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DNA-based vaccine for treatment of intracerebral neoplasms

Research Article

Terry Lichtor[1,3]*, Roberta P Glick[1,3], InSug O-Sullivan[2], Edward P Cohen[2,4]

[1]Department of Neurological Surgery, Rush University Medical Center and John H Stroger Hospital of Cook County

[2]Department of Microbiology and Immunology, University of Illinois at Chicago; Chicago, Illinois

***Correspondence**: Terry Lichtor, MD, PhD, Department of Neurosurgery, 1900 West Polk Street, Chicago, Illinois 60612; Telelphone: 312-864-5120; Fax: 312-864-9606; E-Mail: Terry_Lichtor@rush.edu

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Abbreviations: cytotoxic T-lymphocyte, (CTL); intracerebrally, (i.c.); Mean survival time, (MST); phenazine methosulfate, (PMS); spontaneous breast neoplasm, (SB-5b); tumor associated antigens, (TAA)

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Summary

Antigenic differences between normal and malignant cells of the cancer patient form the rationale for clinical immunotherapeutic strategies. Because the antigenic phenotype of neoplastic cells varies widely among different cells within the same malignant cell-population, immunization with a vaccine that stimulates immunity to the broad array of tumor antigens expressed by the cancer cells is likely to be more efficacious than immunization with a vaccine for a single antigen. A vaccine prepared by transfer of DNA from the tumor into a highly immunogenic cell line can encompass the array of tumor antigens that characterize the patient's neoplasm. Poorly immunogenic tumor antigens, characteristic of malignant cells, can become strongly antigenic if they are expressed by highly immunogenic cells. A DNA-based vaccine was prepared by transfer of genomic DNA from a breast cancer that arose spontaneously in a C3H/He mouse into a highly immunogenic mouse fibroblast cell line, where genes specifying tumor-antigens were expressed. The fibroblasts were modified in advance of DNA-transfer to secrete an immune augmenting cytokine and to express allogeneic MHC class I-determinants. In an animal model of breast cancer metastatic to the brain, introduction of the vaccine directly into the tumor bed stimulated a systemic cellular anti-tumor immune response and prolonged the lives of the tumor-bearing mice.

I. Introduction

An emerging strategy in the treatment of cancer involves stimulation of an immune response against the unique antigens expressed by the neoplastic cells. The expectation is that effectively stimulated, the immune system can be called upon to destroy the malignant cells. In most instances, proliferating tumors do not provoke anti-tumor immune responses, which are capable of controlling tumor growth. The neoplastic cells escape recognition by the immune system in spite of the fact that they form weakly immunogenic tumor associated antigens (TAA). The successful induction of immunity to TAA could result in tumor cell destruction and prolongation of the survival of cancer patients. A number of different techniques have been designed to increase the antigenic properties of tumor cells. The immunogenic properties of tumor cells were increased by modifying neoplastic cells to secrete immune-augmenting cytokines, or by "feeding" antigen presenting (dendritic) apoptotic bodies from tumor cells or tumor cell lysates. Anti-tumor immune responses followed immunization with such vaccines as well as vaccine prepared by introducing tumor cell-derived RNA into dendritic cells. Immunization with dendritic cells "fed" derivates of tumor cells or transfected with tumor-RNA can result in the induction of immune responses against the broad array of tumor antigens expressed by the population of malignant cells including tumors of neuroectodermal origin. In one pre-clinical study, intraperitoneal injection of bone marrow-derived dendritic

cells pulsed with the RNA derived from the GL261 glioma cells induced a T cell response against intracerebrally implanted GL261 cells (O et al, 2002). The efficacy of the vaccine was improved further by administration of recombinant interleukin-12 into the vaccine regimen. In patients, immunization with autologous dendritic cells transfected with mRNA from malignant glioma elicited a tumor specific $CD8^+$ cytotoxic T-lymphocyte (CTL) response against the patient's malignant cells (Kobayashi et al, 2003).

Immunotherapy can result in the selective destruction of the neoplasm with minimal or non-existent toxic effects. Selective tumor regression was observed in experimental animals and patients receiving immunotherapy alone, suggesting the potential effectiveness of this type of treatment for patients with malignant disease (Valmori et al, 2000).

Antigenic differences between normal and malignant cells form the rationale for clinical immunotherapy protocols. Because the antigenic phenotype varies widely among different cells within the same tumor-cell population, immunization with a vaccine that stimulates immunity to multiple TAA expressed by the entire population of malignant cells is likely to be more effective than immunization with a vaccine for a single antigen. Variants that fail to express the antigen chosen for therapy can avoid destruction. Here, in a mouse model, we describe the application of a novel immunotherapeutic strategy to intracerebral breast cancer. The vaccine was prepared by transfer of genomic DNA from breast cancer cells into a highly immunogenic fibroblast cell line, where genes specifying breast cancer antigens are expressed (Cohen, 2001). The vaccine encompasses the array of TAA that defines the patient's neoplasm. Poorly immunogenic TAA, characteristic of malignant cells, become strongly antigenic if they are expressed by highly immunogenic cells. In animal models of melanoma and breast cancer, immunization with DNA-based vaccine was sufficient to deter tumor growth and to prolong the lives of tumor-bearing mice (Cohen, 2001; Whiteside et al, 2002). Previous studies indicated that transfection of genomic DNA from the malignant cells into the cell line resulted in stable integration and expression of the transferred DNA altering both the genotype and the phenotype of the cells that took up the exogenous DNA. The genetically engineered cells were effective stimulators of the anti-tumor immune response. Immunization of tumor-bearing mice with the DNA-based vaccine resulted in the induction of cell mediated immunity directed toward the type of cell from which the DNA was obtained, and prolongation of survival. This was the case for mice with melanoma, squamous cell carcinoma and in mice with breast cancer (de Zoeten et al, 1999). Multiple undefined genes specifying TAA that characterize the malignant cell population were expressed by cells that took up DNA from the tumor. Among other advantages, only microgram quantities of DNA from small amounts of tumor tissue were required to prepare the vaccine. As the transferred DNA is integrated into the genome of the recipient cells, and is replicated as the cells divide, the number of vaccine cells can be expanded as required for multiple immunizations. The recipient cells can also be modified before DNA transfer to increase their immunogenic properties, as for example, to secrete immune-augmenting cytokines or to express allogeneic MHC-determinants. In animal models, injection of cytokine-secreting allogeneic fibroblasts into the tumor bed of intracerebral neoplasms was also effective in the treatment of mice with established brain tumors (Lichtor et al, 2002).

Although immunotherapy with a vaccine prepared by transfer of tumor-DNA into a highly immunogenic cell line has its advantages, there are potential concerns. Genes that specify normal cellular constituents are also expressed by the transfected cells. They may be recognized as 'foreign' by the immune system, provoking an autoimmune disease. Autoimmune disease has not been observed, however, following extensive immunization with the tumor-DNA-transfected fibroblasts. The immune system is normally tolerant to "self" antigens. Mice immunized with DNA-based vaccines have not exhibited adverse effects; they lived their anticipated life spans without evidence of disease. Cellular infiltrates into normal organs or tissues have not been detected. It is also conceivable that the vaccine itself may grow in the recipient, forming a tumor or provoking a neoplasm. However in multiple studies, tumor growth at the vaccination site or elsewhere in the body has not been observed.

II. Materials and methods

A. Preparation of a vaccine for use in the treatment of intracerebral breast cancer by transfection of cytokine-secreting syngeneic /allogeneic fibroblasts with DNA from a breast carcinoma that arose spontaneously in a C3H/He mouse (SB-5b cells)

Cytokine-secreting syngeneic/allogeneic fibroblasts were prepared as described previously (Lichtor et al, 2002). The cells were further modified by transferring DNA from mouse SB-5b breast cancer cells into the fibroblasts (**Figure 1**). Sheared, unfractionated DNA isolated (Qiagen, Chatsworth, CA) from a spontaneous mammary adenocarcinoma (SB-5b) that arose in a C3H/He mouse taken directly from *in vitro* cultured cells, was used to transfect mouse fibroblasts modified to express allogeneic H-$2K^b$-determinants and to secrete IL-2 (LM-IL-$2K^b$ cells), IL-18 (LM-IL-$18K^b$ cells) or GM-CSF (LM-GMCSFK^b cells) or to express H-$2K^b$-determinants alone (LMK^b cells) using the methods described in (Wigler et al, 1979) as modified. Briefly, high molecular weight DNA from each cell type was sheared by passage through the DNA isolation column. The approximate size of the DNA at the time it was used in the experiments was 25 kb. Afterward, 100 μg of sheared DNA was mixed with 10 μg pCDNA6/V5-HisA, a plasmid which gives resistance to the antibiotic Blasticidin, for use in selection. The sheared DNA and plasmid (DNA : plasmid ratio = 10 : 1) were then mixed with Lipofectamine 2000, according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). The DNA/Lipofectamine mixture was added to a population of 1 X 10^7 actively proliferating LM-IL-$2K^b$, LM-IL$18K^b$, LMGMCSFK^b cells, or non-cytokine secreting LMK^b cells divided into ten dishes containing an original inoculum of 1 X 10^6 cells. Eighteen hours afterward, the medium was replaced with fresh growth medium. The fibroblasts were maintained for 14 days in growth medium containing 2-5 μg/ml Blasticidin HCl

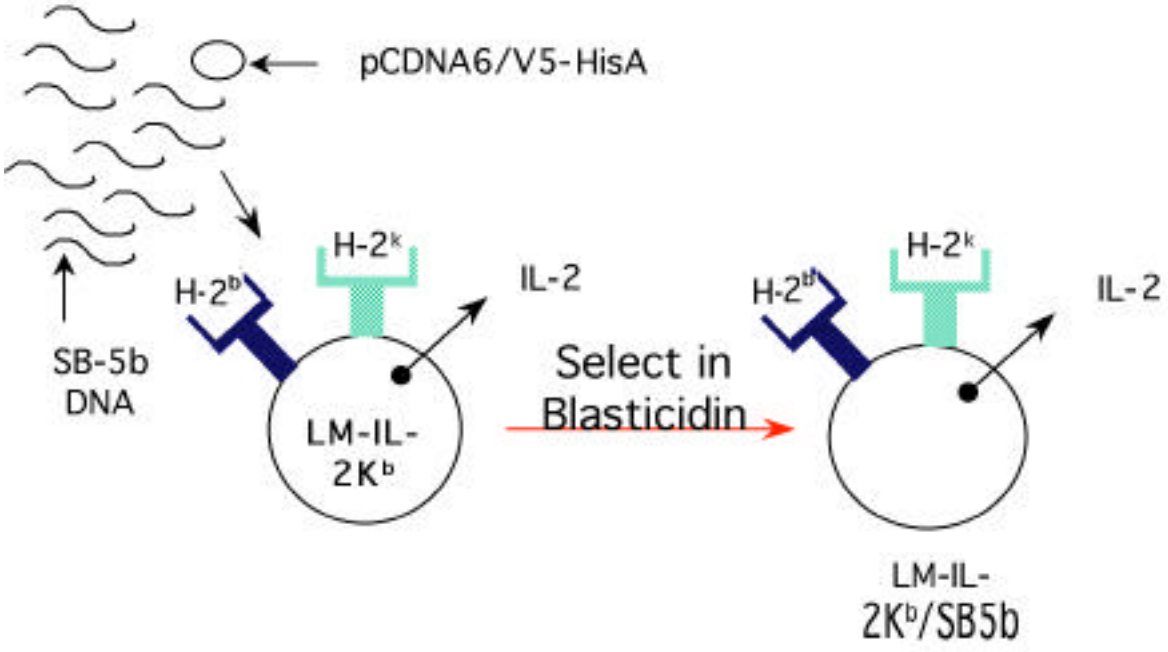

Figure 1. Preparation of the DNA-based vaccine. DNA-based vaccines were prepared by transfection of the fibroblast cell line LM with DNA from mouse breast carcinoma. Briefly, high-molecular weight DNA from SB-5b cells was sheared by passage through the DNA isolation column. Next, 100 µg of the sheared DNA was mixed with 10 µg pCDNA6/V5-HisA, a plasmid that confers resistance to Basticidin. The sheared DNA and the plasmid were then mixed with lipofectamine to facilitate DNA uptake. The DNA-lipofectamine mixture was added to a population of 1 X 10^7 LM fibroblasts modified previously by retroviral transduction to secrete IL-2 and to express H-2K^b-determinants (LM-IL-2K^b cells). The transfected fibroblasts were grown on a tissue culture plate, and Blasticidin was added to the medium to select for cells that had taken up the foreign plasmid DNA.

(Invitrogen, Carlsbad, CA). One hundred percent of the cells transfected with tumor-DNA alone maintained in the Basticidin growth medium died within this period. The surviving colonies in each of the plates (a total of at least 2.5 X 10^4) were pooled and maintained as a cell line for use in the experiments.

B. Intracerebral injection of C3H/He mice with SB-5b breast cancer cells

As a model of intracerebral metastatic breast cancer in patients, C3H/He mice were injected intracerebrally with a mixture of SB-5b breast cancer cells and the DNA-transfected modified fibroblasts. Anesthetized mice were placed into a stereotactic frame. A 1 mm burr hole was introduced into the right frontal lobe in the region of the coronal suture using a D#60 drill bit (Plastics One, Roanoke, VA). A Hamilton syringe containing a 26 gauge needle with a small 2-3 mm piece of solder placed 3-4 mm from the tip of the needle to maintain a uniform depth of injection was used to introduce the breast cancer cells and vaccine into the brain. The total injection volume was 5-10 µl. After injection, the incision over the burr hole was closed with a single 5-O Dexon absorbable suture.

C. T cell mediated cytotoxicity toward breast cancer cells

A CellTiter 96 aqueous non-radioactive cell proliferation assay kit (Promega, Madison WI) was used to measure T cell mediated cytotoxicity toward the breast cancer cells in mice injected intracerebrally with the transfected fibroblasts. T cells from the spleens of mice injected with the transfected cells were co-incubated for 18 hr with SB-5b cells. Afterward, the number of remaining viable cells was measured by MTS, which is bioreduced by cells into a formazan product that can be detected at 490 nm. Effector T cells recovered from the spleens by Histopaque (Sigma) density gradient (Kim and Cohen, 1994) were co-cultured at 37^0 C for 18 hrs with mitomycin C-treated (50 µg/ml for 45 min at 37^0 C) SB-5b target cells. The ratio of spleen cells to SB-5b cells was 30:1. Afterward, the non-adherent cells were removed, washed and viable SB-5b cells were added at various E:T ratios for 4 hrs at 37^0C. Negative control wells were treated with 2% Triton-100 to cause total lysis of the cells. Positive control wells contained SB-5b cells alone. Next 20 µl of MTS and 1 µl of phenazine methosulfate (PMS), an electron coupling reagent, were mixed and added to each well, followed by incubation at 37°C for 1-4 hrs in a 7% CO_2/air atmosphere after which the absorbance was read. The percent specific lysis was calculated from the absorbance using the formula as follows:

$$\frac{\text{Experimental Group} - \text{Negative Control}}{\text{Positive Control} - \text{Negative Control}} \text{ X } 100$$

D. ELISPOT IFN-γ Assay

Spleen cells from C3H/He mice injected i.c. with the various cell constructs were analyzed in ELISPOT IFN-γ assays. This determines the proportion of T cells reactive with SB-5b cells. T cells from the spleens were recovered by Histopaque density gradient and co-incubated with SB-5b tumor cells (the spleen cell: SB-5b cell ratio = 10:1) for 16 hours at 37°C in wells precoated with a high-affinity monoclonal antibody for INF-γ according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). The cells were washed before the addition of biotinylated anti-IFN-γ detection antibody and horse radish peroxidase labeled streptavidin (Streptavidin-HRP). The spots were counted using computer-assisted image analysis (ImmunoSpot Series 2 analyzer: Cellular Technology Limited, Cleveland, OH).

E. Statistical analysis

Student's t test was used to determine the statistical differences between the survival of mice in various experimental and control groups. A *P* value less than 0.05 was considered significant.

III. Results

A. Treatment of mice bearing an intracerebral breast cancer with DNA-transfected syngeneic/allogeneic fibroblasts modified to secrete immune augmenting cytokines

The immunotherapeutic properties of the modified fibroblasts transfected with DNA from a breast cancer that arose spontaneously in a C3H/He mouse were determined in mice with intracerebral breast cancer. C3H/He mice were injected intracerebrally (i.c.) with a mixture of 1.0 X 10^4 SB-5b breast carcinoma cells and 1.0 X 10^6 cytokine-secreting syngeneic/allogeneic fibroblasts transfected with DNA from the breast cancer cells. The results (**Figure 2**) indicated that mice injected i.c. with a mixture of breast cancer cells and transfected syngeneic/allogeneic fibroblasts modified to secrete IL-2 survived significantly longer than mice injected i.c. with a mixture of breast cancer cells and non cytokine-secreting, transfected fibroblasts ($P < 0.005$). Analogous results were obtained for mice injected i.c. with a mixture of breast cancer cells and transfected fibroblasts modified to secrete GM-CSF ($P < 0.05$). The survival of mice injected i.c. with SB-5b cells and transfected fibroblasts modified to secrete IL-18 was not significantly different than that of mice injected with SB-5b cells and non-secreting transfected cells. The experiment was repeated twice with equivalent results.

Thus syngenic/allogeneic fibroblasts modified to secrete IL-2 or GM-CSF that were transfected with DNA from breast cancer cells were effective in prolonging the survival of mice with intracerebral breast cancer. Transfected fibroblasts modified to secrete IL-18 were not effective.

B. T cell mediated toxicity toward breast cancer in mice injected intracerebrally with syngeneic/allogeneic transfected fibroblasts modified to secrete IL-2, GM-CSF or IL-18

An MTS cytotoxicity assay was used to detect the presence of cytotoxic T lymphocytes towards breast cancer in mice injected i.c. with the mixture of SB-5b breast cancer cells and the modified DNA-transfected fibroblasts. The T cells, obtained from the spleens of the injected mice, were analyzed two weeks after the i.c. injection of the cell mixture. The results (**Figure 3**) indicated that, like the survival of mice with i.c. breast cancer treated with the cytokine-secreting fibroblasts, the cytotoxic response of greatest magnitude was in mice injected i.c. with the mixture of SB-5b cells and transfected fibroblasts modified to secrete IL-2 or GM-CSF. Lesser cytotoxic effects were present in mice injected i.c. with SB-5b cells and transfected fibroblasts modified to secrete IL-18.

An Elispot-IFN-γ assay was used to determine the proportion of T cells in the spleen that were reactive with

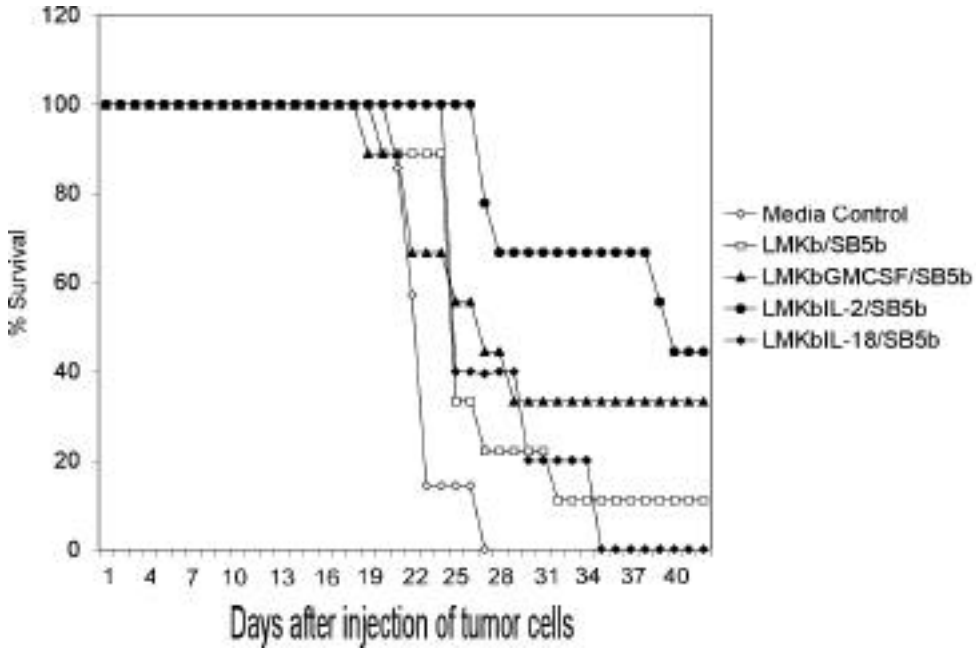

Figure 2. Treatment of C3H/He mice with intracerebral SB-5b breast carcinoma with cytokine-secreting allogeneic fibroblasts transfected with DNA from a spontaneous breast neoplasm (SB-5b). C3H/He mice (nine animals/group) were injected with a mixture of 1.0 X 10^4 SB-5b cells and 1.0 X 10^6 cytokine secreting fibroblasts transfected with tumor DNA or with an equivalent number of non-secreting cells transfected with tumor DNA (LMK^b/SB5b). Mean survival time (MST) in days: Media control, 23.0 ± 1.9; LMK^b/SB5b, 27.3 ± 6.3; LMK^bGMCSF/SB5b, 30.0 ± 9.5; LMK^bIL-2/SB5b, 36.6 ± 7.0; LMK^bIL-18/SB5b, 28.4 ± 4.8. Probability values were as follows: LMK^bIL-2/SB5b vs LMK^b/SB5b or media control, $P < 0.005$; LMK^bIL-2/SB5b vs LMK^bIL-18/SB5b, $P < 0.025$; LMK^bIL-2/SB5b vs LMK^bGMCSF/SB5b, $P < 0.05$; LMK^bGMCSF/SB5b vs media control, $P < 0.05$.

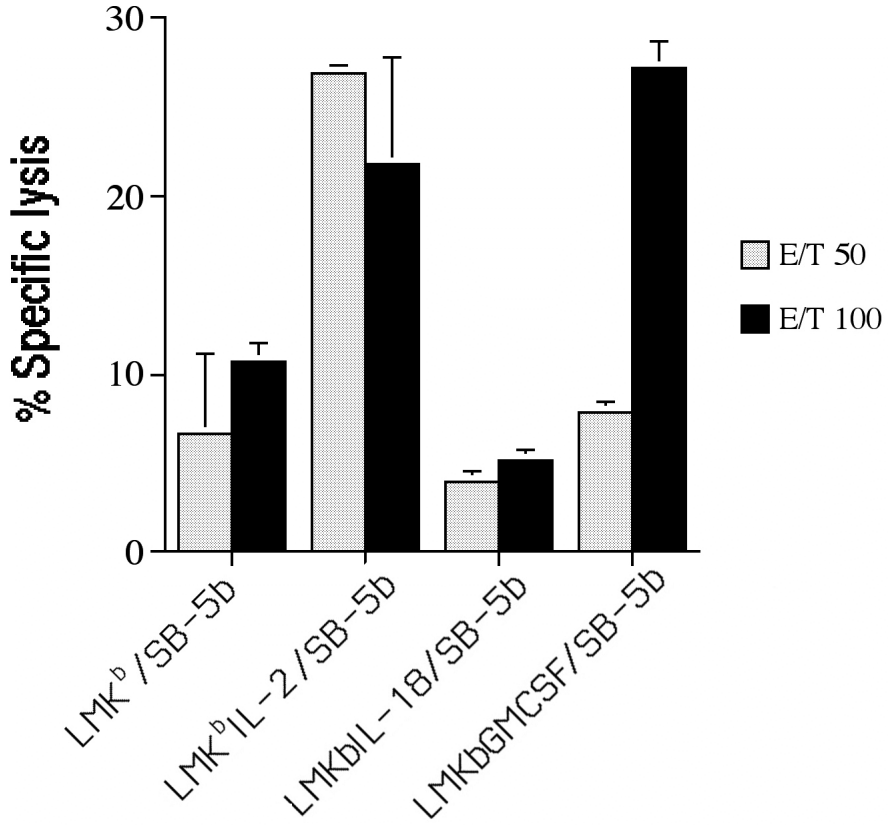

Figure 3. MTS proliferation assay from spleen cells taken from the animals two weeks following a single intracerebral injection of a mixture of tumor and treatment cells. The target cells used in this study were SB-5b breast cancer cells, and the effector (spleen cell) to target cell ratios (E/T) were 50:1 and 100:1. Mononuclear cells from the spleens of the immunized mice obtained through Histopaque centrifugation were used for this assay. The error bars represent one standard deviation.

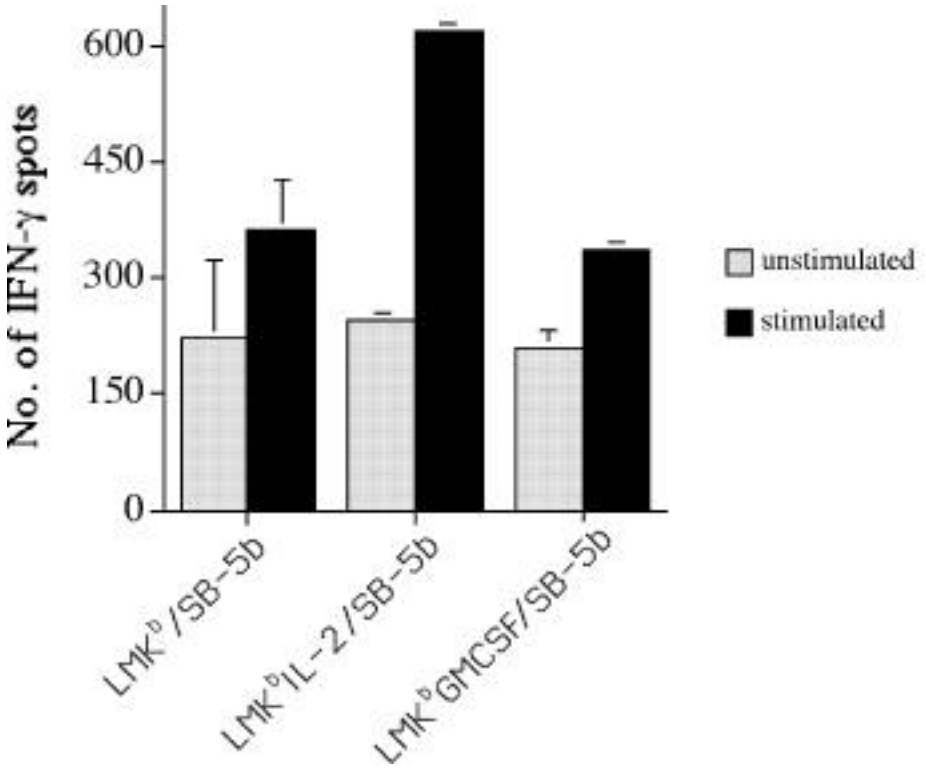

Figure 4. ELISPOT assay detecting INF-γ secretion by spleen cells in the animals that have survived for six weeks following the initial injection of SB-5b tumor cells and allogeneic fibroblasts transfected with tumor DNA. Mononuclear cells from the spleens of the immunized mice obtained through Histopaque centrifugation were used in this assay. The assay was done in the presence (SB-5b stimulated) and absence (unstimulated) of SB-5b tumor cells. The error bars represent one standard deviation.

SB-5b cells in mice immunized with transfected fibroblasts modified to secrete IL-2 or GM-CSF. The assay was performed six weeks after the i.c. injection of the mixture of SB-5b cells and the transfected fibroblasts. The results indicated that the highest proportion of T cells reactive with SB-5b cells was in surviving mice injected with fibroblasts modified to secrete IL-2 (**Figure 4**). Lesser numbers of spots were found in T cells from mice injected with SB-5b cells and non-secreting transfected fibroblasts or SB-5b cells and transfected fibroblasts modified to secrete GM-CSF. The analysis of cells from mice injected i.c. with SB-5b cells and transfected fibroblasts modified to secrete IL-18 was not performed because there were no surviving mice.

IV. Discussion

The prognosis for patients with breast cancer metastatic to the brain is poor, with the survival ranging from eight to thirteen months (Bendell et al, 2003; Ogura et al, 2003). Breast cancer is the second leading cause of cancer-related death in American women, and conventional treatments such as surgery, radiation therapy and chemotherapy have provided little benefit to affect long-term survival. Given the poor prognosis associated with metastatic tumors to the brain, there is urgent need for the development of therapies that can impact on clinical survival rates.

Here, we report the generation of cell mediated immune responses toward breast cancer in mice immunized i.c. with cytokine-secreting syngeneic /allogeneic mouse fibroblasts transfected with DNA from a breast neoplasm that arose spontaneously in a C3H/He mouse (SB-5b cells). Mice injected i.c. with breast cancer cells and the transfected fibroblasts survived significantly longer than mice injected with the breast cancer cells alone, pointing toward the potential of this form of therapy in breast cancer patients whose neoplasm has metastasized to the brain.

Further evidence for the efficacy of the transfected fibroblasts to stimulate an anti-tumor immune response was provided by the results of the *in vitro* studies. Spleen cells from mice injected i.c. with the DNA-based vaccine were responsive to SB-5b breast cancer cells both in ELISPOT IFN-γ and cytolytic T lymphocyte assays. Co-incubation of breast cancer cells and T cells from the spleens of the i.c. injected mice stimulated both CTL-mediated lysis of the breast cancer cells as well as the number of activated T cells as determined by ELISPOT IFN-γ assays. Prior studies by this laboratory have indicated that the introduction of high m.w. genomic DNA from one cell type, using the techniques described in this manuscript, altered both the genotype and the phenotypic characteristics of the cells that took up the exogenous DNA (de Zoeten et al, 1999). No attempt has been made to identify the tumor associated antigens expressed by the transfected cells. The identification of tumor antigens is technically challenging and may not be required in the treatment of breast cancer patients.

Mouse fibroblasts were chosen as recipients of the DNA from the breast cancer cells for several compelling reasons. The cells, maintained as a cell line under conventional laboratory conditions were readily transfected with sheared, genomic DNA from the breast cancer cells. Since the transferred DNA was integrated, and replicated as the recipient cells divided (the transfected fibroblasts were maintained through multiple rounds of cell division before they were used in the experiments), the number of transfected cells could be expanded as necessary. In addition, the fibroblasts could be modified in advance of DNA-transfer to augment their immunogenic properties. In the experiments reported here, the cells were modified to express allogeneic MHC class I-determinants and to secrete IL-2, IL-18 or GM-CSF. Allogeneic class I-determinants are strong immune adjuvants. IL-2 and GM-CSF are growth and activation factors for CTLs. IL-18 stimulates CTLs and augments NK cell mediated cytotoxicity. The immune-augmenting properties of IL-2 and GM-CSF exceeded that of IL-18 in this unique model system. In addition, like dendritic cells, fibroblasts are efficient antigen presenting cells. In particular they express class I-determinants and co-stimulatory molecules required for T cell activation constitutively. The cells used as DNA-recipients expressed H-2^k-determinants and B7.1. Systemic class I restricted cellular breast cancer immune responses were generated in mice injected i.c. with the transfected cells.

Transfection of DNA from the breast cancer cells into a highly immunogenic cell line has additional important advantages. A tumor cell line derived from a primary breast neoplasm does not have to be established if the patient's own tumor is genetically modified to prepare a vaccine for immunization. Preparation of a cell line from a primary neoplasm is technically challenging and, especially in the case of breast cancer, cannot always be accomplished.

Surprisingly, the proportion of the transfected cell population that expressed the products of genes specifying TAA was sufficient to induce the anti-breast cancer immune response. Our observation that the anti-tumor immune response that were sufficient to deter the growth of intracerebral breast cancer, resulting in prolongation of survival may be an indication that multiple and possible large numbers of immunologically distinct TAA, the products of multiple mutant/dysregulated genes were present within the population of breast cancer cells.

The results presented in this study raise the possibility that a human fibroblast cell line that shares identity with the patient at one or more MHC class I alleles may be readily modified to provide immunologic specificity for TAA expressed by the patient's neoplastic cells. Transfection of a highly characteristic fibroblast cell line with DNA prepared from the tumor may capture the array of genes that characterize the neoplasm. It is conceivable that the prolongation of survival noted in the treated animals in this study may be largely due to the expression of potent immunostimulatory cytokines in close proximity to tumor cells and independent of the expression of genomic breast cancer DNA. However in the clinical situation where the treatment cells will be injected into the tumor cavity following surgical resection,

the expression of tumor antigens by the vaccine cells will be more critical.

One concern related to therapy with fibroblasts transfected with DNA from the tumor is that multiple genes specifying normal "self" antigens are likely to be expressed by the transfected cells. There is a theoretical danger that autoimmune disease might develop in breast cancer patients. Vaccines derived from tumor cell-extracts, peptide elutes of tumor cells, or mRNA fed to APCs including dendritic cells are subject to the same concern. However, toxic effects have not been observed. Tumor-free mice injected i.c. with cell-based vaccines including those prepared by transfection of fibroblasts with DNA from the breast cancer cells failed to exhibit adverse effects. They lived their anticipated life spans without evidence of disease.

The ultimate goal of cancer therapy is the elimination of every remaining tumor cell from the patient. It is unlikely that a single form of therapy is capable of achieving this goal. However immunotherapy in combination with surgery, radiation therapy and chemotherapy will likely find a place as a new and important means of treatment for patients with brain tumors.

Acknowledgments

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Terry Lichtor, MD, PhD.