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Exciting new findings in autoimmune disease and cancer have led to the realization that a large set of antigenic determinants of the self have not induced self tolerance. These peptide determinants could provide target structures for autoimmune attack as well as antitumor immune responses. We hypothesized that vaccine strategies can be devised that specifically generate an immune response against breast ductal epithelial cells. Since the overwhelming majority of breast tumors arise in these cells, destroying these cells prior to the development of neoplasia will effectively prevent cancer. We are attempting to augment the immune response to the breast-specific antigen, HER-2/neu, which is expressed by mammary tissue in HER-2/neu transgenic mice prior to mammary tumor development, by enhancing the T cell response using selected vectors that may alter antigen processing, thereby influencing antigen-specific immunity. We have evaluated DNA vectors that express antigen alone or altogether with the cytokine, GM-CSF, to determine if this immunity can be further enhanced. An intramuscular approach was compared with gene gun delivery. We have found that gene gun delivery is superior for enhancing antitumor immunity. We will next compare this vaccine approach with vaccinia vectors carrying the HER-2/neu gene with and without the lysosomal targeting molecule, LAMP-1. This study will provide a paradigm for novel vaccine approaches to breast cancer prevention.

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FOREWORD

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Elizabeth Jaffee 9/23/97

PI - Signature Date

Recombinant Vaccine Strategies for Breast Cancer Prevention

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5. INTRODUCTION.

Breast cancer is a common problem, with an incidence of 182,000 new cases a year, and a mortality rate of 46,000 deaths per year. Overall, women in the United States have a 1 in 9 lifetime incidence of developing breast cancer (1-3). Because of its high incidence, breast cancer is a significant public health problem. Currently, most research is aimed at treating this disease. The time has come to focus efforts on prevention.

Recently, two breast cancer genes, BRCA1 and BRCA2, have been identified which influence susceptibility to breast cancer (4-6). Other genes associated with breast cancer susceptibility are likely to be identified in the future. It is estimated that approximately 5% of all patients who develop breast cancer, and 25% of patients who are diagnosed with breast cancer before age 30, will have a demonstrated susceptibility (7). With the current advances in molecular technology and the intensified effort to sequence the entire human genome, it is also very likely that other breast cancer susceptibility genes will be identified in the near future that are expressed by women at higher risk for breast cancer who do not have a strong family history. The identification of susceptibility genes should allow genetic screening for predisposition to this common deadly disease. Currently, the only option for prevention is surgical removal of the breast, which results in significant dysfiguration and psychological trauma.

The goal of cancer prevention is to develop treatment modalities which specifically target the breast ductile tree, as well as preneoplasia and incipient tumor cells. Vaccine strategies that result in T cell activation directed against self proteins expressed by these cells have the potential to be effective treatment for this purpose because the diversity of the T cell repertoire allows for the recognition of greater than 10^6 distinct peptide determinants. In addition, it is well-established that T cells recognize peptide fragments of cellular proteins bound to major histocompatibility molecules (MHC) on the surface of cells, and any cellular protein can be presented to T cells in this way.

Recent data have demonstrated that many antigenic determinants of the self do not induce self tolerance (8-9). Instead, these peptide determinants can become antigenic targets for autoimmune attack, and therefore, may be potential targets for directing antitumor immune responses. Strong support of this concept comes from data demonstrating that the majority of human melanoma antigens that have been identified are normal tissue peptides that are overexpressed by the melanoma (10-16). Most importantly, melanoma patients receiving *in vitro* expanded tumor infiltrating lymphocytes (TIL) specific for a few of these antigens demonstrated clinical responses against metastases without evidence of significant tissue damage (17-18). Interestingly, *in vitro* cytotoxic T cells (CTL) can be generated against these same antigens from peripheral blood lymphocytes (PBL) of healthy donors without a history of autoimmunity (19). These data are consistent with the

large body of evidence that both a critical density of MHC-peptide complexes on the target cell surface, as well as a critical set of co-stimulatory molecules, are required to activate T cells. As these signals continue to be defined, it should be possible to manipulate them toward beneficial immune responses that result in preventative treatments, without causing unwanted toxicity.

Our recent studies have provided strong evidence that the antitumor response can be considerably enhanced by recruiting subsets of lymphocytes that respond to tumor-specific antigens. Using murine models, we have demonstrated that the injection of tumor cells genetically engineered to produce local concentrations of cytokines, results in the activation of tumor-specific T cells capable of generating systemic antitumor immunity (20-22). In one study that compared over 10 cytokines for their ability to augment antitumor immunity, tumor cells genetically-altered to secrete granulocyte-macrophage colony stimulating factor (GM-CSF) produced the most potent systemic anti-tumor immunity (22). Analysis of the immune response generated has revealed that systemic immunity is dependent on both $CD4^+$ and $CD8^+$ T cells. Vaccination with GM-CSF secreting tumor cells has been shown to immunize mice against subsequent injections of parental tumor, as well as cure mice burdened with a relatively small amount of pre-established tumor. In addition, we have recently completed a phase I trial evaluating this approach in patients with advanced renal cell carcinoma (23). The results of this trial are extremely promising in that they demonstrate both clinical and immunologic activity. In addition, 3/3 patients receiving the bioactive dose of GM-CSF secreting vaccine cells demonstrated a DTH reaction against both autologous tumor cells, and autologous normal renal cells, yet no functional evidence of autoimmune damage was observed. This study provides further support for the existence of tissue-specific antigens that can serve as immune rejection targets in a second human tumor.

Two scientific questions need to be addressed prior to the design of vaccines that can prevent the development of breast cancer. The first requirement is the identification of antigenic targets expressed early by the pre-malignant cell, that can be used for immunization. The second requirement is the identification of optimal methods for delivering these antigens to the immune system for priming antitissue immune responses.

Currently, the Her2/neu gene product, which is overexpressed by 25-40% of invasive malignancies (24), and by 56% of pure ductal carcinoma in situ (DCIS) (25), is the best antigen for targeting immune responses to prevent the development of breast cancer. There are two reasons for this. First, HER2/neu is selectively overexpressed by premalignant and malignant cells, but is also expressed by normal breast ductal tissue (26). Second, HER2/neu encodes a large protein (1255 amino acids) and therefore should contain a range of epitopes capable of binding to different MHC alleles. One major concern is that patients would be immunologically tolerant to self-proteins like HER2/neu and that immunity might be difficult to generate. However,

Disis and colleagues recently described the identification of HER2/neu specific antibodies in 11 of 20 breast cancer patients studied. Interestingly, none of these patients had evidence of autoimmunity (27). A second study demonstrated $CD8^+$ T cell responses against HER2/neu peptides from lymphocytes of a normal donor (28). These studies demonstrate the existence of T cell precursors that have the potential to recognize the endogenous HER2/neu gene product.

Muller and colleagues have developed a transgenic mouse that overexpresses the proto-oncogene Her2/neu under the MMTV promoter (29). The mice develop focal mammary tumors at approximately 6-8 months of age that metastasize with high frequency to lung. Expression of the proto-oncogene product in histopathologically normal mammary epithelium has been demonstrated prior to the development of these focal mammary tumors. There are at least two reasons why this model provides a unique opportunity to study antigen-based vaccine strategies for the prevention of naturally developing mammary tumors. 1) Because the Her2/neu product is overexpressed in the normal ductal epithelium of these mice prior to the development of mammary tumors, this model provides a unique opportunity to evaluate vaccine strategies for preventing the development of spontaneously arising primary tumors. 2) the product of the proto-oncogene HER2/neu is an excellent target for evaluating recombinant vaccine strategies for augmenting antigen-specific immunity, since it is overexpressed in several common human tumors.

The HER2/neu transgenic mouse model is being used to directly compare antigen-based vaccine strategies for the prevention of the development of mammary tumors. Specifically, we are evaluating vectors that express antigen alone or together with co-stimulatory molecules or cytokines to determine if this immunity can be further enhanced. The vectors that are being tested include: 1) plasmid vectors delivered intramuscularly or intradermally using a gene gun; 2) Vaccinia vectors carrying the HER-2/neu antigen alone, or a fusion protein consisting of HER-2/neu and the lysosomal targeting molecule (LAMP-1); 3) Listeria monocytogenes vectors. All three vaccine approaches were chosen based on previous experience demonstrating that these vectors can augment potent specific antitumor immunity against existing cancers (30-33).

One major concern with employing antigen-based vaccine approaches that target rejection antigens expressed by normal tissue prior to the development of malignancy, is that these antigens may go unrecognized by activated T cells at the levels at which they are expressed. Our collaborator, Dr. Sara Sukumar, has developed a gene therapy approach which can selectively ablate mammary epithelial cells in a rat model of breast cancer. Using viral vectors with high efficiency of infection (in particular, vaccinia and adenovirus vectors), her group has demonstrated the feasibility of preferentially targeting the epithelial cells by directly injecting the vectors into the primary mammary duct through the teat. Our working hypothesis is that

we can increase access of activated T cells to normal ductal epithelial antigens by locally infusing vectors carrying a suicide gene such as the Herpes thymidine kinase (HTK) into the primary mammary duct, thereby rendering them susceptible to the toxic lethal effects of an antiviral drug such as gancyclovir (34). Therefore, we are also testing the synergistic effects of combining this unique approach of accessing mammary epithelium with our recombinant vaccine strategies.

6. BODY

A. Hypothesis being tested (Assumptions).

This proposal is testing the hypothesis that the generation of autoimmunity against breast epithelial cell-specific antigens using recombinant vaccines will result in the destruction of the ductile tree, preneoplasias, and incipient tumor cells, thereby preventing breast cancer. The hypothesis is based on the fact a significant number of human melanoma antigens that are the targets of T cells have recently been identified, and the majority of these antigens are normal tissue-specific antigens. These antigens are expressed by 40-60% of other patient's tumors, which provides strong support for the existence of common sets of antigens that can serve as targets for antigen-specific vaccine strategies. Most importantly, these antigens have served as tumor rejection antigens *in vivo*, resulting in clinical responses without functional evidence of destructive autoimmunity.

B. Experimental Methods.

Using the HER2/neu proto-oncogene transgenic mouse model of breast cancer, and the product of HER2/neu as a tissue-specific antigen for targeting immune responses, we are testing three recombinant vaccine strategies for the ability to: 1) activate antigen-specific immune responses and 2) prevent the development of breast cancer. These strategies are first being optimized in the parental FVB/N mouse in which tolerance against the HER-2/neu mouse is not expected. Once the vaccine strategy can prevent the development of mammary tumors in the parental line, we then test the optimized vaccine approach in the HER-2/neu transgenic mouse which demonstrates peripheral tolerance against the HER-2/neu gene product. To accomplish this goal, we are conducting the following experiments.

1. Generation of Recombinant DNA plasmid vectors.

a. Procedure.

We initially tested the plasmid vector pSvneo that expresses the HER-2/neu gene under the SV40 promoter in FVB/N mice for *in vivo* immune priming. We performed intramuscular injections and compared the HER-2/neu expressing plasmid DNA alone with HER-2/neu expressing plasmid DNA combined with the murine GM-CSF expressing plasmid DNA

for protection against subsequent challenge of the NT2 tumor. Ten mice per group were given weekly 100 ug intramuscular injections of the plasmid DNA's in the left flank for three weeks prior to challenge. The plasmid DNA conditions were compared in both the FVB/N mice and in the HER-2/neu Transgenic mice. FVB/N mice were challenged subcutaneously in the right flank with $5x10^6$ NT2 mammary tumor cells. HER-2/neu transgenic mice were challenged with $5x10^4$ NT2 mammary tumor cells. These challenge doses were determined based on the minimal number of tumor cells required for 100% tumor development in naive mice.

b. Results.

We did not demonstrate any protection against challenge with the NT2 mammary tumor lines that derived from spontaneously developing tumors in the HER-2/neu transgenic mice.

c. Discussion.

Because the vector demonstrated high levels of expression *in vitro*, we were concerned that the SV40 promoter was ineffective *in vivo* due to the cell types that were being transfected *in vivo*. Therefore, we decided to subclone the HER-2/neu gene into the pcDNA3 vector (Invitrogen) which would allow expression under the strong and most universal CMV promoter. Using the pcDNA3 plasmid DNA vector from Invitrogen which contains a CMV promoter, we constructed three plasmids: a plasmid containing the entire HER-2/neu gene; a plasmid containing the murine GM-CSF gene; and a plasmid containing the murine B71 co-stimulatory molecule. In addition ,we already had available the pcDNA3 plasmid containing the control antigen influenza A hemagglutinin (HA) which is also a transmembrane protein similar to the product of the HER-2/neu gene. All three plasmids were transfected into COS cells. The HER-2/neu and B7-1 plasmids were confirmed to be expressing by staining with anti-HER-2/neu and B7-1 antibodies, respectively, and analyzing for expression by FACS. The GM-CSF plasmid was confirmed to be producing bioactive GM-CSF using the GM-CSF dependent cell line, NFS-60.

2. *In vivo* testing of Recombinant DNA plasmid vectors.

2.1 Testing of Recombinant DNA plasmid vectors by Intramuscular injection.

a. Procedure.

In our first set of experiments, we performed intramuscular injections and compared the HER-2/neu expressing plasmid DNA alone with HER-2/neu expressing plasmid DNA combined with the murine GM-CSF expressing plasmid DNA for protection against subsequent challenge of the NT5 tumor. As a negative control we compared these mice with mice that received the influenza A HA gene mixed with the murine GM-CSF plasmid. Ten mice per group were given weekly 100 ug intramuscular injections of the plasmid DNA's for three weeks prior to challenge. The plasmid DNA conditions were compared in both the FVB/N mice and in the HER-2/neu

Transgenic mice. FVB/N mice were challenged with $5x10^6$ NT5 mammary tumor cells. HER-2/neu transgenic mice were challenged with $5x10^4$ NT5 mammary tumor cells. These challenge doses were determined based on the minimal number of tumor cells required for 100% tumor development in naive mice.

b. Results.

In the FVB/N mice, thirty percent of the mice receiving the HER-2/neu and GM-CSF expressing plasmids combined were tumor free at 55 days following vaccination. FVB/N mice receiving either the HER-2/neu plasmid vector alone or the control HA vector plus murine GM-CSF vector developed tumors by 50 days following challenge, although there seemed to be a significant delay in tumor development in the mice receiving the HER-2/neu plasmid alone. All of the HER-2/neu Transgenic mice succumbed to tumor by day 30 following challenge, regardless of whether they received the HER-2/neu plasmid alone, the HER-2/neu plasmid mixed with the GM-CSF plasmid, or the HA plasmid mixed with the GM-CSF plasmid.

c. Discussion.

We believe that these studies required further optimization since only a small effect was seen with the HER-2/neu plasmid combined with the GM-CSF plasmid in the FVB/N parental mice which we are using as our control for determining the effectiveness of our vaccine strategies in immunocompetent mice that have not been exposed to the immunizing antigen.

The cell type that is being transfected *in vivo* with intramuscular injections of DNA is unknown. If muscle cells are the cells expressing the gene delivered by plasmid DNA then a significant $CD4^+$ T cell response would not be expected. Yet, $CD4^+$ T cells have been determined to be critical for immune response amplification and memory. It is possible that professional antigen presenting cells (APCs) are being transfected with the DNA or are taking up antigen from the muscle cells by some yet unknown mechanism, but this hypothesis has not been proven. Injection of DNA plasmids with a gene gun may directly deliver the DNA to professional APCs such as langerhans cells that reside at the interface of the epidermis and dermis (which is the greatest delivery depth that the gene gun can achieve). This would allow activation of a $CD4^+$ and $CD8^+$ T cell response and therefore enhance the potency of this approach.

2.2 Testing of Recombinant DNA plasmid vectors by Gene Gun Delivery.

a. Procedure.

In the second set of experiments, ten mice per group were given a single intradermal 1 ug injection using a gene gun of either: 1) the HER-2/neu gene mixed with the pcDNA3 wildtype vector; 2) the HER-2/neu gene mixed with the GM-CSF gene; 3) no vaccination. Mice were challenged 2 weeks later with $5x10^6$ NT5 mammary tumor cells. Mice receiving the

vaccination by gene gun were compared with mice receiving the vaccine by intramuscular injection as described above for the first set of experiments.

b. Results.

The results are demonstrated in **figure 1.** 100% of mice receiving vaccination by intramuscular injection succumbed to tumor by 70 days post challenge with NT5 mammary tumor cells. In contrast, 40% of mice receiving the plasmid DNA by gene gun were tumor free 70 days post-challenge.

c. Discussion.

These studies imply that delivery by the gene gun may be a more efficient way of activating an antitumor immune response in the HER-2/neu mice. However, it would be beneficial to be able to improve the potency of the gene gun delivery by optimizing delivery conditions in the FVB/N mice since the HER-2/neu transgenic mice demonstrate peripheral tolerance to the HER-2/neu antigen. This peripheral tolerance is best demonstrated in the following experiment.

We compared this DNA plasmid strategy with our gold standard vaccination approach with a whole mammary tumor cell genetically modified to express the cytokine GM-CSF. Both the NT2 and NT5 tumor lines were genetically modified using the MFG retroviral gene transfer system to express high levels of GM-CSF. Both tumor lines were demonstrated to express greater than 35 ng/10^6 cells/24 hours of bioactive GM-CSF, which is the minimal level of GM-CSF required to generate an effective antitumor immune response in other murine tumor models in which GM-CSF was demonstrated to be effective. Because of our extensive previous experience demonstrating that whole tumor cells genetically modified to locally express GM-CSF can generate an effective systemic antitumor immune response that can cure mice of established disease, we chose to evaluate the whole tumor cell vaccine in this system against which we will compare all other vaccine strategies. Both HER-2/neu Transgenic mice and the parental FVB/N mice were vaccinated with either irradiated HER-2/neu expressing tumors alone or HER-2/neu expressing tumors genetically modified to express the murine GM-CSF protein in the left flanks subcutaneously on day 0. All mice were challenged in the right flank on day 14, subcutaneously with either $5x10^4$ wild type NT5 and NT2 tumor cells (the HER-2/neu mice) or with 5×10^6 wild type NT5 and NT2 cells (FVB/N mice). Mice were monitored twice a week for the development of tumor. As shown in **figure 2**, FVB/N mice receiving the whole tumor vaccine demonstrated a 100% disease free survival at 60 days when receiving the GM-CSF secreting tumor vaccine and a 80% disease free survival at 60 days when receiving the irradiated NT5 wild type cells. Eighty percent of the FVB/N mice receiving no vaccination developed tumors by day 60 following challenge. In contrast, HER-2/neu mice receiving either no vaccine, the wild type irradiated vaccine, or the NT5 cell genetically modified to express GM-

CSF, all developed tumors by day 35 following challenge. However, there was a two week delay in tumor development between mice that received no vaccine and mice that received the NT5 GM-CSF secreting tumor vaccine. These findings were supported by *in vitro* CTL data (**Figure 3**). T cells from FVB/N animals vaccinated with the NT 5 cells expressing murine GM-CSF showed significant HER-2/neu specific lysis of HER-2/neu expressing NIH3T3 cells relative to T cells from unvaccinated animals. This is compared to minimal lysis seen against wild type NIH3T3 target cells. T cells isolated from splenocytes taken on day 12 following vaccination of HER-2/neu mice with NT5 cells expressing murine GM-CSF also showed Her-2/neu specific lysis of HER-2/neu expressing NIH3T3 cells relative to T cells isolated from unvaccinated animals.

d. Future Studies.

We are planning to optimize conditions for vaccination in FVB/N mice using the gene gun. We will evaluate the following parameters: 1) dose of DNA; 2) number of boost injections; 3) the spatial distribution of simultaneous injections among 3 or more lymph node regions; 4) HER-2/neu antigen given together with the co-stimulatory molecule B7-1, the lysosomal targeting molecule LAMP-1. Once we have optimized conditions in the FVB/N mice we will test the best vaccine method in HER-2/neu transgenic mice with or without local ablation of the mammary tissue.

e. Recommendations in relation to the Statement of Work.

We expected to complete pilot studies testing DNA plasmid vectors by the end of year one. We have accomplished this. We have identified a gene gun delivery approach as being superior to the intramuscular injections. In year two, we will be optimizing this vaccine approach for comparison testing with other vectors.

3. Generation of Recombinant Vaccinia vectors.

a/b. Procedures and Results.

We are in the process of constructing several recombinant vaccinia vectors with and without the lysosomal targeting molecule (LAMP-1) for testing in our mouse model. We initially cut the entire rat HER-2/neu gene out of the psv2/neu vector obtained from William Muller who originally developed the HER-2/neu transgenic mice. However, one recurrent problem that we faced was that all of the restriction sites that could be used to accomplish this could not eliminate the inclusion of a 3′ untranslated region. Although this was adequate for constructing a vaccinia vector containing the HER-2/neu gene alone, we could not use this HER-2/neu gene to create the bicistronic constructs (containing HER-2/neu and the LAMP-1 molecule). We then attempted to amplify the gene using PCR but due to the large size of the

gene (4Kb) repeated attempts resulted in approximately 2 to 3 mutations/500 base pairs. We have therefore decided to use the original HER-2/neu gene which was excised from the psv2/neu vector. We have cloned the entire rat HER-2/neu cDNA into the pSC11 cloning vector for recombination into wildtype vaccinia virus. We are now in the process of cloning the HER-2/neu gene into the vaccinia vector. We will create the bicistronic constructs by performing a second recombination step. In addition to producing recombinant vaccinia which expresses the entire HER-2/neu gene product, we are making a series of constructs consisting of the putative HER-2/neu membrane insertion signal sequence followed by one of ten overlapping 400 base pair segments of the HER-2/neu gene with and without fusion to the LAMP-1 transmembrane and cytoplasmic domain in order to identify portions of this large gene that encodes for the relevant antigenic peptides. So far we have cloned each of the 400 bp segment with and without LAMP-1 into the pSC11 vector. We have confirmed that we have the correct size DNA expected for each clone.

c. Discussion.

These vectors containing minigenes should allow us to identify smaller portions of the gene for insertion into non-vaccinia vectors that do not easily allow cloning of 4 Kb genes. In addition, these vectors should allow us to more easily identify MHC class I and II restricted antigenic peptides for the H-2q background for more specific immune monitoring of the *in vivo* studies.

d. Future Studies.

We are currently cloning HER-2/neu gene and minigenes into the vaccinia vector. We will then test each vector for *in vitro* expression by infecting NIH3T3 cells with the vectors and assaying for surface expression of HER-2/neu. Once expression is confirmed *in vitro*, we will titer the vectors *in vivo* for the generation of an anti-neu response. To accomplish this, 2 or 3 female FVB/N mice will be infected on day 0 and again on day 14 with the each vaccinia construct. Two weeks later, spleens will be excised and lymphocytes isolated for *in vitro* stimulation with neu-expressing targets. Lymphocytes will be tested 5-7 days later for recognition of neu expressing targets relative to wildtype targets. Vectors demonstrating *in vitro* neu-specific T cell generation will be tested in further for the protection against challenge with the NT2 and NT5 mammary tumors. In addition, studies will be set up to determine if these vectors can prevent the development of the spontaneous mammary tumors.

e. Recommendations in relation to the Statement of Work.

We had initially expected to complete pilot studies testing the HER-2/neu only vaccinia construct by the end of year one. Due to difficulties in constructing all of the constructs, we do not expect to complete testing *in vivo* until the end of year two of this proposal. By the end of year two, we will

have tested in pilot studies, the HER-2/neu only construct, and compared it with constructs expressing the HER-2/neu plus LAMP-1 fusion protein.

4. Evaluate recombinant vaccines for synergy with chemical ablation of the mammary ductal system in preventing breast cancer.

We have developed a method for locally ablating the ductal epithelium by injecting via the mammary nipple recombinant vectors carrying the thymidine kinase gene, followed by systemic administration of gancyclovir. We will eventually test whether the combination of this chemical ablation of mammary ductal epithelium with what is identified in the first two specific aims to be the best antigen-specific vaccine, can amplify the HER2/neu T cell response generated, thereby resulting in enhanced long-term prevention of mammary tumors in HER2/neu transgenic mice. During year one, we evaluated methods for optimal mammary duct ablation with a vaccinia vector.

a. Procedures.

One female HER-2/neu mouse each received an intraductal injection of vaccinia/TK in trypan blue into 3 ducts on the left side. Each mouse received a sham injection with trypan blue into 3 ducts on the right side. Five vaccinia doses (10^3, 10^4, 10^5, 10^6, and 10^7 pfu were compared). Five mice per vaccinia dose were injected. One mouse per vaccinia dose was sacrificed and a whole mammary gland mount was performed on days 1, 4, 8, 12 and 16 following intraductal injections. The vaccinia injected glands were compared with the sham injected glands of each mouse.

b. Results.

Mammary ductal destruction was seen in all mammary glands that received the vaccinia vector regardless of dose used. Destruction could be seen for the large ducts as well as the distal branching ducts. Optimal destruction appears to occur with 10^4 pfu or greater. Destruction was not seen in mice receiving the sham injections. Ductal destruction was seen as early as 4 days and persisted for two weeks.

c. Discussion.

These studies demonstrate the feasibility of intraductal injections. Administration of gancyclovir was not required for ductal destruction. Ductal destruction persisted for at least 2 weeks. These studies should facilitate the design of studies evaluating synergy between systemic vaccination and local ablation which are planned in the fourth year of this grant.

d. Future studies.

We will repeat the dose escalation study of intraductal injections of vaccinia/TK and compare destruction with and without gancyclovir. A time course will also be performed and will be extended up to 1 month following ablation.

e. Recommendations in relation to the Statement of Work.

We have completed the initial feasibility studies as planned in year one. We will continue to optimize the intraductal ablation procedure in year two.

7. CONCLUSIONS.

This proposal seeks to develop a vaccine strategy that can specifically generate an immune response to ductal cells (the normal cells from which the majority of breast cancers arise), to preneoplasias, and to incipient tumor cells, by targeting common antigens expressed by these cells, as an alternative therapy for preventing breast cancer development. We are employing the HER-2/neu transgenic mouse model of breast cancer to evaluate vaccine strategies developed in our laboratory. We are in the early stages of evaluating two approaches, plasmid DNA vectors and vaccinia vectors. So far, our data would suggest that plasmid DNA vectors delivered by gene gun can augment antitumor immunity. We are in the process of optimizing this approach and testing it in the HER-2/neu mouse for prevention of spontaneous mammary tumors. During the next year, we should complete testing of the plasmid DNA approach and begin testing of the vaccinia approach.

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9. APPENDICES.

1. **Figure 1.** Gene Gun Delivery of Plasmid DNA Vaccines is Successful at Generating Systemic Antitumor Immunity Against Mammary Tumors. See page 19.

2. **Figure 2.** Tolerance to HER-2/neu gene product in HER-2/neu mice is demonstrated with whole cell GM-CSF secreting mammary tumor vaccine.

3. **Figure 3.** HER-2/neu specific T cells are demonstrated in FVB/N and HER-2/neu transgenic mice following whole cell tumor vaccination.

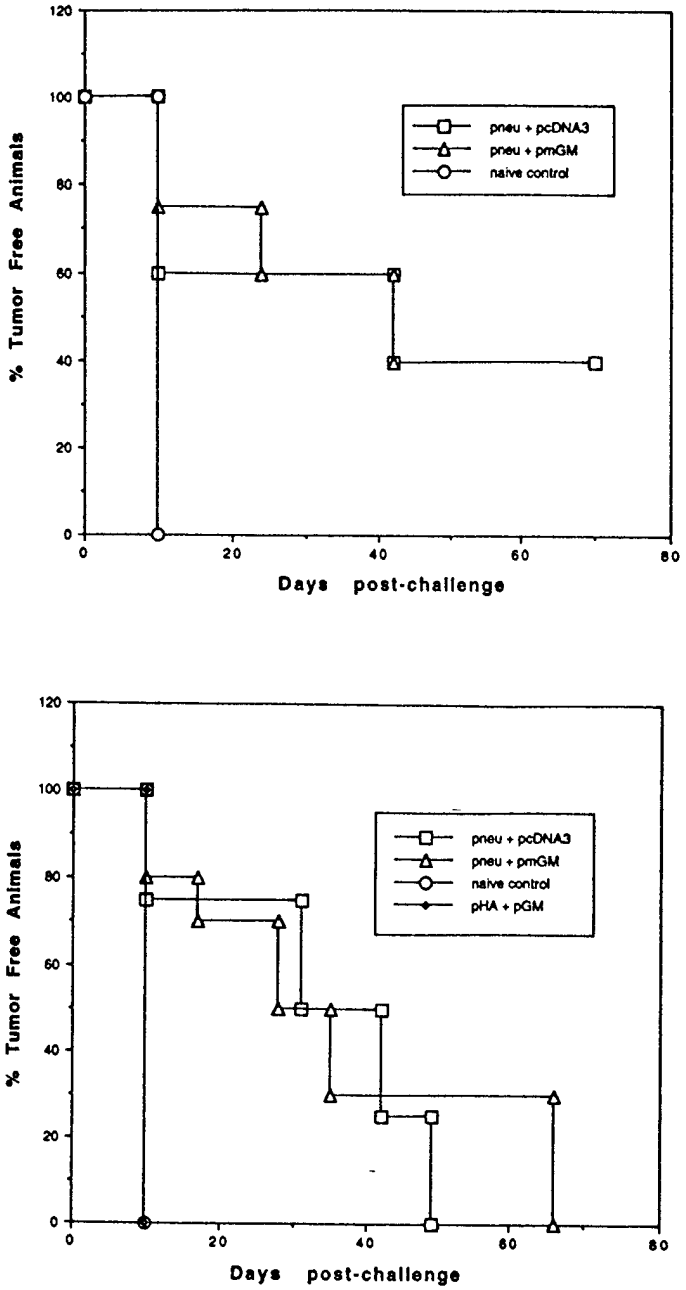

Figure 1. Gene Gun Delivery of Plasmid DNA Vaccines is Successful at Generating Systemic Antitumor Immunity Against Mammary Tumors.
A. 10 FVB/N female mice per group were injected intradermally in the left flank with 2 ug of total plasmid DNA coated onto gold beads using a gene gun on Day 0. Mice received either: 1) 1 ug of HER-2/neu DNA (pneu) + 1 ug of pcDNA3; 2) 1 ug of pneu + 1 ug of murine GM-CSF DNA (pmGM); or 3) beads only (naive mice). **B.** 10 FVB/N female mice per group were injected intramuscularly in the left flank with 2 ug of total plasmid DNA on Day 0. Mice received either: 1) 1 ug of pneu + 1 ug of pcDNA3; 2) 1 ug of pneu + 1 ug of pmGM; 3) 1 ug of control vector Hemagglutinin (pHA) + pmGM; or 4) saline only (naive mice). All mice were challenged with $5x10^6$ NT5 mammary tumor cells subcutaneously in the right flank on day 14. Mice were observed twice a week for the development of tumors.

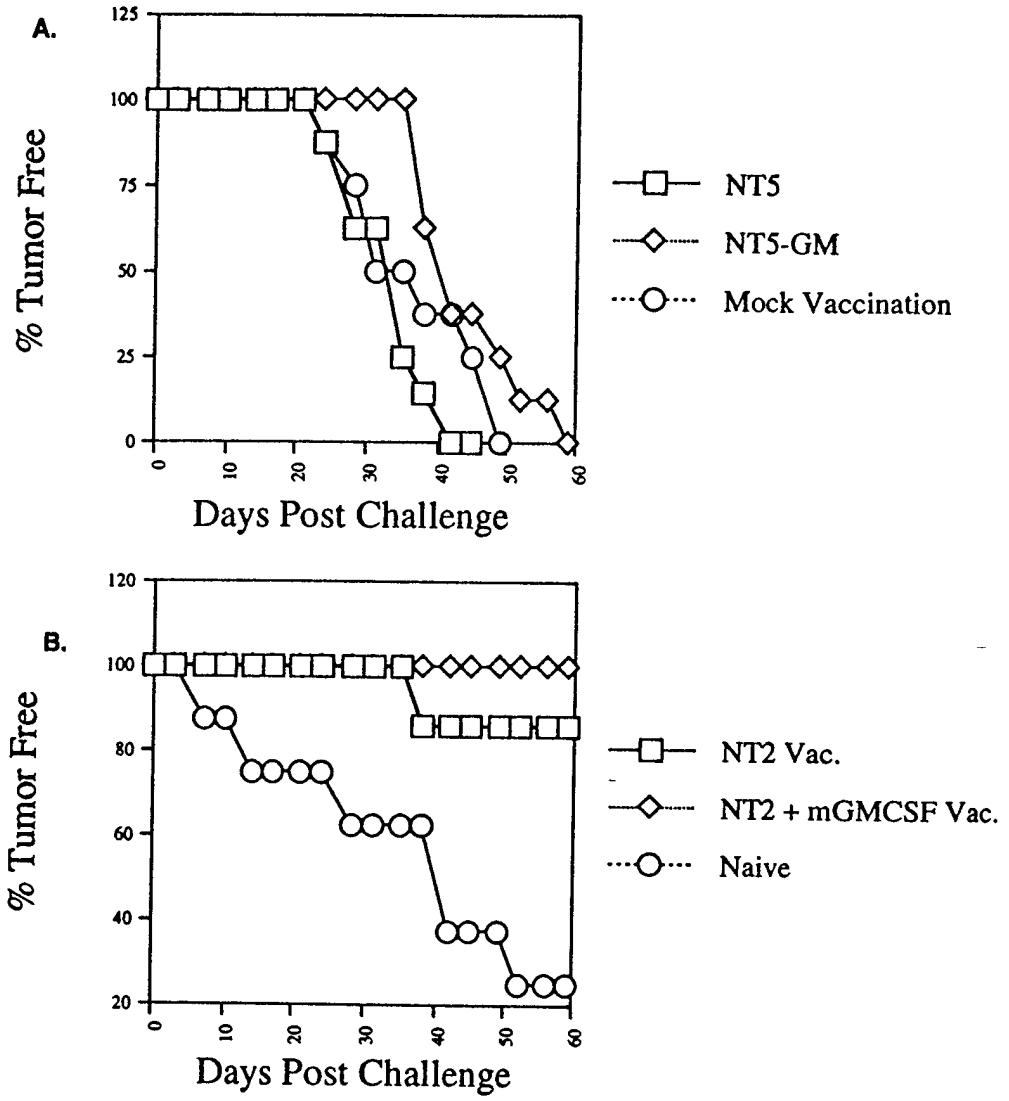

Figure 2. Tolerance to HER-2/neu gene product in HER-2/neu mice is demonstrated with whole cell GM-CSF secreting mammary tumor vaccine. **A.** HER-2/neu female mice (10 mice per group) were vaccinated subcutaneously in the left flank with 10^6: 1) irradiated GM-CSF secreting NT5 mammary tumor cells (NT5-GM); 2) irradiated wildtype NT5 mammary tumor cells (NT5); or 3) saline injection on day 0. Mice were challenged on day 14 subcutaneously in the right flank with $5x10^4$ NT5 tumor cells. **B.** FVB/N female mice (10 mice per group) were vaccinated subcutaneously in the left flank with 10^6: 1) irradiated GM-CSF secreting NT2 mammary tumor cells (NT5-GM); 2) irradiated wildtype NT2 mammary tumor cells (NT2); or 3) saline injection on day 0. Mice were challenged on day 14 subcutaneously in the right flank with $5x10^6$ NT3 tumor cells. All mice were observed twice a week for the development of tumors.

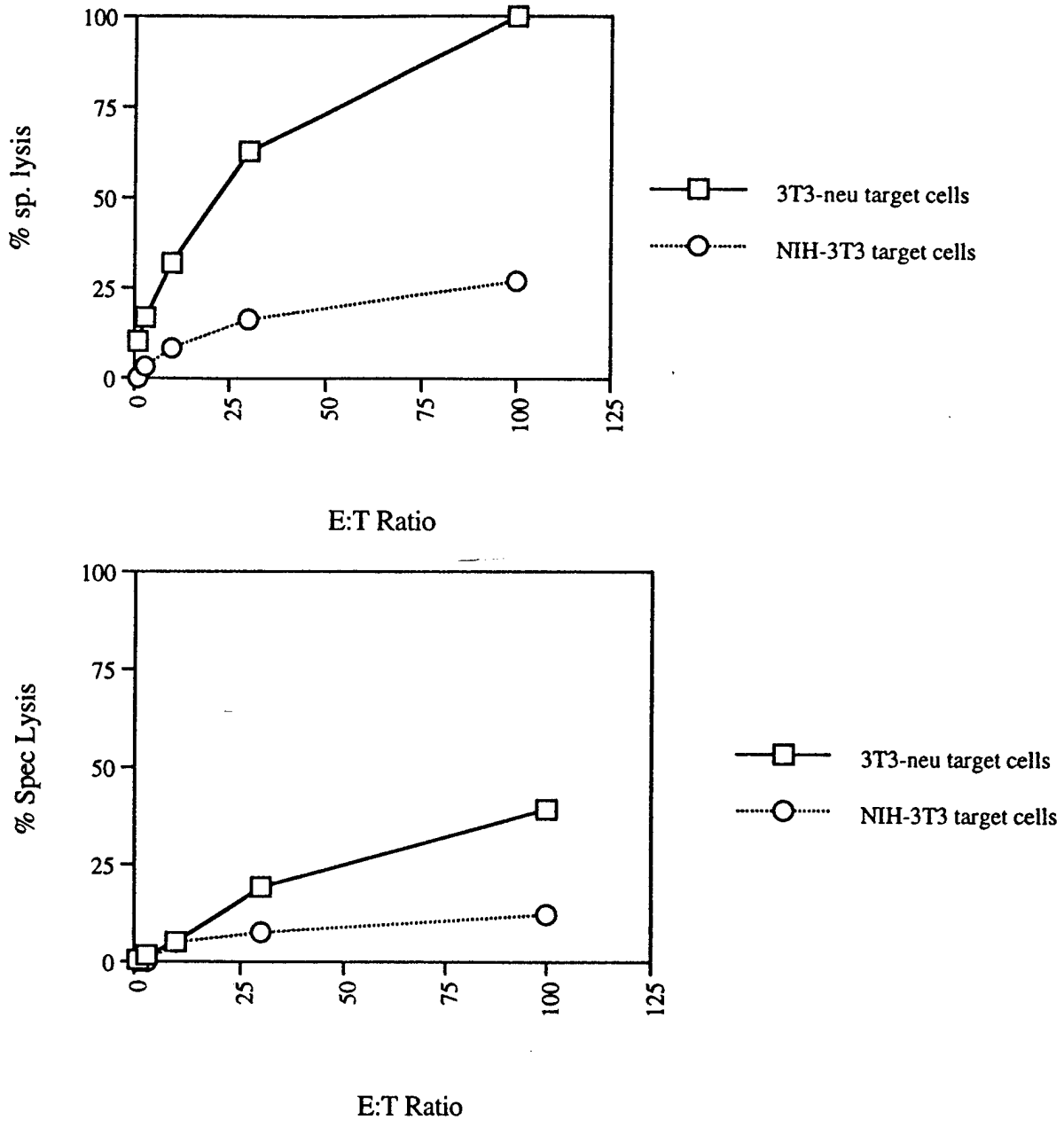

Figure 3. HER-2/neu specific T cells are demonstrated in FVB/N and HER-2/neu transgenic mice following whole cell tumor vaccination. Three mice per group were vaccinated with either: 1) irradiated GM-CSF secreting NT5 mammary tumor cells (NT5-GM); 2) irradiated wildtype NT5 mammary tumor cells (NT5); or 3) saline injection on day 0. Two weeks later, mice were sacrificed, their spleens were excised, and lymphocytes were isolated by ficol gradient. Lymphocytes were stimulated in culture for 5 days with mitomycin-C treated NT5 mammary tumor targets at a 40:1 responder:stimulator ratio. Lymphocytes were assayed in a 4 hr 51Cr release assay for lysis of 51-Cr-labeled NIH-3T3 wildtype targets and NIH-3T3-HER-2/neu expressing targets. Shown in A is the FVB/N mice that received the GM-CSF secreting vaccine. Shown in B is the HER-2/neu mice that received the GM-CSF secreting vaccine. Both HER-2/neu and FVB/N mice receiving the saline vaccine failed to demonstrate an *in vitro* HER-2/neu specific response.