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13. ABSTRACT ***(Maximum 200 Words)***

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. MUC1 has been identified as a breast cancer antigen in breast cancer patients. Transgenic mice were prepared that expressed human MUC1. The mice are naturally tolerant to the molecule providing an opportunity to investigate immunotherapeutic strategies in experimental animals that might eventually be applied to breast cancer in patients. A mouse breast cancer cell line was modified to express human MUC1. If was further modified to secrete various immune augmenting cytokines. Immunization of the transgenic mice with cells modified to secrete IL-12 resulted in the generation of robust, cell mediated immunity toward breast cancer. The immunity was long-term. These data point toward an analogous form of treatment of breast cancer in women. In parallel, we tested a novel vaccine prepared by transfer of DNA from mouse breast cancer cells into a highly immunogenic cell line. The transferred DNA was integrated, and expressed. The DNA-based vaccine was found to be effective in the treatment of breast cancer. This form of vaccine has a number of important advantages over other types of vaccines used in the treatment of malignant disease.

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4. INTRODUCTION

This investigation was prompted by the finding that polymorphic epithelial mucin (PEM), a product of the human MUC1 gene, is expressed in an altered form by human breast cancer cells. In its altered form, MUC1 is a tumor associated antigen. It is uniquely expressed by breast cancer cells, and other types of mucin-producing malignancies, but not by normal, non malignant cells of the cancer patient. Under appropriate circumstances, an immunity can be induced to MUC1 expressed by breast cancer cells. The molecule can be recognized by cytotoxic T lymphocytes of the breast cancer patient, resulting in death of the malignant cells. Breast cancer cells that express MUC1 can become the targets of attack by the immune system. Vaccines that induce immunity to MUC1 may become successful immunotherapeutic agents, as an added option in the treatment of breast cancer patients. The long-term objective of our work is to investigate means of increasing the immunogenic properties breast cancer cells that express MUC1. We wish to develop a safe and effective immunotherapeutic approach that can be used in the treatment of breast cancer patients. The development of transgenic mice that express human mucin by one of us (JTP) enables this important study to be carried out in experimental animals. Like breast cancer patients, MUC1 transgenic mice are naturally tolerant to human mucin. The molecule is treated as "self" by the animal's immune system, just as mucin in breast cancer patients is viewed as "self.". As a model system designed to investigate immunotherapeutic approaches that mimic as closely as possible the treatment of breast cancer in patients, the MUC1 gene was introduced into a highly malignant mouse breast cancer cell line. After the expression of human MUC1 by the mouse breast cancer cells was confirmed, the cells were then further modified to produce various immune-augmenting cytokines, or to express B7.1, a co stimulatory molecule required for T cell activation. We hypothesized that presentation of MUC1 to the immune system in the microenvironment of immune-augmenting cytokines, or by cells that expressed the co stimulatory molecule, would stimulate an immune response to breast cancer cells in MUC1 transgenic mice. The data described below, presented in greater detail in the accompanying published manuscripts, and in manuscripts submitted for publication, indicate the validity of these approaches.

(5) BODY

Overview of the Progress Report

This investigation was carried out in an animal model of breast cancer. The model mimics as closely as possible the treatment of breast cancer in patients. The mice used in the experiments were genetically modified to express a known human breast cancer antigen (MUC1). The highly malignant breast cancer cells used in the experiments were also modified to express the same human breast cancer antigen. The genetic construct used to engineer the mice included promoter elements that restricted expression of MUC1 to the same organs and tissues, including breast epithelia, as in patients. Thus, both the animals, which are naturally tolerant to human MUC1, and the breast cancer cells expressed the same human breast cancer antigen.

The objectives of the Statement of Work originally presented at the time of submission of the proposal have been achieved, or are in the late stages of investigation. The data are presented in five full published manuscripts, one additional full manuscript recently submitted for publication, one review and one manuscript in preparation. Each was supported by the award. The Statement of Work, and an overview of the results are as follows:

1. Introduction of genes for various cytokines into E3 cells. Selection of genetically modified cells. Assays for cytokine-secretion

2. *Comparison of the survival of MUC1 transgenic mice immunized with cytokine-secreting E3 cells challenged with viable E3 cells. Characterization of the anti tumor response*

These, and additional, related objectives have been accomplished. The details are presented in two published manuscripts by Carr-Brendel et al., (1 and 2, below). They indicate that a mouse breast cancer cell line (E3) genetically modified to express human mucin was successfully modified to secrete IL-12, IL-2, IL-4 and interferon-gamma, immune-augmenting cytokines. Of significance, unlike the other cytokines tested, immunization of

syngeneic transgenic mice with E3 cells modified to secrete IL:-12 resulted in immune rejection of the breast cancer cells and the induction of long-term, cellular immunity directed toward both the cytokine-secreting, and non secreting breast cancer cells. Transgenic mice immunized with MUC-1 expressing mouse breast cancer cells modified to secrete IL-12 survived significantly longer than mice in various control groups. At times, they appeared to have rejected the malignant cells and survived indefinitely.

The immune enhancing properties of IL-12 in the treatment of breast cancer in mice were further indicated by the finding (1) that the injection of syngeneic (BALB/c) mice with IL-12-secreting 410.4 cells, a mouse breast cancer cell line, resulted in the induction of immunity toward unmodified 410.4 cells. In this instance, lethal X-irradiation of the IL-12-secreting cells was required to prevent tumor growth in the mice. The IL-12-secreting breast cancer cells retained their immunogenic properties as indicated by the animals' resistance to unmodified breast cancer cells. Taken together, these reports strongly indicate the importance of IL-12 in the development of vaccines useful in the treatment of breast cancer.

The locus of the MUC1 gene maps to chromosome 1q21. The inherent instability of the locus was indicated by findings presented in the work of Waltz et al. (3). Waltz found that a polymorphic microsatellite at intron 6 within the MUC1 locus was altered at high frequency in breast cancer cells from patients. One hundred eighteen paired normal and neoplastic tissue samples were analyzed in this study.

Further studies on the genetic control of expression and control of the distribution of MUC1 were investigated in MUC1 transgenic mice. The results, presented in reference 4, indicate that the tissue specific expression of MUC1 was directed by 1.4Kb of the 5' flanking sequence of the gene. High levels of MUC1 expression were present in the lactating mammary gland, and in mammary tumors that occurred spontaneously in the transgenic mice. This pattern of expression in the transgenic mice is comparable to the pattern of expression found in humans, further supporting the importance of this model system of mammary carcinogenesis.

1a. Introduction of the genes for B7.1 into E3 cells. Selection of genetically modified cells. Assays for B7.1 expression

2a. Determination of the contribution of B7.1 to the immunogenic properties of mouse breast cancer cells modified to express human PEM in MUC1 transgenic mice

4. Detection of an autoimmune response in transgenic mice immunized with breast cancer cells modified to express human PEM and B7

B7.1 is a co stimulatory molecule required for T cell activation. It is normally expressed by antigen presenting cells. The experiments presented in the published manuscript by Smith et al. (reference 5, below) were designed to determine if the expression of B7.1 by mouse breast cancer cells modified to express human MUC1 (PEM) affected the cells' immunogenic properties in MUC1 transgenic mice. As described, MUC1 transgenic mice are naturally tolerant to human mucin.

The results indicated that the expression of B7.1 along with MUC1 by mouse breast cancer cells resulted in a dramatic inhibition of tumor growth. The reduced tumor growth was dependent on the activity of both $CD4^+$ and $CD8^+$ T cells. In an important, related question, mice that rejected the B7.1-positive breast cancer cells showed no evidence of autoimmunity or other signs of toxicity resulting from immunization with the modified cells. The mice lived their anticipated life spans without evidence of disease.

3. *Comparison of the survival of MUC1 transgenic mice bearing PEM-positive breast cancers of varying sizes treated with cytokine-secreting E3 cells*

The results of experiments designed to investigate this question are included in references 1 and 2. As described, an immune response to mouse breast cancer cells modified to express human mucin (PEM) was induced in MUC1 transgenic mice if the cells are modified to

secrete IL-12. The immunity was systemic, long-term and specific. Like the immunity to breast cancer induced in MUC1 transgenic mice immunized with breast cancer cells modified to express B7.1, the immunity induced by the IL-12-secreting breast cancer cells was mediated by CD8+ T cells. Animals that rejected breast cancer cells modified to secrete IL-12 were resistant to a challenging injection of 1 X 10^6 unmodified breast cancer cells. The injection of the unmodified cells was administered 71 days after the first immunizing injection. Further studies to determine the maximum tumor "load" responsive to treatment with the cellular vaccine are in progress.

5. Development of a DNA-based vaccine breast cancer vaccine that prolongs survival of mice with small breast neoplasms

As reported in reference 6, immunizations of mice with breast cancer with a vaccine prepared by transfection of DNA from breast cancer cells into a highly immunogenic cell line resulted in immunity to the breast cancer cells and prolongation of survival. This experimental approach was based on the hypothesis that breast cancer associated antigens including MUC1 are the products of mutant or dysregulated genes that differ from the homologous genes expressed by normal, non malignant cells of the tumor bearing host. Development of a vaccine by transfection of DNA from malignant cells, including DNA from breast cancer cells, into a highly immunogenic cell line is an extension of classic studies indicating that the genotype, and phenotype of one cell type can be altered by transfection of DNA from another. Transfer of DNA from malignant cells into a highly immunogenic cell line resulted in a vaccine that was found to be effective in the treatment of mice with breast cancer. This novel method of vaccine preparation has several important advantages that are described in reference 8. A major advantage is that the cells chosen as recipients of DNA can be selected for special characteristics that will augment their immunogenic properties. The cells can be modified to secrete one or more immune augmenting cytokines, such as IL-2 and IL-12, either singly or in combination. Local delivery of cytokines by the vaccine, for example, mobilizes antigen presenting cells, T cells and B cells to the vaccination site and stimulates their proliferation. Since the cells that take up the exogenous DNA are capable of indefinite division in vitro, and since the transferred DNA is replicated as the cells divide,

only microgram quantities of tumor DNA are sufficient to prepare a vaccine effective in the treatment of breast cancer. A needle biopsy might provide sufficient quantities of DNA for this purpose.

6. Determination of the maximum immunotherapeutic benefit of the DNA-based breast cancer vaccine in mice with breast cancer.

Work on this objective is in progress.

7. Development of a MUC1 transgenic mouse that expresses both human MUC1 alleles

Homozygous mice that express both MUC1 alleles have been prepared. Please see the enclosed manuscript by Graham, RA, Morris, JR, Cohen, EP and Taylor-Papadimitriou J entitled, 1.4kb 5' MUC1 promoter sequence drives tissue specific expression of MUC1 cDNA in MUC1 transgenic mice, and up-regulates expression in the mammary gland at lataction and in malignancy which has been submitted for publication.

8. Development of transgenic mice that express human MUC1 and a human class I MHC allele.

Work on this objective is ongoing. MUC1 transgenic mice are now being crossed with mice transgenic for the human A2 class I allele.

Background

The MUC1 gene codes for a polymorphic membrane associated glycoprotein molecule expressed by epithelial cells that produce mucin. MUC1 is expressed at the apical surfaces of most glandular epithelial cells. It is dramatically up-regulated and overexpressed in breast (and ovarian) carcinoma cells. The glycosylation pattern of MUC1 expressed by mucin-producing carcinoma cells is altered, resulting in the expression of novel T cell epitopes that are potentially immunogenic (9).

The extracellular domain of mucin consists of tandem repeats of twenty amino acids with multiple O-glycans covalently bonded to the amino acid core. In breast and ovarian carcinoma cells, the composition of the carbohydrate side chains is altered, resulting in the

exposure of cryptic peptides that are ordinarily hidden in mucin naturally expressed by non neoplastic cells. Aberrantly expressed breast cancer-associated mucin can become immunogenic and can become the target of cytotoxic T lymphocytes, leading to the rejection of breast cancer cells and the prolongation of survival of tumor-bearing mice. Thus, successful approaches that result in an increase the immunogenic properties of MUC1 could be used to develop a vaccine that might be used in treatment of breast cancer patients.

Increasing the immunogenic properties of breast cancer cells that express human MUC1.

Our initial studies were carried out in transgenic mice injected with a mouse breast cancer cell line (410.4) (10) modified to express human MUC1. (Note-410.4 cells modified to express human MUC1 are designated as E3 cells.) Genes specifying each of several different cytokines known to increase the cells' immunogenic properties were introduced into E3 cells. The immunogenic properties of the cytokine-secreting cells were compared in histocompatible MUC1 transgenic mice.

(6) KEY RESEARCH ACCOMPLISHMENTS

1. A microsatellite within the MUC1 locus in human breast cancer cells was found to be geentically unstable, as indicated by its high frequency of alteration ($P < .001$ relative to two other microsatellites at the same locus). The result is an indication of the high degree of instability a locus that specifies a documented breast cancer antgien. (Waltz, et al., reference 3).
2. A mouse breast cancer cell line (410.4) was successfully modified to express MUC1, a human breast cancer antigen and to secrete immune augmenting cytokines including IL-12. The cytokine-secreting cells were tested for their immunotherapeutic properties in histocompatible transgenic mice that expressed MUC1 as "self" and are naturally tolerant to the molecule. The results indicated that the immunogenic properties of the highly malignant breast cancer cells modified to express MUC1 and to secrete IL-12 exceeded those of MUC1-positive breast cancer cells modified to secrete other immune saugmenting cytokines. The

studies were carried out in MUC1 transgenic mice, mimicking as closely as possible breast cancer in patients.

3. Autoimmunity failed to develop in MUC1 transgenic mice immunized with IL-12-secreting breast cancer cells. The vaccine was found to be non toxic.

3. Modification of breast cancer cells to express MUC1 and to express B7.1, a co stimulatory molecule, dramatically reduced the cells' tumorigenic properties in MUC1 transgenic mice. The latent period following tumor injection was prolonged and the mice survived significantly longer than mice in various control groups. Cytotoxic T lymphocytes mediated tumor rejection in MUC1 transgenic mice.

4. A vaccine prepared by transfer of DNA from adenocarcinoma of the breast that formed spontaneously in a C3H/He mouse into a highly immunogenic cell line was found to be effective in the treatment of mice with breast cancer. This approach toward vaccine development has a number of important advantages over other vaccines.

(7) Reportable outcomes

A list of published manuscripts and manuscripts submitted for publication during the period of grant support is as follows:

1. Carr-Brendel, V, Markovic, D., Smith, M., Taylor-Papadimitriou, J and Cohen, EP. Immunity to breast cancer in mice immunized with X-Irradiated breast cancer cells modified to secrete IL-12. J Immunotherapy, 22: 415-422, 1999. (DAMD 17-96-1-6178)

2. Carr-Brendel, V., Markovic, D., Ferrer, K, Smith, M., Taylor-Papadimitriou, J. and Cohen, E.P. Immunity to murine breast cancer Cells modified to express MUC-1, a human breast cancer antigen, in transgenic mice tolerant to human MUC1., Cancer Research 60: 2435-2443, 2000 (DAMD 17-96-1-6178)

3. Waltz, M.R., S.M., Pandelitis, W. Pratt, D. Barnes, D. M. Swallow, S. Gendler and E.P. Cohen A microsatellite within the MUC1 locus at 1q21 is altered in the neoplastic cells of breast cancer patients. Cancer Genetics and Cytogenetics 100: 63-67, 1998 (DAMD 17-96-1-6178)

4. Graham, RA, Morris, JR, Cohen, EP and Taylor-Papadimitriou J. 1.4kb 5' MUC1 promoter sequence drives tissue specific expression of MUC1 cDNA in MUC1 transgenic mice, and up-regulates expression in the mamamry gland at lataction and in malignancy. Submitted (DAMD 17-96-1-6178).

5. Smith, M., Burchell J.M., Graham, R., Cohen, E.P. and Taylor-Papadimitriou, J. Expression of B7.1 in a MUC1 expressing mouse mammary epithelial tumour cell line inhibits tumorigenicity but does not induce autoimmunity in MUC1 transgenic mice. Immunology 97: 648-655, 1999 (DAMD 17-96-1-6178)

6. de Zoeten, E., Carr-Brendel, V., Markovic, D., Taylor-Papadimitriou and Cohen. E.P. Treatment of Breast Cancer with fibroblasts transfected with DNA from breast cancer cells. Journal of Immunology, 162: 6934-6941, 1999 (DAMD 17-96-1-6178)

7. Markovic D, Taylor-Papaditriou J and Cohen EP. Both CD4+ and CD8+ cytotoxic T lymphocytes mediate the rejection of murine breast cancer cells modified to express MUC1, a human breast cancer antigen, in trangenic mice tolerant to human MUC1. (in preparation). (DAMD 17-96-1-6178)

8. Cohen EP Cancer therapy with DNA-based vaccines. Immunology Letters, "in press"

8. Conclusions

We conclude the following:

1. The immunotherapeutic properties of breast cancer cells that express MUC1, a human breast cancer antigen, are enhanced in MUC1 transgenic mice if the cells are modified to secrete IL-12.

2. A vaccine prepared by modifying breast cancer cells that express MUC1 are also enhanced if the cells are modified to express B7.1, a co stimulatory molecule.

3. Immunizations of mice with the breast cancer cells that express B7.1 or secrete IL-12 does not induce autoimmunity in MUC1 transgenic mice.

4. A breast cancer vaccine that prolongs the survival of mice with breast cancer can be prepared by transfer of DNA from breast neoplasms that arise spontaneously in C3H/He mice. The vaccine is non toxic.

"So what"

The treatment of breast cancer patients has not progressed significantly in many years. Most patients whose tumors have spread beyond the primary site eventually die of their disease. New and innovative types of treatment that differ from prior forms of therapy need to be investigated and eventually brought to clinical trial.

Immune therapy based on tumor cell vaccines is of promise. The treatment is selective in the sense that only the patient's malignant cells are killed. It is non toxic and appears to be without harm.

Two approaches toward vaccine development were evaluated using funds provided by the Department of Defense. Using a unique mouse model of human breast cancer, breast cancer cells that expressed MUC1, modified to secrete IL-12, were found to be highly immunogenic and non toxic to MUC1 transgenic mice with breast cancer. An analogous form of vaccine may be evaluated in breast cancer patients, using breast cancer cells, modified for IL-12-secretion, as a vaccine. Analogous results were obtained if the cells were modified to express B7.1, a co stimulatory molecule. Again, the immunizations were found to be non toxic. An

autoimmune disease did not develop in spite of the fact that the vaccine included many "normal" antigens..

The second approach involved the transfer of DNA from either of two adenocarcinomas that arose spontaneously in C3H/He mice into a highly immunogenic cell line. The use of cells from a primary breast carcinoma (rather than a tumor cell line) closely resembled the disease in patients. Immunizations of mice with breast cancer with the DNA-based vaccine resulted in generalized, long-term immunity to the tumor, and prolongation of survival. This approach has an important practical advantage. Since the transferred DNA is integrated into the genome of the recipient cells, and is replicated as the cells divide, a sufficient quantity of vaccine can be prepared from small surgical specimens. A needle biopsy of tumor could provide enough DNA for this purpose.

Taken together, these findings are the first demonstrations, in a system that closely mimics breast cancer in patients, that a non toxic tumor vaccine can be conveniently prepared that is of use in the treatment of the disease. Immunizations of mice highly susceptible to breast cancer resulted in the induction of immunity to the breast cancer cells and prolongation of survival.

Progress Report

5. Immunity to breast carcinoma cells modified to express human mucin in transgenic mice that express human mucin.

a. Modification of a mouse breast cancer cell line to express human mucin.

410.4 cells, an adenocarcinoma breast cancer cell line is highly tumorigenic in (non transgenic) syngeneic BALB/c mice. One hundred percent of BALB/c mice injected into the fat pad of the breast with 410.4 cells form progressively growing neoplasms that lead to the animals' death.

A retro viral vector (R1-MUC1-pEMSVscribe) that encoded MUC1, the gene specifying mucin expressed by human breast cancer cells, was used to modify the cells to form human MUC1. The vector also specified an antibiotic resistance gene, used for selection. After selection, quantitative immunofluorescent staining was used to confirm the expression of MUC1 by the transduced cells. As indicated in Figure 2 of the enclosed manuscript from our laboratory (Development of Immunity to Murine Breast Cancer Cells Modified to Express MUC1, a Human Breast Cancer Antigen, in Transgenic Mice Tolerant to Human MUC1), which has been submitted for publication.

b. Immunohistochemical staining with monoclonal antibodies for MUC1 was used to determine if selected organs and tissues of MUC1 transgenic mice used in the experiments expressed human mucin. A monoclonal antibody (HMFG1) found previously to react with human mucin (11) was used in the study. The results (Fig 1 in the Appendix and in the enclosed manuscript), indicate that MUC1 was expressed on the apical surfaces of cells in the ductal epithelium of distal tubules of the kidney, cells lining bronchioles of the lung and cells in the liver. No effort was made to test all the organs and tissues of the transgenic mice for the expression of MUC1 as these studies were reported previously (12).

c. E3 cells formed progressively growing neoplasms in MUC1 transgenic mice.

MUC1 transgenic mice express human mucin and are naturally tolerant to human mucin. The data presented in the enclosed (unpublished) manuscript (Fig 3), indicate that the time to first appearance of tumor (the latent period) and the rate of tumor growth in MUC1 transgenic mice injected with E3 cells were not significantly different that the latent period and rate of tumor growth in transgenic mice injected with 410.4 cells, a MUC1-negative breast cancer cell line. Thus, mice that were tolerant to human mucin exhibited no resistance to the growth of breast cancer cells modified to express human mucin. In contrast, the latent period and rate of growth of E3 cells in (non transgenic) BALB/c mice was significantly less than that of 410.4 cells, as presented in Figure 3. Human MUC1 is weakly antigenic in BALB/c mice.

6. Cytokine secretion by E3 cells transduced with retroviral vectors encoding cytokine genes.

Cytokine-secretion by cancer cells is known to augment the cells' immunogenic properties (13-15). Several immune augmenting cytokines were evaluated to determine if cytokine-secretion by E3 cells increased the cells' immunogenic properties in MUC1 transgenic mice. As a first step, retro viral vectors were used to modify the cells to secrete IL-2, IL-4, IFN-g or IL-12. The results presented in the enclosed manuscript confirmed the presence of the relevant cytokine in the culture supernatants of the transduced cells. Passage of the cells in selection medium maintained cytokine-secretion by the transduced cells for more than three months of continuous culture.

7. Secretion of IL-12 by E3 inhibited the cells' tumorigenic properties in MUC1 transgenic mice.

To determine if cytokine-secretion by the cells affected their tumorigenic properties, E3 cells modified to secrete IL-2, IL-4, IFN-g or IL-12 were injected into the fat pad of the breast of MUC1 transgenic mice. As a control, the mice were injected into the fat pad of the breast with an equivalent number of non cytokine-secreting E3 cells transduced with a vector (pZipNeoSV(X) that conferred G418 resistance but did not specify a cytokine gene. The results presented in detail in Figure 5 of the enclosed (unpublished) manuscript indicated that the latent period in the group of MUC1 transgenic mice injected with E3 cells modified to secrete IL-12 (E3-IL-12 cells) was significantly prolonged, relative to that of mice in any of the other groups ($P < .01$). Six of the 8 MUC1 transgenic mice injected with E3-IL-12 cells failed to form tumors and appeared to have rejected the highly malignant breast cancer cells. Two of the mice injected with E3-IL-12 cells formed slowly growing tumors at the injection site. The median survival time (MST) of these mice, approximately 100 days, was significantly ($p < .001$) longer than the MST of mice in any of the other groups including mice injected with E3 cells modified to secrete any of the other cytokines tested. H and E

staining of tissue sections taken from the site of injection of mice injected with E3-IL-12 cells revealed an intense inflammatory infiltrate consistent with the rejection of the cytokine-secreting cells (Figure 1, panels a, b and c, included in the Appendix.).

8. MUC1 transgenic mice that rejected the IL-12-secreting E3 cells developed immunity to breast cancer cells modified to express human mucin.

Tumors failed to form in six of the eight MUC1 transgenic mice injected with IL-12-secreting E3 cells. To determine if the mice that rejected the IL-12-secreting cells developed immunity toward E3 cells, the surviving mice were injected with E3 cells 71 days after the injection of E3-IL-12 cells. As indicated (Fig 6 in the enclosed (unpublished) manuscript), none of these animals formed tumors. Under similar conditions, all of the naïve MUC1 transgenic mice injected with (non cytokine-secreting) E3 cells developed progressively growing neoplasms at the injection site and died from breast cancer.

Thus, the immunogenic properties of E3 cells modified to secrete IL-12 clearly exceeded those of E3 cells modified to secrete any of the other cytokines.

9. Resistance to breast cancer in MUC1 transgenic mice immunized with E3-IL-12 cells was mediated by cellular immune mechanisms.

The failure of E3 cells to grow in MUC1 transgenic mice that rejected E3-IL-12 cells suggested that the resistance was mediated by immune mechanisms. An immunoassay based on the release of cytokine (interferon-g (IFN-g) by antigen-stimulated spleen and lymph node cells was used to investigate this question (16). The results (Table 1 in the enclosed (unpublished) manuscript) indicated that the titers of IFN-g in the culture supernatants of spleen/lymph node cells from MUC1 transgenic mice immunized with E3-IL-12 cells that were co-incubated with E3 cells, or 410.4 cells, were significantly higher than the titers of IFN-g in the culture supernatants of cells co-incubated with B16 cells, an immunologically unrelated mouse neoplasm. IFN-g was undetectable in the supernatants of spleen/lymph

node cell cultures that were incubated without the addition of the tumor cells. Analogous findings were observed if the culture supernatants were analyzed for the presence of MIP1a, a chemokine. The titers of MIP1a in culture supernatants of spleen/lymph node cell suspensions from mice immunized with E3-IL-12 cells co-incubated with E3 cells or 410.4 cells were significantly higher than found in culture supernatants from cell suspensions co-incubated with B16 cells, or from cell suspensions incubated alone. Thus, cellular anti breast cancer immune mechanisms were activated in MUC1 mice immunized with MUC1-positive mouse breast cancer cells modified to secrete IL-12.

An immunofluoresence assay was used to determine if antibodies reactive with E3 cells were present in the sera of MUC1 transgenic mice immunized with E3-IL-12 cells. The results (Table 2 in the enclosed (unpublished) manuscript) indicated that if antibodies reactive with E3 cells were present and that IgG was the major immunoglobulin class reactive with E3 cells in mice immunized with E3-IL-12 cells.

10. Expression of B7.1 by E3 cells augmented the cells' immunogenic properties in MUC1 transgenic mice.

As noted previously, the MUC1 gene is expressed on the apical surfaces of normal glandular epithelial cells of the breast that produce mucin. Mucin is overexpressed and aberrantly glycosylated in breast cancer cells.

The development of a human MUC1 transgenic mouse that expresses MUC1 along with the genetic modification of 410.4 breast cancer cells to express human MUC1 (E3 cells) provides a unique opportunity to investigate the effect of expression of B7.1 by E3 cells on the cells' immunogenic properties. B7.1 is a co stimulatory molecule required for T cell activation (17). It is constitutively expressed by dendritic cells and other types of antigen presenting cells.

Prior reports indicate that the introduction of a gene for co stimulatory molecules such as B7.1 into tumor cells can enhance the cells' immunogenic properties and thus reduce their tumorigenicity. To determine if the expression of B7.1 by E3 cells affected the cells' immunogenic properties in MUC1 transgenic mice, E3 cells were co transfected a cDNA encoding murine B7.1 contained in the plasmid πLN along with a vector that conferred resistance to G418, used for selection. After confirmation of the expression of B7.1 by the breast cancer cells, the cells were tested for their immunogenic and therapeutic properties in MUC1 transgenic mice. The results are summarized in the enclosed (published) manuscript from our laboratory entitled, "Expression of B7.1 in a MUC1-expressing mouse mammary epithelial tumor cell line inhibits tumorigenicity but does not induce autoimmunity in MUC1 transgenic mice," by M. Smith, JM, Burchell, R Graham, EP Cohen and J Taylor-Papadimitriou. Immunology 97: 648-655, 1999. A copy is included in the Appendix.

The results may be summarized as follows:

The expression of B7.1 by E3 cells augmented the cells' immunogenic properties as indicated by a dramatic inhibition of tumor growth in MUC1 transgenic mice injected with the modified cells. The anti tumor activity was mediated by CD+ and CD4+ T cells.
Of special interest, MUC1 transgenic mice immunized with MUC1-expressing breast cancer cells modified to express B7.1 (or to secrete IL-12), did not exhibit signs of autoimmunity. An examination of various organs and tissues from the mice failed to reveal signs of disease. The mice lived their normal life spans without apparent toxic effects.

We believe that the lack of an autoimmune response in MUC1 transgenic mice immunized with MUC1-positive breast cancer cells modified to secrete IL-12, or to express B7.1, is a most important finding. The data indicate that immunotherapy of breast cancer patients who neoplasms express MUC1 with a vaccine prepared by modification of breast cancer cells to secrete IL-12, or to express B7.1 is not likely to induce an autoimmune disease. Tumor regression in the immunized mice suggests that an analogous form of treatment breast cancer patients might offer significant benefit to breast cancer patients.

Treatment of breast cancer with DNA-based vaccines.

In addition to our studies of the immunotherapeutic properties of breast cancer cells modified to express B7.1, or to secrete IL-12, as described above, we are also engaged in complementary studies designed to investigate the immunotherapeutic properties of a DNA-based vaccine in the treatment of mice with breast cancer. The underlying rationale for this approach is that like other types of malignant cells, breast cancer cells are genetically unstable. As a consequence, the cells may specify more than one breast cancer antigen. To "capture" these additional, undefined antigens, we transfer DNA from mouse breast cancer cells into a highly immunogenic cell line. Genes specifying breast cancer antigens are expressed in an immunogenic form by the transfected cells, as indicated by the finding that mice immunized with cells transfected with breast cancer DNA are resistant to challenge with breast cancer cells (please see enclosed (published) manuscript from our laboratory entitled, "Immunity to Breast Cancer in Mice Immunized with Semi-Allogeneic Fibroblasts Transfected with DNA from Breast Cancer Cells." Journal of Immunology, 162: 6934-6941, 1999. A copy is included in the Appendix).

The DNA-based vaccine was prepared by transfection of a mouse fibroblast cell line (LM) with DNA from an adenocarcinoma of the breast that formed spontaneously in a C3H/He mouse. The fibroblasts were first modified to secrete interleukin-2. A retrovirus (pZipNeoSV-IL-2) was used for this purpose. After confirmation that the cells were secreting IL-2, the fibroblasts were further modified to express H-2K^b-determinants. This step was taken because H-2K^b-determinants are allogeneic in C3H/He mice, the original source of the breast cancer cells. Allogeneic determinants are known to act as immune-adjuvants. The modified fibroblasts were then co-transfected with DNA from a spontaneous breast neoplasm that arose in a C3H/He mouse and tested for their immunotherapeutic properties against the growth of the breast cancer cells in the same strain of mice. The results indicated that mice with breast cancer treated solely by immunization with LM fibroblasts transfected with DNA from the breast neoplasm survived significantly ($P < .01$) longer than mice in various control

groups including mice with breast cancer treated by injection with non DNA transfected fibroblasts or fibroblasts transfected with DNA from a heterologous tumor (B16 melanoma). Similar beneficial effects were observed in C57BL/6 mice injected with a syngeneic breast cancer cell line (EO771) and the modified fibroblasts transfected with DNA from E0771 cells. The immunity was mediated by CD8+ T cells since immunized mice depleted of CD8+ cells failed to resist tumor growth.

Figure 2 included in the Appendix illustrates the promise of this approach.

Autoimmunity fails to develop in mice treated with DNA-based vaccines.

Like the treatment of MUC1 transgenic mice with breast cancer cells modified to express B7.1, or to secrete IL-12, the development of an autoimmune disease in mice with breast cancer treated by immunization with a DNA-based vaccine is of concern. Both tumor associated as well as "normal" antigens will be expressed by the DNA-transfected cells. (Other approaches that reply on the transfer of RNA or cDNA from tumor cells into antigen presenting cells, or the "feeding" of peptides eluted from tumor cells to dendritic cells are subject to similar concerns. "Normal" antigens are expressed in an "immunogenic" form by the antigen presenting cells.)

To determine if mice immunized with the DNA-based vaccine exhibited evidence of autoimmunity, various organs and tissues of the immunized mice were analyzed for evidence of lymphoid cell infiltrates. The results are presented in the accompanying Fig 3, included in the Appendix. They indicate that there were no histologic changes in the immunized mice suggestive of autoimmunity. Tumor-free mice injected with the DNA-transfected cells exhibited no ill effects from the vaccine and survived indefinitely.

Plans for future work

We plan two main initiatives in the forthcoming year to further define and expand our previous findings.

For the first, we will modify E3 cells to secrete IL-12 and to express B7.1 The modified cells will be tested in MUC1 transgenic with established breast neoplasms for their immunotherapeutic properties, and compared with the immunotherapeutic properties of E3 modified to secrete IL-12 or to express B7.1 alone. Mice bearing various numbers of tumor cells will be tested so as to define as closely as possible the maximum immunotherapeutic properties of the modified cells. As previously, we will monitor the mice carefully for evidence of autoimmune disease or other types of toxicity to the breast cancer vaccine.

We are also continuing our development of a construct that specifically restricts expression of B7.1 to breast cancer cells. We have defined the region of the MUC1 promoter that is responsible for tissue and tumor specific expression in vivo and have developed a construct in which the B7.1 molecule is driven by the MUC1 promoter.

We are continuing our work on the development of transgenic mice that are homozygously transgenic for MUC1 and are crossing these mice with a mouse that is transgenic for the polyoma T gene. Our preliminary data thus far indicate that these mice develop breast neoplasms within weeks of birth.

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Cancer Therapy with DNA-based Vaccines

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Abstract:

The development of DNA-based vaccines arises from the knowledge that weakly immunogenic, tumor-associated antigens (TAAs), the products of mutant or dysregulated genes in the malignant cells, are expressed in a highly immunogenic form by antigen presenting cells. We successfully prepared vaccines that were effective in the treatment of cancer in mice by transfection of DNA from breast cancer cells into a mouse fibroblast cell line (LM). Fibroblasts express MHC class I-determinants along with B7.1, a co stimulatory molecule. (Classic studies indicate that transfection of genomic DNA can stably alter both the genotype and the phenotype of the cells that take-up the exogenous DNA.) The fibroblasts were transfected with sheared, unfractionated genomic DNA from a breast adenocarcinoma that arose spontaneously in a C3H/He mouse ($H\text{-}2^k$). To increase their non specific immunogenic properties, the fibroblasts were modified before transfection to express allogeneic MHC-determinants ($H\text{-}2K^b$) and to secrete IL-2. Afterward, the IL-2-secreting semi allogeneic cells were co transfected with DNA from the spontaneous breast neoplasm, along with a plasmid (pHyg) conferring resistance to hygromycin. Pooled colonies of hygromycin-resistant cells were then tested in C3H/He mice for their immunotherapeutic properties against the growth of the breast neoplasm. The results indicated that tumor-bearing mice immunized with the transfected cells survived significantly longer than mice in various control groups. Similar beneficial effects were seen in C57BL/6 mice injected with a syngeneic melanoma cells and semi allogeneic, IL-2-secreting fibroblasts transfected with DNA from the melanoma cells. The immunity was mediated by $CD8^+$ T cells and was specific for the type to tumor from which the DNA was obtained.

P.C. Novell first hypothesized that the aggressiveness of growth and the metastatic spread of cancer cells resulted from variations in the genotype of the cells that comprised the population of malignant cells. The genotype of cancer cells was variable, even within a single patient. Since the total number of cancer cells in the patient is so large, a small, barely detectable tumor contains about 10^9 cells, multiple variants are present with unique properties. Altered genes in certain of the malignant cells in the population, for example, genes that control the formation of enzymes such as metalloproteases, enable the cells to degrade matrix proteins including collagen, laminin and fibronectin (Shigemasa et al, 2000). The neoplastic cells invade surrounding tissues and enter lymph channels and capillaries. New blood vessels develop, the result of dysregulation of the malignant cells' usual phenotype to secrete angiogenesis factors (Folkman, 2000; Hahnfeldt et al., 1999). Other genetic changes enable the cells to resist drugs commonly used in chemotherapy (Kolchinsky and Roninson, 1997). Drug resistant cells that survive after chemotherapy form the basis of recurrent tumors. The extraordinarily large number of cells that comprises the primary and metastatic tumor can be characterized by multiple subpopulations, clonal derivatives of the original transformed cell, that vary widely in their phenotypic properties.

The origin of the genetic variation that characterizes the population of malignant cells results from the cells' genetic instability. Mutations are unusually common. Errors during DNA replication occur frequently and go unrecognized. Fishel et al. (1993) among others (Thibodeau et al. 1993; Bonner et al., 1994; Gonzalez-Zulueta et al., 1993, Risinger et al., 1993) described the replication error (RER+) deficient genotype. It was detected in colorectal and breast cancer cells, among types of tumors, and was responsible for the familial pattern of inheritance of the disease (Futreal et al. 1992; Thompson et al., 1993, Gendler et al., 1990). As a result, multiple, largely undefined genes in the malignant cells, not only genes involved in invasion and metastasis, angiogenesis or genes that confer resistance to chemotherapeutic agents, are altered in malignant cells. The phenotype of different cells among the malignant cell population varies widely.

As a result of the frequent alterations in genome of malignant cells, multiple genes in the neoplastic cells differ from the homologous genes in non-malignant cells of the same cancer patient. In some instances, the products of the altered genes are expressed as membrane associated or intracellular proteins. If they differ significantly from proteins expressed by non malignant cells, they become tumor associated antigens (TAAs). Under appropriate circumstances, the altered proteins can become the targets of immune mediated attack. These structural differences between normal and malignant cells of the tumor-bearing host form the rationale of immunotherapeutic strategies that can be used in the treatment of cancer patients.

The antigenic disparity between normal and malignant cells was first described by Prehn and Main (1957), and later confirmed in under different circumstances by van Pel and Boon (1982). Prehn and Main induced a skin cancer in mice by painting a portion of animals' skin with a chemical carcinogen. The painted mice developed a highly aggressive, invasive skin cancers that if left untreated led to the animals' death. Viable cells from the neoplasm could be transferred to naive inbred mice of the same strain, where they would form a tumor. The transferred cells, however, were rejected if the mice had been immunized before transfer with killed cancer cells from the same donor. Thus, the failure of tumor-bearing animal to develop immunity to the tumor was not due to a lack of foreign antigens expressed by the cancer cells, but resulted from the foreign antigen's failure to stimulate an immune response. Of significance, mice immunized to a tumor arising in one mouse were nonetheless susceptible to a tumor arising in a different mouse even if the two mice were of the same inbred strain. The antigenic profile of tumors arising in different mice was unique, consistent with the randomness of the genetic alterations that specified the tumor associated antigens..

Not all tumor antigens are the products of mutant genes. Some are "differentiation antigens," that is, normal gene products that are "reactivated" in malignant cells. TAAs such as MAGE-1, MAGE-3, GAGE 1/2 and BAGE are weakly immunogenic, normal gene products that are shared among different melanoma patients on a background of patient-specific antigens. Others such as P100/Pmel, TRP-2, tyrosinase and Melan A/Mart-1 are tumor antigens associated with both non transformed melanocytes and melanoma cells (Boon et al. 1994; Boon and van der Bruggen, 1996,

Rosenberg, 1999). MUC-1, as an example, is normal gene product which is expressed in an aberrant form in breast cancer cells (Jerome et al., 1991; Miles and Taylor-Papadimitriou, 1998).

If the antigenic phenotype of the transformed cell is sufficiently foreign and can be recognized by the immune system, the cell will be killed, a phenomenon known classically as "immune surveillance." However, other TAAs, insufficiently foreign to provoke immune responses go unrecognized. Malignant cells that express such "weak" antigens are immunologically "silent," and proliferate without inhibition. The end result is that through strong and constant ongoing selection by the immune system, cancer cells that express strong tumor antigens are killed and cancer cells that express weak antigens survive. The cancer patient is therefore populated by proliferating malignant cells whose antigenic profile is insufficiently foreign to provoke the immune system. Under appropriate circumstances, however, an immunity can be induced to weak antigens and the cancer cell can be recognized as foreign with beneficial consequences.

The significance of these findings is that the repertoire of tumor antigens is broad and highly diverse, even within a tumor in a single individual. The population of malignant cells may be characterized by multiple subpopulations that express an undefined, large number of different TAAs. Certain of these antigens are found among cancer cells that are present only in an individual patient. Others are shared among different patients with the same histologic type of tumor.

The characterization and cloning of genes responsible for the formation of TAAs has resulted in new and important approaches in the treatment of cancer patients. Clinical immunotherapy trials are underway based on vaccination with defined genes for TAAs. However, the use of defined genes may not be appropriate since their therapeutic relevance needs to be defined. Some tumor antigens may not be clinically important in the sense that even if the immunization succeeds in inducing an antitumor immune response, the tumor may not be rejected. Furthermore, it is likely that clinically relevant TAAs, that is, defined TAAs that stimulate immune responses capable of leading to tumor rejection, are not expressed by all the cancer cells in the patient. Many cells may be present that express tumor antigens that differ from the antigen chosen for therapy. Just as more than one chemotherapeutic drugs is used, to eliminate a greater proportion of the malignant

cell population, the successful use of a vaccine that includes more than one tumor antigen is likely to result in the killing of a greater number of the diverse population of cancer cells.

Single epitope vaccines can be used in cancer therapy with partial success if the antigenic target is broadly represented among the cells. MUC-1 and Her/2 neu in breast cancer and PSmA in prostate cancer are notable examples of TAAs that are expressed by a large proportion of the patient's cancer cells. Successful immunization against TAAs that are present among the vast majority of the malignant cells can result in the destruction of large numbers of cancer cells, and prolong survival. This was the case in transgenic mice naturally tolerant to human MUC-1, a breast cancer antigen. Mice immunized with a vaccine that stimulated cellular immunity toward human MUC-1 survived longer than mice in various control groups but eventually died from the disease (Carr-Brendel et al., in press).

Nevertheless, greater success will occur if the vaccine encompasses a broader array of tumor antigens expressed by the patient's neoplasm. This point was emphasized by the finding that the expression of known tumor antigens such as gp100 and tyrosinase in melanoma patients was variable even among different melanoma lesions within the same patient. The implication was that different metastatic tumors were derived from clones of cancer cells that varied in their antigenic properties. Strongly antigenic cells were killed; weakly antigenic cells persisted. Further evidence of the diversity of the antigenic phenotype of the cancer cell-population was provided by Anichini et al. (1996) who found CTLs in melanoma patients that were not directed toward known melanoma antigens such as Melan-A/Mart-1, MAGE-3, gp 100 or tyrosinase. Multiple as yet undefined tumor antigens were expressed by different cells that comprised the malignant cell population.

For these reasons, immunotherapy with polyvalent vaccines has a greater rationale. The hope is that by stimulating immunity to a broader representation of the varied antigenic phenotype of the cells that comprise the tumor cell population, greater and greater numbers of cancer cells will be killed. Polyvalent vaccines have been prepared from tumor cell extracts, lysates of tumor cells and by "feeding" apoptotic bodies to autologous antigen presenting (dendritic) cells. Tumor derived RNA and DNA have also been used to induce immune responses toward multiple, different tumor

epitopes within the same tumor-bearing host (Gilboa, 1999; Gilboa et al., 1998). Other means of vaccine preparation include pulsing dendritic cells (DCs) with TAA-derived epitopes (Lotze, 1997). Synthetic peptides from melanoma antigens such as MART1/Melan A, tyrosinase and gp100 have been added to cultures of DCs. The DCs take up and then convert these defined, weak TAAs to TAAs that can stimulate an anti tumor immune response in the host. The rationale is that DCs are the natural and predominant cell type that induces naïve T cells to perform effector functions, in this case, cells that are capable of recognizing and destroying tumor cells. Once activated, the DCs migrate to regional lymph nodes where T cells, predominantly CD8+, are induced to destroy malignant cells.

Although these strategies address the need to immunize with an effective vaccine directed toward multiple T cell epitopes, there are important practical concerns that could limit their wide application. Preparation of a vaccine by exposing antigen presenting cells to tumor peptides or tumor lysates from the patient's tumor requires substantial quantities of tumor cells, limiting its use to patients with extensive disease. Patients in clinical remission may not be candidates for therapy since the quantity of available tumor is small. More than one immunization may not be possible if the entire tumor specimen is used to prepare the first vaccine. In addition, the isolation of DCs from the patient is not trivial. It requires recovery of DCs from peripheral blood by plasmaphoresis, and expansion of the cell number in vitro under carefully controlled conditions.

Immunization by subcutaneous injection of "naked" mRNA or cDNA from the patient's tumor, or by the injection of mRNA-pulsed DC's also addresses the need to immunize a vaccine that encompasses a larger representation of the repertoire of tumor antigens. MRNAs from the neoplastic cells specify various different TAAs along with nucelotide sequences coding for determinants associated with non malignant cells, in varying proportions. However, there is a danger that the injected mRNA will be degraded before it is taken up by DCs, or that it will be sequestered and degraded intracellularly. The quantity of mRNA specifying clinically relevant tumor antigens may be insufficient to induce a vigorous anti tumor immune response, and repeated immunizations of the cancer patient may not be possible if the entire specimen was required to prepare the first vaccine.

Immunization with hybrid cell vaccines prepared by fusion of tumor cells with DCs from the same tumor bearing host resulted in the successful induction of anti tumor immune responses. This approach is an outgrowth of experiments on the immunotherapeutic properties of tumor cell-hybrids first reported by Cohen and his colleagues (Liang and Cohen 1977a; Liang and Cohen, 1977b; Kim and Cohen, 1979). The rationale is that like other genes expressed by hybrid cells, monoclonal antibodies are a notable example, genes specifying undefined weakly immunogenic TAAs that characterize the patient's neoplasm will be expressed in a highly immunogenic form by the hybrid cells. The results (Gong et al., 1997) in a murine breast cancer model system indicated that mice with breast cancer treated solely by immunization with a hybrid cell vaccine prepared by fusion of the breast cancer cells with syngeneic DCs resulted in the induction of strong anti breast cancer immune responses.

The use of hybrid cells as a tumor cell vaccine has certain important advantages. The main advantage is that the hybrid cells combine the desirable features required to successfully induce the anti tumor immune response. The TAAs that characterize the neoplasm are presented to CTLs of the host in the context of class I MHC-determinants, co stimulatory and adhesion molecules contributed by the DCs to the hybrid cells. The use of autologous DCs means that the MHC type is appropriate for direct antigen presentation to naïve T lymphocytes of the host. The main disadvantage, however, is that a cell line derived from the patient's primary tumor must be established if the cells are to be successfully fused with DCs from the patient. This cannot always be accomplished. Cell lines from primary tumors are notoriously difficult to establish. And, in those instances in which a cell line from the patient's tumor can be converted to growth in vitro, there is no assurance that the cells that are capable of proliferation in culture are truly representative of the tumor cell population as a whole. A non representative variant may emerge because it is capable of growth in vitro, and tumor cells that express clinically relevant TAAs may be omitted from the mix. Furthermore, if the hybrid is to be prepared by fusion of tumor cells with DCs from the patient, a requirement for direct antigen presentation through the appropriate class I molecule, then the same technical constraints described previously will apply.

The fundamental and important principle addressed by these methods is that multiple genes specifying most if not all of the total array of TAAs that characterize the weakly antigenic tumor

are likely to be expressed in a highly immunogenic form by the vaccine. The nucleotides and hybrid cells specify the spectrum of mostly undefined antigens that characterize the patient's tumor. A vaccine at least partially effective in cancer therapy can be prepared even if the TAAs have not been identified, or their biological relevance has not been established.

Effective vaccines can be prepared by transfer of genomic DNA from cancer cells into a highly immunogenic cell line.

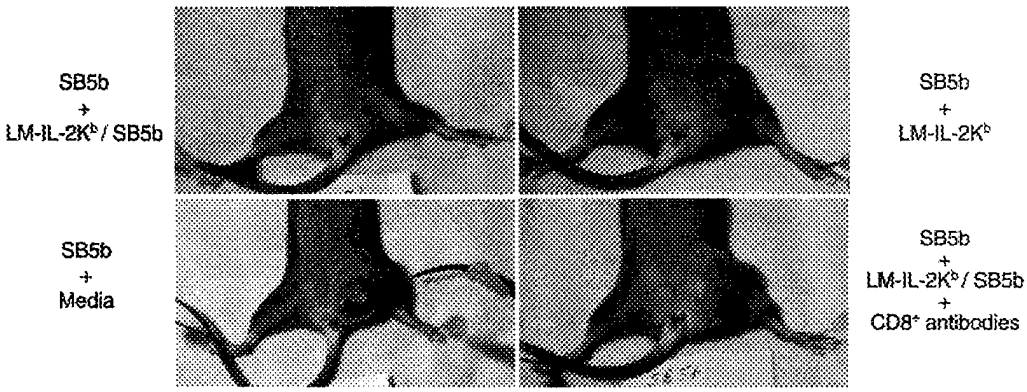
The successful elimination of larger and larger numbers of the patient's tumor cell population, including small, widely dispersed small nests of cancer cells in metastatic sites, will require a polyvalent vaccine that specifies as many of the unique tumor antigens that characterize the patient's neoplasm as possible. Vaccines that induce anti tumor responses to a single TAA are less likely to succeed, even if the TAA is biologically relevant and immunodominant. The heterogeneity of the tumor cell population predicts that variants that do not express the single antigen chosen for therapy will not be killed. To enable large numbers of patients to receive treatment, the preparation of the vaccine should be simple; it should be readily and conveniently prepared, ideally without the need for complex equipment or highly specialized facilities. The vaccine should be able to be prepared with only small amounts of tumor tissue so that even patients with quite small tumor loads and those in clinical remission can receive treatment as well. The vaccine must be nontoxic and without harm, even after multiple immunizations of the cancer patient, and it should induce immunologic memory against the patient's neoplasm.

To address these concerns, we applied classic and well established techniques to prepare a vaccine for use in cancer therapy. Our results in animal models indicate the validity of this approach (Kim and Cohen, 1994; Sun et al., 1995; de Zoeten et al., 1998; de Zoeten et al., 1999). We reasoned that altered genes in cancer cells specifying weak TAAs would expressed in a highly immunogenic form if they were formed by a well characterized cell line that expressed both syngeneic and allogeneic class I MHC-determinants. Genomic DNA from the tumor including genes specifying TAAs that characterized the malignant cell-population were transferred into the recipient cells, modified before DNA transfer to secrete IL-2. The transfected cells were tested for their immunogenic properties in tumor-bearing mice. Modification of the genome by transfer of DNA from one cell type to another is an application of classic studies. Wigler et al. (1979) found that

the genome of mouse cells deficient in the enzyme adenine phosphoribosyltransferase could be modified to express the missing enzyme by transfection of high molecular weight genomic DNA from mouse cells whose genome contained the missing gene. The cells that took up the exogenous DNA, integrated the transferred DNA and expressed the missing enzyme. We confirmed these findings in a related system. A subpopulation of mouse cells deficient in the enzyme thymidine kinase expressed the missing enzyme following transfer of genomic DNA from enzyme positive cells. Analogous findings were observed for genes specifying membrane associated determinants. Mendersohn et al. (1986) converted mouse polio virus receptor-negative cells to cells that expressed the missing receptor by transfection of genomic DNA from receptor positive cells. Hsu et al. (1984) and Kavathas and Herzenberg (1983) generated stable transfectants of mouse LM fibroblasts that expressed the human membrane determinants, HLA and B-2 microglobulin by transfection of DNA from human cells. Approximately 1 in 500 transfected mouse cells expressed the human gene of interest. The transferred genes were integrated into the genome of the recipient cells and were replicated as the cells divided over multiple cell generations. Most notable, Shih and Weinberg (1982) and Shih et al. (1981) used this approach to first identify oncogenes such as *ras* . They transfected DNA from human bladder carcinoma and neuroblastoma cells into premalignant NIH 3T3 cells to identify transforming genes.

We used this approach to develop a DNA-based vaccine that was effective in the treatment of established tumors in experimental animals. We transferred DNA from mouse melanoma, or mouse breast carcinoma cells into a selected mouse fibroblast cell line. Fibroblasts like DCs and macrophages express MHC class I determinants, along with co stimulatory molecules such as B7.1 and can act as efficient antigen presenting cells. The rationale was that multiple genes specifying an array of undefined tumor antigens would be expressed in a highly immunogenic form by the transfected cells.

The results, illustrated in the following figure and in published manuscripts (de Zoeten et al., 1999' de Zoeten et al. 1998) indicate the important potential of this approach.



Legend: Naïve C3H mice received three s.c. injections at weekly intervals of 5 x 10^6 LM fibroblasts transfected with DNA from an adenocarcinoma of the breast that formed spontaneously in a C3H mouse. One week after the last injection, the mice were injected with 5 X 10^3 breast cancer cells from the same neoplasm (SB-5b). The photo was taken 35 days after the injection of the breast cancer cells. SB5b + LM-IL-2K^b/SB5b = Immunized with LM fibroblasts modified for IL-2-secretion and the expression of H-2K^b determinants, transfected with DNA from SB5b, a breast neoplasm that arose spontaneously in a C3H mouse. SB5b + LM-IL-2K^b = Immunized with LM fibroblasts modified for IL-2-secretion and the expression of H-2Kb determinants but not transfected with DNA from the breast cancer cells. SB5b + media = Injected with SB5b cells alone. Sb5b + LM-IL-2K^b/SB5b + CD8 antibodies = The mice were depleted of CD8 cells before immunization with LM-IL-2K^b/5b cells followed by the injection of the breast cancer cells.

Immunization of mice with a cell line transfected with DNA from the neoplastic cells resulted in the induction of strong anti tumor immune responses mediated by CD8+ cells. The immunity was sufficient to result in the regression of small established neoplasms. Immunologic memory was induced as immunized mice rejecting the tumor were resistant to re challenge with the same tumor when challenged three months later. Consistent with the unique antigenic phenotype of independently arising neoplasms, the immunity in the vaccinated mice was specific for the type of tumor from which the DNA was obtained. Mice immunized with a vaccine prepared by transfection of fibroblasts with DNA from a mouse melanoma were susceptible to the growth of breast cancer cells. Immunizations with non DNA-transfected fibroblasts also failed to result in induction of immunity to the tumor.

A vaccine prepared by transfer of tumor DNA into a highly immunogenic cell line has a number of important advantages. A major advantage is that the recipient cells can be selected for special properties that will augment their immunogenic properties. In our experiments, the recipient cell line expressed allogeneic class I determinants along with class I determinants that were syngeneic in the tumor bearing mice. Allogeneic determinants were included because they are strong immune adjuvants. Syngeneic class I determinants provide a restriction element for direct presentation of TAAs to CTLs of the host. Our experience indicates that a vaccine prepared by transfer of tumor DNA into cells that express syngeneic class I-determinants alone, that is, without allogeneic determinants, are not immunogenic and do not stimulate anti tumor immune responses.

(For patient therapy, the recipient cells can be matched for one or more class I alleles with those of the patient. HLA-A2.1, for example, is common. The remaining alleles are expected to be allogeneic. Even though other HLA alleles are present in lesser proportion among the population, there are sufficient types that are commonly found to enable vaccines to be prepared for most if not all patients.)

The use of a selected cell line as the recipient of tumor DNA has other important advantages. The cells can be modified in advance of DNA transfer to further augment their immunogenic properties. Cytokine genes such as IL-2, GM-CSF, IL-12 can be introduced before the cells are transfected with DNA from the neoplasm. Local delivery of IL-2 by the vaccine, for example, mobilizes immune cells to the vaccination site, facilitates the immune response and promotes the development of immunologic memory. GM-CSF stimulates the proliferation and the activity of dendritic cells. Conceivably, modification of the cells to secrete more than one cytokine could augment the cells' immunogenic properties even further. Our prior experience (Kim et al., 1995) indicates that modification of the used as the DNA recipients to secrete both IL-2 and interferon –gamma increased their immunogenic properties even more than that of recipient cells modified to secrete either cytokine alone. And, since the cell line that takes up DNA from the cancer cells is capable of indefinite proliferation in vitro, and the transferred DNA is replicated as the cells divide, only a small quantity of tumor DNA is required to generate the vaccine. Sufficient amounts might be obtained from small surgical specimens. We use 100 ug DNA to prepare an

effective vaccine, equivalent to a neoplasm of approximately 1 mm^3 in size. The vaccine can be prepared from primary tumors, without the establishment of a cell line of the patients tumor.

Although vaccination based on transfer of tumor-derived DNA into a highly immunogenic cell line has a number of advantages, there are concerns as well. Conceivably, genes specifying normal cellular constituents expressed by the transfected cells could be recognized as "foreign", provoking an autoimmune disease. This has not been the case thus far. Mice immunized with the DNA-based vaccines failed to exhibit any adverse effects. Cellular infiltrates into normal organs and tissues have not been observed. The vaccine itself which expresses allogeneic determinants is immunologically foreign and, like any other allograft, is eventually rejected. Other protocols that depend on the use of tumor cell-extracts, mRNAs derived from tumor cells or hybrid cell vaccines are subject to the same concern.

It is also conceivable that the DNA-transfected cells might grow in the recipient, forming a tumor, or that a transforming oncogene or defective tumor suppressor gene might be transferred to a normal cell of the host, provoking a neoplasm. This has not been observed. In multiple experiments, no animal receiving the DNA-based alone vaccine has developed a neoplasm. Our observation that anti tumor immunity developed in mice immunized with the transfected cells is an indication that multiple and possibly large numbers of immunologically distinct tumor antigens, the products of multiple altered genes were present in the tumor from which the DNA was obtained.

The robust anti tumor immune responses that follow immunization with a vaccine prepared by transfection of tumor DNA into the highly immunogenic cell line raise the possibility that an analogous approach can be used to prepare a vaccine for the treatment of patients with malignant disease.

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1.4kb 5′ *MUC1* promoter sequence drives tissue specific expression of *MUC 1* cDNA in *MUC1* transgenic mice, and up-regulates expression in the mammary gland at lactation and in malignancy.

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Summary

This study examines the regulation of expression of the human *MUC1* gene *in vivo*, using *MUC1* transgenic mice. The data show that epithelial specific expression of MUC1 can be directed by just 1.4Kb 5′ flanking sequence using *MUC1* cDNA as a reporter gene *in vivo*. Furthermore high levels of *MUC1* expression were seen in the lactating mammary gland and in spontaneous mammary tumours. This pattern of expression in epithelial tissues is comparable to the expression of MUC1 in humans and also to the expression pattern in another transgenic mouse line developed with a 10.6kb genomic *MUC1* fragment. The study confirms that *MUC1* is a compact gene and demonstrates that the 1.4kb 5′ sequence not only directs epithelial specific expression of MUC1 *in vivo* but also contains the elements governing the up-regulation seen at lactation and in malignancy.

Introduction

The human *MUC1* gene (Gendler et al, 1990) codes for a heavily glycosylated, membrane mucin which is normally expressed by simple epithelial cells lining glands or ducts, including the luminal cells of the mammary gland where expression is dramatically increased at lactation. Increased expression and aberrant glycosylation of this mucin is also seen in more than 90% of breast, ovarian and pancreatic carcinomas and in some lung and colon cancers. Because of the high level of expression and altered glycosylation of the cancer mucin, considerable interest has developed in MUC1 as a target antigen for immunotherapy of various cancers. (see review Graham et al, 1996). This interest has been enhanced by the observation that immunological responses to MUC1 have also been detected in cancer patients (Kotera et al, 1994; Jerome et al, 1989) and pre-clinical and clinical studies have been initiated using various formulations of MUC1 as an immunogen (Graham et al, 1996; Ding et al, 1993; Acres et al, 1993; Xing et al, 1995; Goydos et al, 1996; Reddish et al, 1998; Samuel et al , 1998; Scholl et al, in press).

The *MUC1* promoter is also being studied in the context of directing epithelial and /or tumour specific gene expression. (Kovarik et al, 1993, 1996; Chen et al, 1995; Ring et al, 1997 and Tai et al, 1999). The gene coding for the mucin is located in a gene rich area of chromosome 1q21 (Swallow et al, 1987), where it is flanked 5′ by the thrombospondin-3 gene (Vos et al, 1992), the polyadenylation site of which is just 2.6Kb upstream of the *MUC1* transcription start site. Moreover 1kb downstream of the *MUC1* polyadenylation site lies another gene that gives a 13kb transcript in most

tissues (Gendler and Spicer, 1995). Thus *MUC1* appears to be a relatively compact gene with all the regulatory elements likely to be located within close proximity to the gene itself. Indeed *in vivo* studies in transgenic mice showed that the sequences controlling tissue specificity of expression as well as the increased expression seen upon differentiation of the mammary gland at lactation and in malignancy are contained within a 10.6 kb genomic fragment (Peat et al, 1992; Graham et al, 1995). The fragment used to develop the transgenic mice consisted of the exons and introns of the gene flanked by 1.6 of 5′ sequence and 1.9 kb of 3′ sequence.

In vitro transfection experiments using as little as 0.8kb of 5′ *MUC1* flanking sequences fused to the CAT reporter gene showed CAT expression was restricted to epithelial cell lines. (Kovarik et al, 1993). This sequence contains proximal and distal Sp1 sites, both of which appear to be important in controlling expression (Kovarik et al, 1996, Morris et al, submitted for publication). *In vitro* studies also showed that the 3′ *MUC1* flanking sequence in the fragment used to develop the transgenic mice showed no enhancer activity in an enhancerless SVCAT vector. The importance of the 5′ region is also suggested by the fact that this region (-659/+16) shows 72% homology at the nucleotide level in the mouse *muc1* homologue, and it is likely that the conserved motifs are necessary for correct expression of the gene (Vos et al, 1993).

To determine whether 1.4kb of 5′*MUC1* flanking sequence was capable of regulating expression of the *MUC1* cDNA *in vivo* in a manner comparable to that seen in humans, transgenic mice were generated using a construct where 1.4kb of 5′ flanking

sequence were fused to *MUC1* cDNA sequences. Expression of MUC1 was analysed in normal tissues, lactating mammary gland and in spontaneous mammary gland tumours developing in female mice carrying both the *MUC1* transgene and the *polyoma middle T* oncogene (Guy et al, 1992). The results show that 1.4kb of 5′ sequence contains all the elements necessary for epithelial specific expression of the gene as well as for the up-regulation of gene expression seen at lactation and in tumours.

Materials and Methods

Development of MUC1 transgenic mice

1.4kb 5′*MUC1* sequence (-1401 to +33) previously cloned into a Bluescript vector (Peat et al, 1992), was excised and cloned into the EcoRI-XhoI sites of the promoterless mammalian expression vector pNASSß (Clontech) containing the E. coli ß galactosidase gene. The ß-galactosidase gene was excised using NotI and replaced with the full length *MUC1* cDNA (32 tandem repeats). (Figure 1).

The construct was tested *in vitro* in a transient transfection of the murine mammary carcinoma cell line 410.4 using FuGENE™6 transfection reagent (Boehringer Mannheim), according to manufacturers instructions, using 6µl FuGENE reagent in

94µl Dulbecco's modified Eagles Medium (DMEM) with 2µg DNA. After culturing for 48-72hours at 37°C the transfected cells were fixed for 10 minutes in cold methanol: acetone (1:1), and stained with HMFG2 monoclonal antibody and secondary FITC labelled rabbit anti-mouse immunoglobulins (Dako, 1:50).

A 5.5kb EcoRI fragment containing the *MUC1* promoter and *MUC1* cDNA was purified and dissolved in injection buffer (10mM Tris, 0.1mM EDTA pH7.4 prepared in ultrapure water). The fragment was then centrifuged through a 1ml sepharose G25 column, equilibrated in injection buffer, and sterile filtered by centrifuging through a 0.22µm spin-X Costar filter. DNA (5ng/µl) was injected into the male pronucleus of day1 F1 x F1 hybrid (CBA x C57Bl6) mouse embryos which were subsequently transferred into Day1 plugged pseudopregnant CBA x C57Blk F1 females.

Identification of MUC1 transgenic mice.

Genomic DNA was prepared from tail snips by digesting the tails overnight in proteinase K at 55°C and extracting the DNA twice with phenol: chloroform and once with chloroform. Extracted DNA was digested with Hinf1 restriction endonuclease and run on a 1% agarose gel, blotted onto Hybond N nitrocellulose membrane and subsequently probed with the human p*MUC7* probe. The probe, which corresponds to a 500bp MUC1 tandem repeat fragment, was labelled using random priming using [α-^{32}P]dCTP (Redivue - Amersham International plc). Transgenic founders were then bred to establish whether the transgene was transmitted and expressed.

Immunohistochemical staining of MUC1 transgenic mouse tissues

Tissue samples were collected from mice that were identified as carrying the human *MUC1* gene to establish the pattern of expression of the transgene. Mammary glands were also collected from some female mice at day 9 or 10 of lactation. Tissues were fixed in methacarn (60%methanol: 30% chloroform: 10% acetic acid) and processed for paraffin-wax embedding. MUC1 expression was initially detected in organ sections using biotinylated murine HMFG1 (Burchell et al, 1987) and a peroxidase conjugated avidin-biotin complex (Dako). Sections were developed in Diaminobenzidine tetrahydrochloride (200mg in 400ml PBS + 60µl H_2O_2) and counterstained in haemotoxylin.

Development of spontaneous tumours in MUC1 transgenic mice.

Homozygous mice were made from the founder line showing transmission and epithelial expression of the *MUC1* transgene. Homozygotes were identified by test crossing with non-transgenic mice, and demonstrating the presence of *MUC1* DNA in 100% of the offspring. In order to generate spontaneous tumours in these mice they were mated with heterozygous male *Polyomavirus-Middle-T* oncogene transgenic mice (Guy et al, 1992). The offspring were screened for the *polyoma T* transgene using PCR on 100-500ng genomic DNA prepared from tail snips with the following primers: FWD (5'-3') CCAGAACTCCTGTATCCAGAAGCG and REV(3'-5') GGATGAGCTGGGGTACTTGTTCCCC. 50µl PCR reactions contained 2% deionised formamide, 0.2mMdNTP mix, 1X PCRbuffer (Promega), 1µM forward and reverse primers and 1.75mM $MgCl_2$. After 10 minute hot start at 94°C 0.5µl Taq DNA

polymerase was added and the reaction cycled as follows: 94°C - 1 minute, 55°C - 1 minute and 72°C - 1 minute for 30 cycles followed by 72°C for 10 minutes. Polyoma T antigen positive female mice developed multifocal mammary adenocarcinomas. Tumours were excised when they reached 1cm in diameter, fixed in methacarn, processed for wax embedding and stained for MUC1 using a less sensitive method than described above, employing a humanised HMFG1 monoclonal antibody (1:5,000 (Verhoeyer et al., 1993) and a secondary peroxidase conjugated anti-human IgG (Dako, 1:50). This enabled better differentiation between low and high levels of MUC1 expression while avoiding background staining that was detected when using an unlabelled murine Mab.

Production of RNA from mouse mammary gland and mammary gland tumours.

Normal resting mammary gland, mammary gland at day 9-10 of lactation and 1cm diameter mammary tumours were dissected and immediately snap frozen in liquid nitrogen. Tissue was homogenised in Trizol reagent (Life Technologies) using a polytron homogeniser and total RNA isolated according to manufacturers instructions. Northern blots of 50μg of total RNA were probed with the human p*MUC7* probe.

Results

In Vitro expression of MUC1 cDNA driven b y 1.4kb MUC1 promoter

The *MUC1* construct, where 1.4kb of 5' *MUC1* sequence is driving expression of *MUC1* cDNA, is shown in figure 1. Tissue specificity of expression was first tested *in vitro* by transiently transfecting the 410.4 mammary epithelial tumour cell line (expressing mouse *muc1*). As controls, a construct containing *MUC1* driven by the widely acting β-actin promoter (actin-*MUC1*) and empty vector were also used in the transfections. The mouse homologue muc1 differs sufficiently in the tandem repeat sequence from MUC1 to be unreactive to antibodies reacting with epitopes in the tandem repeat of MUC1 (Spicer et al, 1991) Therefore MUC1 expression was detected by immunofluorescence using the antibody HMFG2 (Burchell et al, 1987) on methanol:acetone fixed cells. Figure 2 shows expression of the construct in the cytoplasm of 410.4 cells 72 hours after transfection. The cells were also positive for MUC1 after transfection with actin-*MUC1*, but no staining was observed after transfection with empty vector.

Tissue specific expression of MUC1 in vivo:

The p*MUC7* probe containing tandem repeat sequence (showing low homology to the mouse tandem repeat sequence) was used to identify 17 possible founder *MUC1* transgenic mice, 7 of which failed to transmit the transgene to their offspring. Tissues were stained from litters of the remaining 10 mice to establish the pattern of expression of the *MUC1* transgene. Progeny from 6 of these lines did not express the transgene. Expression of the human *MUC1* cDNA was seen in the remaining 4 lines,

but 2 of these showed expression in a restricted range of epithelial tissues as compared to expression seen in humans. In these two strains, salivary glands, kidney, uterus, fallopian tubes, lungs, stomach, and testis showed MUC1 expression, but no/ very little expression was seen in the pancreas and normal mammary gland. Furthermore no up-regulation of expression was seen in the mammary gland at lactation. Non-epithelial tissues such as muscle, heart and spleen were negative. The remaining 2 lines however showed a pattern of expression analogous to that seen in humans, with all the above mentioned epithelial tissues showing expression of MUC1 as well as the pancreas and mammary gland, (with expression being up-regulated at lactation). Figure 3 illustrates the epithelial specificity of expression in one of these strains (MUC1.1.4A) showing absence of expression in the mouse muscle (A), and clear expression in the salivary gland (B), the kidney (C) and the pancreas (D). The up-regulation of expression at lactation in the 1.4A mice is illustrated in Figure 4 where the weak expression of MUC1 seen in the resting mammary gland (A) is dramatically elevated in the lactating mammary gland (B). The staining shown in figure 4 was done using the humanised HMFG1 antibody rather than the biotinylated mouse HMFG1 and the avidin–biotin complex, used in figure 3. Staining with humanised HMFG1 is less sensitive than the avidin biotin staining method and allowed the differential expression of MUC1 in the resting and lactating gland to be detected by immunohistochemistry. There is obviously also a dramatic increase in the amount of epithelial tissue present in the lactating mammary gland, but the level of expression at the cellular level does appear to increase at lactation.

Expression of MUC1 glycoprotein in spontaneous tumours:

The mouse line MUC1 1.4A, showing up regulation at lactation, was expanded further and homozygous *MUC1* transgenic mice were developed. Female homozygote *MUC1* 1.4A transgenic mice were mated with heterozygote male *polyoma-middle T* transgenic mice to generate 28 offspring. PCR-based screening identified 14 offspring (9 females and 5 males) expressing both the *MUC1* and *polyoma T* transgenes. All 9 females developed mammary tumours (>5mm) by week 9. Small metastases also developed in the lungs of all 9 females. No tumours developed in the positively identified males by 10 weeks. 32 tumours were collected from the female mice and processed for immunohistochemical analysis of MUC1 expression. One elderly *polyoma middle T* heterozygote male (not expressing the *MUC1* transgene) also developed a tumour, this was used as a negative control.

The *polyomaT* mice were generated on an FVB background and the *MUC1* transgenic mice were created in CBA x C57 F1's. Tissue was therefore also examined from the double transgenic mice to ensure that MUC1 expression remained tissue specific in the FVB x C57 x CBA background. As expected MUC1 was not expressed in tissues collected from male *polyoma middle T* mice on the FVB background, but was expressed in the tissue specific manner already described in *the MUC1/polyoma middle T* double transgenic mice. All 32 tumours collected from the 9 *MUC1/polyoma middle T* female transgenic mice expressed MUC1 as detected with humanised HMFG1 monoclonal antibody. The control tumour from the *polyoma middle T* male mouse did not express MUC1. In the resting mammary gland MUC1 is expressed at low levels on the

apical surface of the gland lumen. This luminal staining is maintained in tumour sections where the tissue remains polarised but is also apparent in the cytoplasm and surface of invasive cells. MUC1 expression within the tumours is heterogenous, with both positive and negative areas. Figure 4C shows MUC1 expression in a spontaneous tumour developing in a polyoma T/1.4A MUC1 transgenic mouse.

MUC1 RNA detection in mammary gland and tumours:

Northern blot analysis was then performed to determine whether this high level of expression of *MUC1* seen with immunohistochemistry, was also seen at the RNA level and was therefore likely to be due to transcription from the *MUC1* promoter. RNA was analysed from resting mammary glands, mammary tumours collected from a further 17 *PolyomaT/MUC1* transgenic female mice and from lactating mammary glands collected from *MUC1* positive/*polyoma T* negative mice. Figure 5 shows that *MUC1* mRNA levels were high in 9/10 tumours and in the four samples prepared from the lactating mammary gland, compared to low or undetectable levels in the resting mammary gland. Phosphor-imager analysis was used to compare the relative radiolabel per lane and the average fold increase of MUC1 in tumours and lactating mammary gland over resting breast was 59 and 51 fold respectively.

Discussion

Earlier *in vitro* data performed in this laboratory suggests that MUC1 is a compact gene, with regulatory elements required for tissue specific expression contained within 1.4kb 5′ flanking sequence. In this study we support this by showing that 1.4kb 5′ *MUC1* flanking sequence is indeed sufficient to direct tissue-specific expression of the human *MUC*1 cDNA *in vivo* in transgenic mice. *MUC1* transgenic mice made in 1992 by Peat et al, also showed tissue specific expression of human MUC1, but these mice were created using a 10.6kb genomic DNA fragment containing the human *MUC1* gene. This study suggests that sequences within the *MUC1* introns or 3′ to the *MUC1* gene are not required for *MUC1* gene expression in epithelial tissues in vivo. The 3′flanking sequences present in the genomic fragment of the original transgenic mice, have also been shown to lack enhancer activity in vitro in experiments using an enhancerless SVCAT vector (Kovarik et al., 1993). While it is formally possible that the *MUC1* cDNA itself could contribute to the regulation of the gene, this is unlikely and mice transgenic for variants of the *RAS* oncogene transcribed from the same 1.4kb *MUC1* promoter fragment express RAS in cells endogeneously expressing *MUC1* (N. Fresney, and Julian Downward Signal Transduction Laboratory, Imperial Cancer Research Fund, personnal communication).

Immunohistochemical studies, using HMFG1 monoclonal antibody, showed that MUC1 was expressed at high levels in the lactating mammary gland of the *MUC1* cDNA transgenic mice. High levels of MUC1 were also detected in spontaneous mammary tumours generated by breeding female homozygous *MUC1* cDNA

transgenic mice with heterozygous male *polyoma middle T* oncogene transgenic mice. Although quantitation of the actual amount of MUC1 expressed by individual cells is difficult using immunohistochemical techniques, there does appear to be an increase in the intensity of staining of the luminal cells seen in the lactating gland and in the malignant cells which are derived from this lineage and found in tumours. Increased transcription of *MUC1* in breast cancers in humans has been demonstrated using *in situ* hybridisation (Ligtenberg et al, 1990). A similar increase in *MUC1* mRNA is seen in the MUC1 transgenic mice described here.

Several studies are in progress evaluating the use of MUC1 based immunogens in developing for immunotherapy of epithelial carcinomas (Graham et al, 1996, Miles and Taylor-Papadimitriou, 1998). However optimal formulations have not yet been defined. The double transgenic mouse produced by breeding homozygous female *MUC1* transgenics with heterozygous male *polyoma T* transgenics should provide a useful model system for evaluating the efficacy of MUC1 based immunogens in tumour rejection studies and would allow the question of tolerance to be addressed. In addition, the demonstration that 1.4kb of *MUC1* 5′ sequence is sufficient to direct epithelial expression in normal and malignant cells suggests that these sequences can be used for the directed expression of specific genes *in vivo.*

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Figure Legends

Figure 1: Diagram of the plasmid designed to produce *MUC1* transgenic mice showing the 1.4kB *MUC1* promoter driving the full length *MUC1* cDNA, based on the Clonetech pNASSβ vector.

Figure 2: Murine mammary carcinoma 410.4 cells transiently transfected with pNASSβ vector containing 1.4kb *MUC1* promoter and *MUC1* cDNA, stained with HMFG2 and FITC anti-mouse secondary antibody.

Figure 3: Expression of human MUC1 in transgenic mouse tissues detected with Biotinylated HMFG1 monoclonal antibody and ABC complex. MUC1 is absent from the muscle (A), but is expressed in the salivary gland (B), kidney (C) and pancreas (D).

Figure 4: MUC1 expression in normal resting mammary gland (A), in the lactating mammary gland (B) and in a spontaneous mammary tumour (C). MUC1 was detected using humanised HMFG1 and peroxidase conjugated anti-human secondary antibody

Figure 5: A - Northern blot analysis of 50μg total RNA from normal resting mammary gland, *Polyoma T* oncogene induced tumours and from day 9-10 post-partum lactating mammary gland, probed with *MUC1* tandem repeat probe. **B** – 5μg total RNA was analysed simultaneously on a formaldehyde gel containing ethidium bromide to show loading.

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FIGURE 1

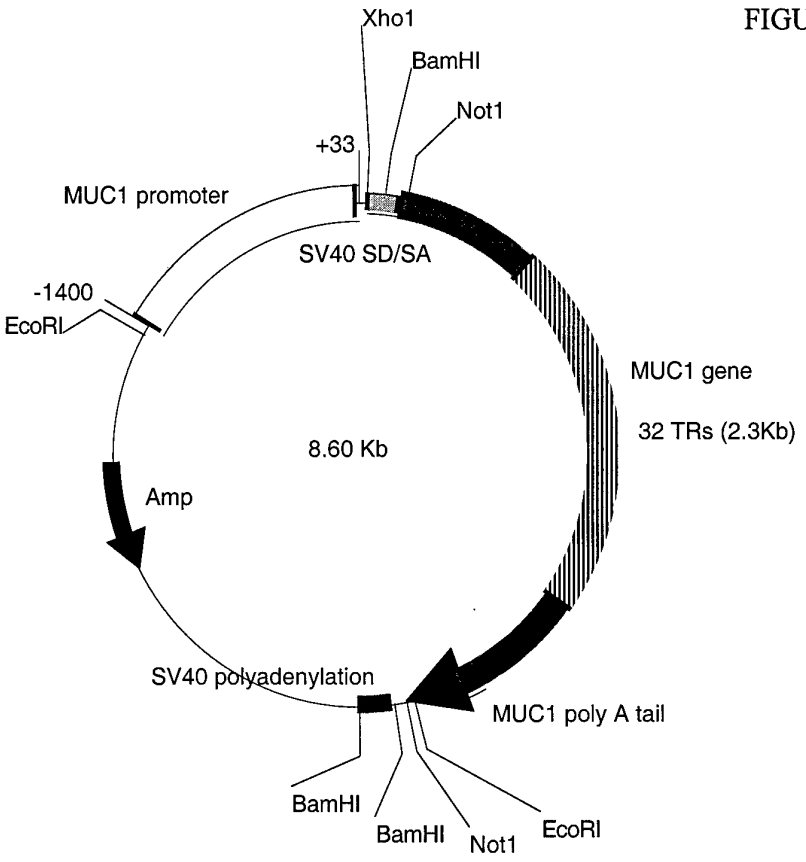

Xho1
BamHI
Not1
+33
MUC1 promoter
SV40 SD/SA
-1400
EcoRI
MUC1 gene
32 TRs (2.3Kb)
8.60 Kb
Amp
SV40 polyadenylation
MUC1 poly A tail
BamHI
BamHI
Not1
EcoRI

FIGURE 2

FIGURE 3

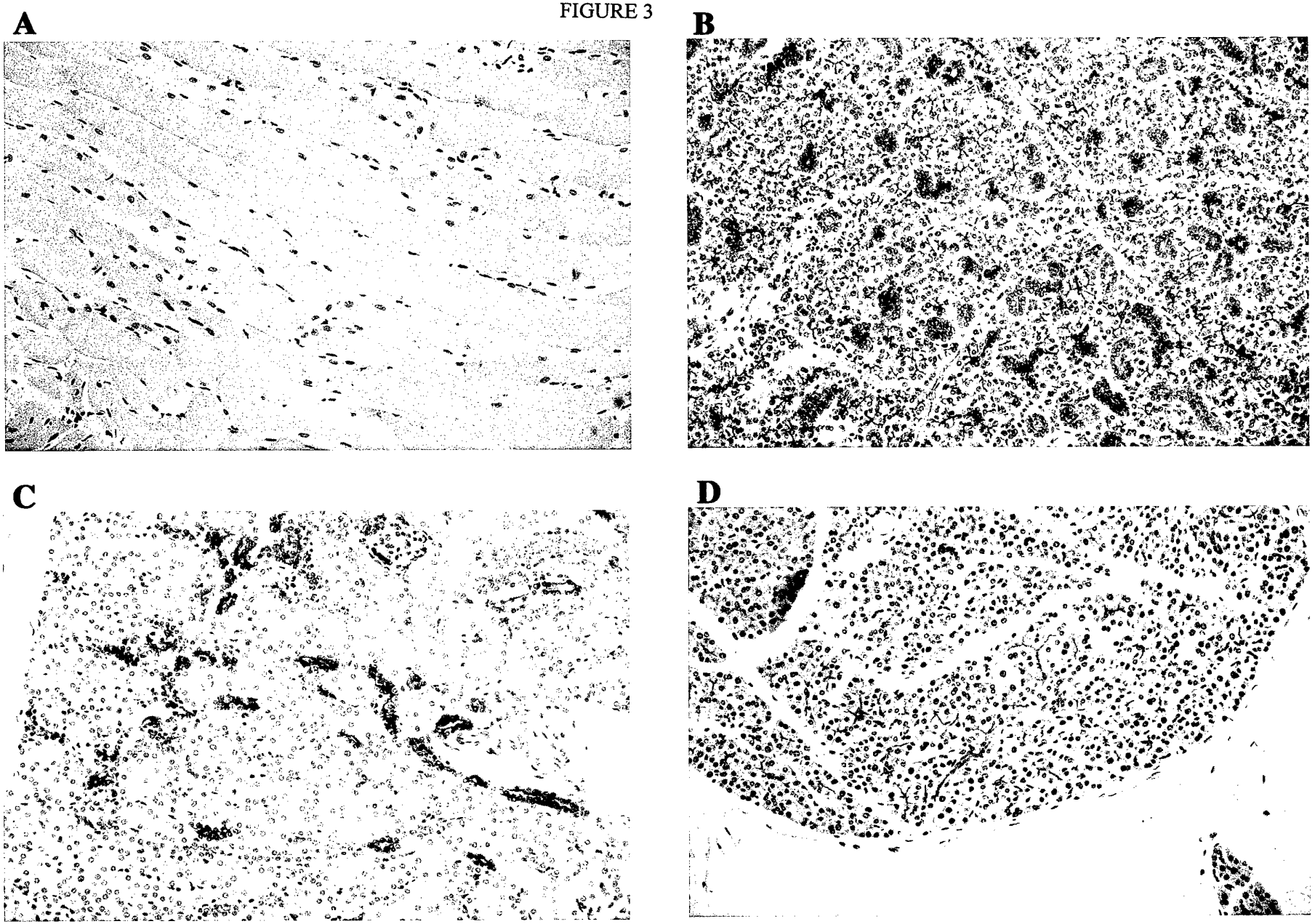
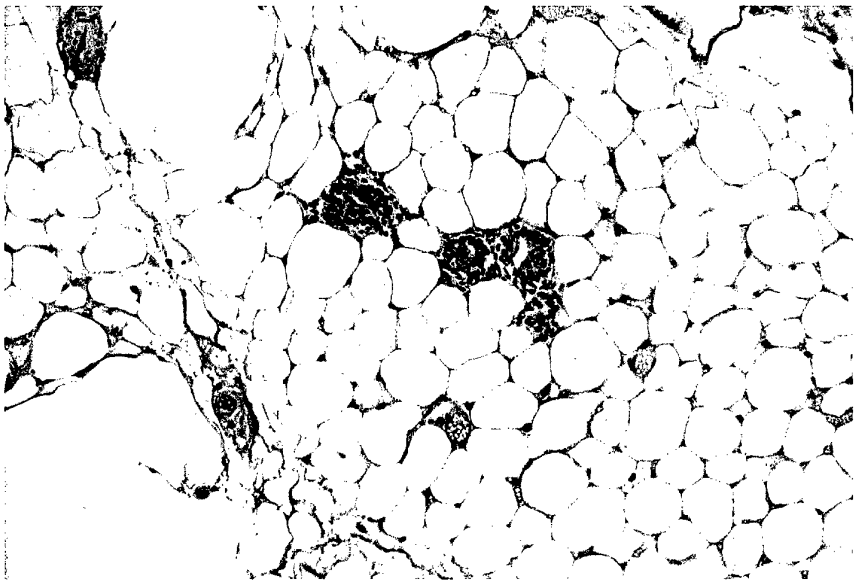


FIGURE 4

A



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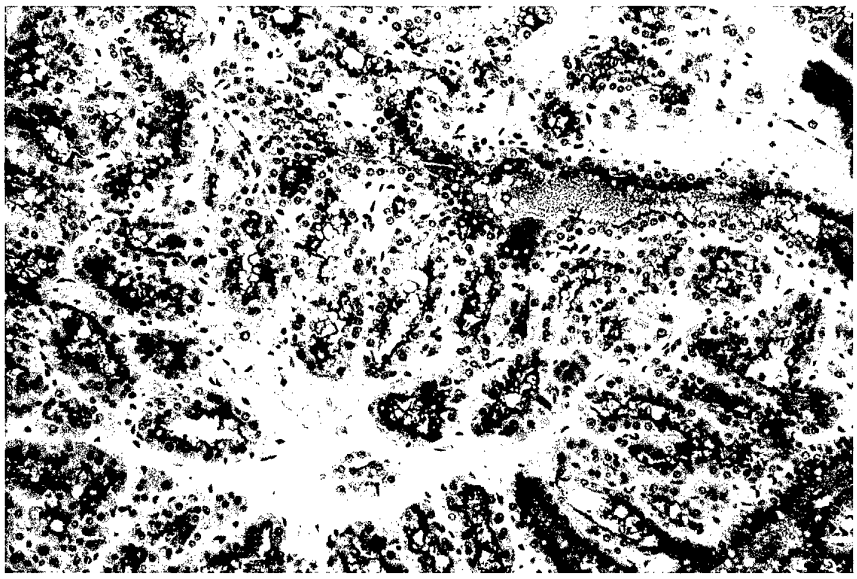

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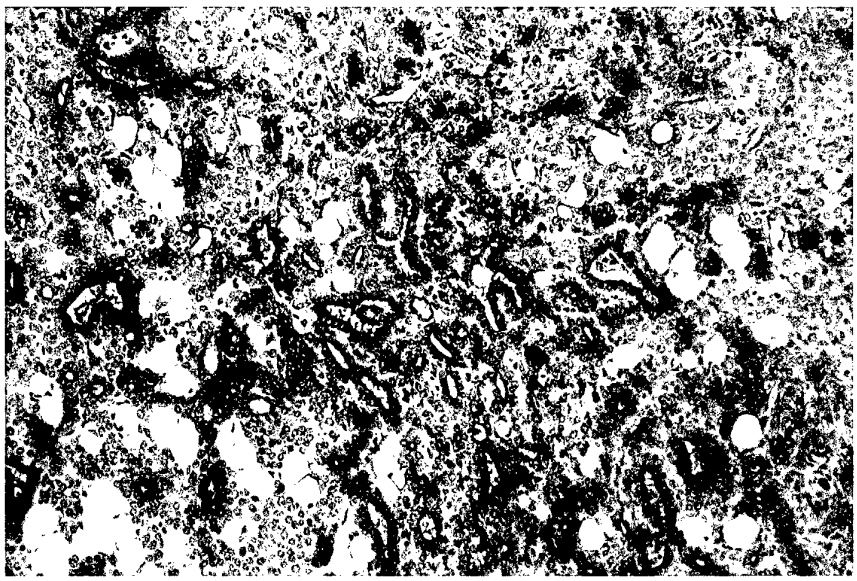
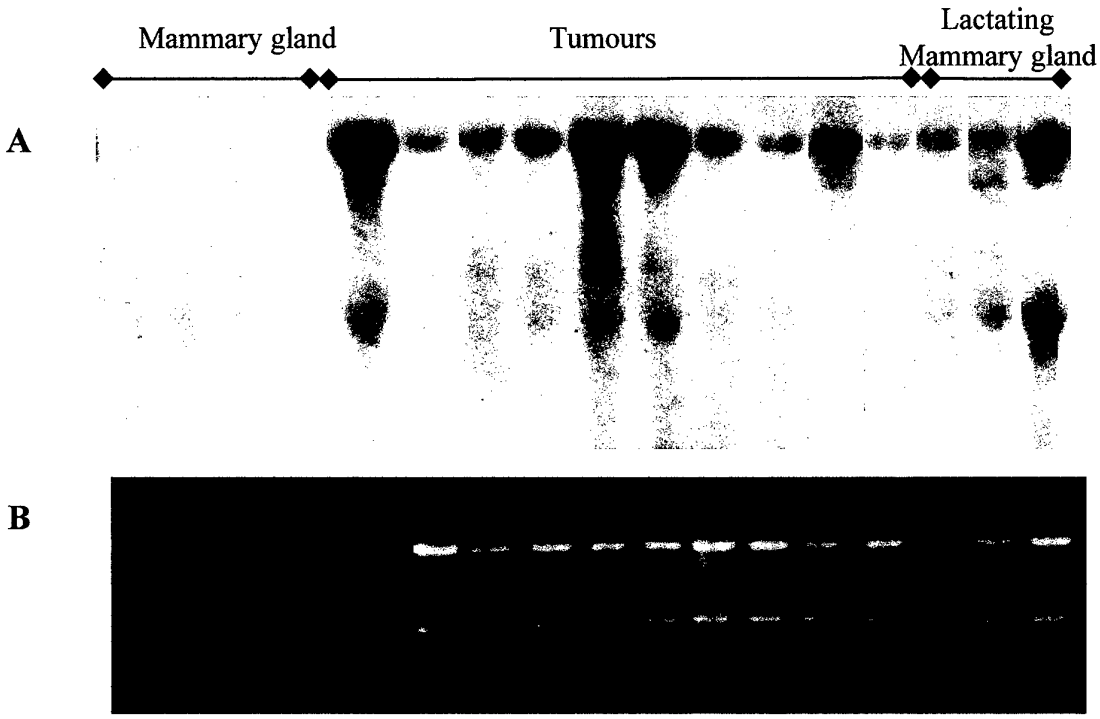


FIGURE 5



ELSEVIER

A Microsatellite within the MUC1 Locus at 1q21 Is Altered in the Neoplastic Cells of Breast Cancer Patients

Margaret R. Waltz, Steven M. Pandelidis, Wendy Pratt, Diana Barnes, Dallas M. Swallow, Sandra J. Gendler, and Edward P. Cohen

ABSTRACT: *Paired DNA samples from the neoplastic and nonneoplastic cells of 118 patients with the sporadic, nonfamilial form of breast cancer were analyzed for evidence of genetic alteration at a polymorphic microsatellite mapped to intron 6 within the MUC1 gene at 1q21. Two other microsatellite loci, D1S104 and APO-A2, which also map to 1q21, were analyzed as well. The frequency of alteration at the microsatellite within the MUC1 locus was significantly higher than D1S104 or APO-A2 ($P < 0.001$). Analysis by Southern blotting of the VNTR region of the MUC1 gene revealed an amplification of one allele in 34 of 54 informative cases (63%). There was no significant association between these alterations and alterations of the microsatellite within the same locus, suggesting independent mechanisms were responsible for the genetic changes. Microsatellite loci D17S579 at 17q21, the site of the BRCA1 gene, and D18S34 at 18q21-qter, the deleted in colorectal cancer locus, were also analyzed by PCR. Alterations at D17S579 and D18S34 were detected in 18.8% and 6.2% of patients, respectively ($P < 0.001$, and $P < 0.1$ relative to the frequency of alteration at D1S104 or APO-A2). A previously described polymorphism of hMSH2 was altered in 16.4% of cases.* © *Elsevier Science Inc., 1998*

INTRODUCTION

An accumulation of genetic changes within an individual somatic cell is responsible for conversion to the malignant phenotype [1–4]. As the malignant cell population increases, genetic instability often results in the appearance of neoplastic cells with varying abnormal properties, such as the capacity for metastasis, and resistance to drugs commonly used in cancer treatment [5–8].

Various genetic loci have been identified that are frequently altered in neoplastic cells. Certain changes are associated with the malignant phenotype. The BRCA1 gene at 17q21, and the VNTR region of the MUC1 gene at 1q21 are notable examples [9–11]. A high frequency of alterations in microsatellite sequences [12–17] in genomic DNA from the tumor can be a first indication of the presence of significant genetic change in neoplastic cells, and may lend insight into the genetic mechanisms involved in mediating the alterations [15].

Pratt et al. [18], recently described the polymorphism of a microsatellite (CA-repeat) sequence within intron 6 of the MUC1 gene. The gene includes a polymorphic VNTR region within exon 2 that is frequently altered in breast cancer cells [11]. Figure 1 illustrates the location of these features of the MUC1 gene. Whether the microsatellite is altered in breast cancer cells and whether such alterations are associated with alterations of the VNTR region has not been determined.

To investigate these questions, we analyzed the microsatellite and VNTR region of the MUC1 gene in paired neoplastic and nonneoplastic cells of 118 patients with the sporadic form of breast cancer. In addition, four other loci were analyzed for microsatellite instability. Two of these have been associated with tumor suppressor genes, *D17S579* at 17q21 [9] and *D18S34* at 18q21-qter [19], whereas the other two, *APO-A2* and *D1S104*, are located near the MUC1 gene, at 1q21.

MATERIALS AND METHODS

Human Tissue Samples

Fresh solid tumor tissues, taken during the usual course of the patients' treatment, were dissected free of fat and stored in liquid nitrogen. Peripheral blood buffy coat cells obtained at the time of surgery were used as the source of

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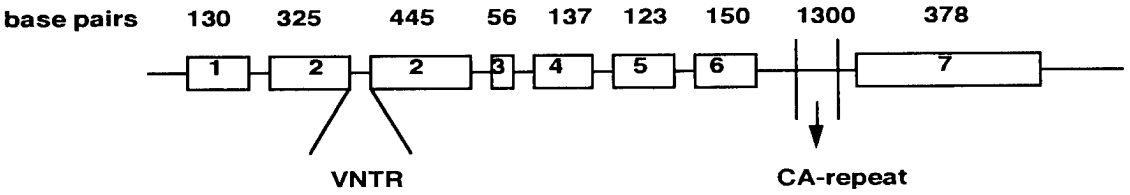


Figure 1 Diagram of the MUC1 gene. The variable number tandem repeat region (VNTR) is located within exon 2 of the gene. The CA repeat is located within intron 6 of the gene.

DNA from nonneoplastic cells from the same individuals. Confirmation of the malignant nature of the tumor was determined in stained paraffin-embedded sections, prepared according to conventional techniques.

Preparation of High–Molecular-weight DNAs from Paired Neoplastic and Nonneoplastic Cells of the Same Individuals with Breast Cancer

Frozen tumor tissues (approximately 0.3 gms) were disaggregated in a Braun (Melsuugen, Germany) Mikro-disembrator II, and the DNA was isolated from the cells in an Applied Biosystems (Foster City, CA) 340A DNA extractor. Nonneoplastic cells from the patients' peripheral blood were lysed with water before addition to the extractor. After dialysis, the DNA concentrations were measured spectrophotometrically. Before the various analyses were performed, aliquots of undigested DNA from the paired samples were subjected to electrophoresis through 0.7% agarose gels. Degraded specimens were not included.

Analysis of Microsatellite DNAs from Paired Neoplastic and Nonneoplastic Specimens of the Same Patients

Analyses of each of five microsatellite loci were performed by PCR, using oligonucleotide primers that flanked the region of interest. The loci investigated and the primer sequences are presented in Table 1. Fifty-microliter reaction mixtures consisted of 300 ng of DNA 10X reaction buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0, and 1% Triton X-100), 2 mM $MgCl_2$, 1.25 mM of each dNTP, 50 pMol of each primer, 3 uCi [a-^{32}P]-dCTP, and 0.5 units *Taq* polymerase (Promega, Madison, WI). The samples were overlaid with oil, placed in a thermocycler (Perkin-Elmer, Norwalk, CT) and subjected to 27 cycles of 94°C, 45 seconds; 55°C, 45 seconds; 72°C, 45 seconds. That the PCR products were of the correct size was confirmed by agarose gel electrophoresis. The PCR products were separated in 8% polyacrylamide denaturing gels and then exposed to XAR-5 film (Kodak, Rochester, NY) at −70°C for 1 to 3 days.

The autoradiographs were compared for differences in the microsatellites from the neoplastic and nonneoplastic cells of the same patients. Changes in the size of a band, deletion or addition of a band in the DNA from the tumor, compared to DNA from the nonneoplastic cells, were considered as evidence of alterations.

Analysis of the VNTR Region of the MUC1 Gene at 1q21 in the Neoplastic and Nonneoplastic Cells of Breast Cancer Patients

Southern blotting was used to detect possible alterations in the polymorphic VNTR region of the MUC1 gene in the neoplastic cells of the breast cancer patients. Approximately 10 μg of DNA from the neoplastic or nonneoplastic cells of the same patients was digested to completion with *Hinf1* (GIBCO BRL, Gaithersburg, MD), and fractionated in 0.7% agarose gels. *HindIII* digested lambda DNA (GIBCO BRL, Gaithersburg, MD) was used as a molecular-weight marker. After electrophoresis, the agarose gels were denatured in alkali and transferred to nylon membranes (Biodyne, Pall, Glen Cove, NY). After transfer, the membranes were incubated for 1 hour at 80°C under vacuum, and then hybridized with a probe homologous to the VNTR region at exon 2 of the MUC1 gene [20]. The probe was labeled to high specific activity with [a-^{32}P]-dCTP by the random priming method [21]. Hybridization was performed at 42°C in the presence of nonspecific DNA (herring testis, Promega, Madison, WI) in a buffer containing 5 × SSC (1 × SSC in 150 mM NaCl, 15 mM trisodium citrate, (pH 7.0), and 50% formamide). After incubation, the filters were

Table 1 Sequence of primers used for analysis of microsatellites

Microsatellite	Primer sequence	Reference
D17S579	5′-AGT CCT GTA GAC AAA ACC TG 5′-CAG TTT CAT ACC AAG TTC CTA	[15]
D18S34	5′-CAG AAA ATT CTC TCT GGC TA 5′-CTC ATG TTC CTG GCA AGA AT	[17]
APO-A2	5′-GGT CTG GAA GTA CTG AGA AA 5′-GAT TCA CTG CYG TGG ACC CA	[10]
D1S104	5′-ATC CTG CCC TTA TGG AGT GC 5′-CCC ACT CCT CTG TCA TTG TA	[23]
Ms within MUC1	5′-AGG AGA GAG TTT AGT TTT CTT GCT CC 5′-TTC TTG GCT CTA ATC AGC CC	[26]

Table 2 Summary of microsatellite alterations

	MUC1	D17S579	D18S34	D1S104	APOA2
No. of cases	104	101	94	74	70
No. of information cases	100 (96.1%)	90 (89.1%)	81 (87.2%)	65 (87.8%)	70 (100%)
Total no. of changes	24 (23.3%)	17 (18.8%)	5 (6.2%)	3 (4.7%)	2 (2.8%)
LOH	5	9	3	0	0
Allelic imbalance	7	4	1	0	0
Size change	4	4	1	2	1
Additional bands	8	0	0	1	1

washed at 55°C and exposed to XAR-5 film (Kodak, Rochester, NY) at −70°C for 1 to 3 days.

Analysis by SSCP of the hMSH2 Locus in the Paired Neoplastic and Nonneoplastic Specimens of the Breast Cancer Patients

Analysis of the conserved region of hMSH2, a replication error repair gene [2], was performed according to the method described by Orita et al. [22], using oligonucleotide primers specific for codons 668-736 of the hMSH2 locus. The primers were:

PF:5′-CGC GAT TAA TCA TCA GTG-3′
PR: 5′-GGA CAG AGA CAT ACA TTT CTA T-3′.

The reaction mixture consisted of 200 ng genomic DNA, 5 ml of 10 × reaction buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 50 pMol of each primer, 25 nMol of each nucleotide, 10 mCi [a-^{32}P]-dCTP, and 2.5 units *Taq* polymerase (Promega, Madison, WI) in a total volume of 50 μl. The reaction mixtures were cycled 27 times, at 94°C, 45 seconds; 57°C, 45 seconds; and 72°C, 45 seconds for each cycle. Afterward, 6 μl of the mixture was heated to 95°C for 3 min before it was analyzed in a neutral 7% polyacrylamide gel containing 10% glycerol. The gels were subjected to electrophoresis at room temperature for 6 hours at 50 W. They were dried and exposed to XAR-5 film (Kodak, Rochester, NY) at −70°C for 1 to 3 days. Sequencing of the PCR products was performed using a double strand DNA Cycle Sequencing System (GIBCO/BRL, Gaithersburg, MD), according to the directions of the supplier.

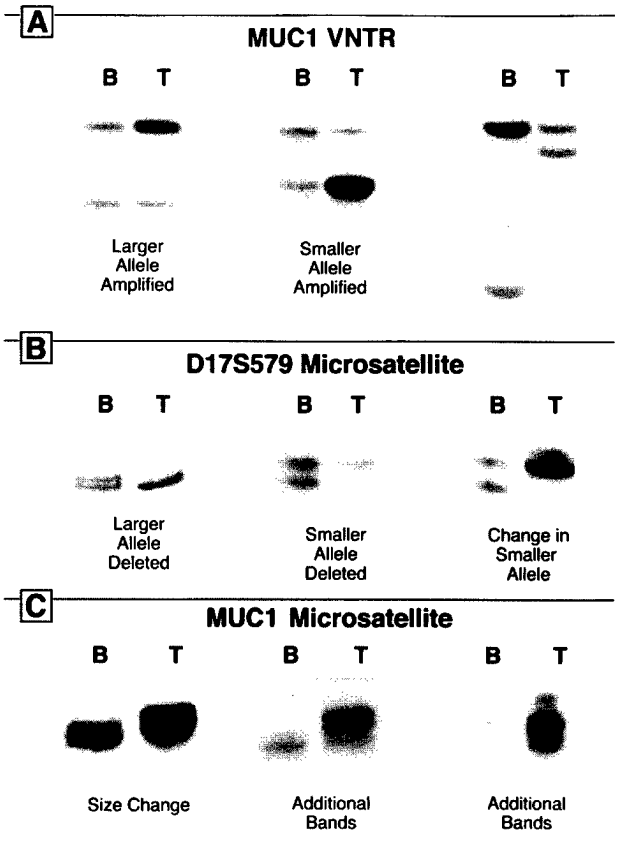
RESULTS

A Microsatellite at Intron 6 within the MUC1 Gene at 1q21 Is Altered in the Neoplastic Cells of Breast Cancer Patients

The microsatellite within the MUC1 gene was analyzed in the paired neoplastic and nonneoplastic specimens from 100 informative patients with the sporadic form of breast cancer. For comparison, two other microsatellite loci mapping to 1q21, and two loci at 17q21 and 18q21, which are associated with tumor suppressor genes, were also analyzed.

Table 2 indicates the changes in microsatellites observed and a breakdown of the changes seen at each locus. The numbers of losses of heterozygosity (LOH), size changes, and additional bands present are indicated. "Allelic imbalance" has been used to describe those microsatellite alterations that appear as an increase or decrease in the band intensity of one allele relative to the other allele [8]. The frequency of alteration of the microsatellite at intron 6 within the MUC1 gene at 1q21 was significantly ($P < 0.001$) higher than that of *APO-A2* and *D1S104*, the other two microsatellites at the same locus. *D17S579* and *D18S34* were also altered at a high frequency relative to *APO-A2* and *D1S104*, 18.8% and 6.2%, respectively. Figure 2B and C presents the types of changes observed at microsatellite loci. The majority (57%) of alterations were

Figure 2 Representative examples of alterations of the VNTR of MUC1 and microsatellite loci. "B" denotes DNA extracted from blood, and "T" denotes DNA extracted from tumor.



LOH, 23% were size changes of an allele, and 20% were indicated by the presence of additional bands.

The VNTR Region within the MUC1 Gene at 1q21 Was Altered in the Neoplastic Cells of the Breast Cancer Patients

An analysis of the polymorphic VNTR within the coding region of the MUC1 gene revealed a high frequency of alteration in the neoplastic cells of the patients. The analysis was performed by Southern blotting. Representative alterations are presented in Figure 2A. The gene was altered in 34 of 54 informative patients. There was LOH in 3 instances (5.2%), amplification of one of the two alleles in 32 cases (56%), and one translocation (1.8%). Amplification of either allele was considered as "allelic imbalance" in Table 2.

Of the three cases of LOH at the VNTR of the MUC1 gene, none showed LOH of the microsatellite in intron 6 of the gene. This was unexpected, as losses of heterozygosity tend to reflect the deletion of large segments of genetic material [26]. If such a loss had occurred at 1q21, both the VNTR and the microsatellite should show LOH. However, an alternate explanation for the LOH at the VNTR is that there has been homologous recombination of the MUC1 gene in the tandem repeat region, resulting in two alleles of the same size. If this were the case, the 1q21 locus may be present, though the VNTR of the MUC1 gene is no longer heterozygous. This would explain how the three individuals with LOH at the VNTR may also be informative for the three microsatellite markers at 1q21.

There was no significant association between an alteration of the VNTR region and an alteration of the microsatellite at intron 6 within the MUC1 gene in the neoplastic cells of the same patient ($P > 1.0$). The likelihood that patients whose neoplastic cells revealed both an alteration at the VNTR region and alteration of the microsatellite within intron 6 was not greater than random.

Alterations of the hMSH2 Gene Were Not Detected in the Neoplastic Cells of the Breast Cancer Patients

The hMSH2 gene encodes a DNA repair protein that binds to base pair mismatches generated during DNA replication [2]. An analysis of the gene for hMSH2 was carried out by SSCP, using primers for codons 668-736 and flanking sequences (corresponding to bps 2072 to 2208) of the hMSH2 locus [2]. This portion of the gene is conserved across species and is thought to encode the DNA binding region of the protein. Sixteen of 97 cases examined (16.4%) revealed an alteration of the hMSH2 gene. The same alteration was present in both the neoplastic and nonneoplastic cells of the same patient. DNA sequencing was carried out to characterize the alteration in greater detail. It revealed a G to T transition at the −6 splice acceptor site. Because the alteration was found in DNA from both normal and tumor cells of the same individuals, it was likely that the variation was a germline, rather than somatic change in sequence. (These data are not presented.)

There was no significant association between a change in the hMSH2 gene and a change in any of the microsatellite loci investigated. DNA from the neoplastic cells of one patient revealed instability of microsatellite DNA at two of the five loci. The hMSH2 gene was not altered in the neoplastic or nonneoplastic cells of this patient.

DISCUSSION

Genetic instability of malignant cells is indicated by widespread alterations in microsatellite DNAs. Because the alterations are not random, they are taken as indications of genetic changes that are invovled in the malignant phenotype. Here, we present the results of a comparative analysis of five microsatellite loci and of the VNTR region of the MUC1 gene in malignant and nonmalignant cells of 118 breast cancer patients. The investigation was an extension of prior studies in which it was found that the MUC1 gene was frequently altered in the neoplastic, but not the nonneoplastic cells of patients with primary breast carcinomas [19].

Both the VNTR region of the MUC1 gene and a microsatellite within the same gene were altered in a high proportion of the patients. However, the likelihood that changes in the VNTR region were accompanied by changes in the microsatellite within the gene was not greater than random. This finding may not be unexpected, as microsatellite and VNTR loci, though both frequent sites of genetic alteration, may be subject to different types of mutation. Indeed, the data were consistent with the presence of different genetic mechanisms responsible for alteration of the VNTR region and a change in the microsatellite. Because the MUC1 gene includes tandemly repeated DNA, it is possible that alteration of the VNTR region was mediated by homologous recombination, whereas alterations in microsatellite DNA may arise from slippage during the replication of simple repetitive sequences, followed by failure of the cell to repair the damage. In support of this hypothesis is a previous finding that VNTR loci are not affected by the replication error phenotype [15]. Why the CA repeat within the MUC1 gene was altered at a higher frequency than two other microsatellites at 1q21 was not determined. Conceivably, a change in the microsatellite conferred an undefined growth advantage to the malignant cells.

The replication error repair (RER^+) phenotype has been found in HNPCC colon cancers as well as in other sporadic tumors associated with HNPCC syndrome [1–3, 23]. In our investigation, microsatellite alterations were occasionally detected, though most were losses of heterozygosity. None of the specimens revealed the ladder-like expansions seen in the microsatellite instability of colorectal cancers [1–3]. Similar changes have been noted previously in studies of patients with the sporadic form of breast cancer [23–26].

Fishel et al. [2] and Leach et al. [3] reported that approximately 60% of patients with colon cancer with the RER^+ phenotype had mutations of the hMSH2 gene. We attempted to detect analogous changes at the hMSH2 locus in the specimens we examined. A T to C substitution at the −6 position of the splice acceptor site was present in 16 of 97 cases. However, because it was found in both the neoplastic as well as nonneoplastic cells of the same individuals, it was likely to have been a ploymorphism. Leach et al. [3] found the same mutation in 2 of 20 tumor-free

individuals. No significant association between this alteration at the hMSH2 locus and alterations of the microsatellite loci was detected.

We conclude that alterations in microsatellites in sporadic breast cancer are likely to result from a different mechanism from that responsible for microsatellite instability in HNPCC. In addition, we confirm the high frequency of alteration in the region of the BRCA1 locus and in the VNTR at exon 2 of the MUC1 gene in sporadic breast cancer. The high frequency of alteration of the CA repeat within the gene was an unexpected finding. Its possible involvement in progression of the tumor remains to be determined.

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Expression of B7.1 in a MUC1-expressing mouse mammary epithelial tumour cell line inhibits tumorigenicity but does not induce autoimmunity in *MUC1* transgenic mice

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SUMMARY

The MUC1 epithelial mucin, which is overexpressed and aberrantly glycosylated in breast and other carcinomas, is also expressed on the apical surface of most normal glandular epithelial cells. Since clinical trials evaluating the efficacy of *MUC1*-based vaccines have been initiated in breast cancer patients, it is important to address the question of whether an effective immune response to the cancer associated mucin can be generated without inducing autoimmunity. Since non-classic cytotoxic T lymphocyte (CTL) responses to MUC1 have been reported, it is also relevant to examine the role of costimulatory molecules in the effective presentation of MUC1 based antigens. We have therefore looked at the effect of expressing B7.1 on the tumorigenicity of a MUC1 expressing mammary epithelial cell line (410.4) in a transgenic mouse expressing MUC1 on its normal glandular epithelial tissues. Coexpression of B7.1 with MUC1 in 410.4 cells resulted in a dramatic inhibition of tumour growth which depended on the activity of $CD4^+$ and $CD8^+$ T cells. The epithelial tissues in the transgenic mice able to reject the B7.1, MUC1-expressing tumours showed no evidence of degeneration and the mice survived their normal life span. The results demonstrate that an immune response to the MUC1 antigen can be induced in *MUC1* transgenic mice and that presentation of the antigen, whether directly or by cross-priming, is markedly enhanced by coexpression of B7.1.

INTRODUCTION

The human *MUC1* gene codes for a type I membrane glycoprotein that is normally expressed on the apical surface of most glandular epithelial cells, but which is upregulated and underglycosylated in breast and other carcinomas.[1] The extracellular domain of the MUC1 mucin is made up largely of exact tandem repeats of 20 amino acids, each repeat containing sites for *O*-linked glycosylation.[2] The shorter carbohydrate side chains which are added in breast cancers result in the exposure of normally cryptic peptide epitopes (such as that recognized by the antibody SM3), and the creation of tumour-associated carbohydrate epitopes.[3] Humoral responses to MUC1 have been detected in breast and other cancer patients[4,5] while both major histocompatibility complex (MHC)-unrestricted and MHC-restricted cytotoxic T lymphocyte (CTL) responses have also been reported.[6–8] Several clinical studies using MUC1-based antigens have been initiated.[9] However, the importance of the different components of the immune response remains unclear.

Because the MUC1 antigen is expressed by normal glandular epithelial cells, the question of whether autoimmunity would occur if the immune responses to the mucin were effective is a question which needs to be addressed. A dominant immunogenic domain of MUC1 is in the tandem repeat sequence,[2,10,11] which has a different sequence in the mouse homologue, Muc1,[12] making the use of the usual mouse strains for the preclinical studies inappropriate. To address this and related questions, we have developed a transgenic mouse expressing the human MUC1 mucin on glandular epithelial tissues with the same profile of expression as is seen in humans.[13] Moreover, the differences in glycosylation of MUC1 seen between normal and malignant breast are also evident in the transgenic mouse: The SM3 epitope is minimally expressed on the mucin expressed by normal glandular tissues,[13] but is expressed on both transplantable tumours expressing MUC1[14] and on spontaneous tumours developing in transgenics cross-fostered on an MMTV carrying mouse strain.[15] Thus, the MUC1 transgenic mouse provides a suitable model for preclinical testing of MUC1-based vaccines.

Introduction of a gene coding for costimulatory molecules such as B7.1 can enhance the immunogenicity of mouse tumour cell lines and thus reduce their tumorigenicity.[16,17] To

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ertain whether an effective immune response to the MUC1 ıcin can be obtained in the *MUC1* transgenic mouse, we ve examined the effect of coexpressing the B7.1 costimuory molecule with MUC1 on the tumorigenicity of a mouse ımmary epithelial cell line in these mice. Our results indicate ıt while coexpression of the B7.1 molecule markedly ɔreases the tumorigenicity of the MUC1-expressing 410.4 ımmary epithelial tumour cell, expression of MUC1 or B7.1 ɔne had no such effect. The effects of B7.1 expression are ated to T-cell responses as no difference in tumorigenicity ıs seen in B7.1/MUC1-expressing cells in the nude mouse. oreover, *in vivo* depletion of $CD4^+$ and $CD8^+$ T cells in the ınsgenic mice abolished the protective effect of coexpression MUC1 and B7.1. Significantly, no evidence of tissue generation was seen in the epithelial tissues expressing UC1 and the mice lived out their natural life span with no idence of disease.

MATERIALS AND METHODS

evelopment of 410.4-derived cell lines

he 410.4H and E3 cell lines were developed from the 410.4 :lls[18] by transfection of the hygromycin resistance gene with :3) or without (410.4H) the MUC1 gene.[14] Calcium phoshate transfection of 410.4H and E3 cells was perfomed using ıe pbabe-neo gene (kind gift of H. Land, ICRF, London) ith or without the cDNA encoding murine B7.1 contained ı the plasmid πLN (a kind gift from P. Linsley, Seattle, WA). he nomenclature of the selected clones (which were grown in)ulbecco's E4 medium containing 600 μg/ml G418, 200 μg/ml ygromycin and 10% fetal calf serum (FCS)) is illustrated ı Fig. 1.

irowth of cell lines in transgenic or nude mice

he transgenic mice homozygous for the *MUC1* gene (desigıated *Sac*II) are $H2^k$.[13] These mice were crossed with BALB/c nice to produce F1 hybrids (haplotype H-2^{kd}) to accept the -10.4 tumour cell derivatives (haplotype H-2^d). *Sac*II ransgenic × BALB/c F1 mice, or nude mice aged between 6 ınd 14 weeks old were injected subcutaneously with a suspenion of cells (in phosphate buffered saline (PBS)), from one)f the 410.4-derived clones. Preliminary titrations in *Sac*II 3ALB/c mice showed that 10^5 was the lowest number of cells vhich consistently induced tumours (the parental tumour, the clone 410.4-H.neo or the MUC1-expressing clone). Mice were examined three times per week and tumour size estimated by measuring the largest diameter and its perpendicular. Mice were euthanized when the largest diameter reached 15 mm, the tumour ulcerated, or the mouse appeared sick. All experiments were conducted according to Home Office Guidelines under an approved project licence (JTP).

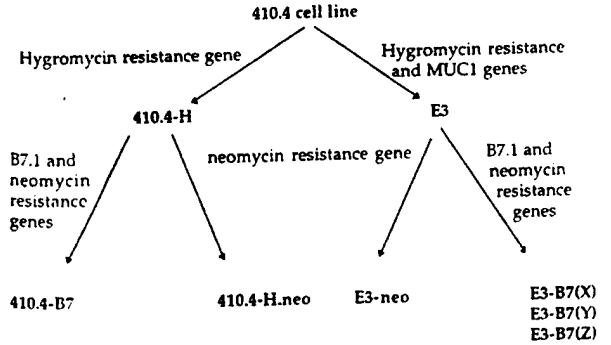

Figure 1. Development and nomenclature of 410.4-derived cell lines transfected with MUC1, B7.1 and selectable markers. For details of transfection and clone isolation see methods.

In vivo *depletion of $CD4^+$ and $CD8^+$ cells*

Five- to 6-week-old *Sac*II × BALB/c F1 mice were thymectomized under general anaesthetic and randomized into one of four groups. One week later they underwent T-cell subset depletion by intravenously injecting the relevant anti-T-cell antibodies in a volume of 200 μl of PBS, three times over a five-day period.[19] $CD4^+$ depletion was achieved by using 1 mg of the synergistic pair of antibodies YTS 191.1.2 (a rat immunoglobulin G2b (IgG2b) antimouse CD4 antibody, epitope a) and YTA 3.1.2 (a rat IgG2b antimouse CD4 antibody, epitope b). $CD8^+$ depletion was achieved by using 1 mg of the synergistic pair of antibodies YTS 169.4.2·1 (a rat IgG2b antimouse CD8α chain, antibody) and YTS 156.7.7 (a rat IgG2b antimouse CD8β chain (or α/β complex) antibody). $CD4^+/CD8^+$ depletion was achieved by using a combination of all the four antibodies to a total of 2 mg of antibody. Mice in the control group received 1 mg of the irrelevant antibody PYLT1 (against the polyoma virus large T antigen). All mice tested had successfully been depleted of the relevant subsets as tested in the spleens of mice killed before injection of the tumour cell lines. One week following T-cell depletion each mouse received a subcutaneous injection of 1×10^5 E3-B7(Z) cells.

Immunoperoxidase staining of sections

Tumours were fixed in methacarn (60% methanol: 30% chloroform: 10% acetic acid) for 4 hr, washed in 70% ethanol, and processed for wax-embedding. Sections were stained with biotinylated affinity purified monoclonal antibodies SM3 (50 μg/ml) or HMFG-1 (100 μg/ml) as previously described.[15]

Examination of tissues for evidence of Autoimmunity

Mice given subcutaneous injections into the flank of 1×10^5 cells of E3-B7 cells were euthanized on day 12 and at monthly intervals following the initial injection. The tumours and organs were analysed histologically for lymphocyte infiltration and evidence of tissue destruction.

Flow cytometry

Cells were screened for membrane expression of murine B7.1 by staining with CTLA4-immunoglobulin.[20] 5×10^5 cells suspended in 100 μl of neat supernatant from the CTLA4-immunoglobulin hybridoma (gift from Dr P. Lane, Basel, Switzerland) were incubated for 1 hr on ice, washed 3 times in cold PBS and resuspended in 100 μl of fluoroscein isothiocyanate (FITC)-conjugated rabbit antihuman antibody (Dako, Denmark, diluted 1:40) for 1 hr. After washing, cells were resuspended in 500 μl of PBS and 5000 cells analysed by a Becton-Dickinson fluorescence-activated cell sorter (FACscan flow cytometer; Becton-Dickinson, Oxford, UK). The clones were also screened in a similar manner for expression of MUC1 with the HMFG-1 mouse monoclonal antibody directed to an epitope in the tandem repeat of MUC1.

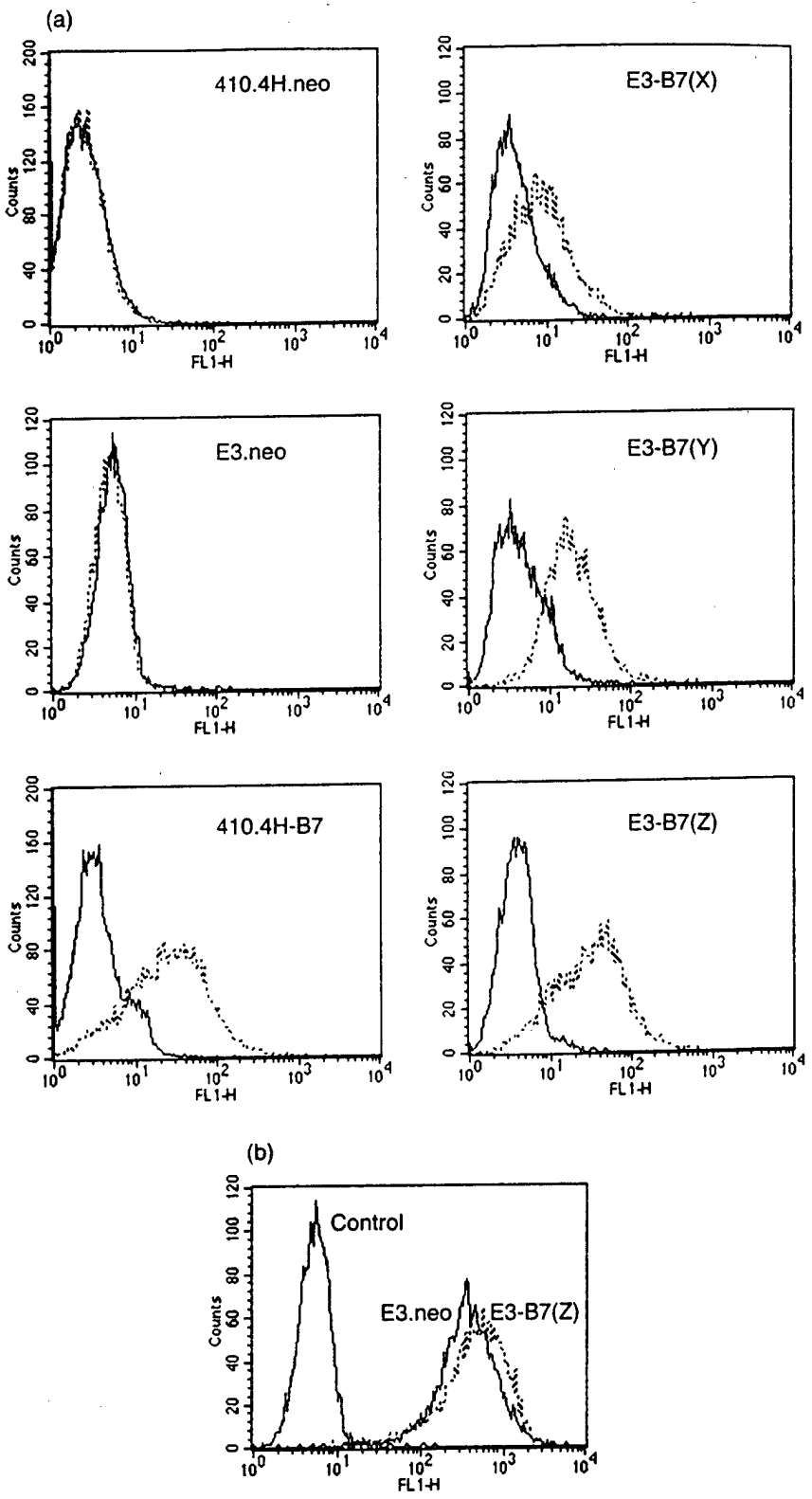

Figure 2. (a) Surface expression of B7.1 on the B7.1 transfected lines 410.4-B7, E3-B7(X), E3-B7 (Y) and E3-B7 (Z) as determined by FACS analysis. CTLA-4 binding (...); control (—). (b) Surface expression of the MUC1 antigen on E3 neo and E3-B7(Z) as determined by FACS analysis.

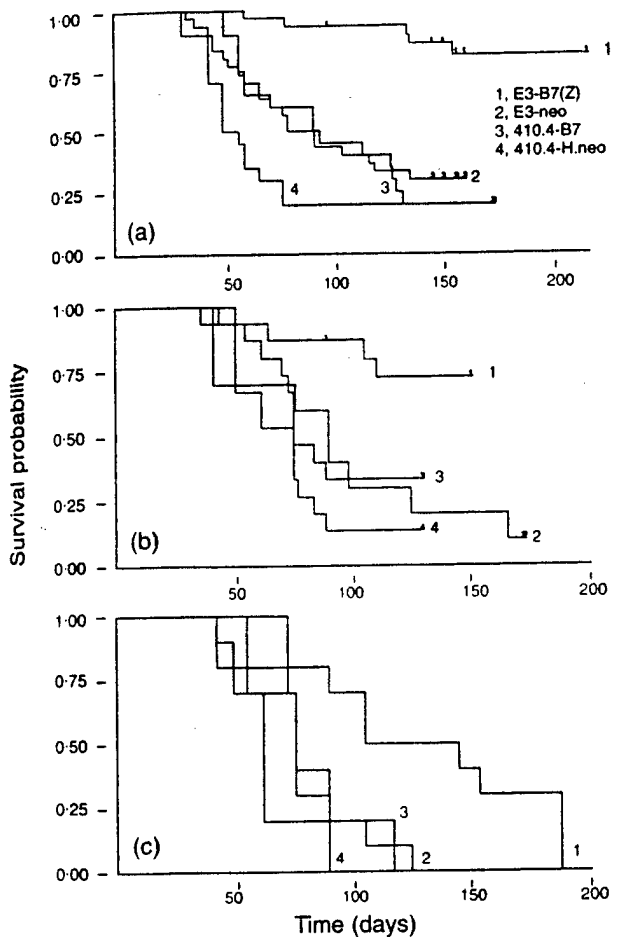

Figure 3. Kaplan–Meier survival curves of mice injected with various doses of 410.4 derived lines. F1 hybrid mice from the MUC1 transgenic mice crossed with BALB/c mice were injected with 10^5 (a), 2×10^5 (b) or 10^6 (c) cells and the development of tumours followed as described in Materials and Methods. The curves represent composites of several experiments, and *P*-values for the log rank heterogeneity test were <0·0001 (a), <0·02 (b) and <0·03 (c).

Cytotoxic T-cell assays

*Sac*II transgenic × BALB/c F1 mice were immunized, in the flank, with 1×10^5 cells of the B7/MUC1 expressing tumour E3-B7(Z). Four to five weeks later splenocytes were isolated and stimulated *in vitro* with mitomycin-C-treated MUC1-expressing P815 cells and after 5 days, the cells were tested for their ability to lyse P815-MUC1 cells in a standard chromium release assay[21] using effector:target ratios from 50:1 to 1·5:1. Control samples to which no lymphocytes had been added, indicated the level of spontaneous lysis and samples to which 100 µl of 10% Triton-X-100 were added indicated maximum lysis. Specific lysis was determined as: (mean sample c.p.m. – mean spontaneous c.p.m.)/(mean maximum c.p.m. – mean spontaneous c.p.m.)

RESULTS

Coexpression of MUC1 and B7.1 in a mouse mammary cell line inhibts tumour growth in MUC1 transgenic mice

The tumour cell line used to express the MUC1 antigen is the 410.4 mammary epithelial cell, originally cultured from a mammary tumour developing in a BALB/c mouse cross-fostered on a mouse mammary tumour virus (MMTV)-carrying mouse strain.[18] The E3 cell line derived from the 410.4 cell line expressing MUC1 and hygromycin (used for selection) and the control 410.4 transfectant expressing only the selectable marker (410.4-H) have been previously described.[14] These two cell lines were transfected with the mouse B7.1 gene together with the neomycin resistance gene or only with the selectable marker. The cell lines developed and their nomenclature is shown in Fig. 1. As shown in Fig. 2(a), the three cell lines expressing both MUC1 and B7.1 express different levels of B7.1 with E3-B7(Z) showing the highest level. Figure 2(a) also shows that the level of expression of B7.1 is comparable in the E3-B7(Z) cell line and in 410.4-HB7 cells not expressing MUC1. Moreover, the levels of expression of MUC1 in the B7.1 tranfectants are the same as those in the E3 neo transfectant (see Fig. 2b). The growth rates of all of the cell lines *in vitro* were not significantly different (data not shown).

The tumorigenicity of the cells lines was tested in the *MUC1* transgenic mice and Fig. 3 shows the Kaplan–Meier survival curves for mice given the four cell lines 410.4-H.neo, 410.4-B7, E3-neo amd E3-B7(Z) at three different doses (10^5 (Fig. 3a), 2×10^5 (Fig. 3b), 10^6 (Fig. 3c)). Figure 4 shows the growth rates for the individual tumours at the higher dose. The data of Fig. 3 show that the expression of the costimulatory molecule together with the MUC1 antigen has a highly significant effect on survival at the two lower challenge doses, while expression of either B7.1 or MUC1 alone has no such effect. Moreover, even though none of the mice survived the challenge dose of 10^6 cells per mouse, there was a strongly significant effect of coexpression of B7.1 and MUC1 on the growth rate of the tumours resulting in a marked delay in the time of death (Fig. 4).

Influence of level of expression of B7.1

The influence of the level of expresson of the B7.1 molecule was examined by following the development of tumours in groups of mice injected with the three cell lines showing different levels of B7.1 expression (see Fig. 2a). Figure 5 shows the Kaplan–Meier survival curve for the mice in the three groups as compared to that for mice given the cell line expressing only MUC1 (E3-neo). Because the strongest effects were seen with the high-expressing E3-B7(Z) cell line, this line was used in subsequent experiments.

Growth of the cell lines in nude mice

The growth of the cell lines was examined in BALB/c nu/nu mice and Fig. 6 shows the survival curves (a) and the growth curves for the individual mice (b). It can be seen that expression of MUC1 with or without B7.1 at any level has no effect on the growth of the 410.4 tumour cell line in these mice. These results suggest that natural killer (NK) cells, which are found in high levels in nude mice, are not responsible for the decrease in tumorigenicity of the E3-B7 cells seen in the MUC1 transgenic mice.

T-cell responses

Attempts were made to isolate cytotoxic T cells from mice injected with E3-B7(Z) cells, using the syngeneic cell line P815

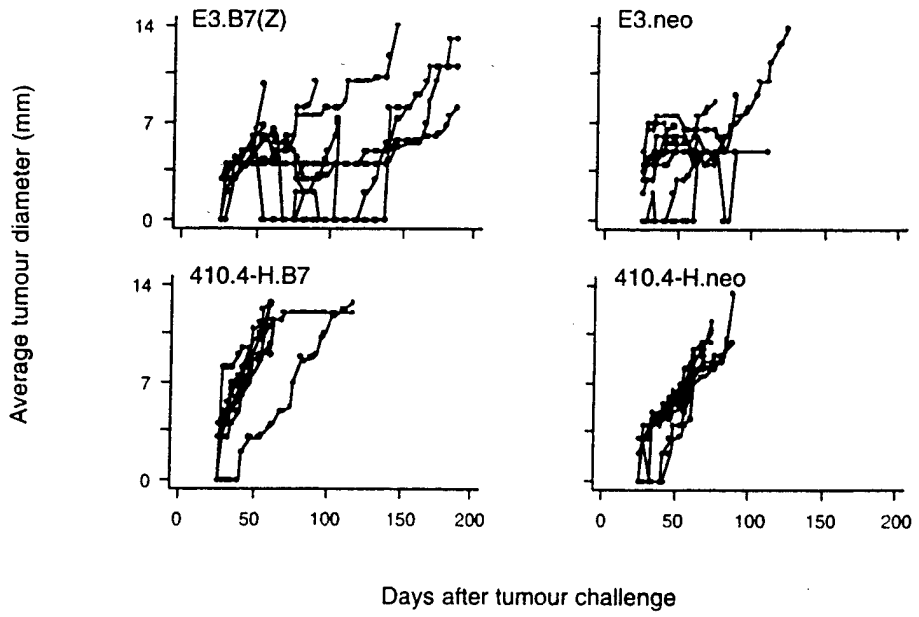

Figure 4. Effect of coexpression of MUC1 and B.7·1 on tumour growth in mice given 10^6 cells (experiment shown in (c). Ten mice were injected in each group. Using the Kruskal–Wallis test for equality of populations, $P = 0{\cdot}0001$.

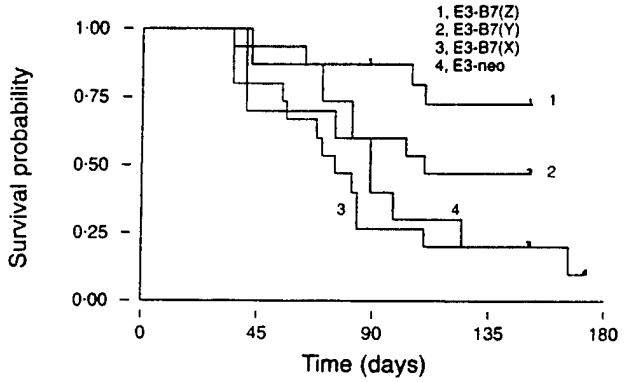

Figure 5. Influence of level of expression of B7.1 on the survival of *Sac*II × BALB/c F1 hybrid mice (15 mice per group) injected with 10^5 E3 cells expressing different levels of B7.1. Log rank test for heteogeneity shows $P = < 0{\cdot}01$.

expressing MUC1 to stimulate T cells from the spleens of the injected mice. A low level of specific killing was observed when the same cell line was used as a target. Although the lysis seen was not very high, the killing observed was specific because untransfected P815 cells were not killed. An example of this effect is shown in Fig. 7.

A synthetic peptide covering three tandem repeats was used to measure antibodies in the serum of mice given the various cell lines. The strongest and most consistent antibody response was seen in the mice given the higher doses (10^6 cells) of the E3-B7(Z)cells (data not shown). However, at the lower doses the antibody response measured in this way was not consistent.

In vivo depletion of CD4$^+$ and/or CD8$^+$ cells stimulates tumorigenicity of E3-B7(Z) cells in the MUC1 transgenic mice

To assess the importance of T-cell responses *in vivo* in the inhibition of tumorigenicity induced by 410.4 cells expressing

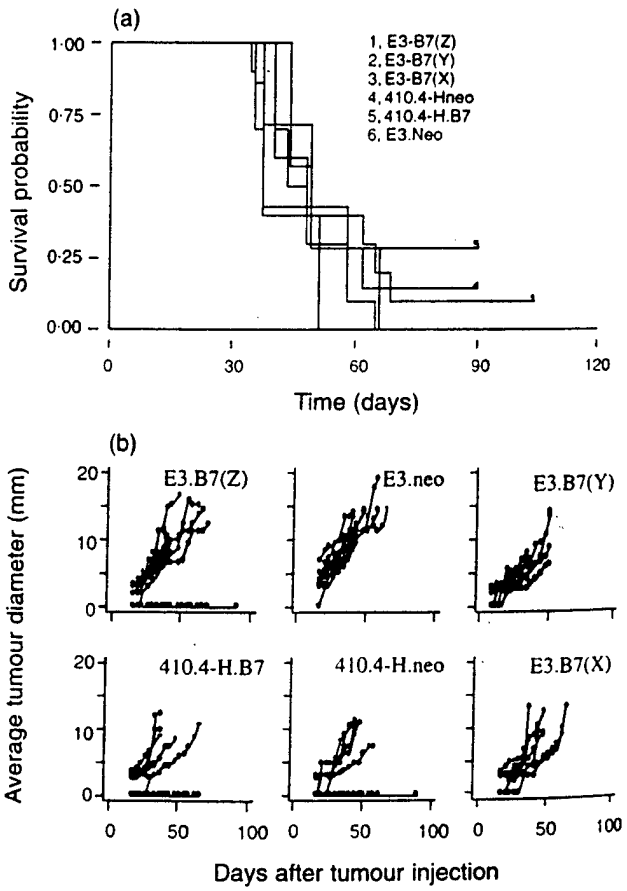

Figure 6. Growth of 410.4 derived lines in nu/nu mice. Cells (10^5) from each of the cell lines indicated were injected into the mice, and the development of individual tumours followed. (a) Survival curves; (b) growth of individual tumours. The log rank test for heterogeneity of the survival curve gives a probability of 0·86, and the Kruskal–Wallis test for equality of populations gives a probability of 0·98.

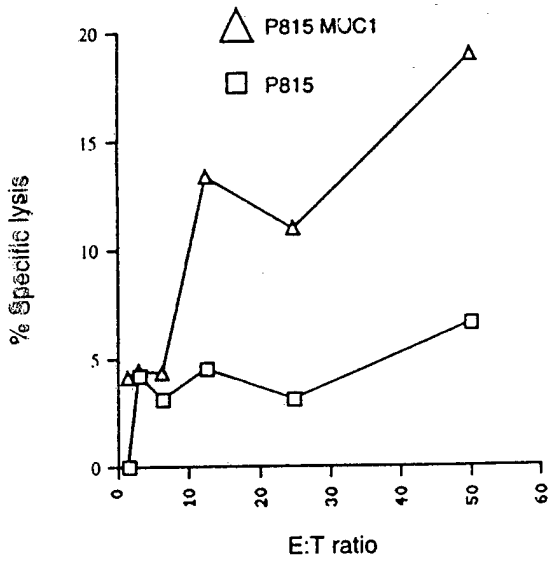

Figure 7. F1 hybrid mice were injected with 1×10^5 E3-B7(Z) cells and after 4 weeks spleens were removed, stimulated with the P815 MUC1 cell line and CTL responses to MUC1 were analysed. Killing was measured in a chromium release assay.

both MUC1 and B7, mice were depleted of $CD4^+$ or $CD8^+$ cells or of both subclasses by injecting antibodies to the CD4 or CD8 molecules after thymectomy (see Materials and Methods). Control mice were thymectomized and treated with an irrelevant antibody. The survival curves and growth of individual tumours are shown in Fig. 8(a,b). Although thymectomy itself showed some inhibitory effect on the ability of the mice to resist the growth of the tumour cells, the effect of depletion of either $CD4^+$ cells or $CD8^+$ cells had a much more pronounced effect, and survival was decreased and rate of tumour growth increased. The effect on both survival and growth rate was marginally stronger in the CD4 depleted mice, however, the mice depleted of both $CD4^+$ and $CD8^+$ cells showed the worst survival. These results demonstrate unequivocally that the protection afforded by expression of B7.1 together with the MUC1 antigen requires stimulation of specific $CD8^+$ and $CD4^+$ T cells.

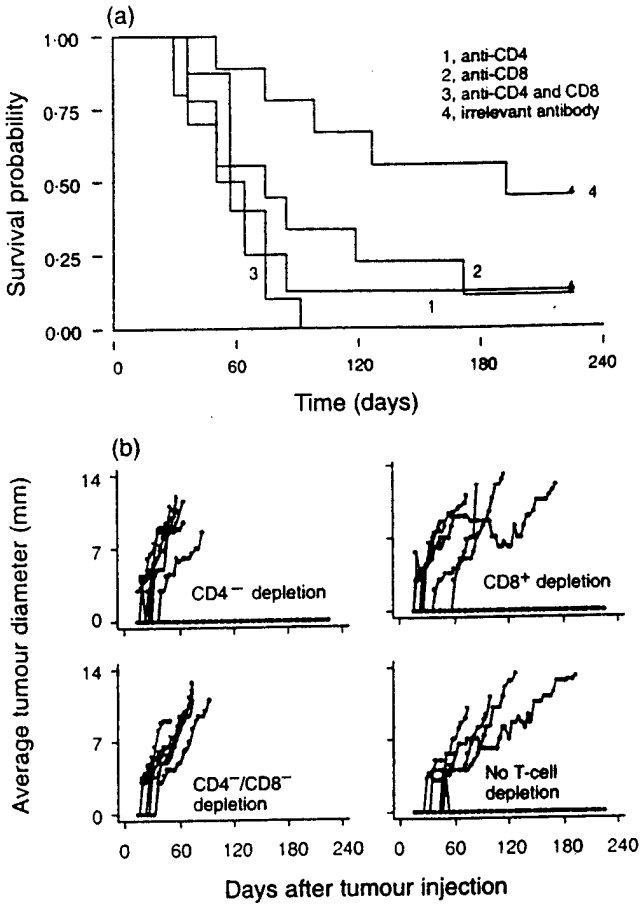

Figure 8. *In vivo* depletion of $CD4^+$ and $CD8^+$ T cells abolishes protective effect of B7.1 expression on tumourigenesis. F1 *Sac*II BALB/c mice were thymectomized and treated with antibodies to CD4, CD8, to both antigens or with an irrelevant antibody, as described in materials and methods, before challenging with 10^5 E3-B7(Z) cells.The survival of the mice (a), and development of tumours (b) was monitored.Using the log rank test for heterogeneity $P = 0{\cdot}027$.

Enhancement of immunogenicity does not affect normal MUC1-expressing epithelial cells

The MUC1 mucin is expressed on most glandular epithelial cells including the pancreas, stomach, lung, kidney and the lactating breast, and this pattern of expression is faithfully reproduced in the MUC1 transgenic mice. The mice injected with the E3-B7(Z) cells survived their natural life span and tissues were taken for examination at monthly intervals after being injected with the tumour cells. An examination of the normal glandular epithelial tissues expressing MUC1 showed no evidence of abnormalities, confirming that the immune response that prevented the growth of the tumour cells did not adversely affect the normal tissues.

DISCUSSION

Several clinical studies are in progress using MUC1-based vaccines, mostly in breast cancer patients with advanced disease.[9] Preclinical testing of these vaccines in model systems should give an indication of both efficacy and potential toxicity, which in this case could relate to autoimmune responses, because the antigen is expressed on normal epithelial tissues. In this paper we have used transgenic mice expressing the human MUC1 mucin as a self antigen to determine if an effective immune response can be generated without the induction of autoimmunity. The data show that coexpression of the MUC1 antigen with B7.1 in a mouse tumour cell line dramatically reduces the tumorigenicity of the cells in the MUC1 transgenic mice without inducing abnormalities in the MUC1 expressing tissues, or affecting the life span of the injected mice.

The MUC1 antigen is normally expressed on the luminal surface of most glandular epithelial cells, but is up-regulated and aberrantly glycosylated in breast and other carcinomas.[1] The molecule is highly repetitive with an extended structure due to the addition of multiple *O*-glycans to serines and threonines found in the tandem repeat sequence. In cancer, the *O*-glycans are shorter[22] so that core protein epitopes are exposed which are normally masked and novel carbohydrate epitopes appear. A similar change appears to occur in mice,

because the same epitope in the tandem repeat recognized by the antibody SM3[23] is masked in normal tissue in the MUC1 transgenic mouse[13] but exposed in tumours developing in these mice.[15]

The changes in glycosylation will affect any immune response which depends on the interaction of the whole MUC1 molecule with an effector cell. This would apply to the induction of antibodies, and possibly to the induction of any MHC unrestricted T-cell responses which have been reported to be specific for the cancer-associated mucin in humans.[5] Moreover, interactions with surface lectins on antigen-presenting cells (APCs) and consequently antigen uptake will also depend on the glycoform, as will the breakdown in the APC[24,25] and the presentation by MHC class II molecules.[26] There are, therefore, multiple ways of recruiting immune responses to MUC1 in addition to the classic stimulation of T cells by specific peptides presented by MHC molecules, which however, have also been described.[7,8,27] It was therefore of interest to ask whether the immune responses which lead to effective tumour rejection are those which require the costimulatory signals generated by the interaction of B7.1 with T cells. Our results clearly show that expression of B7.1 together with the MUC1 mucin in a mouse mammary tumour cell dramatically enhances its immunogenicity and that the immunity requires the activity of $CD8^+$ and $CD4^+$ T cells. Overexpression of B7.1 in the absence of MUC1 had no significant effect on the immunogenicity of the mammary epithelial tumour cells (410.4) used in the experiments. Analogous to the report of Cayeux *et al.*,[28] the data suggest that the MUC1 antigen requires costimulation generated by the B7.1–T-cell interaction to induce an effective response. Alternatively, antigen presentation could occur through cross-priming of tumour-specific T cells stimulated by B7.1 expression.[29] Using a different model system, Gong *et al.* have recently shown that immune responses to MUC1 can be induced by immunizing MUC1 transgenic mice with dendritic cells fused with MUC1-expressing carcinoma cells.[30] The use of professional APC in this system also suggests a role for costimulatory molecules in the effective presentation of MUC1.

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Immunity to Murine Breast Cancer Cells Modified to Express MUC-1, a Human Breast Cancer Antigen, in Transgenic Mice Tolerant to Human MUC-1[1]

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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-γ 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-γ. 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]-2 (1–5), IL-4 (6, 7), granulocyte/macrophage-colony stimulating factor (8, 9), IFN-γ (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-γ. 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_2/air atmosphere) in DMEM supplemented with 10% FCS (Sigma Chemical Co., St. Louis, MO), 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin

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[4] 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 *Sac*II are H-2^k. 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_1 hybrids (H-$2^{k/d}$) to enable them to accept 410.4 cells and E3 cells (both are H-2^d). 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-γ. 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, 5×10^5 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 μg/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 μg/ml of the neomycin analogue, G418. (100% of nontransduced cells die in growth medium containing 400 μg/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-γ. Eukaryote expression vectors pZipNeoSV-IL-2, pZipNeoSV-IL-4, and pZipNeoSV-IFN-γ (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-γ encode human IL-2, mIL-4, and mouse IFN-γ, respectively, and a gene conferring resistance to the neomycin analogue, G418. In brief, 1×10^6 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 μg of the vector in 100 μl of DMEM and 100 μl 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 μg/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 μg/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-γ 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-γ, 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 1×10^4 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^d; 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^d antibodies (PharMingen), to determine the level of H-2K^d class I determinants expressed by E3 cells. For comparison, the level of H-2K^d determi-

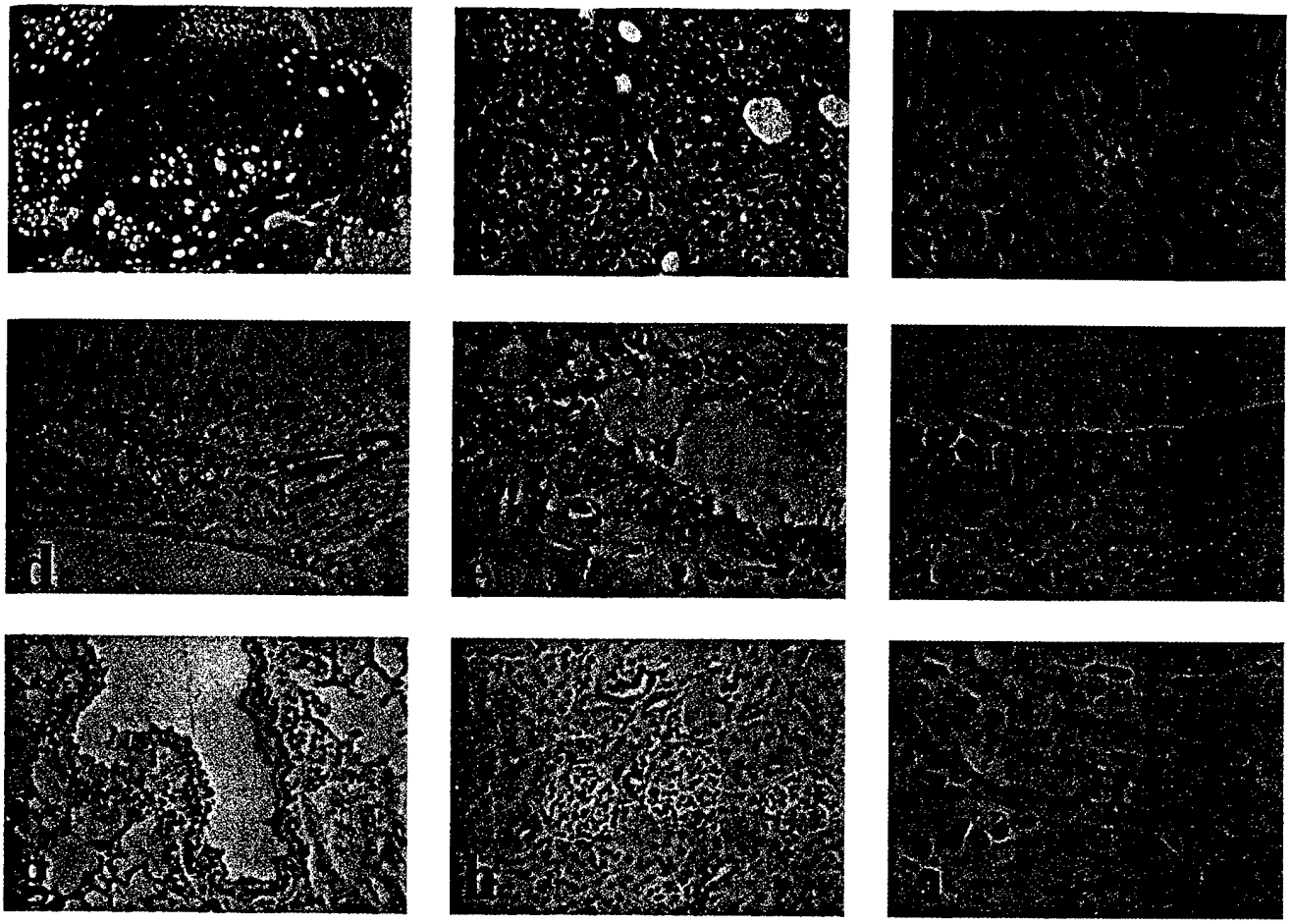

Fig. 1. Immunohistochemical and H&E staining of various tissues of MUC-1 transgenic mice stained with an antibody (HMFG-1) reactive with human mucin. *a*, inflammatory infiltrate at the site of injection of E3-IL-12 cells into breast tissue of MUC-1 transgenic mice. H&E section, ×250. *b*, inflammatory infiltrate at the site of injection of E3-IL-12 cells into breast tissue of MUC-1 transgenic mice. H&E section, ×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, ×400. *d*, immunohistochemical staining of portal tracts in the liver of MUC-1 transgenic mice with immunostaining localized to bile duct epithelium. ×400. *e*, immunohistochemical staining of portal tracts in the liver of MUC-1 transgenic mice, showing strong luminal surface staining. ×1000. *f*, immunohistochemical staining of bronchial epithelium of MUC-1 transgenic mice. ×1000. *g*, immunohistochemical staining of bronchial epithelium of MUC-1 transgenic mice. ×400. *h*, immunohistochemical staining of renal cortex of MUC-1 transgenic showing reaction with cells in the distal convoluted tubules. ×400. *i*, immunohistochemical staining of renal cortex of MUC-1 transgenic mice showing reaction with cells in the distal convoluted tubules. ×1000X. 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^d 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^k antibodies (410.4 cells of BALB/c origin are not expected to form H-2K^k determinants) or with isotype control serum, both followed by FITC rabbit antimouse immunoglobulin. The MFI of E3 cells stained with FITC-labeled H-2K^k 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 1×10^6 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 ± 12 days *versus* 16 ± 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

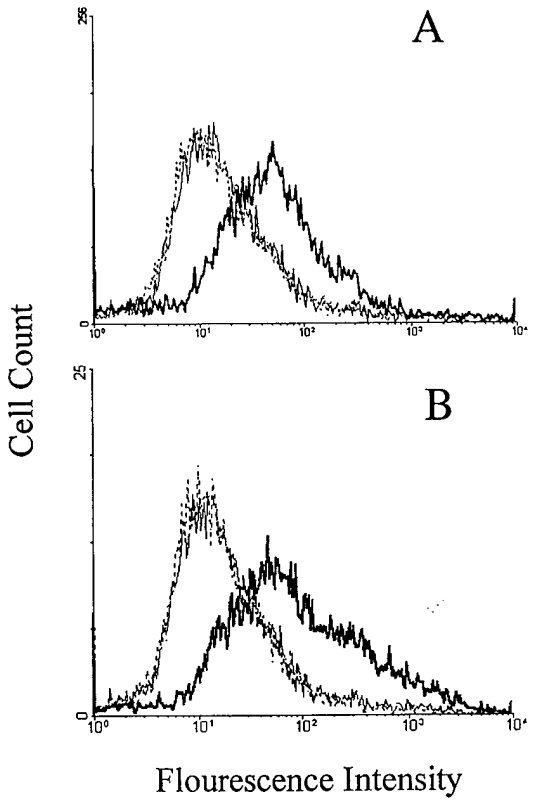

Fig. 2. Expression of mucin by mouse breast cancer cells (410.4) transfected with a plasmid encoding the *MUC-1* gene (E3 cells). E3 cells or 410.4 cells (1×10^6) were incubated for 1 h at 4°C with SM3 (*A*) or HMFG-1(*B*) antibodies or isotype control antibodies. After incubation with antimouse-FITC antibodies and washing as described in "Materials and Methods," the cells were analyzed for fluorescent staining by flow cytofluorography. *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 (~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, 1×10^6 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-γ. 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 μg/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

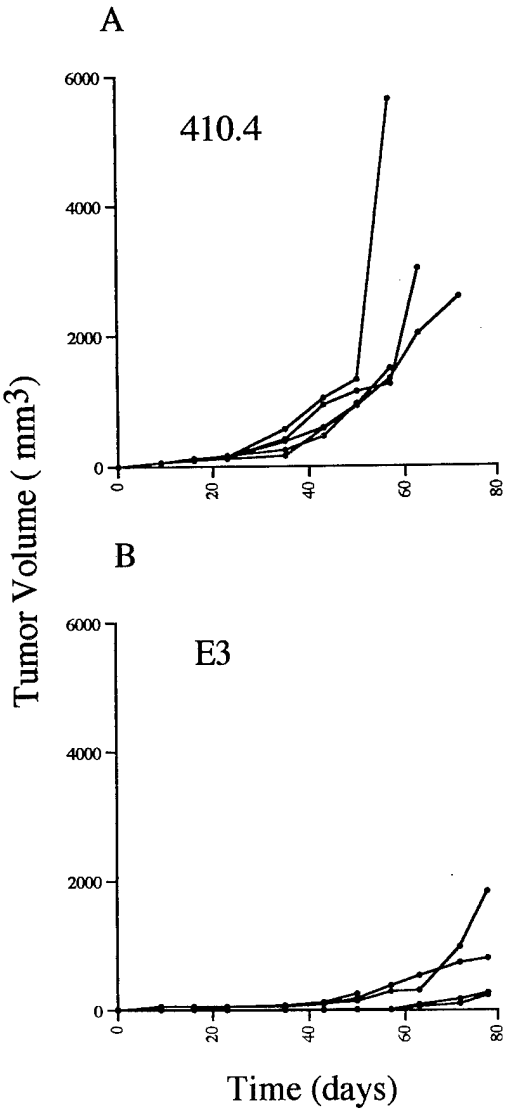

Fig. 3. Tumor growth in BALB/c mice that received injections of the fat pad of the breast with 410.4 cells or E3 cells. Naive BALB/c female mice between 12 and 16 weeks of age received injections into the fat pad of the breast with 1×10^6 E3 cells (*B*) in a total volume of 200 μl of growth medium or, for comparison, with 1×10^6 410.4 cells (*A*) in 200 μl 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.

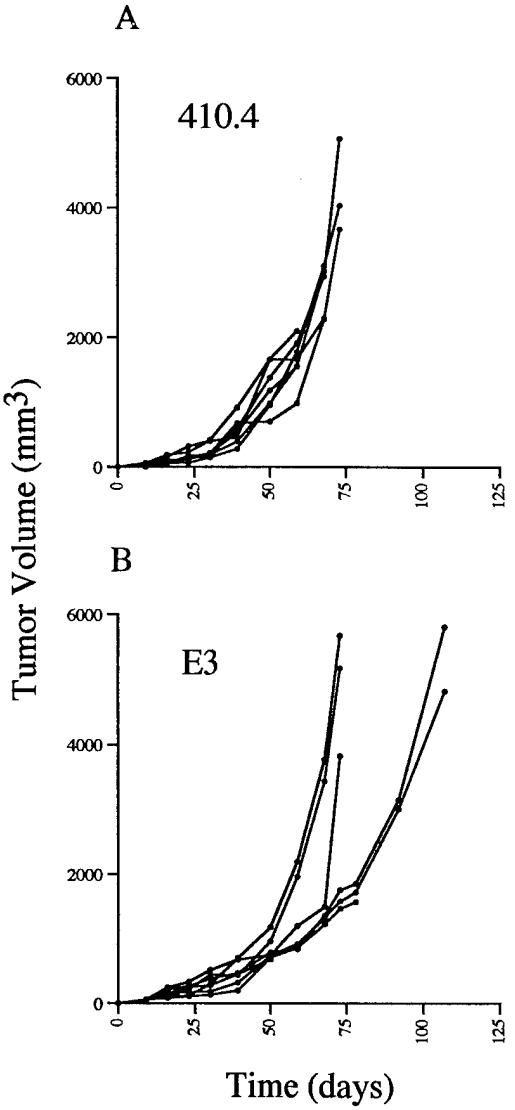

Fig. 4. Tumor growth in MUC-1 transgenic mice that received injections into the fat pad of the breast with 410.4 or E3 cells. Tumor growth was measured in naive MUC-1 transgenic mice that received injections into the fat pad of the breast with 1×10^6 E3 cells (*B*) in a total volume of 200 μl of growth medium. For comparison, naive MUC-1 transgenic mice received injections of 1×10^6 410.4 cells (*A*) in 200 μl 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-γ (pZipNeoSV-IFN-γ) formed 1.2 ng/ml IFN-γ. 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-γ. Every third passage, the cytokine-secreting cells were placed in growth medium containing 400 μg/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 1×10^6 E3 cells modified to secrete IL-2, IL-4, IL-12, or IFN-γ. 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, ~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-γ was found to be less than that of any of the other groups (Fig. 6), suggesting that modification of the cells to secrete IFN-γ 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 1×10^6 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}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-γ. The results (Table 1) indicated that the titers of IFN-γ 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-γ in the culture

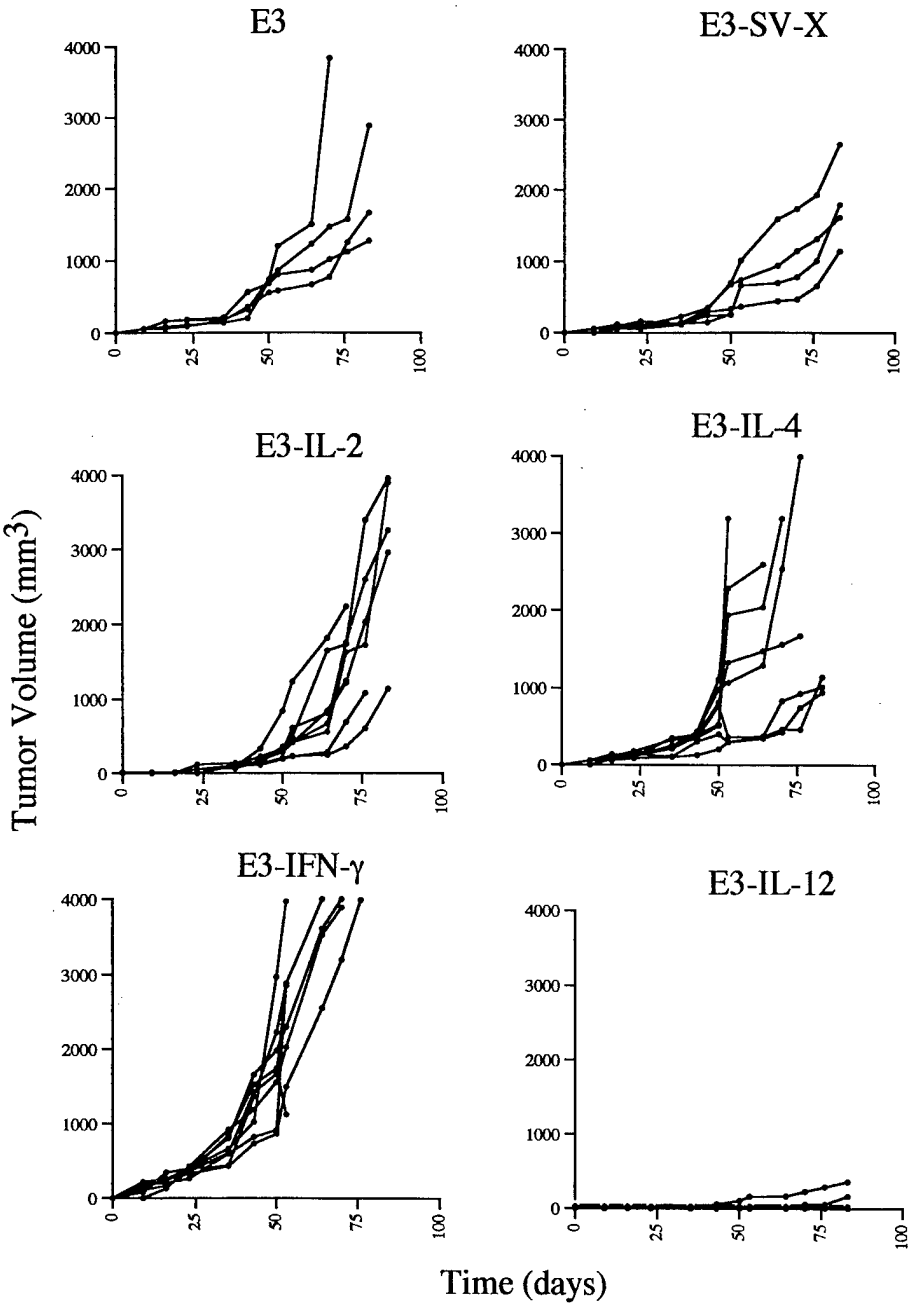

Fig. 5. Tumor growth in MUC-1 transgenic mice that received injections into the fat pad of the breast with E3 cells modified for cytokine secretion. Naive MUC-1 transgenic female mice between 12 and 16 weeks of age received injections into the fat pad of the breast with 1×10^6 E3 cells modified for cytokine 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 μl 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-γ 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 MIP1α, a chemokine (36). The titers of MIP1α 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 1×10^6 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).

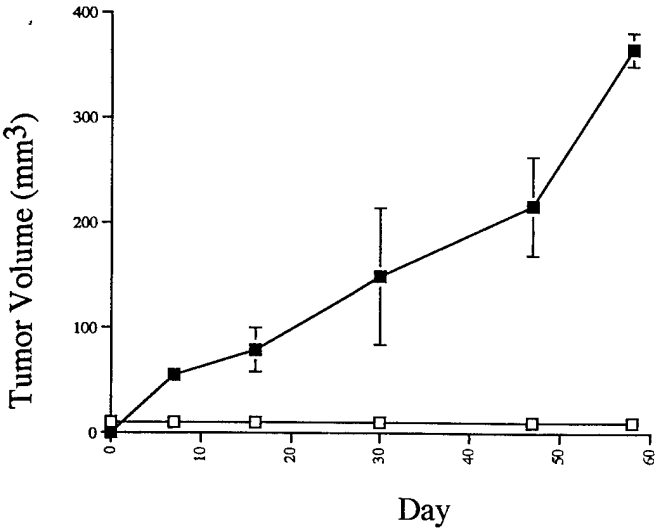

Fig. 6. Tumor growth in MUC-1 transgenic mice surviving a prior injection of E3-IL-12 cells that received injections subsequently with E3 cells. E3-IL-12 cells suspended in 200 μl of growth medium. Seventy-one days later, the mice received injections a second time into the fat pad of the contralateral breast with 1×10^6 viable E3 cells suspended in 200 μl 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 μl 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 coincubation 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 1×10^6 E3-IL-12 cells suspended in 200 μl 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 coincubated for 24 h with X-irradiated (5000 rads from a ^{60}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-γ or MIP1α. 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 ± SD cytokine release from each of three individual mice.

	E3-IL-12	E3	B16	None
	A. IFN-γ (pg/ml)			
24 h				
E3-IL-12	2100 ± 45	1415 ± 94	101 ± 4	0
Naive	505 ± 78	0	0	0
48 h				
E3-IL-12	4260 ± 19	2539 ± 217	1835 ± 120	4 ± 0.1
Naive	2881 ± 198	44 ± 4	0	0
	B. MIP1α (pg/ml)			
24 h				
E3-IL-12	283 ± 7	252 ± 12	37 ± 3	25 ± 8
Naive	168 ± 6	165 ± 11	17 ± 0	14 ± 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^d), 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 1×10^6 E3-IL-12 cells suspended in 200 μl 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 μl of growth medium. The mice in each group were bled 30 days later. A 1:100 dilution of the pooled sera was incubated with 5×10^5 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 cytofluorography using a FACS Calibur cytofluorograph (Becton Dickinson). There were three mice in each group.

	% E3 cells stained		
Serum from mice immunized with	IgG	IgM	IgA
E3-IL-12 cells	72 ± 10	10 ± 3	0.3 ± 0.2
E3 cells	7 ± 3	5 ± 2	0
410.4 cells	6 ± 3	4 ± 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-γ and TNF-α. It also promotes the development of Th1 $CD4^+$ 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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Treatment of Breast Cancer with Fibroblasts Transfected with DNA from Breast Cancer Cells[1]

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This investigation was based on the hypothesis that weakly immunogenic, breast cancer-associated Ags, the products of mutant or dysregulated genes in the malignant cells, will be expressed in a highly immunogenic form by semiallogeneic IL-2-secreting fibroblasts transfected with DNA from breast cancer cells. (Classic studies indicate that transfection of genomic DNA can stably alter both the genotype and the phenotype of the cells that take up the exogenous DNA.) To investigate this question, we transfected LM mouse fibroblasts (H-2^k) modified to secrete IL-2 with genomic DNA from a breast adenocarcinoma that arose spontaneously in a C3H/He mouse (H-2^k). To increase their nonspecific immunogenic properties, the fibroblasts were also modified before transfection to express allogeneic MHC determinants (H-2K^b). Afterward, the IL-2-secreting semiallogeneic cells were cotransfected with DNA from the spontaneous breast neoplasm, along with a plasmid (pHyg) conferring resistance to hygromycin. Pooled colonies of hygromycin-resistant cells were then tested in C3H/He mice for their immunotherapeutic properties against the growth of the breast neoplasm. The results indicated that tumor-bearing mice immunized with the transfected cells survived significantly longer than mice in various control groups. Similar beneficial effects were seen in C57BL/6 mice injected with a syngeneic breast carcinoma cell line (EO771) and semiallogeneic, IL-2-secreting fibroblasts transfected with DNA from EO771 cells. The immunity was mediated by CD8$^+$ T cells since immunized mice depleted of CD8$^+$ cells failed to resist tumor growth. *The Journal of Immunology,* **1999, 162: 6934–6941.**

Cancer cells form weakly immunogenic, tumor-associated Ags (TAAs)[4] (1–4) that can be recognized by CTLs. The TAAs are the products of dysregulated or mutant genes in the neoplastic cells that differ from the homologous genes in nonneoplastic cells of the same individual. Like other neoplasms, breast cancer cells form TAAs. The products of genes specifying HER-2/*neu* (5) MAGE-1 (6), BAGE (7), and MUC-1 (8–10) expressed by breast cancer cells have been identified as targets of CTLs. These may be only several representations of an undefined, and possibly large number of tumor Ags expressed by the malignant cells. Genetic instability is a characteristic phenotype of breast cancer and other types of malignant cells (11–15).

Under appropriate circumstances, tumor-specific cellular immune responses can be induced against TAAs expressed by neoplastic cells. The immune responses can be of sufficient magnitude to prolong the lives of tumor-bearing animals (16–20) and patients (21, 22). Genetic modification of tumor cells to secrete cytokines has been used as one means of augmenting the immunogenic properties of the malignant cells. Expression-competent genes for IL-2 (23–27), IL-4 (28), IL-6 (29), IL-7 (30), IL-12 (31), TNF-α (32, 33), IFN-α and IFN-γ (34, 35), and GM-CSF (36, 37), among others (38), have been introduced into neoplastic cells for this purpose. Immunizations with the cytokine-secreting, tumor cells resulted in cellular immune responses that were directed toward the malignant, but not the nonmalignant cells of the tumor-bearing host. Analogous tumor-specific responses were induced if the neoplastic cells used for the immunizations were modified to express syngeneic or allogeneic MHC determinants (39–42), or to express costimulatory molecules such as B7, required for activation of immune effector cells (43). However, the direct modification of cells from a primary neoplasm requires the establishment of a tumor cell line. This can be technically challenging, and may not always succeed. This is especially the case for breast cancer. Breast cancer cell lines are notoriously difficult to establish from primary breast neoplasms.

In other instances, defined tumor Ags or unfractionated tumor peptides have been used for tumor immunotherapy. However, few defined tumor Ags have been identified and cloned, and immunization with unfractionated tumor peptides requires large amounts of tumor if multiple immunizations are to be performed. Sufficient quantities of tumor tissue may not be available if patients are in clinical remission.

In this study, we tested an alternative approach. Classic studies indicated that transfection of DNA from one cell type can stably alter both the genotype and the phenotype of cells that take up the exogenous DNA. Wigler et al. (44), for example, reported stable integration of the gene for adenine phosphoribosyltransferase into mouse cells deficient in the enzyme by transfection of high m.w. genomic DNA from adenine phosphoribosyltransferase-positive mouse cells. A similar approach was used to convert thymidine kinase-deficient mouse cells to cells that expressed thymidine kinase by transfer of genomic DNA from a variety of thymidine kinase-positive tissues and cultured cells (45). In an analogous manner, Mendersohn et al. (46) reported that polio virus receptor-negative cells could be converted to cells that expressed the

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[4] Abbreviation used in this paper: TAA, tumor-associated Ag.

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receptor by transfection of genomic DNA from receptor-positive cells. The products of single genes specifying the enzymes or membrane-associated determinants were expressed by subpopulations of the transfected cells.

We tested the hypothesis that a cellular vaccine capable of prolonging the survival of mice with breast cancer could be prepared by transfection of a highly immunogenic cell line with DNA from breast cancer cells. We reasoned that genes specifying numerous, undefined, weakly immunogenic TAAs would be expressed in a highly immunogenic form by the transfected cells, and that immunizations with the transfected cells would result in an immune response directed toward the breast cancer cells. We used two types of breast tumors, with analogous results. DNA from an adenocarcinoma of the breast that formed spontaneously in a C3H/He mouse was used to transfect a mouse fibroblast cell line that had been modified to secrete IL-2 and to express allogeneic class I MHC determinants (H-2K^b). A plasmid (pHyg) specifying resistance to hygromycin was included to allow selection of cells that had taken up the exogenous DNA. The antibiotic-resistant, transfected cells were then used to treat mice with breast cancer. The results indicated that mice immunized with the transfected fibroblasts developed generalized, cell-mediated immunity toward the breast cancer cells. The treated animals survived significantly longer than mice in various control groups, including mice with breast cancer treated by immunization with non-DNA-transfected fibroblasts. Similar results were obtained for mice bearing a mammary adenocarcinoma cell line (EO771) of C57BL/6J mouse origin treated with fibroblasts transfected with DNA from EO771 cells. The immunity was mediated by CD8$^+$ T lymphocytes since mice depleted of CD8$^+$ cells failed to resist tumor growth.

The augmented resistance to breast cancer in mice treated with fibroblasts transfected with breast cancer DNA points toward an analogous form of therapy for breast cancer patients.

Materials and Methods

Cell lines and experimental animals

Eight- to ten-week-old pathogen-free C3H/HeJ mice (H-2^k) and eight- to ten-week-old pathogen-free C57BL/6J mice (H-2^b) were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were maintained in the animal care facilities of the University of Illinois, according to National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. They were 8–12 wk old when used in the experiments. EO771 cells, a mammary adenocarcinoma cell line derived from a C57BL/6J mouse, were from the Tumor Repository of the Division of Cancer Treatment, Diagnosis and Centers of the National Cancer Institute (Frederick, MD). SB-1 cells were a breast adenocarcinoma that formed spontaneously in a C3H/HeJ mouse. B16 cells, a melanoma cell line originating in a C57BL/6J mouse, were from I. Fidler (MD Anderson, Houston, TX). EO771 cells were maintained by serial passage in histocompatible C57BL/6J mice. B16 cells were maintained by serial passage in C57BL/6J mice or at 37°C in a humidified 7% CO_2/air atmosphere in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Sigma, St. Louis, MO) and antibiotics (Life Technologies) (growth medium). LM cells, a fibroblast cell line of C3H/He mouse origin, were from the American Type Culture Collection (Manassas, VA). The cells were maintained at 37°C in a humidified 7% CO_2/air atmosphere in growth medium.

Modification of LM mouse fibroblasts for IL-2 secretion

LM fibroblasts were modified for IL-2 secretion by transduction with the retroviral vector pZipNeoSVIL-2 (from M. K. L. Collins, University College, London, U.K.) (LM-IL-2 cells). The vector, packaged in GP+*env* AM12 cells (from A. Bank, Columbia University, New York, NY), included a human IL-2 cDNA and a *neo*^{r} gene, both under control of the Moloney leukemia virus long terminal repeat. The *neo*^{r} gene conferred resistance to the aminoglycoside antibiotic, G418. Virus-containing supernatants of GP+*env* AM12 cells transfected with pZipNeoSVIL-2 were added to LM fibroblasts, followed by overnight incubation at 37°C in growth medium to which polybrene (Sigma; 5 μg/ml, final concentration) had been added. The cells were maintained for 14 days in growth medium containing 400 μg/ml G418 (Life Technologies). One hundred percent of nontransduced LM cells died in the medium supplemented with G418 during this period. Colonies of cells proliferating in the G418-containing growth medium were pooled for later use in the experiments. Every third, fourth, and fifth passage, the transduced cells were cultured in growth medium containing 400 μg/ml G418. IL-2 secretion by LM-IL-2 cells was detected by the capacity of supernatants from the transduced cells to sustain the growth of CTLL-2 cells, an IL-2-dependent cell line (47). Varying dilutions of the filtered culture supernatants (0.2 um nitrocellulose; Gelman, Ann Arbor, MI) were transferred to 96-well plates containing 1 × 10^4 CTLL-2 cells in a final volume of 200 μl of growth medium per well. After incubation for 16 h, 0.5 μCi [^{3}H]thymidine (Amersham, Arlington Heights, IL) was added to each well for additional 6 h of incubation. A standard curve was generated by adding varying amounts of human rIL-2 (Life Technologies) to an equivalent number of CTLL-2 cells. Afterward, the cells were collected onto glass fiber filters (Whittaker M.A. Products, Walkerville, MD) using a PhD multiple harvester (Microbiological Associates, Bethesda, MD). After washing with ethanol (95%), radioactivity in the insoluble fraction was measured in a liquid scintillation spectrometer (Packard Instrument, Downers Grove, IL). One unit of IL-2 resulted in half-maximal proliferation of CTLL-2 cells under these conditions.

Modification of LM-IL-2 cells for the expression of H-2K^b class I determinants

pBR327H-2K^b (Biogen Research, Cambridge, MA), a plasmid encoding MHC H-2K^b (48), was used to modify LM-IL-2 fibroblasts for the expression of H-2K^b determinants (LM-IL-2K^b cells). A total of 10 μg of pBR327H-2K^b and 1 μg of pBabePuro (from M. K. L. Collins), a plasmid conferring resistance to puromycin (49), was mixed with Lipofectin (Life Technologies), according to the supplier's instructions, and then added to 1 × 10^6 LM-IL-2 cells in 10 ml of DMEM without FBS. For use as a control, an equivalent number of LM-IL-2 cells was transfected with 1 μg of pBabePuro alone. The cells were incubated for 18 h at 37°C in a CO_2/air atmosphere, washed with DMEM, followed by the addition of 7 ml of growth medium. After incubation for 48 h, the cell cultures were divided and replated in growth medium supplemented with 3 μg/ml puromycin (Sigma), followed by incubation at 37°C for 7 additional days. The surviving colonies were pooled and tested by staining with specific FITC-conjugated Abs (described, below) for the expression of H-2K^b determinants. One hundred percent of nontransfected LM-IL-2 cells maintained in growth medium containing puromycin died during the 7-day period of incubation.

Immunofluorescent staining and cytofluorometric measurements

Quantitative immunofluorescent staining was used to detect the expression of H-2K^b determinants by LM-IL-2 cells transfected with pBR327H-2K^b. The measurements were performed in an Epic V flow cytofluorograph (Coulter Electronics, Hialeah, FL) equipped with a multiparameter data-acquisition and display system (MDADS). For the analysis, a single cell suspension was prepared from the monolayer cultures of puromycin-resistant cells with 0.1 mM EDTA in 0.1 M PBS, pH 7.4. The cells were washed with PBS containing 0.2% sodium azide and 0.5% FBS. Afterward, FITC-conjugated H-2K^b, H-2K^d, or H-2K^k mAbs (PharMingen, San Diego, CA), or FITC-conjugated IgG2a isotype serum (Dako, Carpenteria, CA) were added to the cells, followed by incubation at 4°C for 1 h. The cells were then washed with PBS containing 0.5% FBS and 0.2% sodium azide. One-parameter fluorescence histograms were generated by analyzing at least 1 × 10^4 cells. Background staining was determined by substituting cells stained with FITC-conjugated mouse IgG2a alone for cells stained with the specific Abs.

Depletion of mice of CD8$^+$ or CD4$^+$ T cells

mAbs were used to deplete naive C57BL/6J mice of CD8$^+$ or CD4$^+$ T cells. The mice were injected i.p. with the Ab-rich fraction obtained from ascites fluid containing anti-CD8 (83-23-5 mouse hybridoma) or from ascites fluid containing anti-CD4 (GK1.5 rat hybridoma) (both hybridomas were from Dr. K. Herald, University of Illinois at Chicago). The mice were injected i.p. with 0.3 ml (5 mg) of enriched 83-12-5 Abs, or i.p. with 0.2 ml (1 mg) of enriched GK1.5 Abs. Depletion of the relevant subset of T cells was verified by flow-cytofluorometric analysis of spleen cell suspensions taken 2 days after the injection of the enriched ascitic fluid. The depleted conditions were maintained in the remaining mice by injections of equivalent amounts of the appropriate Abs every 5 days until the experiments were concluded

Transfection of LM-IL-2K^b cells with DNA from a breast carcinoma that arose spontaneously in a C3H/He mouse (SB-1), from EO771 breast carcinoma cells, or from B16 melanoma cells

Sheared, unfractionated DNA isolated (Qiagen, Chatsworth, CA) from a spontaneous mammary adenocarcinoma (SB-1) taken directly from a C3H/HeJ mouse, or from EO771 cells taken from a C57BL/6J mouse, or from B16 melanoma cells from in vitro culture, was used to transfect LM-IL-2K^b cells. The method described by Wigler et al. (45) was applied, as modified. Briefly, high m.w. DNA from each cell type was sheared by three passages through a 25-gauge needle. Afterward, 100 μg of the sheared DNA was mixed with 10 μg pHyg (from L. Lau, University of Illinois), a plasmid that encoded the *Escherichia coli* enzyme hygromycin B phosphotransferase (52), conferring resistance to hygromycin B. The sheared DNA and pHyg were then mixed with Lipofectin, according to the manufacturer's instructions (Life Technologies). The DNA/Lipofectin mixture was added to a population of 1×10^7 LM-IL-2K^b cells that had been divided into ten 100-mm plastic cell culture plates 24 h previously. Eighteen hours after addition of the DNA/Lipofectin mixture to the cells, the growth medium was replaced with fresh growth medium. For use as a control, DNA from the tumor cells was omitted, and 1 μg of pHyg alone, mixed with Lipofectin, was added to an equivalent number of LM-IL-2K^b cells. The same protocol was followed to transfect LM-IL-2 cells (not transduced with pBR327H-2K^b) with DNA from SB-1 cells. In each instance, the cells were maintained for 14 days in growth medium containing 600 μg/ml hygromycin B (Boehringer Mannheim, Indianapolis, IN). One hundred percent of LM-IL-2K^b or LM-IL-2 cells transfected with tumor-DNA alone maintained in the hygromycin growth medium died within this period. The surviving colonies (at least 2.5×10^4) of LM-IL-2K^b or of LM-IL-2 cells transfected with pHyg and DNA from the tumor cells, or with pHyg alone (LM-IL-2K^b cells), were pooled and used in the experiments.

Results

Modification of LM mouse fibroblasts for IL-2 secretion

A replication-defective retroviral vector, pZipNeoSVIL-2, was used to modify LM fibroblasts (H-2^k) for the secretion of IL-2. The vector specified the gene for human IL-2, along with a gene (neo^r) that conferred resistance to the neomycin analogue, G418. After selection in growth medium containing sufficient quantities of G418 to kill 100% of nontransduced cells, the surviving colonies were pooled and maintained as a cell line. Analysis of the culture supernatants indicated that 1×10^6 retrovirally transduced cells formed 150 U IL-2/10^6 cells/48 h, as determined by the capacity of the supernatants to sustain the growth of IL-2-dependent CTLL-2 cells. IL-2-secreting cells modified to express H-2K^b determinants (LM-IL-2K^b) and IL-2-secreting cells transfected with tumor DNA (described, below) formed equivalent quantities of IL-2. The culture supernatants of LM cells transduced with the IL-2-negative vector (pZipNeoSV(X), or of nontransduced LM cells failed to form detectable quantities of IL-2. Every third, fourth, and fifth passage, the IL-2-secreting cells were placed in medium containing 400 μg/ml G418. Under these conditions, similar quantities of IL-2 were detected in the culture supernatants of cells transduced with pZipNeoSVIL-2 for more than 6 mo of continuous culture (these data are not presented).

Modification of LM-IL-2 cells for the expression of MHC class I H-2K^b determinants

A plasmid, pBR327H-2K^b, was used to modify LM-IL-2 cells for the expression of H-2K^b determinants. LM-IL-2 cells were co-transfected with pBR327H-2K^b DNA along with pBabePuro DNA, used for selection. (A 10:1 ratio of pBR327H-2K^b DNA to pBabePuro DNA was used to increase the likelihood that cells that incorporated pBabePuro DNA took up pBR327H-2K^b DNA as well.) After selection in growth medium containing sufficient quantities of puromycin to kill the nontransduced cells, the surviving colonies were pooled and the cell number was expanded in vitro.

The expression of H-2K^b determinants by the modified cells was measured by quantitative immunofluorescent staining, using FITC-labeled mAbs for mouse H-2K^b determinants. As controls, aliquots of the puromycin-resistant cell suspension were incubated with FITC-labeled IgG2a isotype serum, or with FITC-labeled mAbs for H-2K^d determinants. As an additional control, the cells were incubated with FITC-labeled H-2K^b mAbs (LM cells are of C3H/He mouse origin). The mean fluorescent index of the puromycin-resistant LM-IL-2 cells stained with FITC-conjugated H-2K^b or FITC-conjugated H-2K^k mAbs (0.98 and 7.6, respectively) was significantly ($p < 0.001$) higher than that of cells stained with FITC-conjugated H-2K^d mAbs (Fig. 1). The MFI of cells stained with FITC-conjugated H-2K^d mAbs was approximately the same as that of cells stained with FITC-conjugated IgG2a isotype serum. The expression of H-2K^b determinants was a stable property of the transfected cells. The cells stained with equivalent intensity with FITC-conjugated H-2K^b mAbs after 3 mo of continuous culture (these data are not presented).

Tumor growth and survival of C57BL/6J mice injected with EO771 breast cancer cells and LM-IL-2K^b cells transfected with DNA from EO771 cells (LM-IL-2K^b/EO771)

C57BL/6J mice were highly susceptible to the growth of EO771 cells, a syngeneic breast cancer cell line. One hundred percent of mice injected with EO771 cells died from progressive tumor growth.

The effect of immunization with LM-IL-2K^b/EO771 cells on the growth of EO771 cells in C57BL/6J mice was determined by injecting naive mice into the fat pad of the breast with a mixture of EO771 cells and LM-IL-2K^b/EO771 cells, as described in the legend to Fig. 2. At the same time, the mice received an i.p. injection of 2×10^6 LM-IL-2K^b/EO771 cells alone. The mice then received two subsequent immunizations at weekly intervals with 2×10^6 LM-IL-2K^b/EO771 cells i.p. and an equivalent number of LM-IL-2K^b/EO771 cells injected into the same breast as first injected, without additional EO771 cells. As a control, naive C57BL/6J mice were injected into the breast with EO771 cells alone, followed by the subsequent injections of growth media. As additional controls, naive C57BL/6J mice were injected according to the same protocol with a mixture of EO771 cells and LM-IL-2K^b cells transfected with DNA from B16 melanoma cells (LM-IL-2K^b/B16), with EO771 cells and unmodified LM cells, or with EO771 cells and nontumor-DNA-transfected LM-IL-2K^b cells. The results

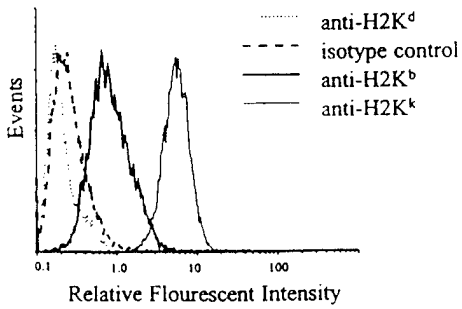

FIGURE 1. The expression of H-2K^b determinants by LM-IL-2 cells transduced with pBR327H-2K^b. A total of 1×10^4 LM-IL-2 cells transduced with the plasmid pBR327H-2K^b (LM-IL-2K^b cells) was incubated for 1 h at 4°C with FITC-conjugated anti-H-2K^b, anti-H-2K^k, or anti-H-2K^d mAbs, as described in *Materials and Methods*. The cells were then analyzed for fluorescent staining by flow cytofluorography.

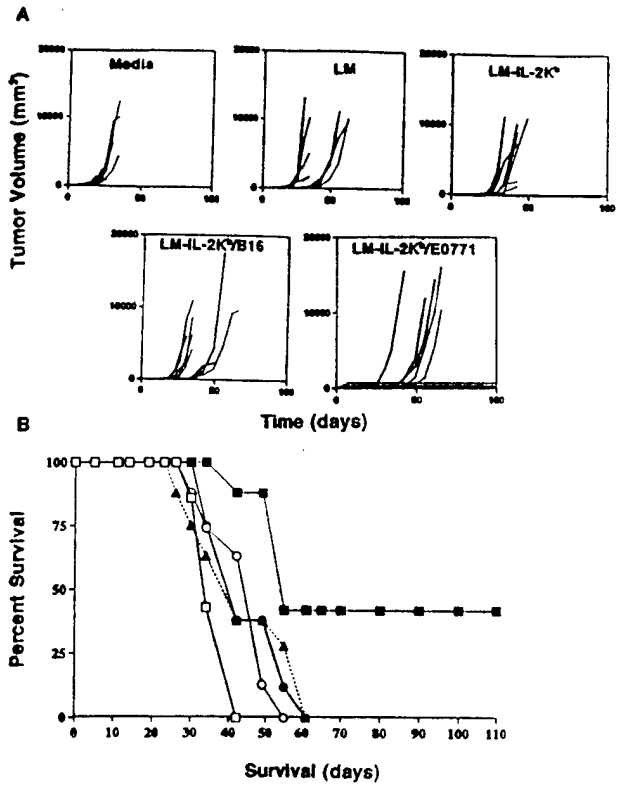

FIGURE 2. *A*, Tumor growth in C57BL/6J mice injected with EO771 breast cancer cells and LM-IL-2K^b/EO771 cells. C57BL/6J mice (seven per group) were injected into the fat pad of the breast with a mixture of 5×10^3 EO771 breast carcinoma cells and 2×10^6 LM-IL-2K^b/EO771 cells in a total volume of 200 μl. At the same time the mice also received an injection i.p. of 2×10^6 LM-IL-2K^b/EO771 cells in 200 μl alone, followed by two subsequent injections at weekly intervals of 2×10^6 LM-IL-2K^b/EO771 cells i.p. and 2×10^6 LM-IL-2K^b/EO771 cells into the fat pad of the same breast as first injected. As controls, other naive C57BL/6J mice were injected according to the same protocol with equivalent numbers of EO771 cells and unmodified LM cells, with EO771 cells and LM-IL-2K^b cells, with EO771 cells and LM-IL-2K^b/B16 cells, or with EO771 cells into the breast alone followed by subsequent injections of growth medium. Mean tumor volume was derived from two-dimensional measurements obtained with a dial caliper. The volume is equal to $0.4ab^2$, where a = length and b = width. *B*, Survival of C57BL/6J mice injected with EO771 breast carcinoma cells and LM-IL-2K^b/EO771 cells. C57BL/6J mice (seven per group) were injected into the fat pad of the breast with a mixture of 5×10^3 EO771 breast carcinoma cells and 2×10^6 LM-IL-2K^b/EO771 cells in a total volume of 200 μl. At the same time the mice also received an injection i.p. of 2×10^6 LM-IL-2K^b/EO771 cells in 200 μl alone, followed by two subsequent injections at weekly intervals of 2×10^6 LM-IL-2K^b/EO771 cells i.p. and 2×10^6 LM-IL-2K^b/EO771 cells into the fat pad of the same breast as first injected. As controls, other naive C57BL/6J mice were injected according to the same protocol with equivalent numbers of EO771 cells and LM cells, with EO771 cells and LM-IL-2K^b cells, with EO771 cells and LM-IL-2K^b/B16 cells, or with EO771 cells into the breast alone with subsequent injections of growth medium. Mean survival times. Mice injected with viable EO771 cells alone, 34.5 ± 5.8 days; mice injected with viable EO771 cells and LM cells, 41 ± 14 days; mice injected with viable EO771 cells and LM-IL-2K^b cells, 44 ± 9 days; mice injected with viable EO771 cells and LM-IL-2K^b/B16 cells, 46 ± 11 days; three mice injected with viable EO771 cells and LM-IL02K^b/EO771 cells, >110 days; mean survival times for remaining mice dying from progressive tumor growth, 54 ± 9. The p value for difference in survival of mice injected with viable EO771 cells and LM-IL-2K^b/EO771 cells, relative to survival of mice in each of the other groups, was <0.01. □, Injected with EO771 cells alone; ○, injected with EO771 cells and LM cells; ●, injected with EO771 cells and LM-IL-2K^b cells; ▲, injected with EO771 cells and LM-IL-2K^b/B16 cells. ■, injected with EO771 cells and LM-IL-2K^b/EO771 cells. The p values are as follows: $p < 0.01$ for difference in survival of mice injected with EO771 cells, and mice injected with EO771 cells and LM-IL-2K^b/EO771 cells; $p < 0.01$ for difference in survival of mice injected with EO771 cells and LM cells, and mice injected with EO771 cells and LM-IL-2K^b/EO771 cells; $p < 0.01$ for difference in survival of mice injected with EO771 cells and LM-IL-2K^b cells, and mice injected with EO771 cells and LM-IL-2K^b/EO771 cells; and $p < 0.01$ for difference in survival of mice injected with EO771 cells and LM-IL-2K^b/B16 cells, and mice injected with EO771 cells and LM-IL-2K^b/EO771 cells.

(Fig. 2*A*) indicate that the first appearance of tumor was significantly delayed ($p < 0.004$) in the group of mice injected with the mixture of EO771 cells and LM-IL-2K^b/EO771 cells, relative to that of mice in any of the other groups. Three mice in the group injected with EO771 cells and LM-IL-2K^b/EO771 cells failed to develop tumors and appeared to have rejected the breast cancer cells.

The development of resistance to EO771 cells in mice immunized with LM-IL-2K^b/EO771 cells was emphasized by the finding that the immunized mice survived significantly ($p < 0.01$) longer than mice in any of the various control groups, including mice injected with EO771 cells and LM-IL-2K^b cells transfected with DNA from B16 melanoma cells. Mice immunized with LM-IL-2K^b cells transfected with DNA from B16 cells failed to resist the growth of the breast cancer cells (Fig. 2*B*). In some instances, mice injected with EO771 cells and LM-IL-2K^b/EO771 cells survived indefinitely, more than 110 days. The injections of LM-IL-2K^b/EO771 cells were without apparent harm. Tumors failed to form in mice injected with LM-IL-2K^b/EO771 cells alone. Since LM cells express foreign histocompatibility determinants in C57BL/6J mice, it is likely that, like other foreign tissue grafts, the cells were rejected.

To determine whether the injections of LM-IL-2K^b/EO771 cells resulted in generalized, long-term immunity toward the breast cancer cells, surviving mice in the group immunized with EO771 cells and LM-IL-2K^b/EO771 cells received a second injection of EO771 cells 110 days after the first immunization. The presence of generalized, long-term immunity to the breast cancer cells was indicated by the finding that mice injected a second time with EO771 cells survived significantly ($p < 0.02$) longer than naive mice injected with an equivalent number of EO771 cells alone (Fig. 3).

CD8$^+$ cells mediate immunity to breast cancer in mice immunized with fibroblasts transfected with DNA from breast cancer cells

T cell depletion was used to determine the subset of T cells that mediated resistance to tumor growth in mice immunized with the DNA-transfected cells. In the experiment, T cell depletion was accomplished by injecting C57BL/6J mice i.p. with CD8$^+$ or CD4$^+$ mAbs, as described in *Materials and Methods*. Two days later, the mice received a second injection of the Abs, followed by an injection into the fat pad of the breast with a mixture of 5×10^3 EO771 cells and 2×10^6 LM-IL-2K^b/EO771 cells. The mice received two subsequent injections of equivalent numbers of LM-IL-2K^b/EO771 cells and additional injections of the mAbs, as described. As indicated, the first appearance of tumor and survival of immunized mice depleted of CD8$^+$ cells (Fig. 4*Aa*) was not significantly different from the first appearance of tumor and survival of mice injected with EO771 cells alone (Fig. 4*Ab*). Depletion of CD4$^+$ cells had no apparent effect on resistance to tumor growth. The first appearance of tumor and survival of CD4$^+$ T cell-depleted mice injected with EO771 cells and LM-IL-2K^b/EO771 cells (Fig. 4*Ac*) was not significantly different from the first appearance of tumor and survival of mice injected with EO771 cells

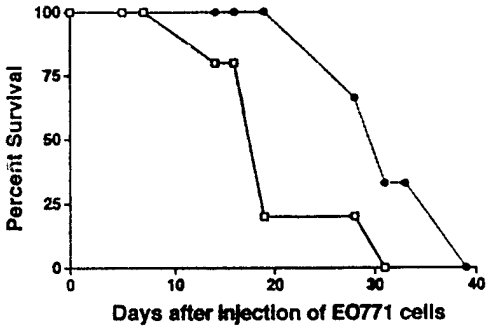

FIGURE 3. Survival of C57BL/6J mice surviving a prior injection of EO711 cells and LM-IL-2K^b/EO771 cells injected with EO771 cells alone. Three C57BL/6J mice surviving 110 days after the prior injection of EO711 cells and LM-IL-2K^b/EO771 cells were injected into the fat pad of the breast a second time with 5×10^3 EO771 cells alone. As a control, five naive C57BL/6J mice were injected into the fat pad of the breast with an equivalent number of EO771 cells; $p < 0.02$ for the difference in survival of mice in the two groups. ●, Surviving mice injected with EO711 cells; □, naive mice injected with EO711 cells.

and LM-IL-2K^b/EO771 cells alone (Fig. 4*Ad*). Thus, depletion of CD8$^+$ T cells, but not CD4$^+$ cells, affected the animals' capacity to resist the growth of the breast cancer cells in mice immunized with the DNA-transfected cells.

CD8$^+$ T cell depletion had analogous effects on the survival of mice injected with the breast cancer cells and the DNA-transfected fibroblasts. As indicated (Fig. 4*B*), the survival of CD8$^+$-depleted mice injected with EO771 cells and LM-IL-2K^b/EO771 cells was significantly ($p < 0.01$) less than the survival of nondepleted mice injected with EO771 cells and LM-IL-2K^b/EO771 cells alone. It was not significantly different from the survival of non-T cell-depleted mice injected with EO771 cells alone. In contrast, depletion of CD4$^+$ cells had no effect on survival. The survival of mice depleted of CD4$^+$ cells injected with EO771 cells and LM-IL-2K^b/EO771 cells was not significantly different from that of non-T cell-depleted mice injected with EO771 cells and LM-IL-2K^b/EO771 cells alone.

Thus, depletion of CD8$^+$ but not CD4$^+$ cells affected both tumor growth and survival of the immunized mice with breast cancer.

Survival of C3H/HeJ mice injected with cells from a spontaneous adenocarcinoma of the breast (SB-1) and LM-IL-2K^b cells transfected with DNA from SB-1 cells

Specific partial immunity toward EO771 cells, a breast cancer cell line, was generated in C57BL/6J mice immunized with semiallogeneic, IL-2-secreting mouse fibroblasts transfected with DNA from EO771 cells. The same protocol was followed to determine whether an analogous response would be obtained in mice immunized with the modified fibroblasts transfected with DNA taken directly from a spontaneous breast adenocarcinoma arising in a C3H/HeJ mouse.

C3H/HeJ mice develop breast cancer spontaneously. A tumor that developed in the breast of a 12-mo-old mouse was excised and used as a source of DNA to develop the vaccine. Histologic sections indicated that it was an adenocarcinoma. In addition, naive C3H/HeJ mice had no apparent resistance to the growth of the breast cancer cells. One hundred percent of mice injected with 1×10^4 SB-1 cells into the fat pad of the breast died from progressive tumor growth in approximately 30 days.

The effect of immunization with LM-IL-2K^b cells transfected with DNA from the spontaneous breast neoplasm (SB-1 cells) on

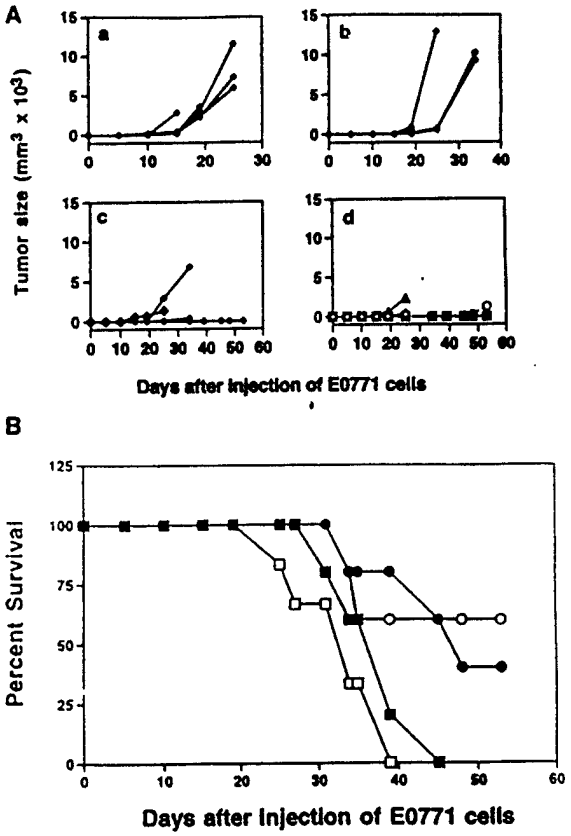

FIGURE 4. *A*, Tumor growth in C57BL/6J mice depleted of CD8$^+$ T lymphocytes injected with a mixture of EO711 breast cancer cells and LM-IL-2K^b/EO771 cells. C57BL/6J mice (seven per group) were injected i.p. with CD4$^+$ (group c) or CD8$^+$ (group b) mAbs, as described in *Materials and Methods*. Two days later, the mice were injected into the fat pad of the breast with a mixture of 5×10^3 EO711 breast carcinoma cells and 2×10^6 LM-IL-2K^b/EO711 cells in a total volume of 200 μl. At the same time, the mice also received an injection i.p. of 2×10^6 LM-IL-2K^b/EO711 cells in 200 μl alone. The mice received additional injections of the relevant Abs as described, and two subsequent injections at weekly intervals of 2×10^6 LM-IL-2K^b/EO771 cells i.p. and 2×10^6 LM-IL-2K^b/EO771 cells into the fat pad of the same breast as first injected. As controls, other C57BL/6J mice (group c) were injected according to the same protocol with EO771 cells and LM-IL-2K^b/EO771 cells, but did not receive mAbs, or with equivalent numbers of EO771 cells into the breast alone followed by subsequent injections of growth medium (group d). Mean tumor volume was derived from two-dimensional measurements obtained with a dial caliper. The volume is equal to $0.4ab^2$, where a = length, and b = width. *B*, Survival of C57BL/6J mice depleted of T cells injected with a mixture of EO771 breast carcinoma cells and LM-IL-2K^b/EO771 cells. The same protocol as described in *A* was followed except that survival of the Ab-treated mice was determined. ■, Injected with CD8$^+$ Abs, EO771 cells, and LM-IL-2K^b/EO771 cells; ●, injected with CD4$^+$ Abs, EO771 cells, and LM-IL-2K^b/EO771 cells; □, injected with EO771 cells alone; ○, injected with EO771 cells and LM-IL-2K^b/EO771 cells alone.

the growth of the breast cancer cells was determined by injecting naive C3H/HeJ mice into the fat pad of the breast with SB-1 cells and LM-IL-2K^b/SB-1 cells, and i.p. with LM-IL-2K^b/SB-1 cells alone. As previously, the mice received two subsequent injections i.p. and two subsequent injections into the same breast as first injected with the same number of LM-IL-2K^b/SB-1 cells. The results (Fig. 5*A*) indicated that the time to first appearance of a palpable tumor in the breasts of mice injected with the mixture of SB-1 cells and LM-IL-2K^b/SB-1 cells was significantly delayed ($p < 0.006$), relative to the first appearance of tumor in mice injected

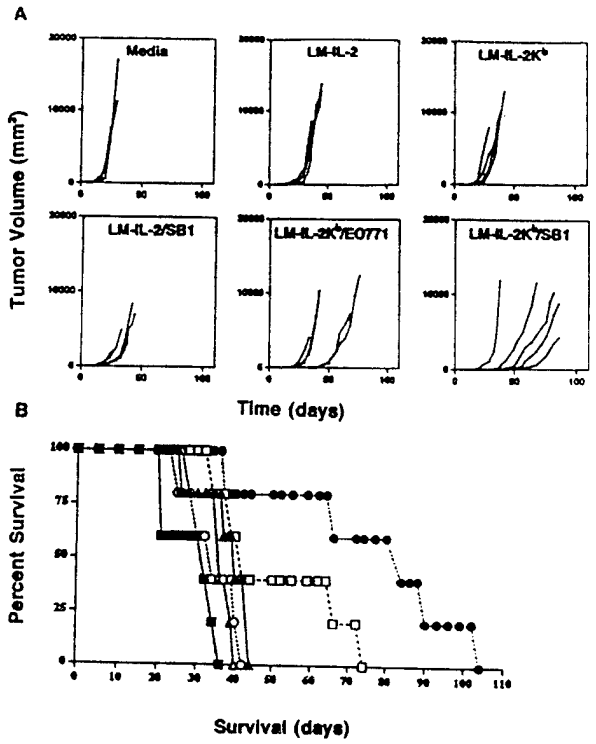

FIGURE 5. *A*, Tumor growth in C3H/HeJ mice injected with cancer cells from a spontaneous breast neoplasm (SB-1) and LM-IL-2K^b cells transfected with DNA from SB-1 cells. C3H/HeJ mice (five per group) were injected into the fat pad of the breast with a mixture of 1×10^6 SB-1 cells and 2×10^6 LM-IL-2K^b/SB-1 cells. At the same time, the mice received an injection i.p. of 2×10^6 LM-IL-2K^b/SB-1 cells alone, followed by two subsequent injections. As controls, the mice were injected according to the same protocol with equivalent numbers of SB-1 cells alone, with SB-1 cells and LM-IL-2 cells, with SB-1 cells and LM-IL-2K^b cells, with SB-1 cells and LM-IL-2/SB-1 cells, or with SB-1 cells and LM-IL-2K^b/EO771 cells. The mice were injected i.p. twice more, at weekly intervals, with the same number of modified cells as in the initial injections, but without additional SB-1 cells. Mean tumor volume was derived from two-dimensional measurements obtained with a dial caliper. *B*, Survival of C3H/HeJ mice injected with a mixture of SB-1 breast carcinoma cells and LM-IL-2K^b/SB-1 cells. C3H/HeJ mice (five per group) were injected into the fat pad of the breast with a mixture of 5×10^3 SB-1 cells and 2×10^6 LM-IL-2K^b/SB-1 cells in a total volume of 200 μl. A the same time the mice received an injection i.p. of 2×10^6 LM-IL-2K^b/SB-1 cells in 200 μl alone, followed by two subsequent injections at weekly intervals of 2×10^6 LM-IL-2K^b/SB-1 cells i.p. and 2×10^6 LM-IL-2K^b/SB-1 cells into the fat pad of the same breast as first injected. As controls, other naive C57BL/6J mice were injected according to the same protocol with equivalent numbers of SB-1 cells and LM-IL-2 cells, with SB-1 cells and LM-IL-2K^b cells, with SB-1 cells and LM-IL-2/SB-1 cells, with SB-1 cells and LM-IL-2K^b/EO771 cells, or with SB-1 cells into the breast alone, without subsequent injections. Mean survival times: Mice injected with SB-1 cells alone, 29 ± 7 days; with SB-1 cells and LM-IL-2 cells, 38 ± 8 days; with SB-1 cells and LM-IL-2K^b cells, 34 ± 7 days; with SB-1 cells and LM-IL-2/SB-1 cells, 36 ± 5 dys; with SB-1 cells and LM-IL-2K^b/EO771 cells, 51 ± 18 days; with SB-1 cells and LM-IL-2K^b/SB-1 cells, 76 ± 26 days. Survival of mice injected with SB-1 cells and LM-IL-2K^b/SB-1 cells, relative to survival of mice in each of the other groups, $p < 0.02$ for difference in survival of mice injected with SB-1 cells alone and mice injected with SB-1 cells and LM-IL-2K^b/EO771 cells. ■, Injected with SB-1 cells alone; △, injected with SB-1 cells and LM-IL-2 cells; ○, injected with SB-1 cells and LM-IL-2K^b cells; ▲, injected with SB-1 cells and LM-IL-2/SB-1 cells; □, injected with SB-1 cells and LM-IL-2K^b/EO771 cells; ●, injected with SB-1 cells and LM-IL-2K^b/SB-1 cells.

with SB-1 cells alone. Once the breast neoplasms first appeared, the rate of tumor growth (two-dimensional measurements) in the treated and untreated groups was approximately the same.

Consistent with the delayed appearance of tumor in the treated group, mice injected with SB-1 cells and LM-IL-2K^b/SB-1 cells survived significantly ($p < 0.006$) longer than mice injected with SB-1 cells alone (Fig. 5*B*). No tumors formed at immunization sites injected with LM-IL-2K^b/SB-1 cells alone.

As controls, naive C3H/HeJ mice were injected according to the same protocol with SB-1 cells and nontransfected LM-IL-2 cells, with SB-1 cells and nontransfected LM-IL-2K^b cells, or with SB-1 cells and syngeneic LM-IL-2 cells transfected with DNA from SB-1 cells (LM-IL-2/SB-1). As indicated (Fig. 5*A*), with the exception of two mice in the group injected with SB-1 cells and LM-IL-2K^b/EO771 cells, the first appearance of tumor, rate of tumor growth, and survival of mice in each group were approximately the same as that of mice injected with SB-1 cells alone. Thus, the greatest immunotherapeutic benefit was in the group of mice injected with the mixture of SB-1 cells and semiallogeneic LM-IL-2K^b cells transfected with genomic DNA from SB-1 cells.

As a means of determining whether immunizations with LM-IL-2K^b cells transfected with DNA from EO771 cells conferred immunity to SB-1 cells, naive C3H/HeJ mice were injected with a mixture of SB-1 cells and LM-IL-2K^b/EO771 cells. As indicated (Fig. 5*B*), although mice injected with SB-1 cells and LM-IL-2K^b/EO771 cells survived longer than mice injected with SB-1 cells alone, they died in significantly ($p < 0.01$) shorter intervals than mice injected with SB-1 cells and LM-IL-2K^b cells transfected with DNA from the same breast cancer.

Discussion

The extraordinarily high incidence of breast cancer in women, approximately one in eight will develop the disease at some point in her life, created an urgent need for new and innovative forms of therapy. Immunotherapeutic approaches, designed to stimulate immunity to autologous tumor, are under active investigation for a number of different types of cancers. The theoretical basis underlying this form of treatment is that neoplastic cells form unique TAAs that can be recognized by CTL, and that cellular immunity to TAAs can follow immunization with tumor vaccines. Malignant cells in the patient can become targets of immune-mediated attack. Like other neoplasms, breast cancer cells form TAAs, several of which have been identified (5–8). However, Ags associated with the proliferating malignant cells are insufficiently immunogenic to generate an effective immune response. Proliferating breast cancer cells fail to elicit antitumor immune responses that can control tumor cell growth.

In this study, we transferred high m.w. DNA from breast cancer cells into a mouse fibroblast cell line to develop a breast cancer vaccine that was effective in the treatment of breast cancer in mice. This approach was based on prior studies that indicated that the introduction of high m.w. genomic DNA from one cell type altered both the genotype and the phenotypic characteristics of the cells that took up the exogenous DNA. This was the case for transfer of single genes specifying enzymes or membrane receptors (44–46). The gene products were expressed by subpopulations of the transfected cells. In an analogous manner, transfer of breast cancer DNA into a highly immunogenic cell line resulted in a cellular vaccine that was effective in the treatment of breast cancer in mice. The results were consistent with the expression in a highly immunogenic form of undefined breast cancer-associated Ags by a subpopulation of the DNA-transfected cells.

Mouse fibroblasts were chosen as the platform for expression of the breast cancer-associated Ags, for several important reasons. The cells, maintained as a cell line in vitro, were readily transfected, using conventional laboratory procedures. And, since the exogenous DNA was replicated as the cells divided, the number of transfected cells could be expanded as might be required for multiple immunizations of the tumor-bearing mice. In addition, like dendritic cells, fibroblasts can act as efficient APCs (53, 54). They constitutively express B7.1, a costimulatory molecule required for T cell activation (55). Class I cellular antitumor immune responses were generated in tumor-bearing mice immunized with fibroblasts transfected with tumor DNA (56, 57).

In this study, DNA was isolated from an adenocarcinoma of the breast that arose spontaneously in a C3H/HeJ mouse (H-2^k). DNA from the breast cancer cells was used to transfect LM cells, a mouse fibroblast cell line of C3H/He mouse origin. To increase their nonspecific immunogenic properties, and to ensure rejection, the fibroblasts were modified to express foreign (allogeneic) H-$2K^b$ determinants, and to secrete IL-2 before they were transfected with the tumor DNA. Antitumor immune responses were generated in mice immunized with the transfected cells. The first appearance of tumor was delayed and the mice survived significantly longer than mice in various control groups, including mice injected with the breast cancer cells and transfected fibroblasts that formed syngeneic MHC determinants alone.

An analogous study was conducted using IL-2-secreting LM fibroblasts modified to express H-$2K^b$ determinants that were transfected with DNA from EO771 cells, a breast cancer cell line of C57BL/6 mouse origin. H-$2K^b$ determinants were syngeneic class I MHC determinants in C57BL/6J mice, providing a restriction element for direct Ag presentation to CTLs of the host (53). Like the survival of C3H/HeJ mice with breast cancer treated by immunization with fibroblasts transfected with breast cancer DNA, C57BL/6J mice injected with EO771 cells and LM-IL-$2K^b$ cells transfected with DNA from EO771 cells survived significantly longer than mice in various control groups, including mice injected with EO771 cells and modified fibroblasts transfected with DNA from mouse melanomas, an unrelated tumor. Some of the mice immunized with the breast cancer DNA-transfected fibroblasts appeared to have rejected the breast cancer cells and survived indefinitely. Immunity failed to develop in mice depleted of $CD8^+$ cells, indicating the essential role of this subset of T cells in mediating tumor rejection.

Whether or not the immunity in mice injected with the DNA-transfected cells was local, or systemic, was not determined. The injections were administered in the vicinity of the tumor. However, several lines of evidence lead us to speculate that systemic immunity to the breast cancer cells may have been engendered by the immunizations. In addition to the involvement of $CD8^+$ cells in mediating the antitumor response, the survival of mice treated previously by immunization with the DNA-based vaccine, and then rechallenged 4 mo later by a second injection of the breast cancer cells was significantly prolonged. Finally, the failure of non-DNA-transfected cells or of cells transfected with DNA from a heterologous tumor (B16 melanoma) to induce an antibreast cancer immune response is consistent with a systemic response. Further studies are required to establish this point.

We conclude that an array of undefined breast cancer-associated Ags was expressed by the modified fibroblasts transfected with breast cancer DNA. No attempt was made to identify TAAs expressed by the transfected cells. The identification of tumor Ags is technically challenging and may not be required in the treatment of breast cancer patients. Immunization with a vaccine that expresses multiple TAAs may have advantages over immunization with one or even several defined Ags. Immunotherapy with defined Ags may not eliminate the entire malignant cell population, as some tumor cells may fail to express the Ag(s) chosen for immunization.

Transfection of tumor DNA into a highly immunogenic cell line has other important advantages. The amount of tumor DNA required to prepare the vaccine can be small, since the transferred DNA is replicated as the cells divide. In addition, a tumor cell line does not have to be established if the patient's own tumor is to be genetically modified for immunization. Tumor DNA can be readily obtained from primary neoplasms. Furthermore, the cells used as recipients of the tumor DNA can be modified in advance for special properties, such as identity with the patient for shared class I determinants, or to secrete one or more cytokines, to further augment their immunogenic properties.

Surprisingly, the number of transfected cells that expressed the products of genes specifying TAAs was sufficient to induce the antitumor immune response. Our observation that antitumor immune responses followed immunizations with the transfected cells may be an indication that multiple, and possibly large numbers of immunologically distinct TAAs, the products of multiple altered genes, were present within the population of breast cancer cells. The prolonged survival of mice injected with cells from a spontaneous breast neoplasm (SB-1) treated with a vaccine prepared with DNA from an independently arising breast cancer cell line (EO771) suggests that the two breast cancers share Ags in common.

The results reported in this work raise the possibility that a human fibroblast cell line that shares identity at one or more MHC class I alleles with the cancer patient may be readily modified to provide immunologic specificity for TAAs expressed by the patient's neoplasm. The data suggest that an optimum response can be obtained if the cellular immunogen is prepared using DNA from the patient's own tumor. Transfection of the cell line with DNA from the neoplastic cells may provide a practical alternative to the modification of autologous malignant cells for the purposes of generating an immunogen that is useful in the overall management of the patient's disease.

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Immunity to Breast Cancer in Mice Immunized with X-Irradiated Breast Cancer Cells Modified to Secrete IL-12

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Summary: A mouse mammary adenocarcinoma cell line (410.4) originating in a BALB/c mouse, was transduced with a retroviral vector (TGF-mIL-12-Neo) that encoded murine IL-12. After confirmation of IL-12-secretion, the cells were tested for their tumorigenic properties in BALB/c mice. The results indicated that unlike other tumors modified for cytokine secretion, modification of 410.4 cells to secrete IL-12 (410.4-IL-12 cells) failed to eliminate the cells' neoplastic growth properties. Progressively growing tumors of 410.4-IL-12 cells invariably formed in syngeneic BALB/c mice and led, eventually, to the animals' death. However, the cells' immunogenic properties were preserved as indicated by the finding that immunizations with 410.4-IL-12 cells, inactivated before injection by X-irradiation, resulted in potent, long-term immunity toward unmodified 410.4 cells and protected the mice against the malignant proliferation of the breast cancer cells. We conclude that modification of 410.4 cells for IL-12-secretion augmented the response of syngeneic BALB/c mice to weakly immunogenic tumor-associated antigens expressed by the cells. The increase in the cells' immunogenic properties, however, was insufficient to prevent tumor growth in the mice. The results point toward the immunotherapeutic potential of X-irradiated tumor cells modified for the secretion of immune augmenting cytokines. **Key Words:** Breast Cancer—Immunotherapy—Immunity—IL-12—Mice.

INTRODUCTION

The tumorigenic properties of highly malignant cells can be altered if the cells are genetically modified to secrete immune-augmenting cytokines. In experimental systems, neoplastic cells modified to secrete interleukin-2 (IL-2) (1–5), interleukin-4 (IL-4) (6,7), granulocyte-macrophage colony-stimulating factor (GM-CSF) (8,9), or interferon-gamma (10,11) were rejected, primarily by cellular immune mechanisms. Animals rejecting the cytokine-secreting tumor cells exhibited strong antitumor immune responses that in some instances resulted in tumor cell rejection. Under analogous circumstances, unmodified tumor cells were insufficiently immunogenic and grew progressively. Immunization with cytokine-secreting tumor cells is under active investigation as a new and important means of tumor therapy (12).

Like other immune augmenting cytokines, modification of tumor cells to secrete interleukin-12 (IL-12) also affected the cells' tumorigenic properties (13–17). Immunizations of mice with IL-12-secreting sarcoma cells (13), melanoma (14), and other types of tumors (15–17) resulted in rejection of the IL-12-secreting cells and the induction of T cell-mediated antitumor immune responses that were directed toward the unmodified tumor as well. As an indication of the potential importance of this form of cancer therapy, mice immunized with the IL-12-secreting tumor cells, challenged by injection of

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viable, unmodified cells, survived significantly longer than naive mice injected with unmodified tumor cells alone.

Here, we report the results of treating mice with a mouse mammary carcinoma cell line (410.4), modified by retroviral transduction to secrete IL-12. 410.4 cells were derived from a spontaneously occurring mammary carcinoma arising in a BALB/c mouse (18). The tumor cells were highly aggressive in syngeneic mice. They metastasized early and secreted immunosuppressive factors, such as prostaglandin-E2, which inhibited IL-12 secretion (18–21). Unlike other tumors modified for cytokine secretion, however, we found that modification of 410.4 cells to secrete IL-12 (410.4-IL-12 cells) failed to eliminate the cells' tumorigenic properties. Tumors of 410.4-IL-12 cells invariably formed in syngeneic BALB/c mice. However, immunizations with 410.4-IL-12 cells, inactivated before injection by X-irradiation, generated immunity toward unmodified 410.4 cells. Mice rejecting X-irradiated 410.4-IL-12 cells were protected against the malignant proliferation of the breast cancer cells. Under similar conditions, antitumor immune responses failed to develop in mice immunized with non-IL-12-secreting 410.4 cells subjected to equivalent amounts of X-irradiation.

MATERIALS AND METHODS

Experimental Animals and Tumor Cell Lines

Six- to eight-week-old BALB/c mice were obtained from Charles River Laboratory (Wilmington, MA, U.S.A.). BALB/c nu/nu (nude) mice were from Jackson Laboratories (Bar Harbor, ME, U.S.A.). The animals were housed in our animal maintenance facility in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

410.4 breast adenocarcinoma cells were obtained from Joyce Taylor-Papadimitriou. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, U.S.A.), 1 m*M* sodium pyruvate, and 100 units/ml penicillin and 100 ug/ml streptomycin (Gibco/BRL, Grand Island, NY, U.S.A.) (growth medium), under standard cell culture conditions (37°C in a humidified 7% CO_2/air atmosphere). LM cells, a mouse fibroblast cell line of C3H/He mouse origin (H-2^k), were from the American Type Culture Collection (ATCC). They were maintained in growth medium under standard cell culture conditions.

Modification of 410.4 Cells for IL-12-Secretion

A retroviral vector (TFG-mIL-12) (22), kindly provided by H. Tahara (University of Pittsburgh, Pittsburgh, PA, U.S.A.), was used to modify 410.4 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′ LTR. 410.4 cells were transduced according to the protocol described by Tahara et al. (15). In brief, 5×10^5 410.4 cells were added to individual wells of a 6 well plate (Falcon). Twenty-four hours later, 1 ml of TFG-mIL-12-Neo retroviral supernatant in the presence of polybrene (8 ug/ml) was added to each well. The cells were maintained in growth medium containing 400 ug/ml of the neomycin analog, 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, England, U.K.) was substituted for TFG-mIL-12 (410.4-SV-X cells). [pZipNeoSV-X specifies a neomycin resistance gene, but not the gene for IL-12.] Lipofectin (Gibco/BRL) was used to facilitate uptake of pZipNeoSV-X by 410.4 cells, according to the manufacturer's instructions.

Detection of IL-12 Formation by 410.4 Cells Transduced with TFG-mIL-12

An enzyme linked immunosorbent assay (ELISA) (Endogen, Woburn, MA, U.S.A.) was used to detect the formation of IL-12 by 410.4 cells transduced with TFG-mIL-12. Cell culture supernatants were analyzed for the p70 heterodimer of IL-12. The ELISA was not cross-reactive with the p40 monomer or homodimer. In brief, 1×10^6 cells 410.4-IL-12 cells were added to cell culture flasks containing 10 ml of RPMI medium (Gibco) supplemented with 2.0 m*M* Na pyruvate, 10% fetal bovine serum (Sigma), 0.15 M Hepes, and antibiotics. After incubation for 48 hours under standard cell culture conditions, the culture supernatant was collected and passed through a 0.45 u nitrocellulose filter before the assay for IL-12 was performed. The same protocol was used to analyze the culture supernatants of 410.4 cells transduced with pZipNeoSV-X, (410.4-SV-X) and nontransduced 410.4 cells.

Growth of 410.4-IL-12 Cells in BALB/c or BALB/c Nude Mice

Two-dimensional measurements were used to measure the growth of tumor in BALB/c or BALB/c nude mice

injected with 410.4-IL-12 cells. The mice were injected in the fat pad of the breast with 1×10^6 410.4-IL-12, 410.4-SV-X, or 410.4 cells suspended in 200 ul of growth medium. Tumor growth was estimated grossly, using a vernier caliper to obtain the two-dimensional measurements. The volume of the tumor was calculated by the formula $0.4ab^2$, where a = length and b = width of the tumor (23).

RESULTS

IL-12 Formation by 410.4 Cells Transduced with the Retroviral Vector, TFG-mIL-12

TFG-mIL-12, a replication-defective retrovirus, was used to modify 410.4 breast adenocarcinoma cells to form IL-12. The vector encoded the p40 and p35 heterodimer subunits of IL-12, along with a gene conferring resistance to the aminoglycoside antibiotic, neomycin-analog, G418. 410.4 cells from in vitro culture were transduced with the virus, and then selected in growth medium containing sufficient quantities (400 ug/ml) of G418 to kill 100% of nontransduced cells. The antibiotic-resistant colonies of cells were pooled and maintained as a cell line. An ELISA with specificity for the p70 heterodimer of IL-12 was used to measure IL-12 formation by the transduced cells. The results indicated that 1×10^6 G418-resistant cells formed 500 pg IL-12/10^6 cells/48 hours. Under similar conditions, the culture supernatants of nontransduced 410.4 cells, or 410.4 cells transduced with pZipNeoSV-X, failed to contain detectable quantities of IL-12. Every third passage, the cells were placed in medium containing 400 ug G418 for the subsequent two passages, after which the cells were placed in growth medium. Under these circumstances, equivalent quantities of IL-12 were detected when the cells were reanalyzed after 3 months of continuous culture (these data are not presented).

Progressively Growing Neoplasms Formed in BALB/c Mice Injected in the Fat Pad of the Breast with 410.4-IL-12 Cells

410.4 cells, an adenocarcinoma breast cancer cell line, is highly tumorigenic in syngeneic BALB/c mice. One hundred percent of the mice injected in the fat pad of the breast with 410.4 cells formed progressively growing neoplasms that led eventually to the animals' death.

To determine if modification of the breast cancer cells to form IL-12 affected the cells' tumorigenic properties, five BALB/c mice were injected in the fat pad of the inguinal breast with 410.4-IL-12 cells or, for comparison, with an equivalent number of unmodified 410.4 cells. As indicated (Fig. 1), progressively growing tumors formed in both instances. The time to first appearance of tumor, however, was significantly ($p < 0.01$) delayed in the group of mice injected with 410.4-IL-12 cells. The delayed appearance of the tumor was reflected in the prolonged survival of mice injected with 410.4-IL-12 cells. Mice injected with 410.4-IL-12 cells survived significantly ($p < 0.001$) longer than mice injected with an equivalent number of non-IL-12-secreting 410.4 cells (92 ± 14 days and 56 ± 7 days, respectively). Eventually, both groups of mice died from progressive tumor growth.

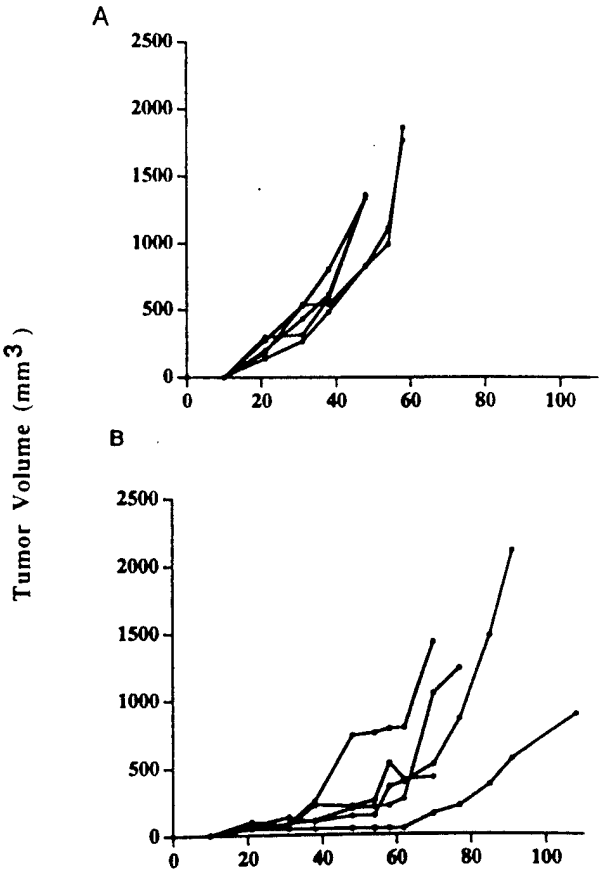

FIG. 1. Tumor growth in BALB/c mice injected in the fat pad of the breast with 410.4-IL-12 cells. Naive BALB/c mice were injected into the fat pad of the breast with 1×10^6 410.4-IL-12 (B) cells or, for comparison, with an equivalent number of unmodified 410.4 cells (A). Each cell type was suspended in 200 ul of growth medium for the injection. Two-dimensional tumor measurements were performed and the volume of the tumor was calculated as described in the Materials and Methods section. Each line represents tumor growth in an individual mouse. The endpoint of the line represents the death of the mouse.

One possible explanation for the delayed appearance and slower growth of 410.4-IL-12 cells in BALB/c mice is that expression of neomycin phosphotransferase, a xenoantigen, by the cells was responsible for the difference in the cells' growth properties. This point was investigated by comparing the survival of BALB/c mice injected with 410.4 cells transduced with pZipNeoSV(X), a retroviral vector that specified neomycin phosphotransferase, but lacked the gene for IL-12, with the survival of mice injected with the IL-12-secreting 410.4 cells. In the experiment, naive mice were injected in the fat pad of the breast with 1×10^6 410.4-IL-12 cells, or with an equivalent number of neomycin-resistant 410.4 cells transduced with pZipNeoSV(X). As an additional control, other naive mice were injected with the same number of nontransduced 410.4 cells. The results indicated that the median survival of mice injected with cells transduced with pZipNeoSV(X), 65 ± 15 days, was significantly less ($p < 0.001$) than that of mice injected with an equivalent number of 410.4 cells modified for IL-12-secretion (94 ± 19 days). It was not significantly different than that of mice injected with unmodified 410.4 cells alone (60 ± 11 days) ($p = 0.1$).

Conceivably, differences in rate of proliferation of 410.4, 410.4 cells transduced with pZipNeoSV(X), and 410.4-IL-12 cells accounted for the differences in the rates of tumor growth and survival of mice injected with 410.4-IL-12 cells. This point was investigated by measuring in vitro the generation times of the three cell types. The results indicated that the doubling times of the cells, approximately 36 hours, were not significantly different from each other.

Progressively Growing Neoplasms Formed in BALB/c Nude Mice Injected with 410.4-IL-12 Cells

The results of the previous experiments indicated that BALB/c mice injected with 410.4-IL-12 cells survived significantly longer than BALB/c mice injected with an equivalent number of 410.4 cells. The prolonged survival of mice injected with 410.4-IL-12 cells may have been an indication the cells' immunogenic properties were enhanced by IL-12-secretion.

To investigate this question, BALB/c nude mice were injected with 410.4-IL-12 or 410.4 cells. The time to first appearance of tumor and survival were compared. As indicated (Fig. 2), unlike immunocompetent BALB/c mice, there were no significant differences in the time to first appearance of tumor and survival of BALB/c nude mice injected with 410.4-IL-12 cells or 410.4 cells. The median survival time, 46 ± 4 days for mice injected with 410.4-IL-12 cells, was not significantly different than the median survival time of mice injected with 410.4 cells (43 ± 9 days; $p = 0.32$). The results were consistent with an enhancement of the immunogenic properties of 410.4 cells by modification for IL-12-secretion.

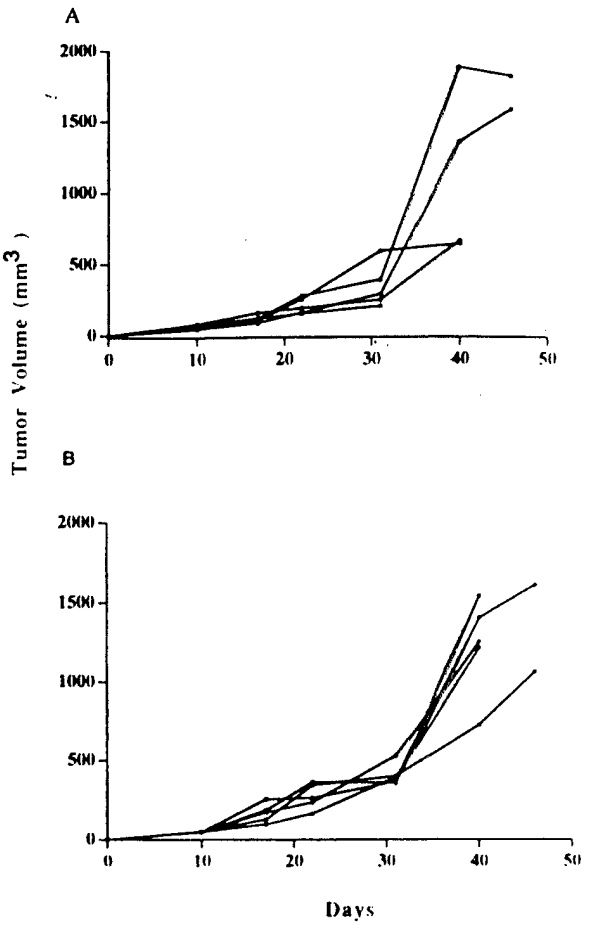

FIG. 2. Tumor growth in BALB/c nude mice injected in the breast with 410.4-IL-12 (A) or 410.4 cells (B). Naive BALB/c nude mice were injected in the fat pad of the breast with 1×10^6 410.4-IL-12 cells or, for comparison, with an equivalent number of unmodified 410.4 cells. Each cell type was suspended in 200 ul of growth medium for the injection. Two-dimensional tumor measurements were performed and the volume of the tumor was calculated as described in the Materials and Methods section. Each line represents tumor growth in an individual mouse. The endpoint of the line represents the death of the mouse.

Progressively Growing Neoplasms Developed in BALB/c Mice Injected with a Mixture of 410.4-IL-12 Cells and 410.4 Breast Cancer Cells

Differences in the time to first appearance of tumor and survival of BALB/c mice injected with 410.4-IL-12 cells suggested that the cells' immunogenic properties were enhanced by IL-12-secretion. This point was investigated further by comparing the time to first appearance

of tumor and survival of BALB/c mice injected in the fat pad of the breast with a mixture of 410.4 cells and 410.4-IL-12 cells with the time to first appearance of tumor and survival of BALB/c mice injected with an equivalent number of 410.4 cells alone. The number of 410.4 cells was the same in both instances. Conceivably, the presence of 410.4-IL-12 cells would delay the growth of 410.4 cells. As indicated (Fig. 3B), the time to first appearance of tumor in mice injected with the mixture of 410.4-IL-12 cells and 410.4 cells was not significantly different than the time to first appearance of tumor in mice injected with 410.4 cells alone (Fig. 3A). Furthermore, the median survival time of mice injected with the mixture of 410.4-IL-12 cells and 410.4 cells was not significantly different than the median survival time of mice injected with 410.4 cells alone (MST = 42 ± 13 days and 60 ± 5 days, respectively; $p = 0.06$). As previously, the time to first appearance of tumor in mice injected with 410.4-IL-12 cells alone was significantly ($p < 0.01$) delayed, relative to that of mice in either of the other groups (Fig. 3C). Thus, if 410.4-IL-12 cells stimulated an immune response toward 410.4 cells, the response was insufficient to inhibit the growth of unmodified 410.4 cells.

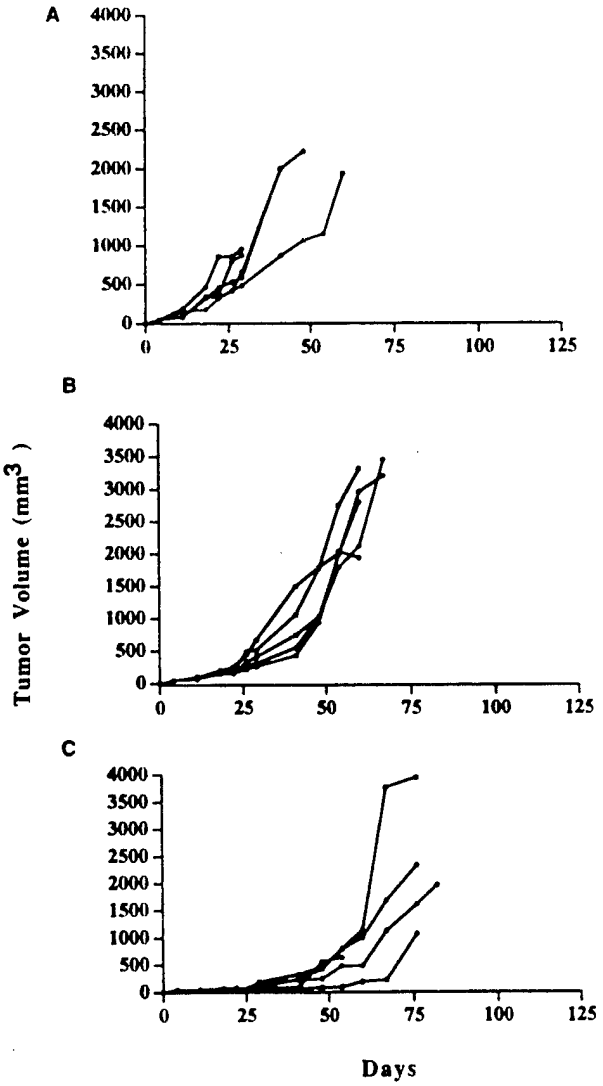

FIG. 3. Tumor growth in BALB/c mice injected in the breast with a mixture of 410.4-IL-12 and 410.4 cells. Tumor growth was measured in naive BALB/c mice injected in the fat pad of the breast with a mixture of 1×10^6 410.4-IL-12 cells and 1×10^6 410.4 cells in a total volume of 200 ul growth medium (B). For comparison, naive mice were injected with 1×10^6 410.4 cells (A) or 1×10^6 410.4-IL-12 cells in 200 ul growth medium alone (C). Two-dimensional tumor measurements were performed and the volume of the tumor was calculated as described in the Materials and Methods section. Each line represents tumor growth in an individual mouse. The endpoint of the line represents the death of the mouse.

One possible explanation for the failure of 410.4-IL-12 cells to control tumor growth is that 410.4 cells secrete immunosuppressive factors such as prostaglandin E2 that inhibit cell-mediated immune responses (19–21). This point was investigated by adding culture supernatants from 410.4 cells to a mixed-cell culture consisting of spleen cells from naive BALB/c mice ($H\text{-}2^d$) and allogeneic LM fibroblasts ($H\text{-}2^k$). The allogeneic cells were mitomycin-C-treated (50 ug/ml; 30 minutes, 37°C) before they were added to the spleen cell suspension. Spleen cell proliferation was determined by adding ^{3}H-thymidine to the mixed-cell cultures for the last 18 hours of the incubation, followed by measuring the incorporation of ^{3}H-thymidine by the proliferating cells. The results indicated that the addition of culture supernatants of 410.4 cells reduced the proliferative response by approximately fivefold (2142 ± 71 cpm in the presence of supernatant vs. 10664 ± 313 in the absence of supernatant), consistent with the secretion of immunosuppressive factors by the breast cancer cells.

Partial Immunity Toward 410.4 Cells Was Generated in BALB/c Mice Injected with X-Ray-Inactivated 410.4-IL-12 Cells

The delayed first appearance of tumor and prolonged survival of BALB/c mice injected with 410.4-IL-12 cells suggested that the weakly immunogenic properties of 410.4 cells had been enhanced by IL-12-secretion, but that the enhanced immunogenic properties were insufficient to control tumor growth. Inactivation of the cells by X-irradiation might preserve the cells' immunogenic properties as it prevented the cells from dividing. This point was investigated by immunizing naive BALB/c mice with X-irradiated 410.4-IL-12 cells and then challenging the mice by an injection of 410.4 cells. In the

experiment, the mice were injected s.c. three times with 1×10^6 X-irradiated (5,000 rads from a ^{60}Co source) 410.4-IL-12 cells at monthly intervals. Thirty days after the last injection, the mice were injected in the fat pad of the breast with 1×10^6 410.4 cells. As controls, naive BALB/c mice were injected according to the same schedule with equivalent numbers of X-irradiated 410.4 cells, or with X-irradiated 410.4-SV-X cells. The results (Fig. 4) indicated that 100% of the mice injected with the irradiated 410.4-IL-12 cells, followed by the challenging injection of 410.4 cells, survived indefinitely—more than 90 days. The mice appeared to have rejected the breast cancer cells. Under similar conditions, all of the mice injected with X-irradiated 410.4 cells, or with 410.4-SV-X cells, followed by the challenging injection of 410.4 cells, developed tumors and died from progressive tumor growth.

Immunizations with X-Irradiated 410.4-IL-12 Cells Induced Long-Term Immunity to 410.4 Cells in BALB/c Mice

BALB/c mice injected with irradiated 410.4-IL-12 cells were resistant to a challenging injection of unmodified 410.4 cells. To determine if mice that survived the first challenging injection were resistant to a second injection of 410.4 cells, the surviving mice were injected a second time with 410.4 cells 100 days after the first injection. As indicated (Fig. 5), BALB/c mice that survived the first injection were resistant to 410.4 cells. They survived significantly ($p < 0.001$) longer than naive mice injected with 410.4 cells alone.

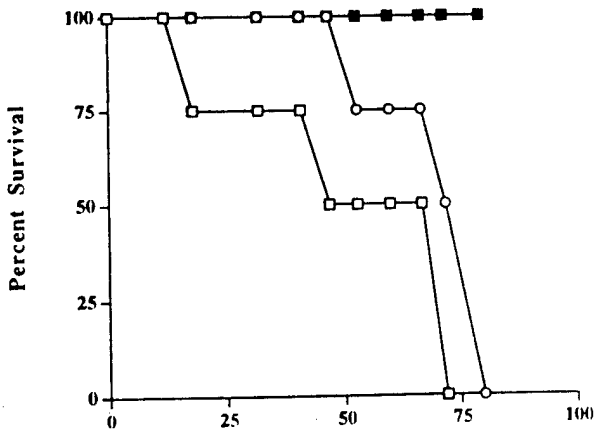

FIG. 4. Survival of BALB/c mice injected with irradiated 410.4-IL-12 cells followed by a challenging injection of 410.4 cells. BALB/c mice were injected s.c. with 1×10^6 X-irradiated (5,000 rads from a ^{60}Co-source) 410.4, 410.4-SV-X, or 410.4-IL-12 cells. The mice received three injections at intervals of 30 days. Thirty days after the last injection, the mice were injected in the fat pad of the breast with 1×10^6 410.4 cells suspended in a volume of 200 ul of growth medium. □ = mice injected with irradiated 410.4 cells, followed by 410.4 cells; ○ = mice injected with irradiated 410.4-SV-X cells, followed by 410.4 cells; ■ = mice injected with irradiated 410.4-IL-12 cells, followed by 410.4 cells.

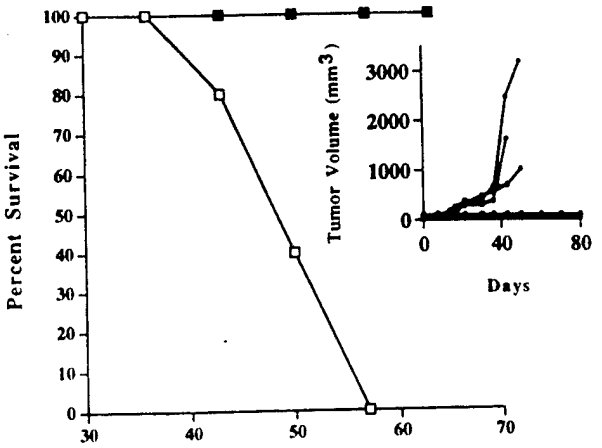

FIG. 5. Tumor growth in surviving BALB/c mice reinjected with 410.4 cells. BALB/c mice injected previously with irradiated 410.4-IL-12 cells, followed by an injection of 410.4 cells, were injected in the fat pad of the breast a second time with 1×10^6 viable 410.4 cells suspended in 200 ul of growth medium. Naive BALB/c mice were injected in the fat pad of the breast with an equivalent number of 410.4 cells suspended in 200 ul of growth medium. □ = naive BALB/c mice injected with 410.4 cells; ■ = surviving BALB/c mice injected with 410.4 cells. The inset represents tumor growth in individual mice. Mice with tumor growth were naive mice injected with 410.4 cells. Mice without tumor growth were immunized mice surviving a prior injection of 410.4 cells, reinjected with 410.4 cells.

DISCUSSION

Under ordinary circumstances, mice injected with viable, syngeneic tumor cells develop progressively growing neoplasms that eventually lead to the animals' death. The mice exhibit no resistance to the growth of the malignant cells. Survival of mice injected with the viable cells is inversely related to the number of cells injected. The tumorigenic properties of the cells can be inhibited, however, if the cells are genetically modified to secrete immune augmenting cytokines such as IL-2, GM-CSF, or IL-12 (12). Normal cells appear to be unaffected. This seminal finding was an indication that tumor cells expressed weakly immunogenic, tumor-associated antigens. They were the products of mutated or dysregulated genes in the tumor cells that differed from the homologous genes in nonneoplastic cells of the tumor-bearing host (24,25). Under appropriate circumstances, such as in the microenvironment of the immune-augmenting cytokines, the tumor-associated antigens can become immunogenic and become the targets of immune-mediated attack. The clinical significance of these findings is that the

experimental animals that rejected the cytokine-secreting tumor cells developed systemic, cell-mediated immunity directed toward both the cytokine-secreting as well as nonsecreting tumor cells (12), pointing toward the important potential of this form of cancer therapy.

Here, we investigated the immunotherapeutic properties of IL-12-secreting 410.4 breast adenocarcinoma cells in BALB/c mice, syngeneic with the tumor. IL-12 is a pleotropic, heterodimeric immune-augmenting cytokine (26–29) that stimulates both natural killer (NK) and T lymphocytes to produce interferon-gamma and tumor necrosis factor-alpha. IL-12 also promotes the development of Th1 $CD4^+$ cells and enhances the activity of tumor-infiltrating lymphocytes (30,31). Our results indicated, however, that in spite of these immune-enhancing effects, IL-12 secretion by the breast cancer cells failed to eliminate the cells' tumorigenic properties. Progressively growing neoplasms formed in mice injected with 410.4-IL-12 cells. However, IL-12 appeared to augment the cells' immunogenic properties since tumor growth was delayed in immunocompetent BALB/c mice injected with the IL-12-secreting cancer cells. In contrast, the rate of tumor-formation in BALB/c nude mice injected with 410.4-IL-12 cells or unmodified 410.4 cells was essentially the same. The unimpaired growth of 410.4-IL-12 cells in nude mice suggested that NK cells were insufficient to control tumor growth (14).

Immunocompetent BALB/c mice immunized with X-irradiated 410.4-IL-12 cells were resistant to a subsequent challenging injection of unmodified 410.4 cells. Under similar conditions, immunizations with X-irradiated (non-IL-12-secreting) 410.4 cells failed to induce tumor immunity. We concluded that IL-12 secretion by 410.4 cells augmented the cells' immunogenic properties, but that the increased immunogenicity of the cells was insufficient to control tumor formation. This may be a unique finding as Kundu et al. (32) reported that 410.4 cells modified to secrete IL-10 failed to form tumors in syngeneic mice.

The potential importance of these findings to breast cancer patients is backed by reports indicating that spontaneous breast neoplasms arising in patients, like other types of cancer, are potentially immunogenic. They form tumor-associated antigens that can be recognized by cytotoxic T lymphocytes. Cytotoxic T lymphocytes were detected in breast cancer patients with specificity for ErbB-2 (33,34), mutant p53 (35), MAGE-1 (36), BAGE (37), and the protein core of mucin expressed in an altered form by breast cancer cells (38–40). These may be only several 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 (41–44).

These data indicate that the immunogenic properties of a cell line derived from an adenocarcinoma of the breast can be enhanced if the cells are modified to secrete IL-12. They point toward the immunotherapeutic potential of X-irradiated breast cancer cells modified for cytokine secretion. The finding may be of importance in the clinical care of breast cancer patients.

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