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

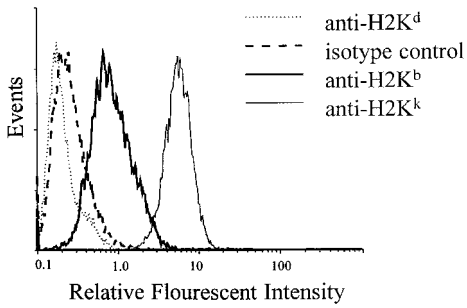

FIGURE 1. The expression of H-2K^b determinants by LM-IL-2 cells transduced with pBR327H-2K^b. A total of 1×10^4 LM-IL-2 cells transduced with the plasmid pBR327H-2K^b (LM-IL-2K^b cells) was incubated for 1 h at 4°C with FITC-conjugated anti-H-2K^b, anti-H-2K^k, or anti-H-2K^d mAbs, as described in *Materials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

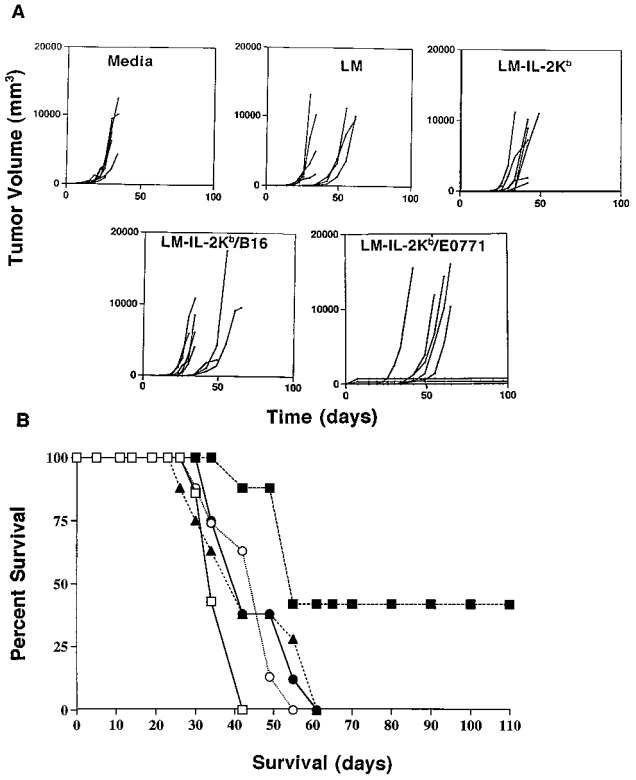

FIGURE 2. *A*, Tumor growth in C57BL/6J mice injected with EO771 breast cancer cells and LM-IL-2K^b/EO771 cells. C57BL/6J mice (seven per group) were injected into the fat pad of the breast with a mixture of 5×10^3 EO771 breast carcinoma cells and 2×10^6 LM-IL-2K^b/EO771 cells in a total volume of 200 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 $\pm$ 5.8 days; mice injected with viable EO771 cells and LM cells, 41 $\pm$ 14 days; mice injected with viable EO771 cells and LM-IL-2K^b cells, 44 $\pm$ 9 days; mice injected with viable EO771 cells and LM-IL-2K^b/B16 cells, 46 $\pm$ 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 $\pm$ 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

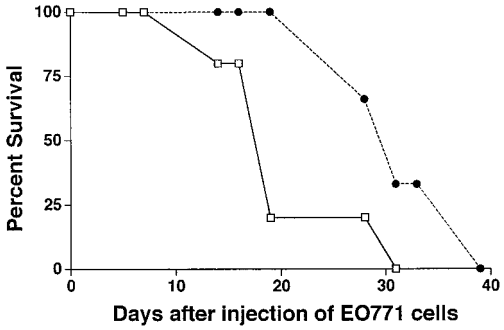

FIGURE 3. Survival of C57BL/6J mice surviving a prior injection of EO711 cells and LM-IL-2K^b/EO771 cells injected with EO771 cells alone. Three C57BL/6J mice surviving 110 days after the prior injection of EO711 cells and LM-IL-2K^b/EO771 cells were injected into the fat pad of the breast a second time 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

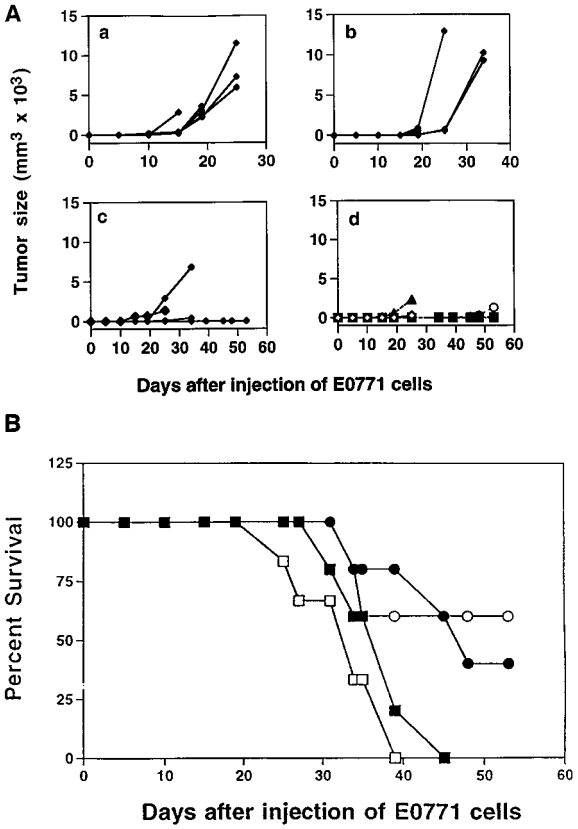

FIGURE 4. *A*, Tumor growth in C57BL/6J mice depleted of CD8$^+$ T lymphocytes injected with a mixture of EO711 breast cancer cells and LM-IL-2K^b/EO771 cells. C57BL/6J mice (seven per group) were injected i.p. with CD4$^+$ (group c) or CD8$^+$ (group b) mAbs, as described in *Materials and Methods*. 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

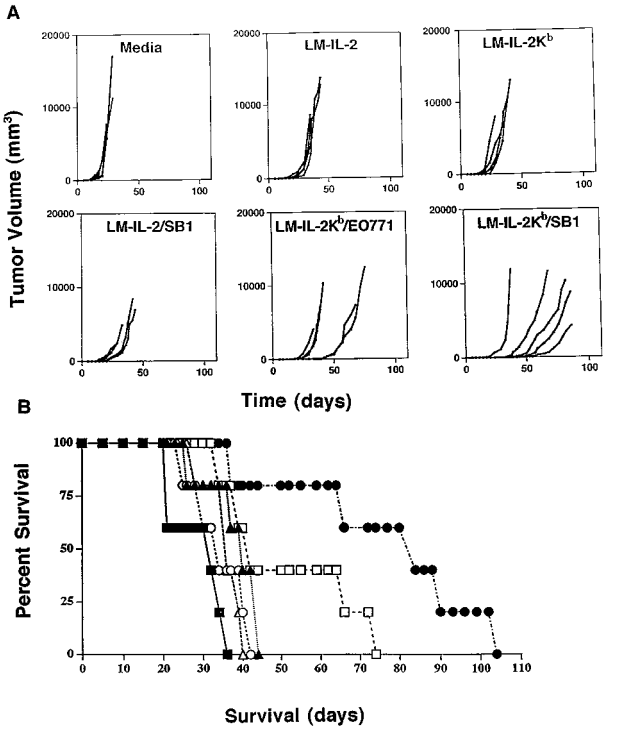

FIGURE 5. *A*, Tumor growth in C3H/HeJ mice injected with cancer cells from a spontaneous breast neoplasm (SB-1) and LM-IL-2K^b cells transfected with DNA from SB-1 cells. C3H/HeJ mice (five per group) were injected into the fat pad of the breast with a mixture of 1×10^6 SB-1 cells and 2×10^6 LM-IL-2K^b/SB-1 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 $\pm$ 7 days; with SB-1 cells and LM-IL-2 cells, 38 $\pm$ 8 days; with SB-1 cells and LM-IL-2K^b cells, 34 $\pm$ 7 days; with SB-1 cells and LM-IL-2/SB-1 cells, 36 $\pm$ 5 dys; with SB-1 cells and LM-IL-2K^b/EO771 cells, 51 $\pm$ 18 days; with SB-1 cells and LM-IL-2K^b/SB-1 cells, 76 $\pm$ 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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