTL that were contained in 1.0 ml. For some experiments, 100 mg/kg cyclophosphamide (Sigma) was injected i.p. 6 h prior to CTL transfer. Tumor growth was monitored by caliper measurements in two perpendicular dimensions, and tumor volume was determined according to the formula:

$$\frac{(\text{short dimension})^2 \times (\text{long dimension})}{2}$$

Statistics

Results were subjected to Student's *t*-test. In addition, a restricted/residual-maximum-likelihood(REML)-based repeated-measures model (mixed model analysis) with various covariance structures was used. The latter procedure is a repeated-measures analysis for correlated continuous-outcome variables, and is designed for longitudinal data analysis with multiple observable vectors for the same subject. SAS version 7.0 was used for all analyses.

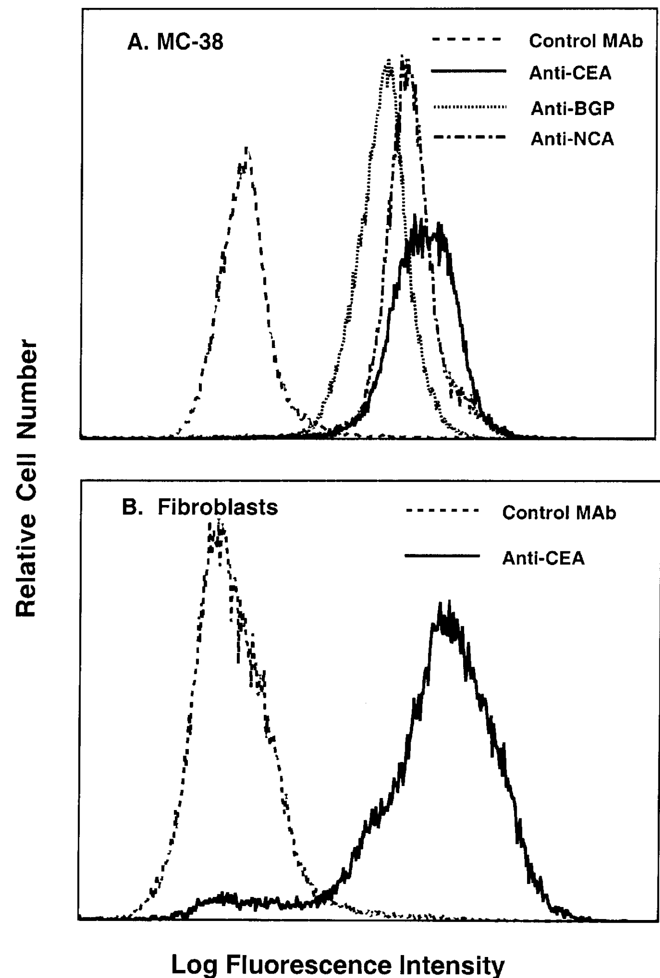

Fig. 1A, B Expression of carcinoembryonic antigen (*CEA*), biliary glycoprotein (*BGP*), or non-specific cross-reacting antigen (*NCA*) on transfected MC-38 cells or fibroblasts measured by flow cytometry. **A** MC-38 cells transfected with CEA, BGP, or NCA. (——) MC-38.CEA cells (clone C15-4.3) stained with CEA-specific T84.66 mAb; MC-38.BGP cells (clone D1) (···) or MC-38.NCA (clone 4-4) (– · –) stained with CEA-cross-reactive T84.1 mAb. (– – –) MC-38.CEA, MC-38.BGP, or MC-38.NCA cells stained with negative control mAb. **B** Fibroblasts transfected with CEA and stained with T84.66 mAb (——) or negative control mAb (– – –)

Results

Expression of CEA, BGP, or NCA by transfectants

MC-38 cells were transfected with either *CEA*, *BGP*, or *NCA* whereas only *CEA* was placed into fibroblasts. Antigen expression was evaluated by flow cytometry utilizing the CEA-specific T84.66 mAb to detect CEA while the CEA-cross-reactive antibody, T84.1, was used to demonstrate BGP or NCA [34]. Both the MC-38.CEA and the fibroblasts.CEA cells displayed high levels of cell-surface CEA with more than 90% of the cells positive (Fig. 1A, B). Over 95% of the MC-38.BGP and the MC-38.NCA cells were also positive although the level of expression of these antigens was somewhat lower than that of CEA on MC-38.CEA cells (Fig. 1A). The CEA-specific T84.66 mAb failed to stain the MC-38.BGP or MC-38.NCA cells. The flow-cytometry results of the transfected cell lines are summarized in Table 1. The transfected cells were routinely maintained on medium containing G418, and no change in antigen expression was observed with the four transfectants.

CTL activity in CEA-transgenic mice

Following immunization of C57BL/6 mice with a single s.c. injection of 5×10^5 irradiated MC-38 cells, over 90% of the mice were resistant to a lethal challenge of a similar number of cells (data not shown). The latter experiments demonstrated the immunogenicity of MC-

38 cells and indicated the need to avoid the generation of CTL against endogenous tumor-specific transplantation antigens that could accompany immunization with CEA-transfected MC-38 cells. Therefore, mice were hyperimmunized with CEA-transfected fibroblasts using *C. parvum* as an adjuvant while CEA-transfected MC-38 cells were subsequently used for restimulation in vitro. The goal of this immunization was to determine if CTL with specificity for CEA could be induced in transgenic mice. Nontransgenic mice were initially immunized to serve as a positive control for experiments with transgenic mice.

Although immunization with a control antigen was not carried out, spleen cells obtained from unimmunized nontransgenic and CEA-transgenic mice did not show specific killing activity against MC-38, MC-38.PHB, and MC-38.CEA cells following in vitro stimulation (data not shown). Occasionally, nonspecific killing activity was observed with either mouse type, unimmunized or immunized, as measured by lysis of YAC-1 cells (data not shown). However, spleen cells from immunized non-

Table 1 Flow cytometry of MC-38 or fibroblast lines transfected with carcinoembryonic antigen (CEA) or CEA-related antigens. The CEA-specific T84.66 and the CEA-cross-reactive mAb T84.1 were used to measure the expression of CEA and CEA-related antigens respectively. Values in parentheses show the mean fluorescent intensity. *ND* not done

Cell line	Gene transfected	Cells staining positive (%) for antibody:		
		MOPC 21	T84.66	T84.1
MC-38.CEA	*CEA*	2.3 (130)	99.6 (319)	ND
MC-38.BGP	*BGP*	2.1 (125)	ND	97.3 (255)
MC-38.NCA	*NCA*	2.4 (129)	ND	99 (290)
Fibroblast.CEA	*CEA*	2.9 (100)	93.7 (300)	ND

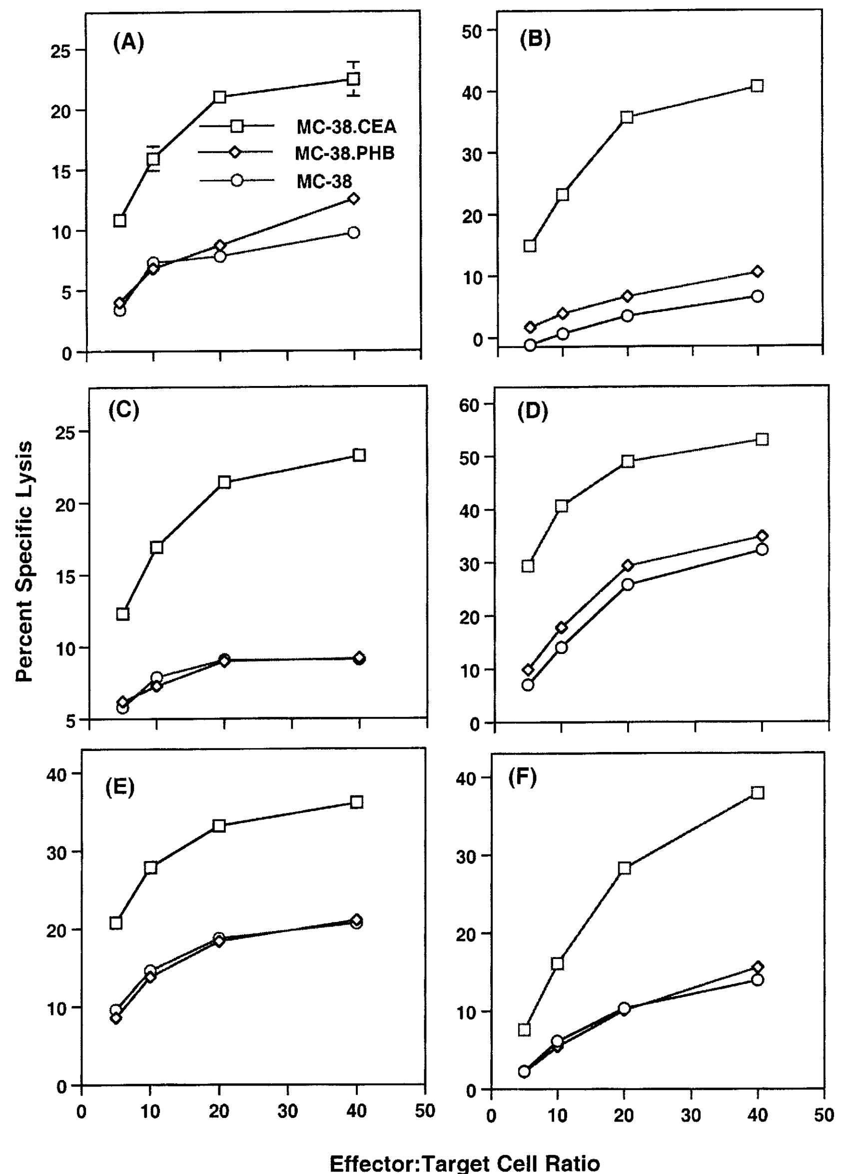

Fig. 2A–F Cytotoxic T lymphocyte (CTL) activity in nontransgenic and CEA transgenic mice immunized with fibroblasts.CEA. CTL measurement in three nontransgenic mice (**A**, **C**, **E**) or three transgenic mice (**B**, **D**, **F**). Target cells were MC-38 (○), MC-38.PHB (◇) and MC-38.CEA (□) cells

transgenic mice showed specific lysis of MC-38.CEA cells after one in vitro stimulation (Fig. 2). From the three mice depicted, lysis of MC-38.CEA cells was between 20% and 35% at a 20:1 effector:target cell ratio. At this ratio, lysis of MC-38.CEA targets was approximately 15% greater than that of the control MC-38.PHB or MC-38 targets. The lysis of MC-38.CEA tumor cells was significantly greater ($P = 0.0003$) than that obtained with either the MC-38 or MC-38.PHB targets with all mice. There was no difference in lysis between the latter control targets. Similar results were obtained when three additional immunized nontransgenic mice were studied.

The above results demonstrated that immunization of nontransgenic mice with the fibroblasts.CEA was effective in priming for CTL specific for CEA. Although it is well-established that CEA is a potent stimulator of a humoral immune response in non-human animal species, these findings show that it can also serve as a target for CTL in mice. When this immunization procedure was applied to transgenic mice, similar specific killing of MC-38.CEA cells was also observed with spleen cells following one in vitro stimulation (Fig. 2). At the 20:1 ratio, killing of MC-38.CEA by CTL from transgenic mice ranged between 30% and 50%. The lysis of MC-38.CEA cells was 20%–30% greater than that of the control targets, MC-38 and MC-38.PHB, at the 20:1 ratio. As with nontransgenic mice, lysis of MC-38.CEA cells with spleen cells from all mice was significantly greater ($P < 0.0001$) than that obtained with the control targets. Spleen cells from an additional seven immunized transgenic mice produced similar results. In the experiment depicted in Fig. 2, the lysis of MC-38.CEA cells by the CTL from the three transgenic mice was significantly higher ($P = 0.02$) than that obtained with CTL from nontransgenic mice. However, analysis of additional nontransgenic mice did not reveal any differences in the specific killing of MC-38.CEA cells between nontransgenic and transgenic mice beyond the variability observed with different mice from the same line. With both mouse types, nonspecific killing of MC-38 and MC-38.PHB targets was very similar, demonstrating that CTL were not induced against any potential peptides derived from the aminoglycoside phosphotransferase conferring resistance to G418. Interestingly, CTL that specifically lysed MC-38.CEA cells did not show specific lysis of CEA.fibroblasts in the same assay. The basis for this difference is unknown.

Effector phenotype and H-2 restriction element of CTL derived from transgenic mice

Antibody blocking experiments with anti-CD8 or anti-CD4 mAb were performed in order to establish the effector cell phenotype responsible for specific killing of MC-38.CEA cells. CTL activity against MC-38.CEA cells was significantly inhibited by pre-incubation of effector cells with anti-CD8 but not anti-CD4 mAb (Table 2). This inhibition ranged from 74% to 89% at the 40:1 effector:target cell ratio shown while other ratios yielded similar results ($P < 0.05$). Likewise, antibody blocking experiments with anti-H-2 D^b or anti-H-2 K^b mAb were performed to determine the H-2 class I locus providing CTL specificity for MC-38.CEA. Inhibition of anti-CEA CTL activity was obtained by treatment with anti-D^b mAb of spleen cells from all three mice shown in Table 2. This inhibition ranged between 20% and 60% while the anti-K^b mAb failed to block lysis ($P < 0.05$). The low level of blocking that was observed in one mouse may have been due to the conditions of the assay coupled with possible higher-affinity T cell receptors on CTL from this mouse. Analysis of additional mice showed that killing of MC-38.CEA cells by CTL from transgenic mice was also inhibited solely by antibody against D^b (data not shown). Blocking studies were not carried out with spleen cells from nontransgenic mice.

Table 2 Phenotype and MHC-H-2 restriction element of cytotoxic T lymphocytes from CEA-transgenic mice. Effector or labeled target cells were preincubated at 37 °C for 30 min, at an E:T ratio of 40:1, with 10 μg/ml antibody prior to the addition of untreated labeled target or effector cells respectively. A 4-h chromium-release assay was then carried out. Results are shown ±SE; values in parentheses show the percentage inhibition of lysis compared to cultures without antibody

mAb treatment	Specific lysis (%) for mouse number:		
	1	2	3
None	23.4 ± 2.9	33.8 ± 6.8	18.5 ± 2.1
Anti-CD4	24.3 ± 1.5 (−4)	35.0 ± 8.2 (−4)	18.0 ± 0.5 (3)
Anti-CD8	6.1 ± 3.3 (74)	6.8 ± 1.5 (81)	2.1 ± 0.2 (89)
None	35.8 ± 4.3	42.0 ± 6.0	34.6 ± 1.0
Anti-K	38.0 ± 3.3 (−6)	42.7 ± 3.8 (0)	32.5 ± 2.8 (6)
Anti-D	14.4 ± 2.6 (60)	33.8 ± 4.4 (20)	15.1 ± 1.3 (56)

Cloning and specificity of anti-CEA CTL from transgenic mice

CTL lines were first produced and then cloned at limiting cell dilution utilizing MC-38.CEA cells for antigen restimulation, B cell blasts as feeder cells, and concanavalin-A-stimulated rat spleen cell culture medium supplement. A stable clone was derived from each of three nontransgenic and transgenic mice. All of these clones were $CD8^+$ by flow cytometry. Specific lysis of MC-38.CEA cells as compared to the antigen-negative parental cells was obtained with clones derived from both nontransgenic and transgenic mice. Figure 3 depicts the results of two clones generated from two transgenic mice. High lytic activity was obtained with both clones, as shown by the lysis observed at low effector:target cell ratios. A similar pattern of reactivity was obtained when additional clones from nontransgenic and transgenic mice were analyzed. Clear differences in the anti-CEA activity of CTL clones derived from nontransgenic and transgenic mice were not observed.

The CEA subgroup gene family is comprised of a number of genes that have 70%–90% sequence homology at the nucleotide level [45, 46]. We therefore examined whether anti-CEA CTL clones derived from both nontransgenic and transgenic mice were also able to recognize MC-38 cells expressing BGP or NCA, two major CEA cross-reacting antigens. Figure 3 shows the specificity of two clones derived from the two transgenic mice. Strong lysis of both MC-38.CEA and MC-38.NCA was obtained with both clones whereas neither lysed MC-38 cells expressing BGP. The lytic activity against MC-38.CEA and MC-38.NCA was significantly higher with CTL derived from both transgenic ($P < 0.0001$) and nontransgenic mice ($P < 0.0001$) than was the lysis of control target cells. A similar pattern of specificity was obtained when additional clones from nontransgenic and transgenic mice were analyzed. As depicted in Fig. 3, there was a tendency for some of the clones to generate stronger lysis of MC-38.NCA cells than of MC-38.CEA cells ($P = 0.003$). Although the cell-surface expression of CEA on MC-38.CEA cells was somewhat higher than the expression of NCA on MC-38.NCA cells, it is possible that the processing of NCA was more efficient than that of CEA for the generation of epitopes recognized by these clones.

Adoptive therapy of MC-38.CEA tumors with anti-CEA CTL clones

The above results demonstrated that expression of CEA by transfected murine tumor cells served as an effective target for killing by CTL clones. Additional experiments were carried out to determine if a CTL clone derived from transgenic mice also expressed antitumor activity in vivo following adoptive transfer. Figure 4 shows the growth of either MC-38.CEA (Fig. 4A) or MC-38.BGP (Fig. 4B) following injection of transgenic mice with the 3H1B CTL clone or phosphate-buffered saline (PBS). The CTL clone induced a significant inhibition of MC-38.CEA tumor growth as compared to the growth of the latter cells after injection of PBS ($P < 0.0008$). Of the eight mice injected with CTL, two remained tumor-free whereas all the control mice developed progressively growing tumors. However, tumor inhibition but no cures were obtained in two additional experiments. The

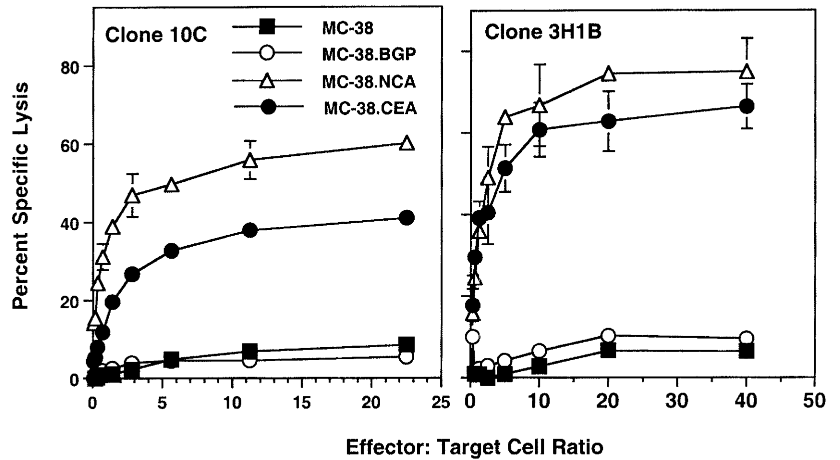

Fig. 3 Reactivity and specificity of anti-CEA CTL clones derived from two transgenic mice. Clones 10C and 3H1B were reacted against MC-38 (■), MC-38.CEA (●), MC-38.BGP (○), and MC-38.NCA (△) targets

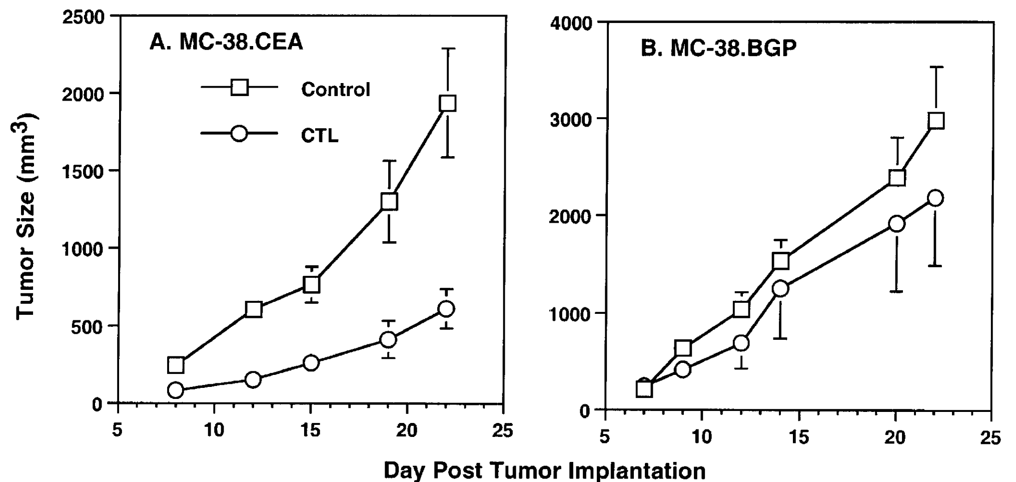

Fig. 4A, B Adoptive therapy with anti-CEA T cell clone. **A** Transgenic mice were injected i.v. two days after s.c implantation of MC-38.CEA cells with 44×10^6 3H1B cloned T cells (○) or phosphate-buffered saline (PBS; □); eight mice/group. **B** Transgenic mice were injected i.v. 2 days after s.c. implantation of MC-38.BGP cells with 43×10^6 3H1B cloned T cells (○) or PBS (□); eight mice/group

mean survival of mice with MC-38.CEA tumors was significantly greater ($P = 0.001$) following administration of the CTL clone (32 ± 2 days) than was survival after PBS injection (20 ± 2 days). In order to determine the specificity of the antitumor effect induced by the 3H1B clone, adoptive transfer was also carried out in mice implanted with MC-38.BGP tumor cells (Fig. 4B). The growth of the latter tumor cells was slightly slower after transfer of the 3H1B CTL clone but this was not significantly different from that of mice injected with PBS. The survival of mice bearing MC-38.BGP tumors was also not significantly different in animals injected with CTL (21 ± 3 days) or PBS (16 ± 3 days). None of the mice bearing MC-38.BGP tumors survived after transfer of the T cell clone. These findings demonstrate that non-specific effects attributed to transfer of the 3H1B clone had only a minor role in the inhibition of MC-38.CEA tumor cells. Similar experiments with the MC-38 tumor cells expressing NCA were not carried out.

Administration of cyclophosphamide can augment the antitumor properties of adoptively transferred CTL by several possible mechanisms [4]. An experiment was carried out to determine if the efficacy of the adoptively transferred 3H1B clone could be enhanced by cyclophosphamide. As shown in Fig. 5, administration of either CTL or cyclophosphamide alone inhibited tumor growth significantly more than injection with PBS ($P < 0.001$). There was no difference between CTL and cyclophosphamide alone in the inhibition of tumor growth. Combined treatment with CTL and cyclophosphamide was more effective in inhibiting tumor growth than CTL ($P < 0.04$) or cyclophosphamide ($P < 0.003$) alone. The combination of the T cell clone with cyclophosphamide was markedly effective in eliciting tumor cures (Fig. 6). Over 80% of tumor-bearing mice treated with the latter combination survived longer than 80 days whereas no cures were obtained in mice treated with CTL alone, and only one mouse survived after CY treatment only. Similar results were observed in a repeat experiment.

Autoimmune reactions

The main site of CEA expression in the transgenic mice used in this study is in the colon, wherein approximately 20% of the epithelial cells are positive [6]. Histopatho-

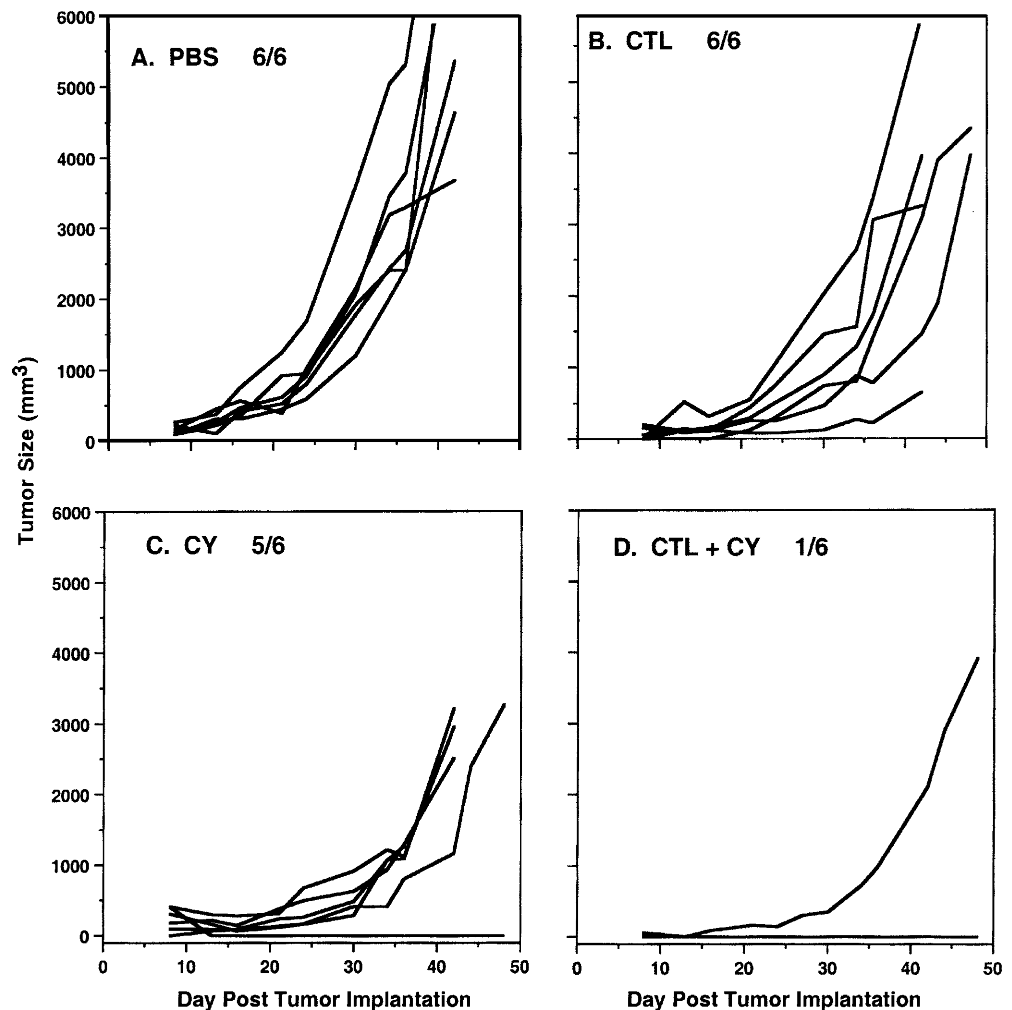

Fig. 5A–D Effect of cyclophosphamide (*CY*) on tumor growth following adoptive therapy with a T cell clone. Transgenic mice were injected 2 days after s.c. implantation of MC-38.CEA cells with (**A**) PBS, (**B**) 45 × 10^6 3H1B cloned T cells, (**C**) 100 mg/kg CY i.p., (**D**) 100 mg/kg CY i.p. followed 6 h later by the i.v. injection of 45 × 10^6 3H1B cloned T cells. Fractions refer to the number of mice with tumors over the total number of mice injected. Six mice/group

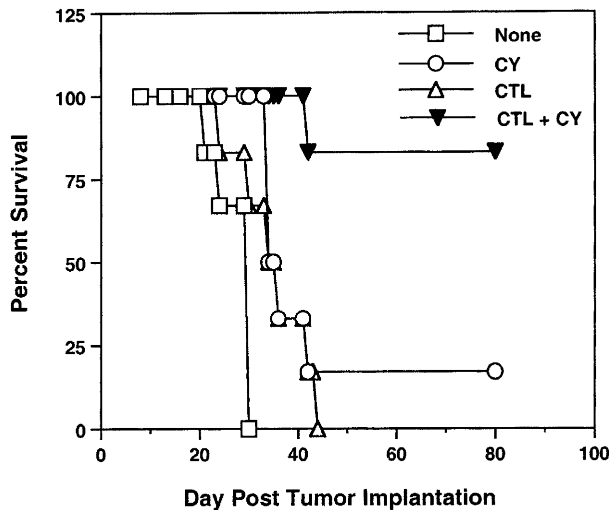

Fig. 6 Effect of cyclophosphamide (*CY*) on survival following adoptive therapy with an anti-CEA T cell clone. Transgenic mice were injected 2 days after s.c. implantation of MC-38.CEA cells with 100 mg/kg CY i.p. followed 6 h later by the i.v. injection of 45×10^6 3H1B cloned T cells. Mice were untreated (□) or treated with CY only (○), CTL only (△), or with CY plus CTL (▼). Six mice/group

logical examination was carried out on colons either from mice immunized with fibroblasts.CEA or from mice receiving adoptively transferred cloned 3H1B T cells. No alterations in morphology or in the incidence of intraepithelial lymphocytes were observed, and all animals remained healthy (data not shown). The latter was found even in mice that received six biweekly injections of $(30–40) \times 10^6$ cloned 3H1B T cells.

Discussion

Our studies demonstrate that anti-CEA CTL can be induced in the line 18 CEA-transgenic mouse following immunization with CEA-expressing cells. The main tolerogenic mechanism responsible for controlling T cells appears to be negative selection of self-reactive clones during their thymic maturation [23, 35]. It is possible that the apparent lack of tolerance to CEA in the $CD8^+$ CTL compartment of these transgenic mice may be due to deficient expression of CEA in the thymus during development, preventing clonal deletion. The tissue-specific expression of CEA lessens the likelihood of its presence in the thymus, which was found to be true for both embryonic and adult thymus of CEA-transgenic mice that were generated with the same genomic CEA clone as used for the preparation of line 18 mice [6, 11]. Likewise, CEA expression has not been observed in human embryonic thymus so that negative selection may not be significant for CEA in humans [34]. However, in transgenic models of T cell tolerance, sensitive reverse transcription/polymerase chain reaction techniques have revealed thymic expression of transgenes driven off from tissue-specific promotors [29]. Although we did not observe a consistent difference between nontransgenic and transgenic mice in their anti-CEA cytotoxicity, characterization and comparison of the epitope specificites of CTL clones derived from these mice is required in order to determine whether certain immunodominant clones may have been deleted in transgenic mice.

Other mechanisms have been proposed to explain why autoreactive T cells that have escaped clonal deletion do not become activated or have not become tolerant to self-antigens in the periphery [36]. For the most part, conditions of antigen exposure have a key role such as the absence of co-stimulatory signals (clonal anergy), sequestration of antigen (clonal ignorance), antigen amount and form [21, 28]. The anatomical confinement of CEA to the epithelial cells of the stomach and large intestine of line 18 mice would encourage a state of clonal ignorance, preventing either activation or tolerance induction [6]. Recently, a CTL response was observed against prostate-specific antigen in transgenic mice showing tissue-specific expression of this antigen [48]. Tumor-infiltrating lymphocytes derived from both nontransgenic and transgenic mice showed similar anti-prostate antigen cytotoxic activity. In the present studies, we also observed similar anti-CEA CTL activity in nontransgenic and transgenic mice. By contrast, widespread expression of a transgene encoding a mutated tumor antigen rendered $CD8^+$ T cells tolerant [1]. In the latter model, provision of a helper antigen on the same antigen-presenting cell resulted in the induction of CTL against the mutated tumor antigen.

Studies in several model systems have shown that autoreactive T cells that have escaped thymic deletion do not precipitate adverse reactions against normal tissues [21, 23]. It is not known if the CEA expressed by the transgenic mice described in this report is in a form recognizable by activated $CD8^+$ T cells. Immunized mice or adoptive-transfer recipients did not display any functional or histopathological abnormalities. Nonetheless, this self-antigen was not able to make $CD8^+$ cells tolerant nor induce immune reactivity. One possibility for this lack of immune reactivity to CEA is the absence of appropriate helper signals that are needed to stimulate $CD8^+$ T cells, as has been previously reported [1]. As shown here, anti-CEA CTL were induced following immunization with SV-40-transformed, CEA^+ fibroblasts using *C. parvum* as an adjuvant. Because of its well-established inflammatory and immunopotentiating properties [27], *C. parvum* could have elicited a cytokine and cellular surroundings capable of inducing $CD8^+$ T cells against CEA. Furthermore, the production of the SV-40 large T antigen by the transfected fibroblasts used for immunization may have contributed to the induction of anti-CEA CTL in transgenic mice. It has been shown that complexes between the T antigen and murine p53 resulted in the generation of autoantibodies to p53 while injection of p53 alone was without effect [10]. Although it appears that $CD8^+$ T cells are minimally tolerant to CEA but functionally silent in the line 18 transgenic mice, studies with antigen conjugates have demonstrated

that helper-dependent antibody responses were markedly suppressed in these mice (Primus FJ, Tompkins K, Dickson, KJ Anti-carcinoembryonic (CEA) antibody and antitumor response in CEA transgenic mice immunized with antigen-KLH conjugates; manuscript in preparation) [6]. This deficiency in CEA-reactive T helper cells supports the notion that lack of helper activity is largely responsible for the absence of activated $CD8^+$ T cells in transgenic mice. Antigen sequestration as well as the inability of the CEA^+ intestinal cells to provide second signals to activate cytotoxic T cells directly are other possibilities that may explain the absence of tolerance or immune reactivity in $CD8^+$ T cells to the CEA self-antigen [36].

Recent studies have shown that in vitro stimulation of peripheral blood lymphocytes from both cancer patients and normal individuals with CEA peptides can elicit $CD8^+$ CTL [3, 47]. In one of these studies [47], cancer patients were immunized with a recombinant CEA vaccinia virus vaccine, but it is uncertain whether vaccination elicited anti-CEA CTL in vivo [30]. Nonetheless, these studies with humans do demonstrate that $CD8^+$ CTL with specificity for CEA have not been negatively selected, similar to our findings described here with the line 18 transgenic mice. Furthermore, we have shown that anti-CEA $CD8^+$ T cells can be produced in vivo since their generation became evident following priming with CEA-transfected fibroblasts. Thus, it appears that the line 18 CEA-transgenic mouse will be valuable for the study of various CEA vaccine approaches that are based on the stimulation of $CD8^+$ T cells. In addition to evaluating antitumor responses, the potentially adverse consequences of eliciting and boosting anti-CEA $CD8^+$ CTL can be followed since the expression of CEA in normal tissues of line 18 mice parallels that found in humans [6]. This transgenic model will become even more beneficial for the latter purposes once combined with mice transgenic for major human HLA class I alleles [42, 44].

The CEA family is composed of several highly homologous members that have a much wider normal tissue distribution, particularly on granulocytes [45, 46]. We found that all the CTL clones derived from different nontransgenic and transgenic mice cross-reacted with one of the major related members of the CEA family, NCA. Although bulk cultures of human anti-CEA CTL obtained from a cancer patient did not show reactivity with target cells pulsed with a cross-reactive peptide [47], Celis et al. [3] did find cytotoxic activity against a peptide shared between CEA, BGP, and NCA. Whereas immunization with defined peptides can help avoid induction of cross-reactive CTL, this may not be true for vaccines that contain or will express the entire molecule [25]. Thus, future vaccine studies with the line 18 transgenic mouse may prove useful for identifying and monitoring the appearance of anti-CEA $CD8^+$ T cells cross-reactive with other members of the family, particularly in CEA/HLA class I double-transgenic mice.

Adoptive-transfer experiments demonstrated that anti-CEA CTL derived from transgenic mice are capable of specifically inhibiting the growth of MC-38.CEA tumor cells. There was also a synergistic effect on survival when adoptive transfer was combined with cyclophosphamide treatment. These studies demonstrate that CEA provides a suitable target for CTL in vivo since the MC-38.CEA tumor cells do not express NCA. However, we could not show that CTL epitopes specific for CEA are also effective targets since clones of this type were not identified. Recently, CTL were derived from another CEA transgenic line that was immunized with a recombinant vaccinia-CEA virus [20]. These CTL were produced following in vitro stimulation with a K^b-restricted CEA octomer peptide that has a conservative change in one residue at position two of a similar sequence in NCA ($NCA_{172-179}$). Since a related sequence is not present in BGP, this peptide may be a good candidate for the peptide specificity of the CTL clones described here. On the basis of known peptide-binding motifs for the MHC class I molecules K^b and D^b [13], one CEA-specific K^b dominant motif is present in the CEA sequence while there is none for the D^b locus. However, there are numerous CEA-specific sequences for both class I loci that contain secondary residues involved in peptide binding [5]. Further studies will need to resolve whether our conditions of immunization and/or cloning favored the selection of CTL that cross-reacted with NCA. Autoimmune reactions against CEA-expressing normal tissues did not ensue following adoptive transfer including mice in which cures were obtained. However, the lack of expression of NCA/CEA cross-reactive epitopes in normal tissues outside of the colon in CEA transgenic mice precludes any determination of adverse reactions induced by the transfer of cross-reactive CTL or by immunization with recombinant vaccinia [20].

We have shown that the line 18 transgenic mouse bears similarities to humans in its tolerance to CEA in the CTL compartment. Although it is too soon to judge how closely the CEA-transgenic mouse matches the immune responsiveness to CEA found in humans, the similarities described in this study suggest that this mouse model will be helpful in identifying optimal means by which reactivity to class-I-binding peptides can be induced in humans. Clinical studies already initiated serve to reinforce the belief that the CEA self-antigen is an appropriate target for the induction and augmentation of antitumor responses [7, 14, 15, 47]. Adverse consequences precipitated by autoimmune reactions have not been noted thus far in clinical trials with CEA vaccines, but it will be important to establish any long-term effects as well as to correlate treatment with antitumor responses that may emerge [25, 31]. Because of the expression of CEA in the large bowel of line 18 mice, it will be possible to determine if immune responsiveness to CEA can be induced in favor of tumor rejection.

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