

# Immunity to Murine Breast Cancer Cells Modified to Express MUC-1, a Human Breast Cancer Antigen, in Transgenic Mice Tolerant to Human MUC-1<sup>1</sup>

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## ABSTRACT

The high incidence of breast cancer in women and the severity of the disease have stimulated a need for improved and novel forms of therapy. The product of the *MUC-1* gene has been identified as a breast cancer-associated antigen in breast cancer patients. The gene has been cloned and sequenced. Transgenic mice were prepared that express human mucin and are naturally tolerant to the molecule, providing a unique opportunity to investigate immunotherapeutic strategies in experimental animals that might eventually be applied to breast cancer patients. A cell line (410.4) derived from a mouse mammary adenocarcinoma that arose in a BALB/c mouse was transduced with a retroviral vector (R1-MUC1-pEMSVscribe) that encoded MUC-1. After confirmation of the expression of human mucin, the cells (E3) were further modified by transduction with retroviral vectors encoding interleukin (IL)-2, IL-4, IL-12, or IFN- $\gamma$  to evaluate the effect of cytokine-secretion on the immunogenic properties of the cells in the MUC-1 transgenic mice. The results indicated that modification of the breast cancer cells to secrete IL-12 reduced and at times eliminated the tumorigenic growth properties of the cells. Under similar circumstances, progressively growing tumors formed in MUC-1 transgenic mice that received injections of unmodified E3 cells or with E3 cells modified to secrete IL-2, IL-4, or IFN- $\gamma$ . Immunity to breast cancer developed in MUC-1 transgenic mice that had rejected IL-12-secreting E3 cells because the animals were resistant to challenge with (non-cytokine-secreting) E3 cells. *In vitro* analyses confirmed the presence of T cell-mediated cytotoxicity toward the breast cancer cells in MUC-1 transgenic mice immunized with the IL-12-secreting cells. Our data obtained in a unique animal model system point toward an analogous form of therapy for breast cancer patients.

## INTRODUCTION

The immunogenic properties of highly malignant cells can be enhanced if the cells are genetically modified to secrete immune-augmenting cytokines. Neoplastic cells modified to secrete IL-4<sup>1–2</sup> (1–5), IL-4 (6, 7), granulocyte/macrophage-colony stimulating factor (8, 9), IFN- $\gamma$  (10, 11), or IL-12 (12, 13), among others (14), are rejected by histocompatible mice. Under analogous circumstances, progressive tumor growth occurs in mice that received injections of unmodified tumor cells. The growth of the tumor leads, eventually, to the animal's death. The immunity in mice rejecting the cytokine-secreting cells, mediated primarily by cellular immune mechanisms, is directed toward unique tumor-associated antigens expressed by both the cytokine-secreting as well as nonsecreting tumor cells. For this reason, cytokine-secreting tumor cells are under evaluation as potential immunotherapeutic agents (15–17).

Similar to other types of malignant cells, human breast cancer cells form unique antigens. Under appropriate circumstances, the antigens can become the targets of immune-mediated attack. One such antigen, breast cancer-associated mucin, has been characterized extensively and has been identified as a breast cancer-associated T-cell epitope (17). Mucin is the product of the *MUC-1* gene. Antibody-mediated (18) and both MHC-unrestricted (19, 20) as well as MHC-restricted class I CTL responses (21) directed toward mucin have been identified in patients bearing breast neoplasms that express MUC-1.

Mucin specified by the *MUC-1* gene is a type I membrane glycoprotein that is naturally expressed on the apical surfaces of most glandular epithelial cells, including the ductal epithelium of the breast. In contrast to mucin formed by nonneoplastic cells, MUC-1 expressed by breast cancer cells is aberrant; it is underglycosylated and therefore differs antigenically from mucin expressed by nonneoplastic cells (22, 23). Breast cancer-associated mucin is antigenically weak, because breast neoplasms that express MUC-1 proliferate without apparent inhibition in breast cancer patients. Thus, the development of techniques that can successfully increase the antigenic properties of mucin-expressing breast carcinomas could be of importance in the treatment of patients with the disease.

Here, we took advantage of the development of transgenic mice that have been genetically modified to express human MUC-1. The mice provide a unique opportunity to investigate the effect (on the immunogenic properties of the cells) of cytokine secretion by breast cancer cells modified to express human MUC-1. MUC-1 transgenic mice express human MUC-1 on glandular epithelial cells that produce mucin and are naturally tolerant to the molecule. The profile of expression and tissue distribution of mucin in MUC-1 transgenic mice as well as the differences in the glycosylation pattern seen between normal breast epithelial cells and the malignant breast cancer cells are analogous to those found in humans (24, 25).

To determine whether cytokine secretion affected the immunogenic properties of breast cancer cells in MUC-1 transgenic mice, a mouse breast cancer cell line (410.4) was first modified to express human MUC-1 (410.4 cells that express human MUC-1 are designated as E3 cells). The cells were then further modified to secrete IL-2, IL-4, IL-12, or IFN- $\gamma$ . The immunogenic properties of the cytokine-secreting cells were then tested in MUC-1 transgenic mice. The results indicated that unlike the other cytokines tested, immunization of the mice with E3 cells modified to secrete IL-12 resulted in generalized, long-term immunity toward the breast cancer cells and prolongation of survival of mice with breast cancer.

## MATERIALS AND METHODS

**Mouse Mammary Carcinoma Cell Lines.** A mouse mammary carcinoma cell line, 410.4, originally isolated from a single, spontaneous mammary tumor that arose in a BALB/c mouse that was cross-fostered on an mouse mammary tumor virus-carrying C3H mouse (26) was obtained from Bonnie Miller (Michigan Cancer Foundation, Detroit, MI). The cells were maintained under standard cell culture conditions (37°C in a humidified 7% CO<sub>2</sub>/air atmosphere) in DMEM supplemented with 10% FCS (Sigma Chemical Co., St. Louis, MO), 1 mM sodium pyruvate, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin

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<sup>4</sup> The abbreviations used are: IL, interleukin; mIL, murine IL; TNF, tumor necrosis factor; MFI, mean fluorescence index.

(growth medium; Life Technologies, Inc., Grand Island, NY). The cells were modified to express human mucin, as described previously (27). In brief, 410.4 cells were cotransfected (calcium phosphate coprecipitation) with plasmid DNA specifying MUC-1 (R1-MUC-1-pEMSVscribe) and PY3, a plasmid specifying the hygromycin resistance gene (28). Colonies of hygromycin-resistant transfected cells were pooled and used in the experiments described below. The cells were maintained in growth medium under standard cell culture conditions. B16 cells, a melanoma cell line, were kindly provided by Isiah Fidler (The University of Texas M. D. Anderson Cancer Center, Houston, TX). Like 410.4 cells, they were maintained as a cell line under standard conditions.

**Experimental Animals.** BALB/c mice, 6–8 weeks of age, were from Charles River Laboratory (Wilmington, MA). Transgenic mice homozygous for the *MUC-1* gene and designated *SacII* are H-2<sup>k</sup>. Southern blotting using a human probe that corresponded to ~500 bp of the tandem amino acid repeat domain of MUC-1 was used to identify transgenic mice that had integrated the transferred human DNA sequence. The transgenic mice were then crossed with BALB/c mice to produce F<sub>1</sub> hybrids (H-2<sup>k/d</sup>) to enable them to accept 410.4 cells and E3 cells (both are H-2<sup>d</sup>). All animals in the experiments were housed in our animal maintenance facility in accordance with the NIH guide for the Care and Use of Laboratory Animals.

**Modification of E3 Cells to Form IL-12, IL-2, IL-4, or IFN- $\gamma$ .** A retroviral vector (TFG-mIL-12; Ref. 29), kindly provided by H. Tahara, (University of Pittsburgh, Pittsburgh, PA) was used to modify E3 cells to secrete IL-12. The vector specified the murine heterodimeric subunits (p40 and p35) of IL-12 and a gene conferring neomycin resistance. Both were under control of the TFG vector 5' long terminal repeat. E3 cells were transduced with TFG-mIL-12, according to the protocol described by Tahara *et al.*, (29). In brief,  $5 \times 10^5$  cells in growth medium were added to individual wells of a six-well plate (Falcon). Twenty-four h later, 1 ml of TFG-mIL-12-Neo retroviral supernatant in the presence of Polybrene (8  $\mu\text{g}/\text{ml}$ ) was added to each well. After 24 h additional incubation, the cells were washed, and the medium was replaced with growth medium containing 400  $\mu\text{g}/\text{ml}$  of the neomycin analogue, G418. (100% of nontransduced cells die in growth medium containing 400  $\mu\text{g}/\text{ml}$  G418.) After a 14-day period of incubation, cells proliferating in the G418-containing medium were pooled and maintained as a cell line. As a control, the same protocol was followed except that a plasmid (pZipNeoSV-X from M.K.L. Collins, University College, London, United Kingdom) was substituted for TFG-mIL-12. (pZipNeoSV-X specifies a neomycin resistance gene, but not the gene for a cytokine.) Lipofectin (Life Technologies, Inc.) was used to facilitate uptake of pZipNeoSV-X by E3 cells, according to the manufacturer's instructions (Life Technologies, Inc.).

A similar procedure was followed to modify E3 cells for the secretion of IL-2, IL-4, or IFN- $\gamma$ . Eukaryote expression vectors pZipNeoSV-IL-2, pZipNeoSV-IL-4, and pZipNeoSV-IFN- $\gamma$  (obtained from M.K.L. Collins, University College, London, United Kingdom) were used for this purpose. pZipNeoSV-IL-2, pZipNeoSV-IL-4, and pZipNeoSV-IFN- $\gamma$  encode human IL-2, mIL-4, and mouse IFN- $\gamma$ , respectively, and a gene conferring resistance to the neomycin analogue, G418. In brief,  $1 \times 10^6$  E3 cells were seeded into 100-mm cell culture dishes in 10 ml of growth medium. After incubation for 18 h, the cells were washed with DMEM (Life Technologies, Inc.), followed by the addition of 10  $\mu\text{g}$  of the vector in 100  $\mu\text{l}$  of DMEM and 100  $\mu\text{l}$  of Lipofectin, prepared according to the manufacturer's instructions (Life Technologies, Inc.). After 24 h additional incubation, the cells were washed, and the medium was replaced with growth medium containing 400  $\mu\text{g}/\text{ml}$  G418 (Life Technologies, Inc.). Colonies of cells proliferating in G418-containing growth medium were pooled and maintained as cell lines. Every third passage, the transfected cells were cultured in growth medium containing 400  $\mu\text{g}/\text{ml}$  G418.

**Detection of Cytokine Formation by Retrovirally Transduced E3 Cells.** An ELISA was used to detect the formation of IL-12, IL-2, IL-4, or IFN- $\gamma$  by the transduced cells. In brief,  $10^6$  cells transduced with the relevant vector were added to cell culture flasks containing 10 ml of RPMI 1640 (Life Technologies, Inc.) supplemented with 2.0 mM sodium pyruvate, 10% FCS (Sigma), 0.15 M HEPES, and antibiotics. After incubation for 48 h, the cell culture supernatants were assayed for the presence of the relevant cytokines, following the directions of the supplier (Endogen, Woburn, MA).

**Quantitative Immunofluorescent Staining and Cytofluorometric Measurements.** Quantitative immunofluorescent measurements were used to detect the expression of human MUC-1 by E3 cells transduced with pZipNeoSV-

IL-2, pZipNeoSV-IL-4, pZipNeoSV-IFN- $\gamma$ , or TFG-mIL-12. The measurements were performed in a FACS Calibur (Becton Dickinson). For the analysis, a single-cell suspension was prepared from the monolayer cultures of the retrovirally transduced cells using 0.1 mM EDTA in 0.1 M PBS (pH 7.4). The cells were washed with growth medium containing 0.2% sodium azide and 0.5% FCS. Afterward, a monoclonal antibody (SM3), or HMFG-1, which reacts with mucin expressed by human breast cancer cells (30), was added to the cells, followed by incubation at 4°C for 1 h. After incubation, the cells were washed with PBS containing 0.2% sodium azide and 0.5% FCS, followed by the addition of rabbit antimouse immunoglobulin antibody conjugated to FITC (Sigma) and additional incubation at 4°C for 1 h. After additional washing, the cells were analyzed by quantitative immunofluorescent staining. One parameter fluorescence histograms were generated by analyzing  $1 \times 10^4$  cells. Background staining was determined by substituting cells stained with rabbit antimouse immunoglobulin antibody conjugated to FITC alone.

**Measurement of Tumor Growth in MUC-1 Transgenic Mice.** Two-dimensional measurements were used to determine the growth of tumor cells that were injected into breast tissues of MUC-1 transgenic mice. A vernier caliper was used for this purpose. The volume of the tumor was calculated by the formula  $0.4ab^2$ , where  $a$  = length and  $b$  = width of the tumor.

## RESULTS

**MUC1 Transgenic Mice Express Human Mucin.** Immunohistochemical staining for mucin specified by the human *MUC-1* gene was used to determine whether the MUC-1 transgenic mice used in the experiments expressed human mucin. Selected organs of the mice were tested, using a monoclonal antibody (HMFG-1) found previously to react with the amino acid sequence Pro-Asp-Thr-Arg-Pro of the core protein of human MUC-1. The results (Fig. 1) indicate that mucin was expressed on the apical surfaces of cells in the ductal epithelium of distal convoluted tubules of the kidney, epithelial cells lining bronchioles of the lung, and bile duct epithelium of cells in the liver. Under similar conditions, sections of the same tissues from transgenic mice incubated with the secondary antibody alone failed to stain.

**410.4, a Mouse Breast Cancer Cell Line, Transduced with R1-MUC1-pEMSVscribe Expresses Human Mucin.** The protein core of mucin formed by human breast cancer cells specifies the immunodominant epitope (30–32). It is “exposed” because mucin is aberrantly expressed by mucin-producing epithelial carcinoma cells. A mouse breast cancer cell line (410.4; H-2<sup>d</sup>; Ref. 26) was modified to express human MUC-1 by transduction with a vector that specified the coding sequence. Quantitative immunofluorescent staining was used to determine whether MUC-1 was expressed by the transduced cells.

Either of two antibodies, SM3 and HMFG-1, were used in the study. (Similar to HMFG-1, SM3 is specific for the amino acid sequence Pro-Asp-Thr-Arg-Pro of the core protein of mucin). As indicated (Fig. 2), the MFI of the transduced cells incubated with SM3 monoclonal antibodies, followed by incubation with FITC-conjugated rabbit antimouse immunoglobulin (Sigma), was significantly ( $P < 0.01$ ) higher than the MFI of cells incubated with FITC-conjugated rabbit antimouse immunoglobulin alone, taken as “background.” The MFI of nontransduced 410.4 cells stained with SM3 antibodies was not above this “background.” The MFI of transduced cells stained with HMFG-1 antibodies was equivalent to that of the cells stained with the SM3 antibody.

**E3 Cells Express Low Levels of MHC Class I Determinants.** Neoplastic cells may express low levels of MHC class I determinants, a documented means of tumor cell “escape” from immune-mediated destruction (reviewed in Ref. 33). Quantitative immunofluorescent measurements were performed, using FITC-labeled H-2K<sup>d</sup> antibodies (PharMingen), to determine the level of H-2K<sup>d</sup> class I determinants expressed by E3 cells. For comparison, the level of H-2K<sup>d</sup> determi-



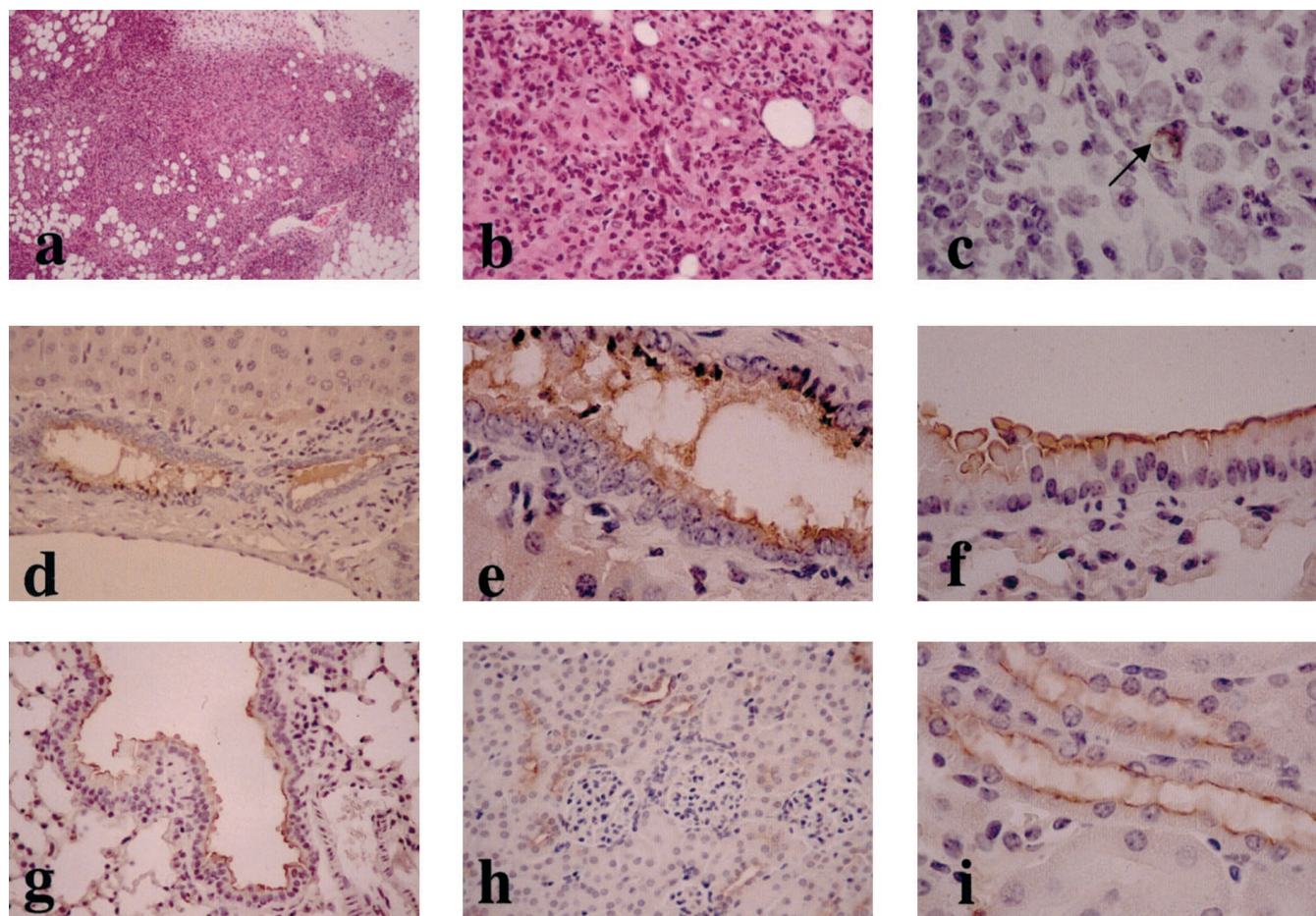


Fig. 1. Immunohistochemical and H&E staining of various tissues of MUC-1 transgenic mice stained with an antibody (HMF-G-1) reactive with human mucin. *a*, inflammatory infiltrate at the site of injection of E3-IL-12 cells into breast tissue of MUC-1 transgenic mice. H&E section,  $\times 250$ . *b*, inflammatory infiltrate at the site of injection of E3-IL-12 cells into breast tissue of MUC-1 transgenic mice. H&E section,  $\times 400$ . *c*, inflammatory infiltrate and a mucin-positive breast cancer cell (arrow) in breast tissue from MUC-1 transgenic mice injected with E3-IL-12 cells. Immunohistochemical staining,  $\times 400$ . *d*, immunohistochemical staining of portal tracts in the liver of MUC-1 transgenic mice with immunostaining localized to bile duct epithelium.  $\times 400$ . *e*, immunohistochemical staining of portal tracts in the liver of MUC-1 transgenic mice, showing strong luminal surface staining.  $\times 1000$ . *f*, immunohistochemical staining of bronchial epithelium of MUC-1 transgenic mice.  $\times 1000$ . *g*, immunohistochemical staining of bronchial epithelium of MUC-1 transgenic mice.  $\times 400$ . *h*, immunohistochemical staining of renal cortex of MUC-1 transgenic showing reaction with cells in the distal convoluted tubules.  $\times 400$ . *i*, immunohistochemical staining of renal cortex of MUC-1 transgenic mice showing reaction with cells in the distal convoluted tubules.  $\times 1000$ . For the immunohistochemical staining, the tissues were fixed in methanol:chloroform:acetic acid (60:30:10) and paraffin embedded for sectioning. Dewaxed paraffin sections were incubated with 50% FCS in PBS for 30 min to prevent nonspecific binding of antibodies. The blocking solution was then removed and replaced by neat hybridoma culture supernatant, followed by incubation at room temperature for 60 min. After three 5-min washes with PBS, the sections were incubated for 1 h with peroxidase-conjugated rabbit antimouse immunoglobulin antiserum (Dako; diluted 1:50 in PBS with 15% FCS). The slides were then washed three times with PBS. The substrate solution consisting of 0.03% hydrogen peroxide in PBS and 1 mg/ml diaminobenzidine (Sigma) was added, and the reaction was allowed to continue for 5–8 min. After final washing with PBS, the slide were counterstained with H&E and mounted.

nants expressed by nonneoplastic nucleated spleen cells from MUC-1 transgenic mice was determined. The results indicated that, similar to other types of cancer cells, E3 cells formed lesser quantities of class I determinants than nonneoplastic cells from MUC-1 transgenic mice. The MFI of E3 cells stained with H-2K<sup>d</sup> antibodies was significantly less than that of nucleated spleen cells from the mice (86.4 and 281, respectively;  $P < 0.01$ ). As controls, E3 cells were stained with FITC-labeled H-2K<sup>k</sup> antibodies (410.4 cells of BALB/c origin are not expected to form H-2K<sup>k</sup> determinants) or with isotype control serum, both followed by FITC rabbit antimouse immunoglobulin. The MFI of E3 cells stained with FITC-labeled H-2K<sup>k</sup> antibodies was not significantly different from that of E3 cells stained with the isotype control serum alone.

**E3 Cells Formed Slowly Growing Tumors in Immunocompetent BALB/c Mice.** Human MUC-1 is immunologically foreign in (nontransgenic) BALB/c mice. To determine whether the molecule was sufficiently antigenic to inhibit the tumorigenic properties of the cells, *i.e.*, to lead to rejection of the tumor cells, immunocompetent BALB/c mice received injections into the fat pad of the breast with

$1 \times 10^6$  E3 cells, or for comparison, with an equivalent number of (MUC-1-negative) 410.4 cells. The animals were monitored for the time to first appearance of tumor (the latent period) and the rate of tumor growth at the injection sites. The results (Fig. 3) indicated that progressively growing breast neoplasms formed at the injection sites in both instances. However, the latent period was significantly prolonged in the mice injected with E3 cells relative to that of BALB/c mice injected with 410.4 cells ( $50 \pm 12$  days *versus*  $16 \pm 1$  day, respectively;  $P < 0.01$ ). Once the tumors appeared, however, the rate of tumor growth in mice injected with E3 cells was not significantly different from the rate of tumor growth in mice injected with 410.4 cells.

Cells recovered from the injection sites were re established as breast cancer cell lines. An analysis by ELISA of the culture supernatants was performed to determine whether the cells were producing cytokines. The results (not presented) indicated that the cells were producing equivalent quantities of the same cytokine as the cells first injected.

Conceivably, the generation time of E3 cells was longer than the

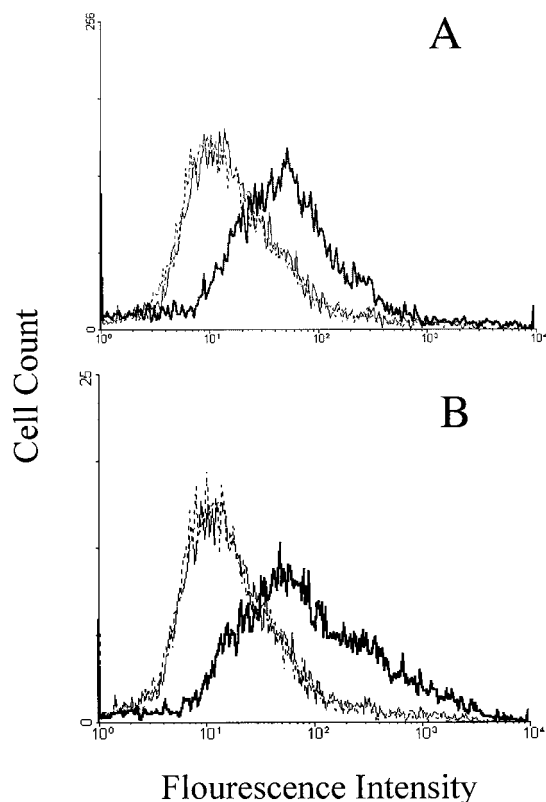


Fig. 2. Expression of mucin by mouse breast cancer cells (410.4) transfected with a plasmid encoding the *MUC-1* gene (E3 cells). E3 cells or 410.4 cells ( $1 \times 10^6$ ) were incubated for 1 h at  $4^\circ\text{C}$  with SM3 (A) or HMFG-1 (B) antibodies or isotype control antibodies. After incubation with antimouse-FITC antibodies and washing as described in "Materials and Methods," the cells were analyzed for fluorescent staining by flow cytometry. A: *light line*, cells incubated without the primary antibody; *dotted line*, 410.4 cells incubated with SM3 antibodies; *bold line*, E3 cells incubated with SM3 antibodies. B: *light line*, cells incubated without the primary antibody; *dotted line*, 410.4 cells incubated with HMFG-1 antibodies; *bold line*, E3 cells incubated with HMFG-1 antibodies.

generation time of 410.4 cells, and this difference was responsible for the delayed appearance of the tumor of E3 cells in the BALB/c mice. This question was investigated by comparing the proliferation rates of E3 cells and 410.4 breast cancer cells *in vitro*. The results indicated that the generation times ( $\sim 24$  h in each instance) of the two cell types were not significantly different from each other. As noted previously, the rates of tumor growth in mice injected with E3 or 410.4 cells were not significantly different.

**E3 Cells Formed Progressively Growing Tumors in MUC1 Transgenic Mice.** MUC-1 transgenic mice express human mucin and would be expected to be naturally tolerant to histocompatible cells that express human MUC-1. To determine whether E3 cells formed tumors in the transgenic mice,  $1 \times 10^6$  viable E3 cells were injected into the fat pad of the breast of MUC-1 transgenic mice, and the latent period and the rate of tumor growth were compared with the latent period and rate of tumor growth in MUC-1 transgenic mice injected with an equivalent number of 410.4 cells. As indicated (Fig. 4), unlike the injections in BALB/c mice, the latent period and rate of tumor growth in MUC-1 transgenic mice injected with E3 cells were not significantly different that the latent period and rate of tumor growth in transgenic mice that received injections of 410.4 cells. The mice exhibited no resistance to the growth of breast cancer cells modified to express human mucin.

**Cytokine Secretion by E3 Cells Transduced with Retroviral Vectors Specifying Cytokine Genes.** Cytokine secretion by cancer cells augments the immunogenic properties of the cells (1–14). Several immune augmenting cytokines were evaluated to determine

whether cytokine secretion by E3 cells affected the immunogenic properties of the cells in MUC-1 transgenic mice. As a first step, the cells were modified to secrete IL-2, IL-4, IL-12, or IFN- $\gamma$ . Retroviral vectors encoding the relevant cytokine gene and a gene conferring resistance to the neomycin analogue, G418, were used for this purpose. As a control, E3 cells were transduced with a vector (pZipNeoSV-X) that specified the neomycin resistance gene but did not encode a cytokine gene.

After selection in growth medium containing sufficient quantities (400  $\mu\text{g/ml}$ ) of G418 to kill 100% of nontransduced E3 cells, the antibiotic-resistant cells were maintained as cell lines. After 48 h incubation, culture supernatants from the cells were analyzed by ELISA for the presence of the relevant cytokine. The results indicated that  $10^6$  cells transduced with a vector encoding the gene for IL-2 (pZipNeoSV-IL-2) formed 72 units/ml IL-2, cells transduced with a vector encoding the gene for IL-4 (pZipNeoSV-IL-4) formed 5 pg/ml

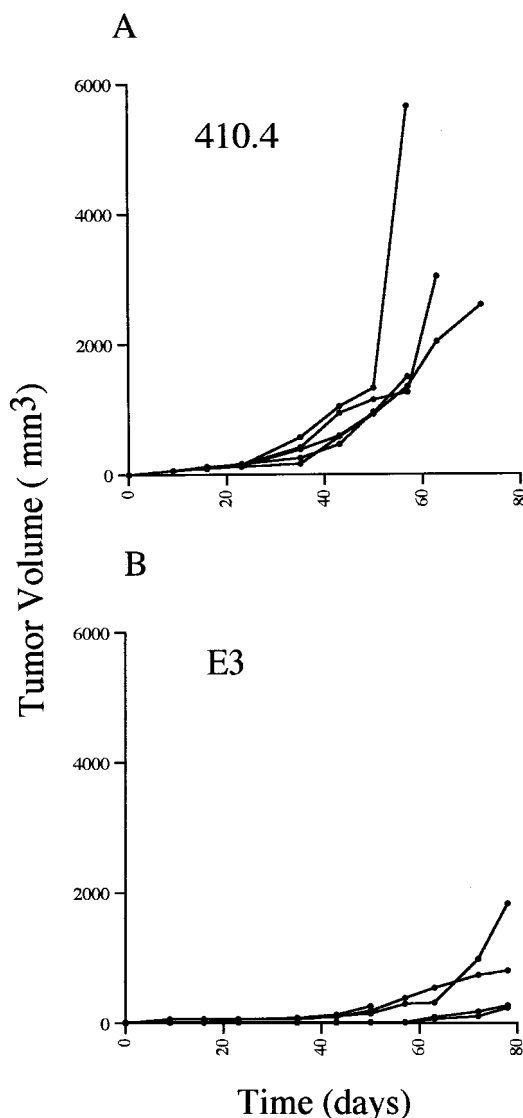


Fig. 3. Tumor growth in BALB/c mice that received injections of the fat pad of the breast with 410.4 cells or E3 cells. Naive BALB/c female mice between 12 and 16 weeks of age received injections into the fat pad of the breast with  $1 \times 10^6$  E3 cells (B) in a total volume of 200  $\mu\text{l}$  of growth medium or, for comparison, with  $1 \times 10^6$  410.4 cells (A) in 200  $\mu\text{l}$  of growth medium. Two-dimensional tumor measurements were performed, and the volume of the tumor was calculated as described in "Materials and Methods." Each line represents tumor growth in an individual mouse. The end point of the line represents the death of the mouse.



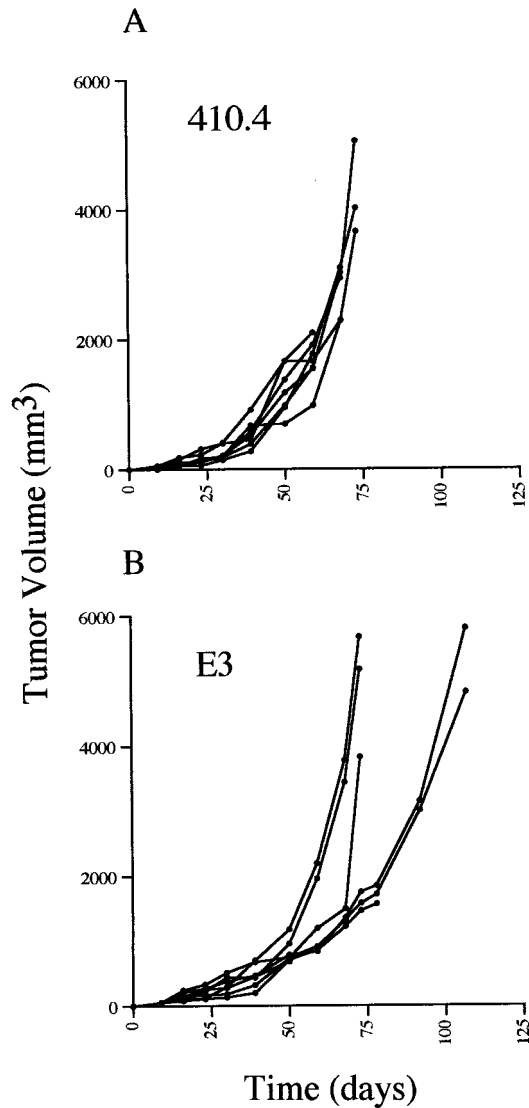


Fig. 4. Tumor growth in MUC-1 transgenic mice that received injections into the fat pad of the breast with 410.4 or E3 cells. Tumor growth was measured in naive MUC-1 transgenic mice that received injections into the fat pad of the breast with  $1 \times 10^6$  E3 cells (B) in a total volume of 200  $\mu$ l of growth medium. For comparison, naive MUC-1 transgenic mice received injections of  $1 \times 10^6$  410.4 cells (A) in 200  $\mu$ l of growth medium. Two-dimensional tumor measurements were performed, and the volume of the tumor was calculated as described in "Materials and Methods." Each line represents tumor growth in an individual mouse. The end point of the line represents the death of the mouse.

IL-4, cells transduced with a vector encoding the gene for IL-12 (TFG-mIL-12) formed 2.54 ng/ml IL-12, and cells transduced with a vector encoding the gene for IFN- $\gamma$  (pZipNeoSV-IFN- $\gamma$ ) formed 1.2 ng/ml IFN- $\gamma$ . Under similar conditions, the culture supernatants of nontransduced E3 cells, or E3 cells transduced with pZipNeoSV-X, failed to contain detectable quantities of IL-2, IL-4, IL-12, or IFN- $\gamma$ . Every third passage, the cytokine-secreting cells were placed in growth medium containing 400  $\mu$ g/ml G418. Under these circumstances, equivalent quantities of the relevant cytokines were detected when the cells were reanalyzed after 3 months of continuous culture. In addition, reestablishment in culture of E3-IL-12 cells recovered from injection sites indicated that the cells continued to secrete IL-12 (these data are not presented).

**IL-12 Secretion by E3 Cells Inhibited the Tumorigenic Properties of the Cells in MUC-1 Transgenic Mice.** To determine whether cytokine secretion by E3 cells affected their tumorigenic properties, MUC-1 transgenic mice received injections into the fat pad

of the breast with  $1 \times 10^6$  E3 cells modified to secrete IL-2, IL-4, IL-12, or IFN- $\gamma$ . For comparison, the mice received injections of an equivalent number of non-cytokine-secreting E3 cells transduced with the vector [pZipNeoSV(X)] that conferred neomycin resistance but did not specify a cytokine gene. As indicated (Fig. 5), the latent period was prolonged in the group of MUC-1 transgenic mice that received the injections of IL-12-secreting E3 cells (E3-IL-12 cells), relative to that of any of the other groups ( $P < 0.01$ ). Six of the eight mice that received injections of E3-IL-12 cells failed to form tumors and appeared to have rejected the breast cancer cells. Two mice that received injections of E3-IL-12 cells formed slowly growing tumors at the injection sites that led eventually to the animals' deaths. The median survival time of these animals,  $\sim 100$  days, was significantly ( $P < 0.001$ ) longer than the median survival time of mice in any of the other groups (Fig. 6). H&E staining of tissue sections taken from the sites of injection of mice that received injections of E3-IL-12 cells revealed an intense inflammatory infiltrate consistent with the rejection of the cytokine-secreting cells (Fig. 1, a-c).

The latent period in MUC-1 transgenic mice that received injections of E3 cells modified to secrete IFN- $\gamma$  was found to be less than that of any of the other groups (Fig. 6), suggesting that modification of the cells to secrete IFN- $\gamma$  augmented the tumorigenic properties of the cells. This point was not investigated further but is consistent with the report of Puisieux *et al.* (34), who noted a similar phenomenon.

**Immunity to Breast Cancer Developed in MUC-1 Transgenic Mice That Rejected E3 Cells Modified to Secrete IL-12.** Tumors failed to form in the majority of MUC-1 transgenic mice that received injections of IL-12-secreting E3 cells. To determine whether the mice that rejected the IL-12-secreting cells developed immunity to E3 cells, that is, whether they were resistant to the growth of (non-cytokine-secreting) E3 cells, the surviving mice received a challenging injection of E3 cells 71 days after the injection of E3-IL-12 cells. As indicated (Fig. 6), none of the animals formed tumors. Under similar conditions, 100% of naive MUC-1 transgenic mice that received injections of non-cytokine-secreting E3 cells developed progressively growing neoplasms at the injection sites that led to the animals' deaths.

Conceivably, differences in the growth rates of E3 cells and E3-IL-12 cells affected the results we obtained. An analysis of the generation times of the cells indicated that they did not differ significantly (these data are not presented).

**Immunity to E3 Cells in Transgenic Mice Injected with E3-IL-12 Cells.** Inhibition of the growth E3 cells in the MUC-1 transgenic mice that rejected E3-IL-12 cells suggested that the resistance was mediated by immune mechanisms. An immunoassay that uses cytokine release by antigen-stimulated spleen and lymph node cells as an indication of an immune response (35) was used to investigate this question. In the experiment, naive MUC1 transgenic mice received a single s.c. injection of  $1 \times 10^6$  E3-IL-12 cells. Two weeks later, the mice were sacrificed, and pooled cell suspensions were prepared from the spleens and regional lymph nodes. The cell suspensions were coincubated for 24 h under standard cell culture conditions with X-irradiated (5000 rads from a  $^{60}\text{Co}$  source) E3 cells or, for comparison, with X-irradiated B16 cells, a melanoma cell line used as a specificity control. As an additional control, the spleen/lymph node cell suspensions were incubated under the same conditions without the addition of the X-irradiated cells. After incubation, the culture supernatants were analyzed by ELISA for the presence of IFN- $\gamma$ . The results (Table 1) indicated that the titers of IFN- $\gamma$  in the culture supernatants of cells from the immunized mice coincubated with X-irradiated E3-IL-12 cells or with X-irradiated E3 cells were significantly higher ( $P < 0.01$ ) than the titers of IFN- $\gamma$  in the culture

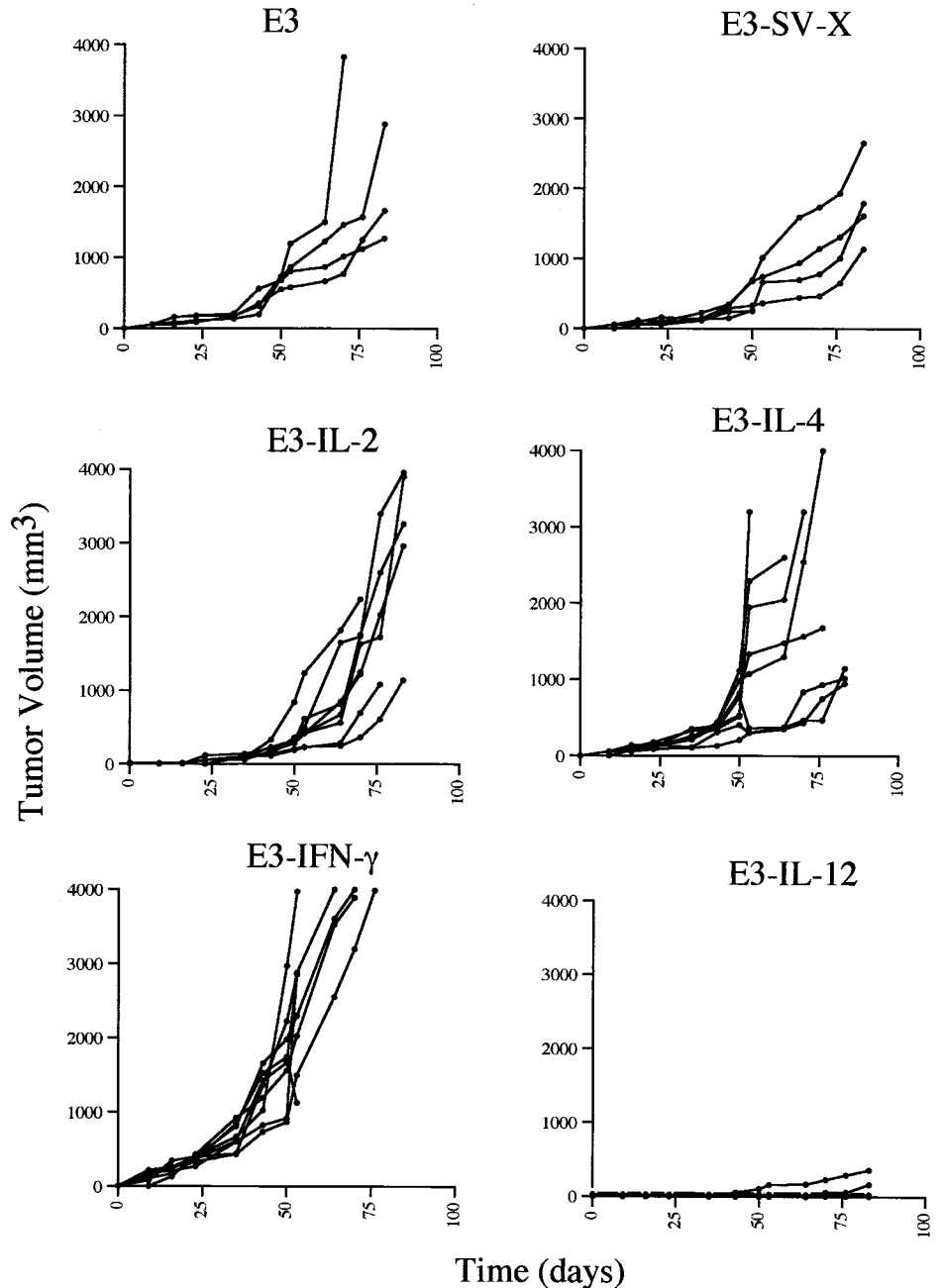


Fig. 5. Tumor growth in MUC-1 transgenic mice that received injections into the fat pad of the breast with E3 cells modified for cytokine secretion. Naive MUC-1 transgenic female mice between 12 and 16 weeks of age received injections into the fat pad of the breast with  $1 \times 10^6$  E3 cells modified for cytokine secretion. For comparison, the mice received injections of an equivalent number of unmodified E3 cells or E3 cells transfected with a vector (pZiPNeoSV-X) that specified a neomycin resistance gene but did not encode a cytokine gene. Each cell type was suspended in 200  $\mu$ l of growth medium for the injection. Two-dimensional tumor measurements were performed, and the volume of the tumor was calculated as described in "Materials and Methods." Each line represents tumor growth in an individual mouse. The end point of the line represents the death of the mouse. There were eight mice in each group.

supernatants of cells coincubated with X-irradiated B16 cells. IFN- $\gamma$  was undetectable in the supernatants of spleen/lymph node cell cultures that were incubated alone, *i.e.*, without the addition of X-irradiated tumor cells. Analogous findings were observed if the culture supernatants were analyzed for the presence of MIP1 $\alpha$ , a chemokine (36). The titers of MIP1 $\alpha$  in culture supernatants of spleen/lymph node cell suspensions from mice immunized with E3-IL-12 cells coincubated with X-irradiated E3 cells were significantly higher ( $P < 0.01$ ) than present in culture supernatants from cell suspensions coincubated with X-irradiated B16 cells or cell suspensions incubated alone (Table 1). These results indicated that immunization with E3-IL-12 cells specifically enhanced the immune response toward E3 cells.

An immunofluorescence assay was used to determine whether antibodies reactive with E3 cells were present in the sera of MUC-1 transgenic mice immunized with E3-IL-12 cells. Thirty days after a

single injection of  $1 \times 10^6$  E3-IL-12 cells, pooled sera from the mice were incubated with E3 cells, followed by further incubation with FITC-conjugated antimouse IgG, IgM, or IgA to determine the isotype of the antibodies that reacted with E3 cells. The results (Table 2) indicated that E3 cells incubated with sera from mice immunized with E3-IL-12 cells, followed by FITC-conjugated antimouse IgG, reacted positively. Under similar conditions, E3 cells incubated with sera from mice immunized with E3-IL-12 cells, followed by FITC-conjugated antimouse IgM or FITC-conjugated antimouse IgA, failed to react. Thus, IgG was the major immunoglobulin class reactive with E3 cells in mice immunized with E3-IL-12 cells. The requirement for IL-12-secretion by E3 cells in the generation of the antibody response was supported by the finding that immunization of MUC-1 transgenic mice with (non-cytokine-secreting) E3 cells or with (non-cytokine-secreting) 410.4 cells failed to generate an antibody response toward the breast cancer cells (Table 2).

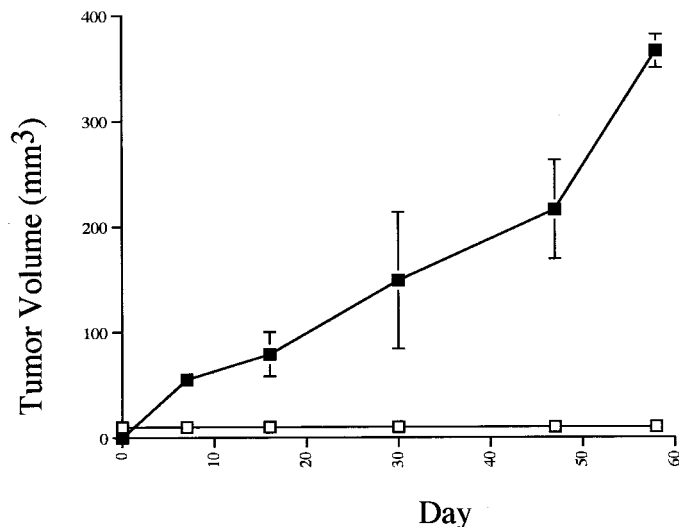


Fig. 6. Tumor growth in MUC-1 transgenic mice surviving a prior injection of E3-IL-12 cells that received injections subsequently with E3 cells. E3-IL-12 cells suspended in 200  $\mu$ l of growth medium. Seventy-one days later, the mice received injections a second time into the fat pad of the contralateral breast with  $1 \times 10^6$  viable E3 cells suspended in 200  $\mu$ l of growth medium (□). As a control, naive MUC-1 transgenic female mice of the same age received injections into the fat pad of the breast with an equivalent number of E3 cells suspended in 200  $\mu$ l of growth medium (■). Bars, SD.

Table 1 Cytokine release from spleen and lymph node cells of MUC-1 transgenic mice immunized with E3-IL-12 cells after coinubation with X-irradiated E3 cells

MUC-1 transgenic female mice between 12 and 16 weeks of age received injections into the fat pad of the breast with  $1 \times 10^6$  E3-IL-12 cells suspended in 200  $\mu$ l of growth medium. Fourteen days later, the animals were sacrificed, and a pooled cell suspension was prepared from the spleen and lymph nodes of individual mice. The nucleated cells (Histopaque) were coinubated for 24 h with X-irradiated (5000 rads from a  $^{60}\text{Co}$  source) E3 cells. The ratio of spleen/lymph node cells to E3 cells was 3:1. At the end of the incubation, the culture supernatants were analyzed by ELISA for the presence of IFN- $\gamma$  or MIP1 $\alpha$ . As controls, X-irradiated B16 cells were substituted for the E3 cells, or the spleen/lymph node cell suspensions were incubated alone. The results represent the mean  $\pm$  SD cytokine release from each of three individual mice.

	E3-IL-12	E3	B16	None
A. IFN- $\gamma$ (pg/ml)				
24 h				
E3-IL-12	2100 $\pm$ 45	1415 $\pm$ 94	101 $\pm$ 4	0
Naive	505 $\pm$ 78	0	0	0
48 h				
E3-IL-12	4260 $\pm$ 19	2539 $\pm$ 217	1835 $\pm$ 120	4 $\pm$ 0.1
Naive	2881 $\pm$ 198	44 $\pm$ 4	0	0
B. MIP1 $\alpha$ (pg/ml)				
24 h				
E3-IL-12	283 $\pm$ 7	252 $\pm$ 12	37 $\pm$ 3	25 $\pm$ 8
Naive	168 $\pm$ 6	165 $\pm$ 11	17 $\pm$ 0	14 $\pm$ 1

**DISCUSSION**

Under ordinary circumstances, inbred mice that received injections of histocompatible breast cancer cells develop progressively growing neoplasms that lead, eventually, to the animals' deaths. The malignant cells do not provoke effective antitumor immune responses because the duration of survival can be (inversely) related to the number of cancer cells injected.

The studies reported here were prompted by the finding that human breast cancer-associated mucin, the product of the *MUC-1* gene, is expressed in an altered form by breast cancer cells and is a breast cancer antigen. Under appropriate circumstances, breast cancer cells that express mucin can be recognized by CTLs and can become targets of immune-mediated attack. Clinical studies are in progress to

test the immunotherapeutic benefits of tumor vaccines that express mucin (15-17).

*MUC-1*, a gene for mucin, codes for a heavily glycosylated macromolecule found on the surface membranes of both normal and malignant mucin-producing epithelial cells. The glycosylation pattern of mucin expressed by breast cancer cells can be distinguished from the glycosylation pattern of mucin expressed by nonmalignant cells of the same individual. Mucin formed by breast cancer cells is overexpressed and underglycosylated and differs antigenically from mucin expressed naturally by nonmalignant epithelial cells of the breast. As a consequence, novel T-cell epitopes are exposed that are potentially antigenic. The underglycosylated mucin can be a target for both MHC-unrestricted as well as MHC-restricted class I CTL responses. T cell-mediated cytotoxicity responses toward mucin have been identified in patients bearing breast neoplasms that express MUC-1. However, the natural antigenic properties of tumor-associated mucin are insufficient to stimulate immune responses that are capable of leading to tumor cell rejection.

The long-term objective of our work is to investigate various strategies to increase the immunogenic properties of mucin expressed by breast cancer cells, with a view toward developing an immunotherapeutic agent that can be used in the treatment of breast cancer patients. The successful development of MUC-1 transgenic mice that express human mucin in a tissue-specific manner enables the study to be carried out in experimental animals. Similar to breast cancer patients, the mice are naturally tolerant to human mucin. As with other cellular constituents, MUC-1 is expressed most strongly at the apical surfaces of ductal epithelial cells of the lactating breast (37) and is viewed as "self" by the animal's immune system. The injection of a highly malignant mouse breast cancer line modified to express human MUC-1 into the breast of MUC-1 transgenic mice mimics, as closely as possible, mucin-producing breast cancer in patients.

Here, the *MUC-1* gene was introduced into 410.4 cells, a highly malignant breast cancer cell line of BALB/c origin (H-2<sup>d</sup>), and the cells (E3) were tested for their immunogenic properties in MUC-1 transgenic mice. The validity of the model was emphasized by the finding that the latent period and rate of tumor growth of E3 cells in MUC-1 transgenic mice were essentially the same as the latent period and rate of tumor growth of 410.4 cells.

The mouse breast cancer cells modified to express human MUC-1 were further modified to produce various cytokines known to augment the immunogenic properties of malignant cells. We hypothesized that presentation of MUC-1 to the immune system in the microenvironment of immune-augmenting cytokines would generate an immune response to MUC-1 expressed by the breast cancer cells.

The results clearly indicated that modification of the MUC-1-expressing breast cancer cells to secrete IL-12 increased the immu-

Table 2 Isotype of antibodies to E3 cells in MUC-1 transgenic mice immunized with E3-IL-12 cells

Naive MUC-1 transgenic female mice between 12 and 16 weeks of age received injections into the fat pad of the breast with  $1 \times 10^6$  E3-IL-12 cells suspended in 200  $\mu$ l of growth medium. As controls, naive MUC-1 transgenic mice received injections of an equivalent number of E3 cells, or 410.4 cells, suspended in 200  $\mu$ l of growth medium. The mice in each group were bled 30 days later. A 1:100 dilution of the pooled sera was incubated with  $5 \times 10^3$  E3 cells for 45 min at 4°C. At the end of the incubation, the cells were washed and then incubated for 45 min at 4°C with FITC-conjugated goat antimouse IgG, antimouse IgM, or antimouse IgA serum. The intensity of immunofluorescent staining was measured by flow cytometry using a FACS Calibur cytofluorograph (Becton Dickinson). There were three mice in each group.

Serum from mice immunized with	% E3 cells stained		
	IgG	IgM	IgA
E3-IL-12 cells	72 $\pm$ 10	10 $\pm$ 3	0.3 $\pm$ 0.2
E3 cells	7 $\pm$ 3	5 $\pm$ 2	0
410.4 cells	6 $\pm$ 3	4 $\pm$ 3	0

nogenic properties of the cells in MUC-1 transgenic mice. Six of eight mice that received injections of the IL-12-secreting cells failed to form tumors and appeared to have rejected the IL-12-secreting cells. The remaining two mice that eventually formed tumors did so after a prolonged latent period. When tested at a later time, the animals that failed to form tumors were completely resistant to (non-cytokine-secreting) MUC-1-positive breast cancer cells. They developed cellular immune responses toward the cells, as indicated by studies performed *in vitro*, and the presence of an intense inflammatory infiltrate at the site of injection of the IL-12-secreting breast cancer cells. IgG antibodies reactive with the cells developed in MUC-1 transgenic mice that received injections of E3-IL-12 cells. Whether the antibodies were specific for MUC-1 or reactive with other, as yet undefined, determinants associated with the cells was uncertain. Whether they contributed to the eradication of the breast cancer cells was not determined.

IL-12 is a pleiotropic, heterodimeric cytokine that stimulates both natural killer and T lymphocytes to produce IFN- $\gamma$  and TNF- $\alpha$ . It also promotes the development of Th1 CD4<sup>+</sup> cells, which are also involved in the induction of cellular immunity (38, 39). IL-12 also enhances the activity of tumor-infiltrating lymphocytes and has been described previously as a strong immune-augmenting cytokine (40–42). Our results extend these studies to a mouse model of breast cancer in patients.

There are significant differences between the structure of mouse and human mucin that emphasize the importance of the use of transgenic mice in this study. Mouse mucin is only 34% homologous within the tandem repeat domain to human MUC-1 (43). Furthermore, the number of tandem repeats in the core of human mucin is greater than that of the mouse equivalent. Thus, the two molecules are structurally distinct. How these structural differences might affect the immune response toward mucin in the mouse or human breast cancer patient is unknown. Studies in transgenic mice that express human breast cancer associated mucin as "self" mimic the equivalent disease in humans and obviate these concerns.

The potential importance of these findings to the treatment of breast cancer patients is supported by reports indicating that spontaneous breast neoplasms arising in patients, similar to other types of cancer, are potentially immunogenic. The malignant cells form various tumor associated antigens such as HER-2/neu (44, 45), mutant p53 (46), MAGE-1 (47), and BAGE (48), as well as the protein core of mucin that can be recognized by CTLs. These may be only several examples of an array of breast cancer-associated antigens that arise from altered genes in the malignant cells. Genetic instability is a common characteristic of breast cancer and other types of neoplastic cells (49–51).

In an experimental system that mimics breast cancer in patients, the data presented here indicate that the immunogenic properties of an adenocarcinoma of the breast that expresses mucin can be enhanced if the cells are modified to secrete IL-12. They point toward the possible immunotherapeutic potential of breast cancer cells modified for IL-12 secretion. Our findings may be of importance in the clinical care of breast cancer patients.

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