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

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 attempted 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 together with the cytokine, GM-CSF, to determine if this immunity can be further enhanced. An intramuscular approach was compared with gene gun delivery. Due to suboptimal results, we then explored vaccinia vectors containing the HER-2/neu gene with or without co-stimulatory molecules. We found that vaccinia containing the antigen plus the GM-CSF gene was most potent in delaying the development of spontaneous mammary tumors. We have found that gene gun delivery is superior for enhancing antitumor immunity. Two additional findings that arose from these studies include: 1) The requirement for vaccine induced antibody responses; the requirement for vaccination early prior to significant overexpression of the antigen. We will follow up on these findings ultimately to design the optimal vaccine for the prevention of breast cancer.

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04. 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 (1). Currently, most research is aimed at treating this disease. The goal of this proposal was to develop vaccine strategies aimed at cancer prevention. To accomplish this goal, we employed the HER-2/neu transgenic mouse model of breast cancer developed by Muller (2). 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 HER-2/*neu* transgenic mouse model was used to directly compare antigen-based vaccine strategies for the prevention of the development of mammary tumors. Specifically, we evaluated vectors that express antigen alone or together with co-stimulatory molecules or cytokines to determine if this immunity can be further enhanced (3-9). The vectors that were tested included: 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). These vaccine approaches were chosen based on previous experience demonstrating that these vectors can augment potent specific antitumor immunity against existing cancers.

5. BODY

A. Hypothesis being tested (Assumptions).

This proposal tested 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 (10-19). 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. Research Accomplishments Associated with Each Task Outlined in the Statement of Work.

Using the HER-2/*neu* proto-oncogene transgenic mouse model of breast cancer, and the product of HER-2/neu as a tissue-specific antigen for targeting immune responses, we tested three recombinant vaccine strategies for the ability to: 1) activate antigen-specific immune responses and 2) prevent the development of breast cancer. These strategies were first optimized in the parental FVB/N mouse in which tolerance against the HER-2/neu mouse is not exhibited. Once the vaccine strategy was shown to prevent the development of mammary tumors in the parental line, we then tested the optimized vaccine approach in the HER-2/neu transgenic mouse which we demonstrated does exhibit peripheral tolerance against the HER-2/neu gene product. To accomplish this goal, we conducting the following experiments (listed as originally described in the Technical Objectives and Statement of Work).

The original Technical Objectives were as follows:

1. Construct recombinant vaccines encoding for the expression of the HER-2/neu proto-oncogene product plus cytokines.

We had constructed several different vectors carrying the HER-2/neu proto-oncogene, the cytokine GM-CSF, and the co-stimulatory molecules, $B7_1$ and $B7_2$. The choice of vectors included vaccinia, *L. monocytogenes*, and plasmid DNA. We successfully constructed and tested the vaccinia and plasmid DNA constructs as described below. In addition, we constructed a GM-CSF secreting and HER-2/neu expressing whole cell vaccine which we used as our original gold standard vaccine. The results of testing of these vaccines are described below and in the appended publications and submitted manuscripts. We were unable to complete construction of a functioning *Listeria Monocytogenes* vector. Although such a vector was provided to us by our collaborator, Dr. Yvonne Patterson. It turned out that this vector did not function due to the large size of the inserted HER-2/neu gene. We have since identified a 500 Kb HER-2/neu gene fragment in the extracellular domain that encodes for the relevant HER-2/neu immunizing epitopes in this model system. Dr. Patterson, through a collaboration with us, is in the process of constructing a new *Listeria Monocytogenes* vector containing this smaller fragment.

2. Evaluate recombinant vaccines for the induction of antigen-specific immunity potent enough to prevent breast cancer development.

Initially, *in vivo* HER-2/neu transgenic mouse studies were designed to directly compare antigen-based vaccines using plasmid DNA, recombinant vaccinia vectors, and the HER-2/neu and GM-CSF expressing whole cell vaccine. Additional studies were then designed to directly compare the most potent vaccine strategies identified, with vaccines that combined antigen with either GM-CSF, or the co-stimulatory molecules B7-1 and/or B7-2, for the ability to further influence the immunity generated. These studies are described below and in the appended publication.

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

In collaboration with Dr. Sara Sukumar, we developed a method for locally ablating the ductal epithelium by injecting via the mammary nipple, recombinant vectors carrying the various genes. We tested whether the combination of this local ablation of mammary ductal epithelium with what was identified in the first two specific aims to be the best antigen-specific vaccine, can amplify the HER-2/neu T cell response generated, thereby resulting in enhanced long-term prevention of mammary tumors in HER-2/neu transgenic mice. We tested the systemic vaccinia and whole cell vaccines with local administration by wildtype vaccinia (non-specific local ablation), and by vaccinia containing specific immune modulating molecules (B71 and B72) for their ability to delay spontaneous mammary development. Unfortunately, we were unable to show that combining local ablation with systemic vaccination is superior to systemic vaccination alone. These studies are described in detail below.

Specific Studies Related to the Statement of Work.

1. Generation and testing of Recombinant DNA plasmid vectors.

Summary of findings.

During the first year of this proposal, we tested two different naked DNA plasmid vectors for immune priming in the parental FVB/N mice. The first plasmid, pSvneo, expressed the HER-2/neu gene under the SV40 promoter. 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. 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. Although we detected good expression of the neu gene *in vitro*, we were concerned that the SV40 promoter was not being expressed well *in vivo*. In addition, we were concerned that this vector did not contain the bacterial immune stimulating sequences recently reported to be required for optimal immune priming . We therefore cloned theHER-2/*neu* gene into the pcDNA3 vector (Invitrogen) under the CMV promoter, which allows for expression under the strong and most universal CMV promoter. We constructed and tested 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 pcDNA-3 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 confirmed to be functional by transfection into COS cells. In our first set of experiments which we reported in the first year of funding, 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. We have now generated five mammary tumor lines from endogenously developing mammary tumors in the HER-2/*neu* transgenic mice. All of these lines express equivalent levels of HER-2/neu protein. We have chosen to use NT5 for all of our *in vivo* studies because it is the most aggressive 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. 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.

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 in theory allow activation of a $CD4^+$ and $CD8^+$ T cell response and therefore enhance the potency of this approach.

We tested the gene gun approach in the second set of experiments. 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. 100% of mice receiving vaccination by intramuscular injection succumbed to tumor by 70 days post challenge with NT5 mammary tumor cells. In contrast, 50% of mice receiving the plasmid DNA by gene gun were tumor free 70 days post-challenge. 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, we still believed that 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, as discussed in the first year report, the HER-2/*neu* transgenic mice demonstrate peripheral tolerance to the HER-2/*neu* antigen.

At the conclusion of year one (10/97), we planned to optimize conditions for vaccination with naked DNA plasmids in FVB/N mice using the gene gun. We planned to 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, B7-2, GM-CSF, and with the lysosomal targeting molecule LAMP-1. To optimize immunization with gene gun delivery of naked DNA, a set of experiments were performed to compare immunization with either: a single intradermal 1 ug injection of the DNA vaccine given once; a single intradermal 1 ug injection of the DNA vaccine given twice, each one month apart; three 1 ug injections of DNA vaccine given simultaneously at three different lymph node regions. 10 mice per group received 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) the HER-2/*neu* gene mixed with the B7.1 gene; 4) the HER-2/*neu* gene mixed with the B7.2 gene; 5) the HA gene mixed with the GM-CSF gene as a negative control; 6) no vaccination. Mice were challenged 2 weeks after the last vaccination with $5x10^6$ (parental mice) and $5x10^4$ (transgenic mice) NT5 mammary tumor cells.

After repeating several experiments, we found that a single dose of vaccine at a single site is just as potent at generating anti-HER-2/*neu* specific immunity when compared with either two vaccinations 2 weeks apart, or the spatial distribution of the vaccinating plasmid. The results of the subsequent experiments demonstrated that 100% of the FVB/N mice receiving the HER-2/*neu* plus GM-CSF DNA vaccine remained tumor free for more than 60 days before this cohort began developing tumors. This is in contrast to mice receiving no vaccine or the HA plus GM-CSF control DNA vaccine. 100% of mice in these two control groups developed tumors by day 28 following inoculation with tumor cells. In addition, the HER-2/*neu* plus GM-CSF combination appeared to be more potent than combination of the HER-2/*neu* plasmid with either B7.1 or B7.2 co-stimulatory genes. Unfortunately, none of these vaccines were potent enough to prevent tumor development in the transgenic mice. The data for these studies were presented in the first grant update.

These studies imply that delivery by the gene gun is potent enough to significantly delay tumor development in the FVB/N mice. We do not believe that the eventual development of tumors is a result of a less than optimal vaccine. Rather, we have found that the tumors that grow out no longer express HER-2/*neu* surface protein and are they recognized by HER-2/neu specific T cells. *In vitro* growth of explanted tumor cells regain surface HER-2/neu expression after one week in culture and are subsequently detected and lysed by HER-2/neu specific T

cells. To confirm that the FVB/N mice were still adequately immunized against the HER-2/neu protein at the time the original tumor developed, we rechallenged the vaccinated mice with $5x10^6$ HER-2/neu expressing tumors on the opposite limb to which the original tumor was given on day 35 following the original inoculation with tumor. Normally, vaccinated mice begin to develop tumors starting on day 40. All of the mice that were vaccinated developed tumors at the original site of vaccination. However, none of these mice developed tumors at the rechallenge site in the opposite limb. Thus, we believed that we had optimized this approach adequately. We therefore evaluated other approaches for prevention of tumor development in the transgenic mice.

2. Generation of Recombinant Vaccinia vectors.

Summary of Findings.

At the end of year one we were 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 had difficulty constructing a vaccinia vector containing the HER-2/*neu* gene. In particular, we 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 successfully cloned the entire rat HER-2/*neu* cDNA into the pSC11 cloning vector for recombination into wildtype vaccinia virus. We then cloned the HER-2/*neu* gene into the vaccinia vector. We created the bicistronic construct containing the LAMP-1 targeting molecule and the HER-2/*neu* gene by performing a second recombination step. In addition to producing recombinant vaccinia which expresses the entire HER-2/*neu* gene product, we made 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. We confirmed that we had the correct size DNA expected for each clone and that they expressed the fragment. All of these fragments were cloned into the pcDNA3 plasmid and were transfected into NIH3T3 cells. Transfected clones were tested for integration of the HER-2/*neu* segment by PCR and for recognition by our HER-2/*neu* specific T cells.

As discussed in the 1999 update, we also confirmed the sequence and function of the bicistronic HER-2/*neu*/LAMP-1 construct that had been cloned into vaccinia. In addition, we cloned the HER-2/*neu* minigenes (extracellular and intracellular fragments) into the vaccinia vector. Fluorescent microscopy confirmed localization of the HER-2/*neu* gene in the lysosomal compartment demonstrating in vitro expression of the HER-2/neu/LAMP-1 construct. NIH3T3 cells infected with the vaccinia constructs containing the HER-2/*neu* minigenes were shown to express the HER-2/neu protein by T cell recognition.

3. Testing of vaccinia constructs *in vivo.*

Summary of Findings.

As discussed in the previous update report, we initially performed studies to test the potency of the vaccinia vectors we constructed. Five mice per group were vaccinated on day 0 with a single intraperitoneal injection of either: 1) 10^7 pfu of the HER-2/neu gene mixed with $2x10^7$ pfu wildtype vector; 2) 10^7 pfu HER-2/neu gene mixed with 10^7 pfu of the GM-CSF gene and 10^7 pfu of the wildtype vector; 3) 10^7 pfu HER-2/neu gene mixed with 10^7 pfu of the GM-CSF gene and 10^7 pfu of the B7.1 vector; 4) 10^7 pfu HER-2/neu gene mixed with 10^7 pfu of the GM-CSF gene and 10^7 pfu of the B7.2 vector; 5) 10^7 pfu of the control HA gene mixed with 10^7 pfu of the GM-CSF vector and 10^7 pfu of the wildtype vector. Mice were challenged 2 weeks after vaccination with $5x10^6$ (parental mice) and $5x10^4$ (transgenic mice) NT5 mammary tumor cells subcutaneously in the right flank. Mice were monitored twice a week for the development of tumors. We did not observe significant protection with the vaccinia constructs. However, we did observe that many of the mice in each group became ill. We believed that mixing the recombinant vaccinia vectors with wildtype vector to standardize the concentration of total vaccinia vector given per mouse explains the illness of the mice and the poor outcome of this study. The recombinant vectors are more attenuated than the wildtype vector and therefore require a higher concentration for immunization and result in less toxicity than the wildtype vector. We therefore performing a series of titering experiments with each recombinant vaccinia construct. We also diluted each construct with the attenuated control HA vector rather than wildtype vector in subsequent studies to prevent toxcity.

During the third and fourth years of this grant (1998-2000), we completed testing and optimization of the vaccinia vaccines. In addition, we compared the vaccinia constructs with the 3T3-neu/GM vaccine as a gold standard. Transgenic mice received either a 3T3-neu/GM vaccine given s.c. (open squares) (n = 10 mice) or neu recombinant vaccinia virus (open triangles) (n = 10 mice) followed two weeks later by a s.c. NT2 challenge in the right hind limb. The data demonstrated that it is possible to significantly delay tumor development in the transgenic mice. However, GM-CSF, B7-1, and B7-2 do not seem to significantly improve on the HER-2/*neu* vaccinia vector alone (data not shown). The data also demonstrates that the HER-2/*neu* protein can serve as a mammary tumor rejection target. Finally, we evaluated the vaccinia vaccine in combination with anti-CTLA-4, anti-CD40, and

anti-OX40 for the ability to immune modulate the vaccine induced responses. However, all three immune modulating agents failed to demonstrate an improved systemic response.

The above described data has recently been published and can be found **Appendix #5.**

Dissection of the immune response demonstrated that HER-2/*neu* specific T cells were indeed amplified with the vaccinia vaccine. However, we were surprised to find that HER-2/neu-specific IgG antibody responses were also induced. As shown in **figure 1**, HER-2/neu-specific vaccines elicit an antibody response in both FVB/N and neu-N mice. Studies were performed to further evaluate these antibody responses. In particular, SCID mice lacking B and T cells were reconstituted with either HER-2/neu specific T cells plus irrelevant antibody, HER-2/neu specific T cells plus vaccine induced HER-2/neu specific antibodies, irrelevant HA specific T cells plus irrelevant antibodies, or HA specific T cells plus HER-2/neu antibodies. Only mice receiving both the HER-2/neu specific T cells and antibody could completely reject pre-established tumors. These studies are described in the submitted manuscript by Reilly, et al. in the appendix. These studies are the first to suggest that vaccine induced antibody responses may play a role in vaccine induced antitumor immunity.

HER-2/*neu*-specific vaccination of transgenic mice also results in an increase in HER-2/neu-specific T cells. Furthermore, as described in detail in Reilly et al., Cancer Research, 2000, depletion of mice of either CD4 or CD8 T cell subsets abrogates the vaccine induced protective immunity.

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

We had 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 tested whether the combination of this chemical ablation of mammary ductal epithelium with several HER-2/*neu* vaccines, could amplify the HER-2/neu T cell response generated, thereby resulting in enhanced long-term prevention of mammary tumors in HER-2/neu transgenic mice. During year one, we evaluated methods for mammary duct ablation with a vaccinia vector. During year 2, we tested this approach in an initial study together with the DNA plasmid vaccine. During year 3 we tested this approach with our best systemic vaccinia vector. Unfortunately, the addition of local ablation did not enhance the systemic vaccine's ability to prevent spontaneous mammary tumors (**figure 2**). However, the vaccinia vector given systemically does induce HER-2/neu specific immunity potent enough to significantly delay spontaneous mammary tumors in post-lactational mice (**figure 3,**

Reilly et al., Cancer Research, 2000). Interestingly, it is possible to improve upon vaccine efficacy if the vaccine is given prior to lactation **(figure 4)**.

5. **Key Research Accomplishments Emanating From This Research.**

- A HER-2/neu containing vaccinia vector can significantly delay the development of spontaneously developing mammary tumors in HER-2/neu transgenic mice (Reilly et al. Cancer Research, 60:3569-76, 2000.
- Vaccine induced HER-2/neu specific immunity is dependent on both T cell and antibody dependent immune responses (Reilly et al., submitted).
- Immune modulating doses of chemotherapy can enhance the potency of HER-2/neu specific vaccinations and result in long-term cure of mammary tumors in the transgenic mice (Machiels et al. Submitted).
- HER-2/neu transgenic mice exhibit HER-2/neu directed peripheral tolerance that is the result of the deletion of high affinity HER-2/neu specific T cells and the peripheral suppression of low affinity HER-2/neu specific T cells (Ercolini et al, Abstract; Manuscript in preparation).

7. **Reportable Outcomes.**

A. Publications/Abstracts.

1 Publication.

Reilly RT, Gottlieb, MBC, Ercolini AM, Machiels J-P, Kane CE, Okoye FI, Muller WJ, Dixon KH, **Jaffee, EM**. HER-2/neu is a tumor rejection target in the HER-2/neu transgenic mouse model of breast cancer. Cancer Research 60:3569-3576, 2000.

2 Submitted Manuscripts.

Machiels JP, Reilly RT, Emens L, Ercolini A, Okoye F, **Jaffee EM**. Cyclophosophamide, Doxorubicin, and Paclitaxel enhance the antigen-specific antitumor immune response of GM-CSF secreting whole cell vaccines in tolerized mice. Submitted.

Reilly RT, Machiels J-P, Emens LA, Ercolini AM, Okoye FI, Lei RY, Weintraub D, Jaffee EM. The collaboration of both humoral and cellular HER-2/neu targeted immune responses is required for the complete eradication of HER-2/neu-expressing tumors. Submitted.

2 Oral Abstracts.

Reilly, R.T., Machiels, J-P.H., Ercolini, A.M., Gottlieb, M.B.C., Kane, C.E., Okoye, F.I., and Jaffee, E.M. Antibody-mediated Down-regulation of Oncogene Expression as a Mechanism of Tumor Escape from Immune Recognition Following Antigen-specific Vaccination. AACR Meeting (Minisymposium), Philadelphia, PA, April 10-14, 1999.

Ercolini, A.M., Reilly, R.T., Machiels, J-P.H., Lei, R., and Jaffee, E.M. HER-2/neu transgenic mice use an alternate neu-specific T cell Repertoire relative to the parental strain which can be induced to prevent neu-expressing tumors. Keystone Symposia on Cellular Immunity and

Immunotherapy of Cancer (Poster), Santa Fe, NM, January 21 – January 27, 2000.

5 Poster Abstracts.

Reilly RT, Machiels J-P, Ercolini AE, Gottlieb MBC, Kane CE, Okoye FI. and **Jaffee EM.** Antibody-mediated down-regulation of oncogene expression as a mechanism of tumor escape from immune recognition following antigen-specific vaccination. Proceedings of the American Association for Cancer Research, April 10-14, 1999 vol. 40, 512 #3383.

Machiels J-P,

Gottlieb, M.B.C., Dixon, K.H., Reilly, R.T., Kane, C.E., and Jaffee, E.M. Immunologic Characterization of a HER-2/neu Transgenic Mouse Model for Breast Cancer: Peripheral Tolerance Mimicking Human Disease. Abstr. 10415, AACR Meeting (Poster), San Diego, CA, April 12-16, 1997.

Reilly, R.T., Gottlieb, M.B.C., Kane, C.E., and Jaffee, E.M. The Generation of a HER-2/neu-specific Immune Response in the *neu/neu* Transgenic Mouse Mammary Tumor Model. Keystone Symposia on Molecular and Cellular Biology (Poster), Keystone, CO, January 26 – February 1, 1998.

Reilly, R.T., Okoye, F.I.., and Jaffee, E.M. Active immunization against HER-2/neu proto-oncogene demonstrates the importance of both antibody- and T cell-mediated immune responses in tumor eradication. Keystone Symposia on Cellular Immunity and Immunotherapy of Cancer (Poster), Santa Fe, NM, January 21 – January 27, 2000.

Ercolini, A.M., Reilly, R.T., Machiels, J-P.H., Lei, R., and Jaffee, E.M. HER-2/neu transgenic mice use an alternate neu-specific T cell Repertoire relative to the parental strain which can be induced to prevent neu-expressing tumors. Cancer Research Institute, Cancer Vaccines 2000 (Poster), New York, NY, October 2 – October 4, 2000.

B. Degrees/Research Opportunities.

1. Dr. Richard Todd Reilly completed his postdoctoral fellowship and was promoted to Instructor in the Department of Oncology at the Johns Hopkins University. Dr. Reilly is continuing work on the HER-2/neu transgenic mouse model of breast cancer.

2. Anne Ercolini is a fifth year graduate student in the Immunology Program. She is expected to receive her doctorate degree in 2001.

C. Development of Cell Lines.

Five mammary tumor cell lines were generated from spontaneously developing murine mammary tumors in the HER-2/neu transgenic mice. These lines have been characterized and found to overexpress the HER-2/neu proto-oncogene. Two of these lines have been reported in the literature (Reilly RT, et al. Cancer Research 60:3569-3576, 2000).

D. Funding Applied For Based On The Work Performed In This Grant.

1. National Cancer Institute NCDDG program grant awarded 9/2000. Title: Antigen specific vaccines for breast cancer. PI: Dr. Jaffee.
2. National Cancer Institute SPORE in Breast Cancer. Awarded 9/2000. Dr. Jaffee has a project on this program grant entitled: Vaccines: A new paradigm for Breast Cancer Prevention.
3. National Cancer Institute RO1. Pending. Title: Vaccine and Immune Modulation for the Treatment of Breast Cancer.

8. CONCLUSIONS.

This proposal attempted 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 successfully constructed several HER-2/neu targeted vaccines and tested these vaccines in the HER-2/*neu* transgenic mouse model of breast cancer. We also successfully demonstrated that one of these approaches can significantly delay spontaneous mammary tumor development. However, local ablation of ductal tissue did not add to the delay induced by the systemic vaccine. We attempted to improve on our systemic vaccine in two ways. First, we tested several immune modulating agents together with our best systemic vaccine. Unfortunately, combining anti-CTLA-4, anti-CD40, and anti-OX40 did not significantly improve on the vaccine alone results. Second, we tried to construct a *listeria monocytogenes* vector carrying the HER-2/*neu* gene. Unfortunately, the vector did not function. We are still working on constructing a vector carrying the minigenes. Testing will be performed in the next year. We have also found that vaccine induced HER-2/neu specific antibody responses contribute to the systemic antitumor immune response. Follow-up studies are underway to design new vaccines that specifically target antibody induction. Once we have improved on our systemic vaccine approach, we will revisit combining local mammary tissue modulation and ablation with the systemic vaccine. Finally, we have recently found that the HER-2/neu specific CD8 T cells isolated from the transgenic mice are lower affinity than the CD8 T cells isolated from the parental mice. These findings strongly support the need to identify additional requirements for enhancing low affinity T cells. We have found that it is possible to accomplish this by combining vaccination with immune modulating doses of chemotherapy. We will continue to understand the mechanism of inducing lower affinity T cells and to identify improved methods for activating these T cells.

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10. APPENDICES.

1. **Figure 1.** A neu-specific whole-cell vaccine elicits an antibody response in both FVB/N and neu-N mice.

2. **Figure 2.** Pilot Study to Evaluate the Effects of a Plasmid DNA Vaccine Combined with Local Ductal Ablation to Prevent Breast Cancer Development.

3. **Figure 3.** Neu-specific vaccination is protective against spontaneous tumor formation in neu-N transgenic mice.

4. **Figure 4.** Vaccine efficacy is decreased in post-lactational females.

5. Reilly RT, Gottlieb, MBC, Ercolini AM, Machiels J-P, Kane CE, Okoye FI, Muller WJ, Dixon KH, Jaffee, EM. HER-2/neu is a tumor rejection target in the HER-2/neu transgenic mouse model of breast cancer. Cancer Research 60:3569-3576, 2000.

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HER-2/neu targeted immune responses is required for the complete eradication of HER-2/neu-expressing tumors. Submitted.

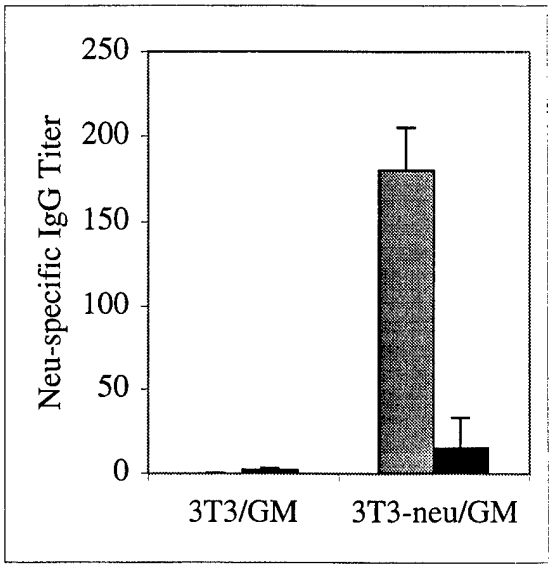

Figure 1. A neu-specific whole-cell vaccine elicits an antibody response in both FVB/N and neu-N mice. Serum was obtained from FVB/N (gray bars) and neu-N mice (black bars) 14 days after vaccination with irradiated 3T3/GM or 3T3-neu/GM cells. Serial dilutions of serum were then used as the primary antibody solution in a FACS analysis of both 3T3 and 3T3-neu cells using a secondary antibody against murine IgG. Neu-specific serum IgG titers were determined based on the greatest dilution of serum that gave a significant shift when staining 3T3-neu cells relative to background staining of 3T3 cells.

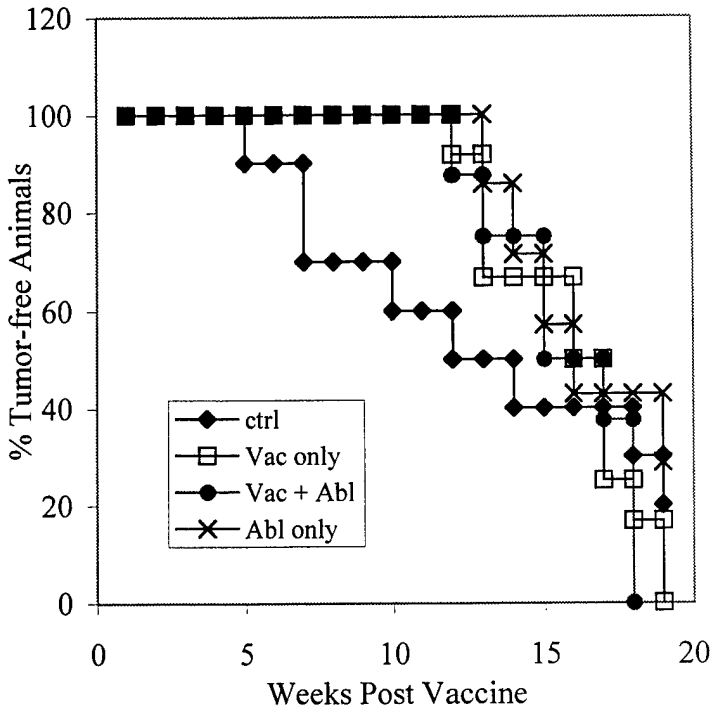

Figure 2. Study to Evaluate the Effects of the optimal VacciniaVaccine Combined with Local Ductal Ablation to Prevent Breast Cancer Development.
Eighteen mice per group were received either: 1) Vaccinia carrying the irrelevant HA gene (diamonds); 2) vaccinia carrying the HER-2/neu gene (open square); 3) vaccinia carrying the HER-2/neu gene + local ablation with wildtype vaccinia (closed circles); 4) local ablation with wildtype vacinnia only (X). Mice were monitored weekly for the development of endogenous mammary tumors.

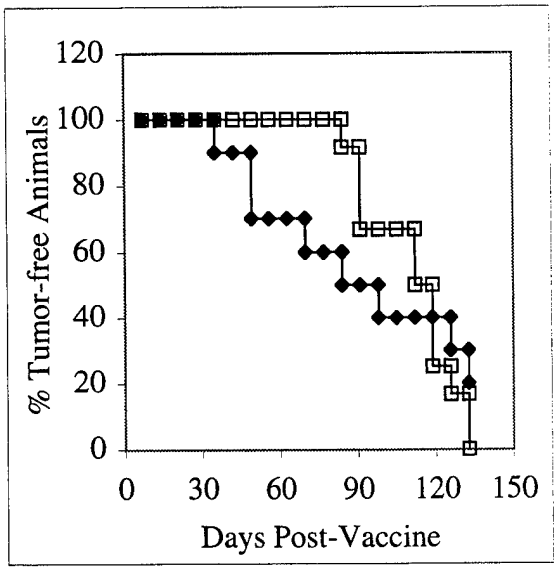

Figure 3.Neu-specific vaccination is protective against spontaneous tumor formation in neu-N transgenic mice. A series of 5 neu rVV vaccinations were given i.p. to neu-N mice beginning at 20 weeks of age (3 weeks after weaning from pups) and the mice monitored for spontaneous mammary tumor formation. Vaccinated animals (open squares) (n = 12) showed a significant delay in the onset of tumor formation relative to age-matched mock-vaccinated post-lactational control animals (filled diamonds) (n = 12) (p = 0.02 at day 84). These data represent the results from one of two duplicate experiments, each with essentially identical results. Similar results were obtained with 3T3-neu/GM vaccination (n = 12) (data not shown).

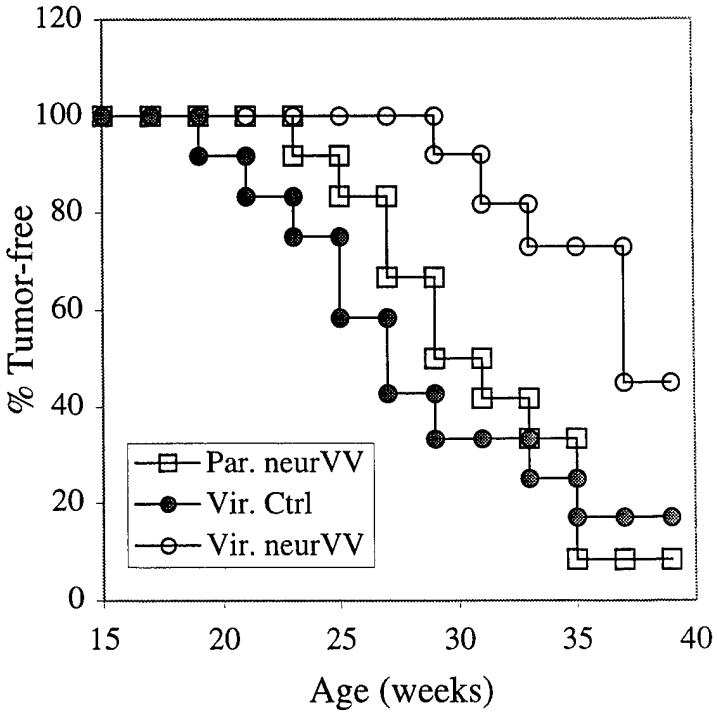

Figure 4. Vaccine efficacy is decreased in post-lactational females. Homozygous HER-2/neu transgenic littermates were divided into breeding (parous) and non-breeding (virgin) groups. One week after the first litter of pups was weaned from the parous females, animals received 1 x 10^7 pfu of either HA rVV (virgin ctrl) or neu rVV (virgin neu and par neu). Animals were boosted with the same dose of rVV every other week for a total of 6 vaccinations. Animals were monitored for the development of spontaneous mammary tumors.

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Appendix 5

HER-2/*neu* Is a Tumor Rejection Target in Tolerized HER-2/*neu* Transgenic Mice[1]

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ABSTRACT

HER-2/*neu* (*neu*-N) transgenic mice, which express the nontransforming rat proto-oncogene, develop spontaneous focal mammary adenocarcinomas beginning at 5–6 months of age. The development and histology of these tumors bears a striking resemblance to what is seen in patients with breast cancer. We have characterized the immunological responses to HER-2/*neu* (neu) in this animal model. neu-positive tumor lines, which were derived from spontaneous tumors that formed in *neu*-N animals, are highly immunogenic in parental, FVB/N mice. In contrast, a 100-fold lower tumor challenge is sufficient for growth in 100% of transgenic animals. Despite significant tolerance to the transgene, neu-specific immune responses similar to those observed in breast cancer patients can be demonstrated in *neu*-N mice prior to vaccination. Both cellular and humoral neu-specific responses in transgenic mice can be boosted with neu-specific vaccination, although to a significantly lesser degree than what is observed in FVB/N mice, indicating that the T cells involved are less responsive than in the nontoleragenic parental strain. Using irradiated whole-cell and recombinant vaccinia virus vaccinations we are able to protect *neu*-N mice from a neu-expressing tumor challenge. T-cell depletion experiments demonstrated that the observed protection is T cell dependent. The vaccine-dependent neu-specific immune response is also sufficient to delay the onset of spontaneous tumor formation in these mice. These data suggest that, despite tolerance to neu in this transgenic model, it is possible to immunize neu-specific T cells to achieve neu-specific tumor rejection *in vivo*. These transgenic mice provide a spontaneous tumor model for identifying vaccine approaches potent enough to overcome mechanisms of immune tolerance that are likely to exist in patients with cancer.

INTRODUCTION

The development of vaccine approaches that induce antigen-specific antitumor immune responses against solid tumors is an active area of immunological research. There are many reports demonstrating the induction of antigen-specific T-cell responses potent enough to eradicate murine tumor cells that have been genetically modified to express model antigens such as β-galactosidase (1, 2), influenza A nuclear protein (3, 4), and the SV40 large T antigen (5–7). Whereas these model systems support a potential clinical role for vaccines, they fail to address the issue of host tolerance to naturally expressed TAs[3] that is likely to exist in patients with cancer. Rather, these model systems target an immune response against a protein that is artificially overexpressed by the model tumor and not normally expressed in these animals during T-cell development and selection. Therefore, tolerance to the TA is not expected to occur and create the same complications that are encountered in the treatment of human cancers.

MART-1, gp-100, tyrosinase, and neu[3] are among an increasing number of naturally occurring human TAs that have been demonstrated to be T-cell targets (8–12). These antigens have the potential to serve as tumor rejection antigens *in vivo*. Interestingly, these antigens are tumor-associated or differentiation antigens rather than antigens that are only expressed by the tumor. Thus, these antigens are self-antigens against which natural mechanisms of T-cell deletion or peripheral tolerance are expected to occur. These findings provide strong evidence that T cells that target antigens expressed by spontaneously arising tumors may be susceptible to tolerizing mechanisms in the host.

TA transgenic mouse models are being developed to specifically dissect the mechanisms of TA-directed tolerance and to identify more potent vaccine strategies that have the potential to overcome these mechanisms of tolerance. TA transgenic mice, such as those that express carcinoembryonic antigen (13–15), prostate-specific antigen (16), HA (17), Friend murine leukemia virus envelope protein (18), and MUC-1 (19) seem to be more clinically relevant models because antigen-specific tolerance has been shown to occur against the protein encoded by the transgene. However, these mice fail to demonstrate antigen-associated spontaneous tumor development. In contrast, TA transgenic mice that express the v-Ha-*ras* oncogene develop spontaneous breast cancers. However, these spontaneous breast cancers are highly immunogenic, and the T-cell response is not directed against the transgene-encoded *ras* oncogene (20). Spontaneous tumor development is also seen in the TRAMP mouse model for prostate cancer (21, 22), however, the immunological response to tumor in this model has not been fully characterized.

neu is an attractive target for enhancing antitumor immunity because neu-specific antibody (23, 24) and T-cell (25–29) responses have been demonstrated in patients with neu-expressing mammary and ovarian cancers. Yet, neu-expressing tumors in these same patients continue to grow and metastasize, indicating that immune tolerance exists to the proto-oncogene. Two types of neu transgenic mice have also been developed. *neu*-N transgenic mice developed by Guy *et al.* (30), which were derived from the FVB/N strain, overexpress the nontransforming rat *neu* cDNA under the control of a mammary-specific promoter. As a consequence, these mice develop spontaneous focal mammary adenocarcinomas beginning at approximately 125 days, with the majority of mice developing spontaneous tumors by 300 days. In a similar transgenic strain expressing the activated *neu* oncogene *neu*-T (31, 32), in which a point mutation renders the *neu* gene product constitutively active, animals rapidly develop spontaneous mammary tumors (100% of animals develop tumors by ~30 weeks of age) with total glandular involvement, indicating that *neu*-T overexpression alone is sufficient for mammary carcinogenesis. These mice do not seem to exhibit tolerance to neu (33, 34).

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[3] The abbreviations used are: TA, tumor antigen; neu, HER-2/*neu*; *neu*-N, wild-type HER-2/*neu*; *neu*-T, mutated transforming HER-2/*neu*; NK, natural killer; FACS, fluorescence-activated cell sorter; ELISPOT, enzyme-linked immunospot assay; rVV, recombinant vaccinia virus; pfu, plaque-forming unit; mGM-CSF, murine granulocyte-macrophage colony-stimulating factor; HA, hemagglutinin; 3T3, NIH-3T3; 3T3/GM, 3T3 cells producing mGM-CSF; 3T3-*neu*, NIH-3T3 cells expressing rat *neu*-N; 3T3-*neu*/GM, 3T3-*neu* cells producing mGM-CSF; ATCC, American Type Culture Collection; RT-PCR, reverse transcription-PCR.

The stochastic appearance of spontaneous mammary tumors in the *neu*-N mice suggests that, as in the clinical setting, mammary carcinogenesis occurs through a multistep process in which *neu*-N overexpression is an early and necessary event (30, 35, 36). Because neu is expressed as a transgene in *neu*-N mice, these animals would, likewise, be expected to demonstrate similar tolerance to neu. We describe here the immunological characterization of the *neu*-N transgenic mouse model of breast cancer. Our findings demonstrate that tolerance to neu exists in these mice relative to nontransgenic mice. In addition, it is possible to induce neu-targeted protective immunity in the *neu*-N mice potent enough to overcome tolerance and significantly delay transplantable and spontaneous neu-expressing tumor development.

MATERIALS AND METHODS

Mice. *neu*-N transgenic mice (line 202; Ref. 30) were bred to homozygosity as verified by Southern blot analysis (data not shown). FVB/N mice were obtained commercially from the National Cancer Institute (Bethesda, MD). All experiments involving the use of mice were performed in accordance with protocols approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine (Baltimore, MD).

Cell Lines and Media. Five neu-expressing mammary tumor cell lines, designated NT1–NT5, were derived from spontaneous mammary tumors in female *neu*-N mice. *In vitro* cell lines were established by digestion of spontaneous tumors with dispase and collagenase (Roche Molecular Biochemicals, Indianapolis, IN), followed by differential trypsinization to remove fibroblasts (37). NT lines were grown in our defined Breast Media, which consisted of RPMI (Life Technologies, Inc., Grand Island, NY) with 20% fetal bovine serum (Hyclone Laboratories, Logan, UT), 1% L-glutamine, 1% nonessential amino acids, 1% Na pyruvate, 0.5% penicillin/streptomycin, 0.02% gentamicin (JRH Biosciences, Lenexa, KS), and 0.2% insulin (Eli Lilly and Co., Indianapolis, IN), and maintained at 37°C in 5% CO_2. NIH-3T3 cells (ATCC, Manassas, VA) were grown in 3T3-media: DMEM (Life Technologies, Inc.) with 10% bovine calf serum, 1% L-glutamine, 1% Na pyruvate, 1% non-essential amino acids, and 0.5% penicillin/streptomycin at 37°C and 10% CO_2. The 3T3-neu cell line ATCC CRL-1915 (ATCC), which expresses rat neu (38), was grown in 3T3 media + 0.3 μM methotrexate at 37°C in 10% CO_2. The 3T3 and 3T3-neu lines were genetically modified to express mGM-CSF by retroviral transduction with the MFG murine GM-CSF-encoding retrovirus, using previously described methods (39) to produce the 3T3/GM and 3T3-*neu*/GM cell lines. This method of mGM-CSF gene transfer results in high-efficiency gene delivery (~100% of cells) and, therefore, does not require subsequent cell selection (39, 40). mGM-CSF production was assessed with a commercially available ELISA kit (Endogen, Woburn, MA) and was determined to be 200–250 ng/10^6 cells/24 h for 3T3/GM and 3T3-neu/GM cells. mGM-CSF production by the parental and tumor cell lines (3T3, 3T3-neu, NT2, and NT5) was not detectable by ELISA. Functional assessment of mGM-CSF production was performed using the mGM-CSF-dependent cell line NSF-60 (39). The NT2 and NT5 tumor lines were expanded to large numbers to produce master cell banks of each line to avoid extensive *in vitro* passage and to insure the reproducibility of each *in vivo* study. Production was performed at the NIH cGMP facility (Frederick, MD). neu and MHC I levels were tested by FACS and confirmed to be stable before freezing and storage in liquid nitrogen.

Flow Cytometry Analysis. NT cell lines were assayed by flow cytometry using antibodies against murine MHC I (28-14-8), human MHC I (W632), and the neu protein (neu Ab4; Oncogene Science, Cambride, MA). A fluorescein-conjugated goat antimouse IgG2a secondary antibody was used to detect expression of surface molecules. To determine neu-specific serum IgG titers, 3T3 cells, and 3T3-*neu* cells were stained using mouse serum as the primary antibody. A fluorescein-conjugated goat antimouse γ-chain pan IgG secondary antibody was used to detect bound serum IgG (Vector Laboratories, Inc., Burlingame, CA). A FACScan Flow Cytometer and CellQuest Software (Becton Dickinson, San Diego, CA) were used to acquire and analyze data.

PCR Primers and RT-PCR. The thymus was removed from *neu*-N mice at gestational day 18, age 7 days, age 10 weeks, or age 15 weeks (1 week after weaning from pups); and the thymocytes were separated from the thymic stroma using a cell strainer. RNA was then isolated using RNAzol, and reverse transcription was performed using DNase I, RNase Inhibitor, and AMV Reverse Transcriptase (Life Technologies, Inc.) according to the manufacturer's specifications. The 5′- and 3′-neu-specific primers 5′-ATTCATCATTGCAACTGTAGA-3′ and 5′-AAGCACCTTCACCCTTCCTTA-3′, respectively, amplify a 222-bp region between bases 2140 and 2362 of the rat *neu*-N cDNA. Primers that amplify a 260-bp fragment of β-2 microglobulin (41) were used as standard and positive control in these assays.

Whole-Cell Vaccinations. On the day of vaccination, cells grown *in vitro* were trypsinized, washed three times in HBSS (pH 7.4), and counted. The cells were resuspended in HBSS at 10^7 cells/ml and irradiated with 5,000 rad from a ^{137}Cs source discharging ~1400 rad/min. *neu*-N or FVB/N mice, 8 weeks of age, were given three 100-μl s.c. injections (right and left forelimb, left hind limb) using a 1-ml tuberculin syringe with a 27-gauge needle.

rVV Vaccinations. To generate recombinant vaccinia expressing *neu*-N (*neu* rVV), the 5-kb *Hin*dIII/*Sal*I fragment from pSV2-*neu*-N was cloned into pSC11–1, and rVV was prepared and amplified as described (42). neu expression was verified by FACS analysis of neu rVV-infected 3T3 cells. *neu*-N mice, 8 weeks of age, received four weekly injections of 3×10^7 pfu/animal given i.p. using a 1-ml tuberculin syringe with a 27-gauge needle. rVV expressing the influenza HA protein HA rVV, used as a negative control, was a gift from Dr. Hyam Levitsky (Department of Oncology, The Johns Hopkins School of Medicine, Baltimore, MD).

Tumor Challenge. To titrate NT cell lines *in vivo*, *neu*-N or FVB/N mice, 8 weeks of age, were given s.c. injections of NT cells in HBSS at doses ranging from 1×10^3 cells to 5×10^5 cells for *neu*-N mice and 5×10^5 to 1×10^7 cells for FVB/N mice. Animals were then monitored twice weekly for the development of palpable (>5 mm diameter) tumors at the challenge site. For tumor challenge experiments, *neu*-N mice were given 5×10^4 NT cells s.c. in the right hind limb 14 days after receiving a whole-cell vaccine or 7 days after the final rVV vaccination.

T-Cell Assays. ELISPOT analysis for neu-specific T cells was performed using splenocytes isolated from *neu*-N mice. Mice were given a s.c. NT2 challenge, followed 3 days later by vaccination with 3T3-*neu*/GM or 3T3/GM. On day 12 after the vaccine, splenocytes were isolated by Ficoll separation and passed through a nylon wool column to remove B-cell and macrophage contaminants. neu-specific IFN-γ production was determined by a standard ELISPOT protocol (42) following a 24-h incubation of 5×10^5 NT2 target cells/well with serial dilutions of T cells (1×10^5 cells/well to 1×10^3 cell/well). Reagents used in the assay were: 10 μg/ml rat antimouse IFN-γ (PharMingen, San Diego, CA), 2 μg/ml biotinylated rat antimouse IFN-γ (PharMingen), 2 μg/ml avidin-alkaline phosphatase (Sigma Chemical Co., St. Louis, MO), and 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (Sigma Chemical Co.).

neu-specific lysis was determined as described previously (42). Briefly, spleens were excised from mice vaccinated 2 weeks before assay. Splenocytes were isolated by Ficoll separation and incubated for 5 days in the presence of murine IL-2 and IFN-γ and mitomycin-C-treated NT2 cells. Lytic function was determined against 3T3 and 3T3-*neu* cells in a 4-h chromium-51 release assay with and without the CD8-blocking rat monoclonal antibody 2.43 and the CD4-blocking rat monoclonal antibody GK1.5. The percentage of neu-specific lysis was determined by the following formula: % neu-specific lysis = (% lysis against 3T3-*neu* targets) − (% lysis against 3T3 targets).

T-Cell Depletion. The depletion of $CD4^+$ and $CD8^+$ T-cell subsets was accomplished by i.p. injection of 500 μg GK1.5 (anti-CD4) or 2.43 (anti-CD8) antibody, respectively, given every other day for 3 days. Depletion of $CD4^+$ and $CD8^+$ T cells was verified by FACS analysis of splenocytes 1 day after the three injections and maintained by continuing the antibody injections twice weekly for the duration of the tumor challenge experiment. Spleens were excised from additional animals in each group throughout the experiment to confirm the depletion of lymphocyte subsets. The anti-NK cell antibody pk136 was used as a negative control.

Prevention of Spontaneous Tumor Formation. Because lactation hastens spontaneous tumor formation in *neu*-N mice,[4] females, 8–10 weeks of age, were allowed to breed and the pups were weaned 3 weeks after birth. The parous *neu*-N females (~20 weeks of age, 3 weeks after weaning from pups)

[4] R. Reilly, unpublished observations.

were given a series of five weekly injections of 1×10^7 pfu neu rVV (vaccine group) or no treatment (control group) and were monitored for the development of spontaneous mammary tumors.

Statistical Analyses. Statistical analyses were performed using the Statview software program. Kaplan-Meier nonparametric regression analyses for tumor-protection and tumor-prevention experiments were performed, and significance was determined using the Mantel-Cox log rank test.

RESULTS

Establishment of neu-expressing Mammary Tumor Cell Lines. *neu*-N transgenic mice spontaneously develop focal neu-expressing mammary adenocarcinomas (30). These mice provide a more relevant model in which to identify antigen-specific vaccines that are potent enough to prevent natural tumor development. To facilitate the identification of neu-specific vaccine strategies that are potent enough to prevent spontaneous tumor formation, neu-expressing transplantable tumor lines were derived from five different spontaneous mammary tumors arising in these mice. Tumors were excised, digested with dispase and collagenase, and established *in vitro* using defined breast media as described in "Materials and Methods." Tumor cells were separated from untransformed epithelial cells by differential trypsinization (37), and the resulting neu-expressing tumor cell lines were given the designation NT1–NT5. FACS analysis of the NT lines showed similar levels of surface neu and MHC class I (data for NT2 and NT5 are shown in Fig. 1). NT2 and NT5 cells were each expanded to generate a certified master cell bank of each line at the National Cancer Institute cGMP laboratory (Frederick, MD). Each lot demonstrated stable neu and MHC I expression when grown *in vitro* for up to 3 months (data not shown). Frozen aliquots of each lot were thawed, expanded for 1 week *in vitro*, and used in tumor challenge experiments. Both cell lines were tested at least twice in each of the experiments described below.

Spontaneous Tumors are Highly Immunogenic in Parental, But Not Transgenic Mice. To evaluate the immunogenicity of the neu tumors *in vivo*, naïve FVB/N and *neu*-N mice were given a s.c. tumor challenge consisting of either NT2 or NT5 cells in HBSS and monitored for the development of palpable tumors (>5 mm mean diameter). FVB/N mice were able to reject an NT5 challenge at doses below 5×10^6 cells/animals (Fig. 2). In contrast, *neu*-N mice demonstrated significant tolerance to the neu-expressing tumor line; the minimum dose required for tumor growth in 100% of animals was at least 100-fold lower for the transgenic mice compared with FVB/N mice. Data obtained using the NT2 cell line were virtually identical to what was seen with an NT5 tumor challenge (data not shown).

Analysis of Transgene Expression in the Thymus of *neu*-N Mice. To determine whether central deletion contributes to the neu-specific T-cell tolerance observed in *neu*-N mice, we examined transgene expression in the thymus of fetal, neonatal, and adult *neu*-N mice by RT-PCR. Samples of mRNA were obtained from both the thymic stroma and thymic epithelium, and primers were used to amplify a 300-bp fragment from the rat *neu*-N cDNA, using primers for β2-microglobulin as a control. As shown in Fig. 3, the data indicated that neu expression is seen in parous adult thymi but not in samples from fetal or virgin adult mice. In addition, low-level neu expression was also found in newborn mice. The apparent discrepancy in thymic expression of neu in newborn and virgin adult mice may be explained by the exposure of the newborns to hormones in their milk that activated the MMTV promoter driving the rat *neu*-N transgene. Control samples taken from parental FVB/N animals showed no detectable neu expression.

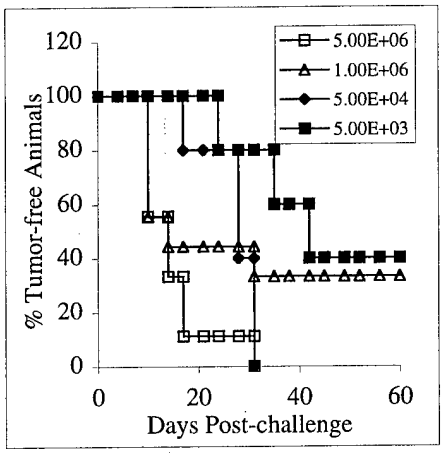

Fig. 2. neu tumor cells are immunogenic in parental but not transgenic mice. FVB/N mice (*open symbols*; $n = 10$ mice/dose of cells) and neu mice (*closed symbols*; $n = 10$ mice/dose of cells) were given a s.c. tumor challenge in the right hind limb that consisted of the indicated number of NT5 cells suspended in HBSS. Animals were then monitored for the development of palpable tumors. This experiment was repeated at least two times using either NT5 or NT2 cells, with similar results.

Transgenic Mice Demonstrate neu-specific Immune Responses. Vaccination of FVB/N mice with a neu-specific irradiated whole-cell vaccine consisting of 3T3-*neu* cells transduced to secrete murine GM-CSF results in the induction of a neu-specific antibody response (Fig. 4), as well as the generation of neu-specific CTLs (data not shown). Because the transgenic mice demonstrated profound tolerance to neu-expressing tumors *in vivo*, we sought to determine whether or not a neu-specific immune response analogous to that observed in the parental animals could be induced by vaccination. As with the FVB/N animals, *neu*-N mice were vaccinated with either 3T3/GM or 3T3-*neu*/GM, and serum samples were obtained 14 days after vaccination. Titers of neu-specific serum IgG were determined

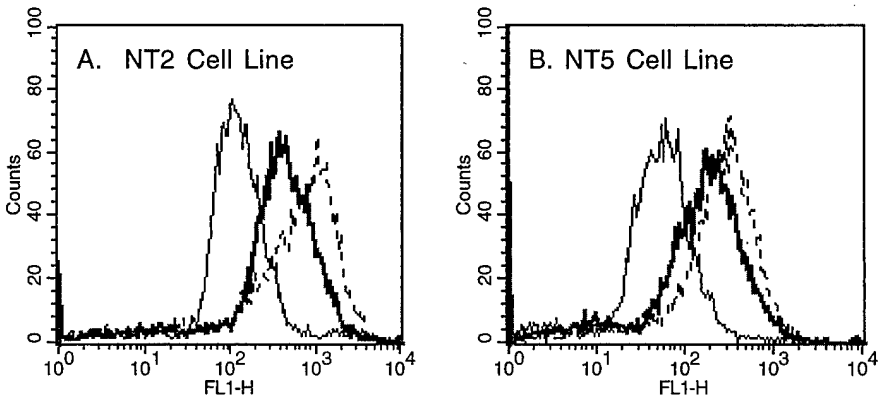

Fig. 1. Transplantable mammary tumor lines express similar levels of neu and MHC class I. Tumor cells, derived from spontaneous mammary tumors in transgenic mice and established *in vitro*, were analyzed by FACS for surface protein expression using primary antibodies against neu (c-*neu* Ab4) and MHC class I (28-14-8 s). *A*, NT2 cell line 1 week after thaw from cell bank. *B*, NT5 cell line 1 week after thaw from cell bank. Levels of neu (*dashed line*) and MHC I (*heavy gray line*) show a significant shift in fluorescence relative to the curve obtained using an irrelevant antibody (W632, *black line*).

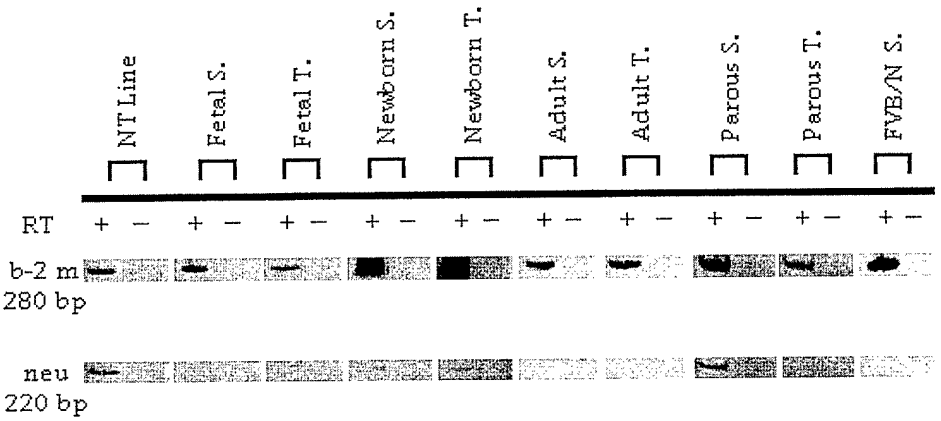

Fig. 3. HER-2/*neu* expression is not detected in the thymus during the peak of T-cell development. The thymic stroma (*S*) and thymocytes (*T*) were obtained at gestational day 18 (fetal), age 7 days (fetal), age 10 weeks (adult), and postlactational (parous) *neu* mice and mRNA was extracted. RT-PCR was then performed with primers for neu, using primers for β-2 microglobulin as a positive control. Neu expression was detected in mRNA samples from a neu-expressing cell line (NT5 line) as well as in the thymic stroma of parous females and in both the stroma and thymocytes of newborn mice. No neu signal was detected in thymic stroma from nontransgenic mice (FVB/N S.).

by FACS analysis of serial dilutions of mouse serum. In contrast to FVB/N mice, transgenic mice demonstrate low basal levels of neu-specific IgG (Fig. 4). Vaccination with a GM-secreting 3T3-*neu* vaccine resulted in an increase in neu-specific IgG relative to mock-vaccinated animals, although the level of induction was much less than that seen in FVB/N mice.

Having demonstrated that vaccination can boost the low levels of neu-specific antibody in transgenic mice, we then sought to determine whether or not neu-specific T-cell responses could also be induced in these mice. Transgenic mice were given a s.c. tumor challenge (5×10^4 NT2 cells), followed 3 days later with either a 3T3-*neu*/GM vaccine (vaccine group) or a 3T3/GM vaccine (control group). On day 12 after the vaccine, splenocytes were isolated, passed through a nylon wool column to remove B-cell and macrophage contaminants, and assayed for neu-specific IFN-γ production by ELISPOT assay. As shown in Fig. 5*A*, mice receiving a neu-specific vaccine showed a 3–4-fold increase in the number of T cells producing IFN-γ in response to NT2 cells relative to mock-vaccinated mice. Further evidence of neu-specific effector function was demonstrated by chromium-51-release assay using 3T3-*neu* cell targets. Animals were vaccinated with 1×10^6 irradiated 3T3 or 3T3-*neu*/GM cells, and splenic T cells were isolated 14 days later. T cells, cultured for 5 days in the presence of interleukin 2 and IFN-γ and mitomycin-C-treated NT2 cells, were assayed for the lysis of 3T3 or 3T3-*neu* target cells in a 4-h chromium-release assay. As shown in Fig. 5*B*, splenocytes from 3T3-*neu*/GM-vaccinated mice demonstrated $CD8^+$ T cell-dependent lysis that is not seen with splenocytes from 3T3/GM vaccinated control mice. This neu-specific lysis is almost completely abrogated by the addition of the CD 8-blocking antibody 2.43.

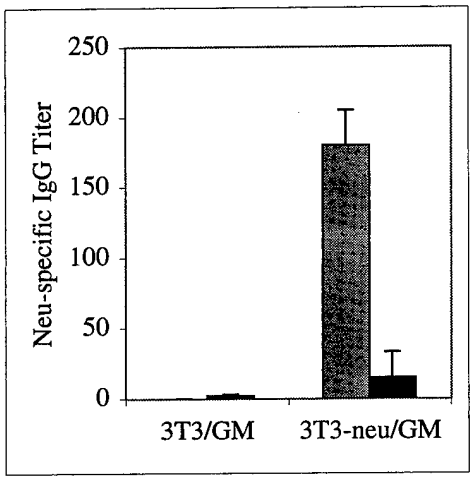

Fig. 4. A neu-specific whole-cell vaccine elicits an antibody response in both FVB/N and *neu*-N mice. Serum was obtained from FVB/N mice (▨) and *neu*-N mice (■) 14 days after vaccination with irradiated 3T3/GM or 3T3-*neu*/GM cells. Serial dilutions of serum were then used as the primary antibody solution in a FACS analysis of both 3T3 and 3T3-*neu* cells using a secondary antibody against murine IgG. Neu-specific serum IgG titers were determined based on the greatest dilution of serum that gave a significant shift when staining 3T3-*neu* cells relative to background staining of 3T3 cells.

neu Can Serve as a Tumor Rejection Target in Vaccinated Transgenic Mice. Because we were able to demonstrate inducible neu-specific immune responses in the form of antibody production and CTL generation, we next sought to determine whether a protective *in vivo* antitumor response could be directed against neu-expressing tumors despite tolerance to neu in the transgenic mice. To ensure that neu was the *in vivo* tumor rejection antigen, we used two vaccination methods in which neu was the only target antigen. The vaccines tested, whole-cell (3T3-*neu*/GM) and *neu* rVV, each express only rat neu in common with the mammary tumor. As shown in Fig. 6*A*, *neu*-N mice given a 3T3-*neu*/GM vaccine demonstrated a significant delay in NT2 tumor development relative to mice treated with the control 3T3/GM vaccine ($P < 0.0005$). Although unlikely, 3T3 cells may express antigens other than neu in common with the mammary tumors. However, this delay in transplantable tumor development was confirmed to be neu-specific by vaccinating *neu*-N mice with rVV-expressing neu. Mice vaccinated with one i.p. injection of rVV-expressing neu demonstrated as significant delay in NT2 tumor growth compared with mice vaccinated with a control rVV expressing the irrelevant HA antigen ($P < 0.01$). Similar results were obtained in two additional experiments in which animals received an NT5 challenge (data not shown). In contrast, FVB/N mice, which do not exhibit tolerance to neu, were completely protected from tumor challenge after identical vaccine protocols (Fig. 6*B*). These data clearly demonstrate that, although profound tolerance to neu exists in *neu*-N mice, neu-specific vaccination can generate immune responses that are potent enough to significantly delay the growth of transplantable neu-expressing tumors.

Both $CD4^+$ and $CD8^+$ T Cells Are Necessary for the Induction of Antitumor Immunity. Next, we depleted *neu*-N mice of either $CD4^+$ T cells or $CD8^+$ T cells prior to vaccination with 3T3-*neu*/GM to determine whether the protection we had observed in the above experiments was, in fact, T-cell mediated. Animals were given i.p. injections of either the anti-CD4 GK1.5 or the anti-CD8 antibody 2.43 to deplete the respective T-cell subset. Depletion was verified by FACS analysis of splenic T cells prior to vaccination. Two weeks after vaccination with 3T3-*neu*/GM cells, animals were given an NT2 challenge and monitored for the development of palpable tumors. The data, summarized in Table 1, show that mice depleted of $CD4^+$ T cells develop tumors with kinetics that are similar to that seen in unvacci-

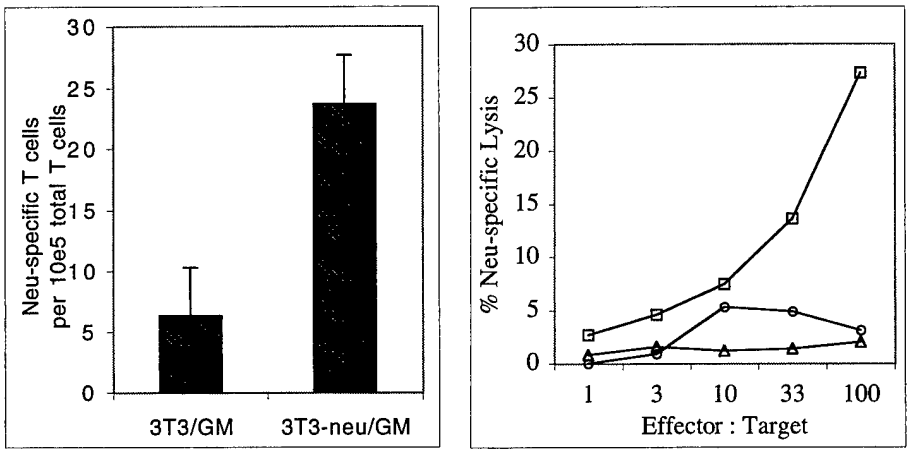

Fig. 5. neu-specific vaccination of transgenic mice results in an increase in neu-specific T cells. *A*, ELISPOT analysis was used to determine the number of neu-specific T cells induced in neu mice with a 3T3-*neu*/GM vaccine. Transgenic mice were given a s.c. NT2 challenge, followed 3 days later by vaccination with 3T3-*neu*/GM or 3T3/GM. Spleens were removed 12 days later, and splenocytes were obtained by Ficoll separation. ELISPOT analysis was then used to detect the production of IFN-γ in response to NT2 cells. The data shown are from two separate experiments. *B*, a 4-h chromium-release assay was performed using splenocytes isolated from transgenic mice to determine the relative increase in neu-specific lysis of 3T3-*neu* cells after vaccination. Splenocytes were isolated from neu mice 14 days after vaccination with irradiated 3T3/GM or 3T3-*neu*/GM cells, incubated 5 days in the presence of mitomycin-C-treated and IFN-γ-treated NT5 cells, and used in a 4-h chromium-release assay. Both 3T3 and 3T3-*neu* cells were used as targets in the assay. Neu-specific lysis is shown for neu vaccinated (□) and control (△) groups. ○, neu-specific lysis in the presence of the CD8-blocking antibody 2.43. This experiment was repeated three times with similar results.

nated, undepleted controls. The deletion of $CD8^+$ T cells has a less dramatic effect on tumor growth, although NT2 growth is statistically distinct from vaccinated, undepleted animals. The tumor-free survival of animals given the NK cell-depleting antibody pk136 and vaccine were identical to that of undepleted, vaccinated mice. These data establish the importance of both $CD4^+$ and $CD8^+$ T cells in inducing neu-specific immunity.

neu-specific Vaccination of Parous Mice Results in Delayed Spontaneous Tumor Growth. Next, we sought to evaluate the neu-specific vaccines for their effectiveness in the prevention of spontaneous tumor formation in *neu*-N mice. Because postlactational *neu*-N females show a slightly earlier onset of spontaneous tumor formation relative to age-matched virgins,[4] parous *neu*-N females were used. After their first litter was weaned, transgenic females were given five weekly injections consisting of 3×10^7 pfu neu rVV (beginning at ~20 weeks of age). The animals were then monitored weekly for the development of spontaneous tumors. As shown in Fig. 7, there was a significant delay in the onset of tumor formation in the vaccinated mice by 7 weeks after the initial vaccination, relative to unvaccinated controls ($P = 0.02$). Nearly identical results were obtained using whole-cell vaccination (3T3-*neu*/GM; data not shown), demonstrating that the neu-specific vaccines can delay tumor onset and suggesting that more complete protection may be possible.

DISCUSSION

Here, we describe the immunological characterization of the transgenic mouse model of mammary cancer developed by Guy *et al.* (30) in which the nonactivated form of neu is overexpressed in the mammary tissue. These mice demonstrate tolerance to neu relative to parental animals. However, targeted vaccine approaches can induce neu-specific T-cell responses potent enough to significantly delay the development of transplantable neu-expressing tumors. These vaccine strategies are also potent enough to induce a significant delay in the

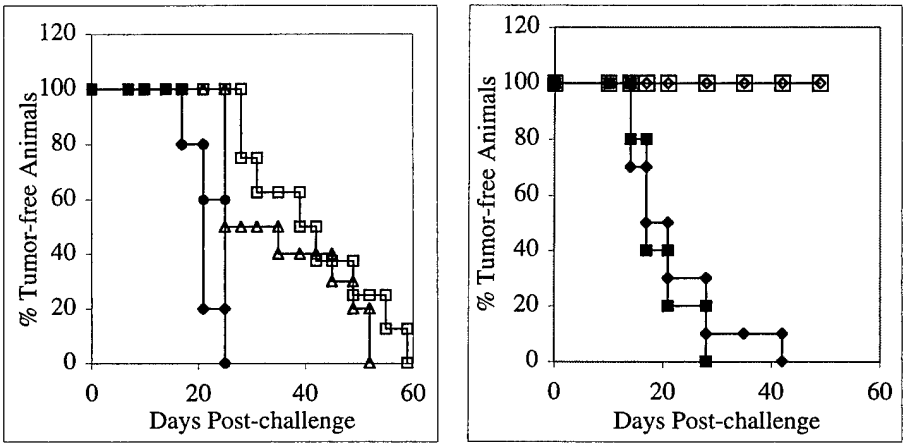

Fig. 6. neu is the *in vivo* rejection antigen in transgenic mice. *A*, transgenic mice received either a 3T3-*neu*/GM vaccine given s.c. (□; $n = 10$ mice) or neu rVV (△; $n = 10$ mice), followed 2 weeks later by a s.c. NT2 challenge in the right hind limb. Animals were then monitored for the formation of palpable tumors. Control animals received either 3T3/GM (◆) or HA rVV (●; $n = 10$ mice/group), with *P*s of 0.005 and 0.01 for the whole-cell and rVV groups, respectively. The data shown are combined from two separate experiments. Similar results were obtained with an NT5 challenge (data not shown). *B*, FVB/N mice vaccinated with irradiated 3T3-*neu*/GM vaccine given s.c. (□; $n = 12$ mice) or neu rVV given i.p. (◇; $n = 12$ mice) on day 0 were protected from a s.c. NT2 challenge given in the right hind limb on day 14. Control mice received mock vaccinations with either s.c. 3T3/GM (■) or i.p. HA rVV (◆; $n = 12$ mice/group). Similar results were obtained with identical vaccination protocols, followed by an NT5 challenge (data not shown).

spontaneous development of tumors. These findings demonstrate that despite tolerance to the transgene, neu can serve as a tumor rejection target recognized by T cells *in vivo*. Therefore, it should be possible to use this model to develop more potent vaccine approaches for the treatment and prevention of neu-expressing tumors.

It is clear from our data that neu tumors are highly immunogenic in nontransgenic mice in which tolerance to neu is absent. This is consistent with the findings of Bernards *et al.* (43) and Chen *et al.* (44), who induced protective immunity in nontransgenic NFS and FVB/N mice, respectively, that was sufficient to give protection from a neu-expressing tumor challenge. Similarly, Cefai *et al.* (34) and Amici *et al.* (33) demonstrated potent antitumor immunity using the FVB/N *neu*-T mammary tumor model, in which mice are not tolerant to the transgene-encoded *neu*-T oncoprotein. In separate studies using neu-specific whole-cell or plasmid DNA vaccinations, it was shown that *neu*-T mice were completely protected from neu-expressing tumor challenge (34), and spontaneous tumor development could also be prevented (33, 34). Thus, our data confirm that neu is a relevant tumor rejection target in nontransgenic immunocompetent mice. However, our data also demonstrate that neu is a relevant rejection target recognized by T cells in tolerized transgenic mice.

The presence of tolerance to a TA *in vivo* represents a significant challenge to successful immunotherapy of human cancers. For example, patients with breast cancer show neu-specific responses in the form of antibody and CTL, but these responses are not sufficient to prevent tumor progression (23, 24, 29). We have made a similar observation in the *neu*-N mice. Despite the fact that we were unable to detect neu expression in the thymus of fetal *neu*-N mice, the central deletion of high-avidity neu-specific T-cell populations is implicit in the qualitative and quantitative differences in neu-specific immune responses seen in FVB/N mice *versus neu*-N mice. It is possible that neu expression in the thymus, although below the detection level of RT-PCR, was sufficient to cause the deletion of high-avidity neu-specific T-cell precursors. Alternatively, because neu expression is seen in a number of tissues in *neu*-N mice (30), it is conceivable that neu antigen was acquired by antigen-presenting cells in the periphery and trafficked to the thymus for presentation during T-cell development. neu expression is demonstrated in the thymus at times when the mice have higher levels of peripheral neu expression (*i.e.*, when prolactin levels, an inducer of the MMTV promoter, are high during nursing or pregnancy). Therefore, it is probable that higher-avidity T cells that are present in the FVB/N mice and are responsible for the tumor immunogenicity observed have undergone deletion in the transgenic mice (45). The fact that antibody and T-cell responses are induced in *neu*-N mice after vaccination but are not fully protective strongly suggests that the T cells present in *neu*-N mice may well be of lower avidity and have more stringent requirements for activation and effector function. Consistent with this interpretation is our recent finding that there is a predominant oligoclonal TCR Vβ usage among the vaccine-induced FVB/N-derived T cells that is very different from the predominant oligoclonal TCR Vβ usage observed among the vaccine-induced transgenic-derived T cells.[5] It is also possible that the lower-avidity T cells undergo peripheral anergy induction in the transgenic mice.

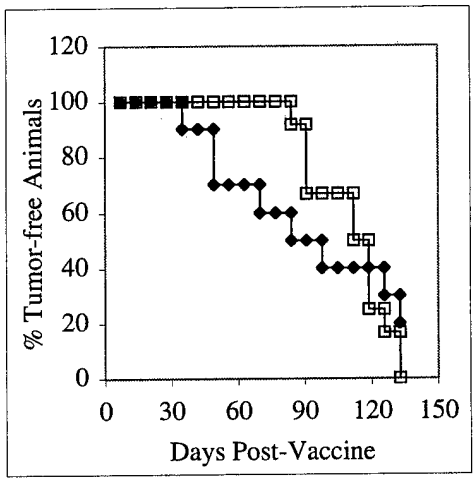

Fig. 7. neu-specific vaccination is protective against spontaneous tumor formation in *neu*-N transgenic mice. A series of five neu rVV vaccinations were given i.p. to *neu*-N mice beginning at 20 weeks of age (3 weeks after weaning from pups), and the mice were monitored for spontaneous mammary tumor formation. Vaccinated animals (□; $n = 12$) showed a significant delay in the onset of tumor formation relative to age-matched mock-vaccinated postlactational control animals (♦; $n = 12$; $P = 0.02$ at day 84). These data represent the results from one of two duplicate experiments, each with essentially identical results. Similar results were obtained with 3T3-*neu*/GM vaccination ($n = 12$; data not shown).

Table 1 *Effects of T-cell depletion in neu-N mice*

Transgenic mice were depleted of T-cell subsets, and depletion was maintained by twice-weekly i.p. injections of anti-CD4 antibody (GK1.5–CD4 depletion), anti-CD8 antibody (2.43–CD8 depletion), anti-NK antibody (pk136–NK depletion), or control antibody (W632–mock depletion). T-cell depletion was verified by FACS analysis of splenic T cells isolated from members of each group. Control animals (no vaccine) received HA rVV given i.p., and all other groups (vaccine) received neu rVV given i.p. after verification of T-cell depletion. Animals received a s.c. tumor challenge consisting of 5×10^4 NT2 cells injected s.c. 14 days after vaccination. Statistical significance values were determined using the log rank test as compared with the control group. These data represent the combination of at least three separate experiments.

Group (number of animals)	% Tumor-free animals (day 24 after challenge)	*P*
No depletion, no vaccine ($n = 30$)	0	—
Mock depletion, vaccine ($n = 31$)	75	0.007 (*vs.* no vaccine)
CD4 depletion, vaccine ($n = 22$)	0	0.31 (*vs.* no vaccine)
CD8 depletion, vaccine ($n = 33$)	40	0.02 (*vs.* no vaccine)
NK depletion, vaccine ($n = 30$)	68	0.004 (*vs.* no vaccine)

We have shown that two neu-specific vaccines can generate antitumor immunity in *neu*-N mice capable of significantly delaying tumor growth. These results clearly implicate neu as the *in vivo* rejection antigen. The fact that FVB/N mice are fully protected from a 100-fold greater tumor burden after an identical vaccination is indicative of the profound tolerance to neu in the *neu*-N mice. Depletion experiments carried out in *neu*-N mice demonstrated that the significant delay in tumor development is both $CD4^+$ and $CD8^+$ T cell mediated. It is unlikely that $CD4^+$ T cells play a direct role in tumor cell lysis because significant MHC II levels cannot be induced on NT cells, even after treatment with γ-IFN (data not shown). Thus, the role of $CD4^+$ T cells in tumor rejection seems to be at the level of T-cell help. Likewise, $CD8^+$ T-cell depletion abrogates the tumor-protective effects of neu-specific vaccination, although the delayed tumor growth observed in $CD8^+$ T cell-depleted mice is not as great as that seen in the undepleted, vaccinated mice. This may reflect the fact that the undepleted, vaccinated mice are capable of both neu-specific antibody production and CTL induction. It is likely that the vaccine-induced $CD4^+$ T-cell response up-regulates neu-specific humoral and CTL responses and that each contributes to the retardation of tumor growth.

Our studies also show that it is possible to induce protective immunity capable of delaying the development of spontaneous mammary tumors in the transgenic mice. The modest delay in tumor onset

[5] A. Ercolini and E. Jaffee, personal communication.

seen in our prevention studies is consistent with the delays seen in the tumor challenge model using virgin *neu*-N mice, thus giving validation to the use of transplantable tumors for the initial evaluation of vaccine approaches for tumor prevention. It is unlikely that neu-specific immune responses differ greatly in virgin *neu*-N mice vaccinated before inoculation with transplantable tumors *versus* vaccinated postlactational *neu*-N mice in which spontaneous tumors develop. Our investigation of *neu*-specific T-cell and antibody responses in virgin *neu*-N females, 8–10 weeks of age, clearly indicates these mice are already profoundly tolerant to neu. The prevention of spontaneous tumor formation has been reported in the *neu*-T transgenic mouse model using i.p. injections of antibody against neu (46) as well as whole-cell and plasmid DNA immunization (33, 34), demonstrating the successful use of neu-specific immunotherapy for tumor prevention in the absence of tolerance to neu. The fact that we were able to delay tumor onset in *neu*-N mice is significant in that it demonstrates that we can generate a neu-specific response in *neu*-N transgenic mice that is capable of overcoming *in vivo* tolerance and is sufficient to significantly delay spontaneous tumor development. Esserman *et al.* (47) also demonstrated vaccine-mediated prevention of spontaneous tumors using *neu*-N mice. Vaccination of mice that were heterozygous for the *neu*-N proto-oncogene with the neu extracellular domain was sufficient to prevent spontaneous tumor development in 50% of transgenic mice. The improved vaccine efficacy in their tumor prevention studies could be due to a number of factors. First, in our studies, we used mice that were homozygous for the *neu*-N transgene. It is possible that differences in neu expression in heterozygous *versus* homozygous mice results in different levels of tolerance to neu. Second, our protection experiments used postlactational *neu*-N mice. Whereas tolerance to neu is unlikely to differ significantly in postlactational *versus* virgin females, the increase in hormones such as prolactin that are associated with milk production are known to increase expression by the MMTV promoter, which drives neu expression in these mice. neu expression in the thymus, which is not detected in fetal or virgin females, is clearly up-regulated in nursing and postlactational mice. Third, these studies use whole-cell and rVV vaccines expressing the entire neu protein as opposed to plasmid DNA vaccination with the neu extracellular domain. Fourth, the timing of neu-specific vaccination may be important for vaccine efficacy. Esserman *et al.* (47) vaccinated heterozygous virgin *neu*-N females very early, at 10 weeks of age, well before the onset of tumorigenesis, and boosted at 24 weeks of age. In our model, animals were not vaccinated until 20 weeks of age, possibly at a time when these animals have significant, although not macroscopic, tumors.

Additional work is required to determine whether the level of transgene expression influences the extent of tolerance to neu, or whether lactational status has an effect on vaccine-mediated antitumor immunity. Furthermore, now that it is clear that neu-specific antitumor immunity can be induced in *neu*-N mice despite tolerance, the next step is to determine what factors are necessary for the improvement of vaccine efficacy.

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Cyclophosphamide, Doxorubicin, and Paclitaxel Enhance the Antitumor Immune Response of GM-CSF Secreting Whole-cell Vaccines in HER-2/*neu* Tolerized Mice.

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The abbreviations used are: *neu*, HER-2/*neu*; DOX, Doxorubicin; CIS, Cisplatin; PTX, Paclitaxel; CTX, Cyclophosphamide; MHC, major histocompatibility complex; CTL, cytolytic T lymphocyte; γ-IFN, gamma interferon; IL, interleukin; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; ELISPOT, enzyme-linked immuno-spot assay; mGM-CSF, murine granulocyte-macrophage colony-stimulating factor; T helper, Th; HBSS, Hank's balanced salt solution; 3T3, NIH-3T3; 3T3/GM, 3T3 cells producing mGM-CSF; 3T3-*neu*, NIH-3T3 cells expressing rat HER-2/*neu* cDNA; 3T3-*neu*/GM, 3T3-*neu* cells producing mGM-CSF; NT cells, neu-expressing tumor cell line derived from spontaneous tumor of *neu* transgenic mice; NT-B7, NT cells expressing human B7 co-stimulatory molecule.

ABSTRACT

Tumor-specific immune tolerance limits the effectiveness of cancer vaccines. In addition, tumor vaccines alone have a limited potential for the treatment of measurable tumor burdens. This highlights the importance of identifying more potent cancer vaccine strategies for clinical testing. We tested immune-modulating doses of chemotherapy in combination with a GM-CSF secreting HER-2/neu (neu) expressing whole-cell vaccine as a means to treat existing mammary tumors in antigen-specific tolerized *neu* transgenic mice. Earlier studies have shown that *neu* transgenic mice exhibit immune tolerance to the neu-expressing tumors similar to what is observed in patients with cancer. We found that Cyclophosphamide, Paclitaxel, and Doxorubicin, when given in a defined sequence with a GM-CSF-secreting, neu-expressing whole-cell vaccine, enhanced the vaccine's potential to delay tumor growth in *neu* transgenic mice. In addition, we showed that these drugs mediate their effects by enhancing the efficacy of the vaccine rather than via a direct cytolytic effect on cancer cells. Furthermore, Paclitaxel and Cyclophosphamide appear to amplify the T helper 1 neu-specific T cell response. These findings suggest that the combined treatment with immune-modulating doses of chemotherapy and the GM-CSF-secreting neu-vaccine can overcome immune tolerance and induce an antigen-specific antitumor immune response. These data provide the immunologic rationale for testing immune-modulating doses of chemotherapy in combination with tumor vaccines in patients with cancer.

INTRODUCTION

Cytokine-secreting, whole-cell cancer vaccines are currently being investigated for the treatment of solid tumors (1-3). In particular, tumor cells genetically modified to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) induce a systemic antitumor T cell response potent enough to cure mice with pre-established micrometastases (4). GM-CSF recruits dendritic cells to the vaccine site where they take up and process tumor antigens, subsequently presenting them in a form that can induce effective systemic T cell responses (5-7). Clinical trials testing both autologous and allogeneic tumor cells engineered to secrete GM-CSF for the treatment of a variety of human cancers have already been completed or are underway (8-11). Although induction of antitumor immunity and clinical responses have been demonstrated in some patients, it is unlikely that this current form of the vaccine is potent enough to be effective in the majority of patients with minimal residual disease or small numbers of metastases (8-11).

Studies aimed at identifying tumor-associated T cell antigens (12-13) and understanding antigen-specific T cell regulation (14-16) have provided new insights into the mechanisms of immune tolerance that may limit the effectiveness of cancer vaccines (17-20). For example, a number of non-mutated, tissue-specific proteins have been identified as T cell targets recognized on human tumors (12-13, 21-22). This implies that mechanisms are in place to delete or suppress high avidity T cells specific for these antigens that would otherwise be capable of inducing autoimmunity. This also implies that T cells with lower avidity for these same antigens may have escaped tolerance and are capable of being activated. This would explain reports describing the existence of ineffective antibody and T cell responses directed at specific antigens

expressed by simultaneously progressing cancers in patients (11, 23).

Several groups have observed that some chemotherapeutic agents can modulate the immune response (24-26). A number of reports have demonstrated that some chemotherapeutic agents can enhance the antitumor activity of adoptively transferred T cells (24-26), tumor vaccines (27), and macrophages (28). Other studies have revealed the synergistic effect of chemotherapy with passive immunotherapy using the HER-2/neu targeted antibody, trastuzumab (29-30). However, the potential interactions of chemotherapy with vaccines that induce T cell responses, particularly in models that exhibit tumor-specific tolerance, is relatively unexplored.

Mice transgenic for the non-transforming rat *neu* proto-oncogene expressed under the control of a mammary-specific promoter (*neu* transgenic mice) develop spontaneous focal mammary adenocarcinomas (31). We recently described the immunological characterization of these mice and found that T cell tolerance to neu exists in these mice relative to the parental non-transgenic mice (32). Despite the existence of tolerance, it was possible to induce neu-targeted immunity potent enough to overcome this tolerance and significantly delay both transplantable and spontaneously arising tumors. In this report, we have employed the *neu* transgenic mouse model to identify chemotherapeutic agents that, when given sequentially with a neu-expressing GM-CSF secreting whole tumor vaccine, can enhance vaccine efficacy. Our findings suggest that combined treatment with immune-modulating doses of chemotherapy and the GM-CSF-secreting neu vaccine can overcome immune tolerance and induce an antigen-specific antitumor immune response.

MATERIALS AND METHODS

Mice. *Neu* transgenic mice developed by Guy and colleagues (line #202) were bred to homozygosity as verified by Southern blot analysis (31). FVB/N mice were obtained commercially from Jackson Laboratories. All experiments involving the use of mice were performed in accordance with protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Cell lines and media. NT cells were derived from spontaneous mammary tumors of female *neu* transgenic mice as previously described (32). NT cell line overexpresses the rat *neu* cDNA and these levels remain stable (32). NT cell line was grown in our defined Breast Media, which consists of RPMI (Life Technologies, Grand Island, NY) with 20 % FBS (Hyclone, Logan, UH), 1% L-Glutamine (JRH Biosciences, Lenexa, KS), 1 % NEAA, 1 % Na Pyruvate, 0.5 % Pen/Strep, 0.02 % Gentamycin (Sigma, St. Louis, MO), and 0.2 % Insulin (Lilly, Indianapolis, IN), and maintained at 37° C in 5 % CO_2. The NT cell line was expanded to large numbers to produce master cell banks of each line to avoid extensive *in vitro* passage thereby maintaining reproducibility of each *in vivo* study. Production was performed at the NIH cGMP facility (Frederick, MD). Neu and Major Histocompatibility Complex (MHC) class I levels were tested by FACS and confirmed to be stable before and after freezing. NT-B7 cells were produced via retroviral transduction of NT cells with a human B7-1 encoding retrovirus as previously described (4). NT-B7 was maintained in our defined Breast Media supplemented with neomycin (Life Technologies, Grand Island, NY). NIH-3T3 cells (3T3) (ATCC, Rockville, MD) were grown in DMEM (Life Technologies, Grand Island, NY) with 10 % BCS (Hyclone, Logan, UH) 1 % L-Glutamine, 1 % NEAA, 1 % Na Pyruvate, 0.5 % Pen/Strep at 37 C and 10 % CO_2. The

NIH-3T3 derivative, ATCC CRL-1915, (3T3-*neu*) (ATCC, Rockville, MD), which overexpresses the rat HER-2/*neu* proto-oncogene, was grown in 3T3 media + 0.3 μM methotrexate at 37°C in 10 % CO_2. NIH-3T3 cells and 3T3-*neu* were genetically modified to express the murine cytokine GM-CSF using the MGF retroviral gene transfer system as previously described (4) resulting in 3T3/GM and 3T3-*neu*/GM cell lines, respectively. Murine GM-CSF production was tested with a commercially available ELISA kit (Endogen,Woburn, MA), and was determined to be between 200-250 ng/10^6 cells/24hrs for 3T3/GM and 3T3-*neu*/GM. GM-CSF bioactivity was confirmed using the GM-CSF dependent cell line, NFS-60 as previously described (4). Production of GM-CSF by untransduced NT cell line is not detected as determined by ELISA.

Chemotherapeutic agents. Paclitaxel (PTX) (Bristol-Myers Squibb, Princeton, NJ), Doxorubicin (DOX) (Gensia, Irvine,CA), and Cisplatin (CIS) (Bristol-Myers Squibb, Princeton, NJ) were diluted in Hank's balanced salt solution (HBSS) before injection. Cyclophosphamide (CTX) (Bristol-Myers Squibb, Princeton, NJ) was diluted in sterile water before injection. PTX, CTX, and CIS were injected intraperitoneally (i.p.), DOX was injected intravenously (i.v.).

Vaccination and tumor challenge. On the day of vaccination, vaccine cells grown *in vitro* were trypsinized, washed three times in HBSS (pH 7.4) (Life Technologies, Grand Island, NY) and counted. The cells were resuspended in HBSS at 10^7 cells/ml and irradiated with 50 Gy from a ^{137}Cs source (Nordion, Toronto, Canada) discharging 1400 rad/min. Eight-week-old *neu* transgenic or FVB/N mice were given three simultaneous 100 μl subcutaneous injections (right and left hind limbs and left arm) using a 1-ml tuberculin syringe with a 27-gauge needle (33). The mice in the vaccine group received three simultaneous injections of 10^6 3T3-*neu*/GM cells. To insure that the effect observed was *neu*-specific, all control mice as well as the mice in the

chemotherapy group alone received a mock vaccination consisting of three subcutaneous injections of 10^6 3T3/GM cells. 3T3/GM mock vaccination did not delay the occurrence of tumor growth compared with mice injected only with HBSS (data not shown). On the day of the tumor challenge, NT cells thawed from frozen bank stores and grown *in vitro* for 1 or 2 weeks, were trypsinized, washed three times in HBSS, and injected into the right upper mammary fat pad. Mice were challenged with 5×10^4 (*neu* transgenic) or 5×10^6 (FVB/N) NT tumor cells. In treatment experiments, mice were challenged with NT cells on day 0 and vaccinated on day 3 unless otherwise specified. In prevention experiments, mice were challenged with NT cells two weeks after vaccination. Tumor occurrence (shown as the tumor free probability) or changes in tumor growth were monitored twice a week. Changes in tumor growth (mm^2) were determined by multiplying the two perpendicular diameters.

T cell Assays. Neu-specific interferon gamma (γ-IFN) or interleukin (IL)-4 producing T cells were quantified by enzyme-linked immuno-spot assay (ELISPOT) analysis. *Neu* transgenic mice were given a subcutaneous challenge with NT cells followed three days later by vaccination with 3T3-*neu*/GM or 3T3/GM with or without chemotherapy. On day 12 post-vaccine, T cells were isolated from splenocytes by Ficoll (Amersham, Sweden) separation and passed over a nylon wool column to remove B cells and macrophages. $CD4^+$ cells were positively selected with dynabeads® and detachbead® mouse CD4 according to the manufacturer's instructions (Dynal, Lake Success, NY). After one round of $CD4^+$ positive selection, more than 98 % of cells were shown to be $CD4^+$ by FACS. Neu-specific γ-IFN or IL-4 production was determined by a standard ELISPOT protocol and as previously described (32, 34). 10^4 γ-IFN-treated NT-B7 cells per well were used as stimulators and serial dilutions of unfractionated lymphocytes or $CD4^+$ T cells were added to the wells for an 18-hr incubation. Each condition was tested in triplicate.

Reagents and materials used in the assay were the following: 96-well filtration plate (MAIPS4510, Millipore, Molsheim, France), rat anti-mouse γ-IFN at 10 μg/ml (Pharmingen, San Diego, CA), rat anti-mouse biotin γ-IFN (Biosource International, Camarillo, CA), rat anti-mouse Il-4 at 10 μg/ml (Pharmingen, San Diego, CA), rat anti-mouse biotin Il-4 (Biosource International, Camarillo, CA), avidin-alkaline phosphatase at 2 μg/ml (Sigma, St. Louis, MO), and 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (Sigma, St. Louis, MO).

Neu specific lysis was determined by Chromium-51 release assay as previously described (32). Briefly, spleens were removed and splenocytes isolated by Ficoll separation. The splenocytes were incubated for 5 days in the presence of murine IL-2 and γ-IFN and Mitomycin-C (Sigma, St. Louis, MO) treated NT-B7 cells. Stimulated splenocytes were harvested, washed twice with HBSS, and incubated with ^{51}Cr-labeled 3T3 and 3T3-*neu* target cells to assess neu-specific lytic function. Neu-specific lysis was determined by subtracting the background lysis of 3T3 cells from the lysis obtained against 3T3-*neu* targets.

Statistical analysis. Unpaired Student's t-tests were performed to analyze tumor size and ELISPOT data. Kaplan-Meier analyses were used to analyze tumor-free survival and the log-rank test was used for comparisons. A p value $< .05$ was considered as statistically significant.

RESULTS

A neu-targeted vaccine is highly effective at preventing and treating neu-expressing tumors in parental FVB/N but not in *neu* transgenic mice.

We have previously shown that *neu* transgenic mice demonstrate immune tolerance to neu : the dose of tumor cells required for tumor growth in 100 % of animals was at least 100-fold lower for the *neu* transgenic mice when compared with parental FVB/N mice (32). Non-transgenic and *neu* transgenic mice were compared for their ability to respond to a neu and GM-CSF expressing whole-cell vaccine (3T3-*neu*/GM) given before or after a tumor challenge with NT tumor cells. Non-transgenic FVB/N mice vaccinated once with 3T3-*neu*/GM demonstrated an impressive antitumor response capable of preventing and treating large tumor burdens **(figure 1A&B)**. *Neu* transgenic mice given the same vaccine demonstrated a small but significant and reproducible delay in transplantable tumor growth when the tumor challenge was given two weeks after the vaccine **(figure 1C)**. However, in treatment experiments, a statistical difference in the rate of tumor growth between the control and the vaccine groups was not detected in *neu* transgenic mice even if the vaccine was administered as early as one day after the tumor challenge **(figure 1D)**. These results provide further evidence that the *neu* transgenic mice demonstrate an immune tolerance to neu.

Identification of a chemotherapy dose range and sequence of administration that may enhance the antitumor effects of the 3T3-*neu*/GM vaccine.

The data presented in **figure 1** show that our vaccine approach is potent enough to eradicate large established tumor burdens in a host that does not exhibit antigen-specific immune tolerance to tumor. In contrast, the same vaccine is not potent enough to prevent tumor

development in the *neu* transgenic mice (32, 35). Therefore, immune-modulating agents that can overcome the mechanisms of tolerance may enhance the effectiveness of neu-specific immunization.

One immune-modulating approach is to test selected chemotherapeutic agents at non-immune suppressing doses for their ability to enhance the potency of the vaccine. We therefore tested four chemotherapeutic agents (Cyclophosphamide, Doxorubicin, Paclitaxel, and Cisplatin) in combination with the 3T3-*neu*/GM vaccine. These four drugs were chosen for evaluation for three reasons. First, these agents are commonly employed for the treatment of human cancers. Second, each drug represents a different class of chemotherapeutic agents and would be expected to interact with the vaccine by distinctly different mechanisms. Third, there is data in the literature suggesting that each of these agents have immune-modulating effects (24-30, 36-37).

Initially, feasibility studies were performed in FVB/N mice to identify a dose range and optimal sequence of administration for each chemotherapeutic agent when combined with the vaccine. Mice were given $5x10^6$ NT cells inoculated into the right upper mammary fat pad on day 0 and vaccinated three days later with three simultaneous subcutaneous injections of $1x10^6$ irradiated 3T3-*neu*/GM cells given into the left and right hind limbs and the left upper limb. Each of the four chemotherapeutic agents were given either one day prior to vaccination (at the time of immune priming) or seven days after vaccination (at the time of initial T cell activition and expansion). **Table 1** summarizes the dose range for each agent used as well as the type of effect observed.

When either PTX (dose range between 20 and 30 mg/kg) or CTX (dose range between 50-150 mg/kg) were given prior to the vaccine, the combination of chemotherapy plus vaccine was better to control tumor growth than treatment with either modality alone **(Table 1)**.

However, when these two chemotherapeutic agents were given at the same doses seven days after vaccination, the combination chemotherapy/vaccine was not superior to chemotherapy alone. In contrast, DOX (dose below 10 mg/kg) and CIS (doses below 5 mg/kg) neither inhibited nor significantly enhanced the vaccine's potency when given either one day before or one week after vaccination.

Cyclophosphamide, Paclitaxel, and Doxorubicin enhance the vaccine's antitumor effects and significantly delay transplantable tumor progression in *neu* transgenic mice.

Next, we tested the dose and schedule of each chemotherapeutic agent found to be effective in the non-tolerized mice, for the ability to enhance the vaccine's potency in the *neu* transgenic mice. Mice were inoculated with 5×10^4 NT cells in the right upper mammary fat pad on day 0 and vaccinated three days later. As shown in **figure 2**, when either CTX or PTX was given one day before vaccination, they enhanced the vaccine's potential to delay tumor growth. In contrast, DOX had no effect when administered prior to vaccination, but could enhance the antitumor effect when administered one week following the vaccination. CIS was the only drug of the four that did not appear to enhance the vaccine's antitumor immune response at all at the dose range and schedules studied (data not shown).

Since CTX and PTX enhance the effect of the vaccine at a different time point than DOX, it is likely that the mechanisms by which they interact with the vaccine also differ. If this is indeed the case, then it is possible that administering either CTX or PTX in sequence with both the vaccine and DOX would further enhance the antitumor immune response in the *neu* transgenic mice. We therefore tested the combination of CTX (100 mg/kg) and DOX (5mg/kg) given respectively 1 day before and 7 days after vaccination. This polychemotherapy regimen induced a mild leukopenia ranging between 4000 and 5000 white blood cells (normal between

8,000 and 12,000). As shown in **figure 3**, the association of CTX/DOX and vaccine was significantly more effective at controlling the tumor occurrence than either treatment modality alone. This chemotherapy/vaccine regimen cured 20% of the *neu* transgenic mice in two similar experiments.

Chemotherapy appears to enhance the potency of the neu-targeted vaccine through a mechanism distinct from direct tumor lysis

Prevention experiments were performed to determine whether the mechanism by which the chemotherapeutic agents enhance the efficacy of the vaccine is through direct tumor killing or through amplification of the antitumor immune response. Mice received three simultaneous subcutaneous injections of 10^6 3T3-*neu*/GM vaccine in the right and left hind limb and right upper limb on day 0. PTX or CTX were given i.p. one day prior and DOX was given i.v. 7 days after vaccine. All mice were challenged with $5x10^4$ NT cells 14 days after vaccination. This experimental design makes it unlikely that the chemotherapy can directly reduce the tumor burden since the chemotherapy dose was administered 7 days prior to tumor challenge for DOX and 15 days prior to tumor challenge for PTX and CTX. As shown in **figure 4**, the three chemotherapy and vaccine combinations induced a more potent antitumor response than either vaccine or chemotherapy alone. These data therefore support the hypothesis that these chemotherapeutic agents increase the potency of the vaccine via mechanisms that are distinct from direct tumor lysis.

Paclitaxel and Cyclophosphamide given in sequence with the neu-targeted vaccine results in an increase in neu-specific T cells in *neu* transgenic mice

ELISPOT analysis was used to quantitate neu-specific T cell induction in *neu* transgenic mice following 3T3-*neu*/GM vaccine with and without chemotherapy. Mice were challenged

with NT cells followed three days later with either a 3T3-*neu*/GM vaccine or a mock vaccination (3T3/GM). CTX, PTX, and DOX were given either one day before the vaccine or one week after the vaccine. The mice were sacrificed 12 days after vaccine administration and unfractioned T cells were isolated from spleen as described in materials and methods. As shown in **figure 5**, CTX and PTX administered one day before the vaccine increased the number of neu-specific T cells when compared with mice that received 3T3-*neu*/GM vaccine alone. PTX and CTX injected after the vaccine significantly decreased the number of neu-specific T cells when compared with the mice that received vaccine alone. In contrast, DOX given one day before the vaccine or one week after the vaccine did not decrease or increase the number of neu-specific T cells. This supports the hypothesis that the DOX given after the vaccine increased its efficacy through a different mechanism than PTX or CTX.

Cyclophosphamide and Paclitaxel appear to specifically enhance the T helper 1 (Th) response of the 3T3-*neu*/GM vaccine in *neu* transgenic mice.

We have previously described the importance of $CD4^+$ T cells in orchestrating the host response to tumor after vaccination with whole-cell vaccines engineered to secrete GM-CSF (4, 38). To study the Th1 and Th2 balance in *neu* transgenic mice given chemotherapy in sequence with the vaccine, γ-IFN and IL-4 ELISPOT analyses were performed on $CD4^+$ T cells isolated and purified from spleen 12 days after vaccination. As shown in **figure 6**, the Th1 but not the Th2 response was increased when PTX or CTX were given before a 3T3-*neu*/GM vaccine compared with the group that received the vaccine only. In contrast, DOX given after the vaccine did not increase or decrease the Th1 or Th2 response (data not shown). These data therefore suggest that CTX and PTX, when given prior to the vaccine, enhances the Th1 T cell response.

Chemotherapy does not impair the neu-specific Cytolytic T Lymphocyte (CTL) immune response in *neu* transgenic mice.

In vivo lymphocyte depletion studies have demonstrated a critical role for both $CD4^+$ and $CD8^+$ T cells as effectors of the antitumor immune response induced by GM-CSF whole-cell tumor vaccines (4, 32). The data shown in **figures 5 and 6** provide evidence that the chemotherapy (PTX and CTX) administered prior to the vaccine can enhance T cell cytokine production. We next evaluated T cells isolated from mice treated with the chemotherapy and vaccine combination for the ability to lyse neu-expressing targets. The lymphocytes used for this analysis were isolated from mice that were part of a larger *in vivo* treatment study so that the *in vivo* and *in vitro* results could be directly correlated. Tumors were established and mice were subsequently treated in sequence with CTX on day 2, vaccination on day 3, and DOX on day 10, respectively. Splenocytes were isolated one month after completing the treatment regimen and stimulated *in vitro* for two weeks in the presence of Mitomycin-C and γ-IFN treated NT-B7 cells. Following two *in vitro* stimulations, lymphocytes were harvested and tested for the ability to lyse 3T3-*neu* and 3T3 target cells in a 4-hour ^{51}chromium-release assay. Neu-specific lysis was calculated by subtracting the percent lysis against 3T3 from the percent lysis of 3T3-*neu* cells. As shown in **figure 7**, the polychemotherapy regimen did not impair the neu-specific CTL response generated by the vaccine. In addition, there appeared to be an enhanced *in vitro* neu-specific lytic response observed for splenocytes isolated from polychemotherapy and vaccine treated mice that did not develop tumors when compared with splenocytes isolated from treated mice that developed tumors (**figure 7**). CTL responses similar to the vaccine alone were observed using splenocytes isolated from mice treated with single agent CTX 100 mg/kg or PTX 20 mg/kg given before vaccine as well as DOX 5 mg/kg given one week after vaccine (data not

shown). In addition, 5/5 mice that remained tumor free successfully rejected a rechallenge of 5 x 10^4 NT tumor cells given 3-6 months post-vaccine (data not shown). This finding provides support that neu-specific T cell memory responses are also induced.

DISCUSSION

The data presented in this study support the following two conclusions. First, CTX, PTX, and DOX, when given in a defined sequence with a murine GM-CSF secreting neu-expressing whole-cell vaccine, enhance the vaccine's potential to delay tumor growth in tolerized *neu* transgenic mice. The optimal immune-modulating dose for each chemotherapeutic agent appears to be just above doses that begin to induce cytopenias. Second, the enhanced antitumor response appears to be mediated at least in part by an increase in number and function of antigen-specific T cells (CTX and PTX). These findings suggest that combined treatment with immune-modulating doses of chemotherapy and the GM-CSF-secreting neu vaccine can overcome immune tolerance and induce a more potent antigen-specific antitumor immune response than vaccine alone.

Neu transgenic mice offer the opportunity to test vaccine strategies in the context of tumor-specific immune tolerance (32). Our previous studies have demonstrated that *neu* transgenic mice exhibit a neu-specific immune tolerance similar to what is observed in patients with breast cancers that overexpress HER-2/*neu* (32). Although neu-targeted vaccination was able to eradicate large burdens of pre-established tumors in the non-tolerized parental mice in this study, these same vaccines could only significantly delay the development of transplantable neu-expressing tumors in a prevention model in the *neu* transgenic mice (32). Furthermore, we did not observe a significant difference in tumor growth between the control and vaccine groups in the treatment experiments. This reinforces previously reported data demonstrating that tumor vaccines alone have a

limited potential for the treatment of measurable tumor burdens and highlights the importance of identifying more potent vaccine strategies for clinical testing.

We evaluated the possible integration of chemotherapy and vaccine to treat transplantable mammary tumors in *neu* transgenic mice. We found that, when given in the proper sequence and at immune-modulating doses, systemic administration of CTX, PTX, and DOX can enhance rather than inhibit the antitumor immunity generated by the vaccine. The fact that this finding is also observed in prevention experiments in which the tumor challenge is given seven days after the last dose of chemotherapy, suggests the antitumor effect cannot be explained only by a direct chemotherapy induced cytolytic effect on the tumor cells. Rather, CTX, PTX, and DOX appear to also have a direct immune augmenting effect. This immune enhancing effect appears to be due in part to an augmentation of the number and activity of antigen specific T cells. Furthermore, the data suggest that PTX and CTX may amplify the Th1 T cell response. In contrast to CTX and PTX, DOX does not appear to significantly enhance the number of neu-specific T cells in our model. It is still possible that it acts by enhancing T cell function. However, alternative mechanisms, including recruitment and activation of professional antigen presenting cells, and enhancement of innate immune responses also require consideration.

Previous studies have already demonstrated that pre-treatment with CTX prior to T cell adoptive transfer enhance T cell efficacy (25-26). There are also reports suggesting that CTX can enhance the antitumor immune response of whole-cell vaccination (39) and induce a Th1 immune response in tumor models (40). Other studies have suggested that pre-treatment with CTX can overcome tolerance (41-42). Yoshiba and colleagues successfully provoked significant delayed-type hypersensitivity footpad reactions against

syngeneic and autologous testicular cells in mice pre-treated with CTX (41). In addition, Polak and colleagues demonstrated that acquired tolerance to 2,4-dinitrochlorobenzene can be reversed by a single treatment with CTX just prior to administration of the allergen (42). Our results are consistent with these earlier findings and confirm that CTX can break tolerance and augment the antigen-specific antitumor immune response induced by a GM-CSF secreting whole-cell vaccine in a murine model that exhibits tumor-specific tolerance. However, an earlier study performed by our group failed to demonstrate a synergistic effect between pre-treatment with CTX and immunization with a GM-CSF secreting whole-cell vaccine in the murine CT26 colorectal carcinoma model (27). The discrepancy between the results of the earlier study and this current study may be explained in part by the difference in the tumor models, since tolerance has not been demonstrated in the CT26 tumor system. In fact, the interactions of each chemotherapeutic agent with vaccine were more evident in the *neu* transgenic mice than in the parental FVB/N mice. The differences may also be explained by the timing and dose of CTX tested in the two studies (27).

The exact mechanisms by which CTX enhances antitumor immunity are still undergoing debate. Many studies have reported that CTX may delete or inhibit tumor-induced suppressor or immunoregulatory T cells (43, 44). Others have suggested that CTX may release soluble factors, which may sustain the proliferation, survival, and activity of the transferred immune T cells (26). Recently, Schiavoni and colleagues demonstrated that CTX induces type 1 IFN secretion *in vivo* and enhances the number of T cells exhibiting the $CD44^{hi}$ memory phenotype (45, 46). We were able to abolish the CTX effects on vaccine efficacy by adoptively transferring splenocytes from tumor

bearing mice into CTX/vaccine treated mice (Machiels and Jaffee, unpublished observations). This finding provides further support that CTX removes a T cell population capable of suppressing anti-tumor immune responses (43-44). In contrast, adoptive transfer of splenocytes isolated from tumor bearing mice into DOX/vaccine and PTX/vaccine treated mice did not abrogate the antitumor response induced by the vaccine (Machiels and Jaffee, unpublished observations).

To our knowledge, this is the first study to evaluate the potential synergy between PTX and an antigen-specific whole-cell vaccine for the ability to induce T cell responses. As with CTX, we observed that PTX was synergistic with the vaccine only when given prior to vaccination. Multiple immunostimulatory functions have been previously attributed to PTX *in vitro* and *in vivo* (28, 47-49). PTX can enhance the tumoricidal activity of murine macrophages by inducing nitric oxide production and secretion of TNF-alpha, IL-1 beta, and superoxide anions (48). PTX has also been reported to enhance macrophage IL-12 production, a Th1 type cytokine (49). This finding provides one explanation for the observed PTX/vaccine induced increase in number of neu-specific Th 1 cells in our studies. The fact that PTX inhibited the *in vivo* activity of the vaccine when given after vaccination is not surprising since PTX has been shown to impair the proliferation of T cells by stabilization of the microtubules (50). Importantly, the observed abrogation of *in vivo* activity also correlated with a lack of Th 1 induction when PTX was given post-vaccination.

Among the chemotherapeutic drugs tested, DOX was the only one that enhanced the *in vivo* antitumor response when given after the vaccine. This observed *in vivo* response could not be correlated with an increase in the number of neu-specific T cells.

Although an earlier report from our group suggested that DOX could enhance tumor-specific T cell activity, this finding was only based on an observed increase in CTL activity *in vitro* (27). Others have reported that splenic and tumor-infiltrating mature T cells were completely insensitive to DOX cytotoxicity and showed increased CTL activity when examined *ex vivo* (51). However, CTL activity is not quantitative, and has not been rigorously evaluated for its ability to correlate with *in vivo* antitumor activity. Other reports have suggested that DOX can modulate monocyte/macrophage activity in an antigen-independent manner (24). Mihich and colleagues have demonstrated a two-fold increase in the number of splenic macrophages as early as five days after DOX administration (52-53). DOX has also been shown to increase macrophage tumoricidal activity (54). Early studies from our group have also defined a non-antigen-dependent role for macrophages induced by the GM-CSF whole-cell vaccine (4, 38). Macrophages have been shown to infiltrate the site of tumor challenge as early as 1 day after immunization (4). These macrophages release nitric oxide and probably collaborate with other immune cells infiltrating the site to cause its destruction (38). Further investigation of DOX's effects on macrophages when given with the GM-CSF secreting vaccine is underway.

Finally, CIS did not seem to enhance the efficacy of the vaccine in *neu* transgenic mice despite the fact that the doses tested did not inhibit the vaccine's ability to induce antitumor immunity in the non-transgenic mice. CIS has been previously shown to have both immune stimulatory and immune inhibitory effects, depending on the tumor model in which it was tested (37). This drug was not rigorously evaluated in our model because

the initial studies failed to identify a dose or sequence of administration that enhanced the GM-CSF secreting vaccine.

We successfully combined immune-modulating doses of chemotherapy and an antigen-specific vaccine to treat neu-expressing tumors in *neu* transgenic mice. The doses of chemotherapy that appear to enhance the vaccine are clearly inferior as tumor lytic agents to the conventional cytoreductive doses currently employed in the clinic. To date, the combination of chemotherapy and active immunotherapy for the treatment of cancer is relatively unexplored in the clinic. For patients with metastatic breast cancer, Trastuzumab, a recombinant humanized monoclonal antibody to HER-2/neu, administered as a single agent, produces durable objective responses in women with HER-2/neu-overexpressing breast cancer (27). More recently, synergistic or additive clinical responses have been observed for trastuzumab when combined with chemotherapy (29). Further studies are needed to evaluate the impact of conventional chemotherapy doses on the potency of antigen-specific vaccines.

In conclusion, our data support a role for immune-modulating doses of chemotherapy in overcoming immune tolerance when combined with antigen-specific vaccination. These data provide the rationale for testing immune-modulating doses of chemotherapy in sequence with antigen-specific cancer vaccines in patients with cancer.

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Table 1 *Dose and schedule-dependent associations between chemotherapy and the GM-CSF secreting whole-cell vaccine in FVB/N mice*[a]

		T cells count (nadir) number/μl (normal range: 4000-9000)[c]	Chemotherapy 1 day before vaccine	Chemotherapy 7 days after vaccine
CTX	50 mg/kg	6128	+[b]	-
	100 mg/kg	5120	+	-
	150 mg/kg	1559	+	NT
	200 mg/kg	1100	+/-	NT
	250 mg/kg	989	+/-	NT
PTX	20 mg/kg	4365	+	-
	30 mg/kg	4200	+	NT
	35 mg/kg	3600	+/-	NT
	40 mg/kg	3451	+/-	NT
DOX	4 mg/kg	6265	+/-	+/-
	8 mg/kg	5586	+/-	+/-
	15 mg/kg	4180	-	-
CIS	2 mg/kg	6320	+/-	+/-
	3 mg/kg	6200	+/-	+/-
	5 mg/kg	3679	-	-
	10 mg/kg	3400	-	-

Table 1. Dose and schedule-dependent associations between chemotherapy and the GM-CSF secreting whole-cell vaccine in FVB/N mice.

[a] FVB/N mice were implanted with 5 x 10^6 NT cells in the right upper mammary fat pad on day 0. Groups of 5 to 10 mice received either: 1) HBSS as a control; 2) 3T3-*neu*/GM vaccine cells; 3) chemotherapy; or 4) chemotherapy and 3T3-*neu*/GM vaccine cells. 3T3-*neu*/GM vaccine cells were injected subcutaneously at three sites (10^6 cells per site), the left and right hind limb and left upper limb, on day 3. All mice were monitored twice a week for a change in tumor growth as determined by multiplying the 2 perpendicular diameters. p value was determined by unpaired Student's t-test.

[b] + = additional effect (vaccine/chemotherapy group statistically superior ($p<.05$) to each treatment modality alone); +/- = no effect (vaccine/chemotherapy group statistically superior ($p<.05$) to chemotherapy group but not to vaccine group); - = inhibition effect (no statistical difference between vaccine/chemotherapy group and chemotherapy group); NT = not tested.

[c] Blood was drawn four days after chemotherapy administration (nadir) and T cells were automatically counted (ANTECH Diagnostics, New-York). Data shown are the mean of T cell counts for three mice at day four after chemotherapy injection (nadir).

Legends of figures

Figure 1. Neu-specific vaccination can prevent and treat neu-expressing tumors in the parental FVB/N but not in *neu* transgenic mice.

Parental FVB/N (A) and *neu* transgenic (C) mice were vaccinated with three simultaneous subcutaneous injections of 10^6 3T3-*neu*/GM vaccine cells (Right and left hind limbs and left upper limb) on day 0 and challenged with 5 x 10^6 (FVB/N mice) or 5x 10^4 (*neu* transgenic mice) NT cells into the right upper mammary fat pad on day 14. In a treatment experiment, a second group of parental FVB/N (B) and *neu* transgenic (D) mice were first implanted with 5 x 10^6 (FVB/N) or 5 x 10^4 (*neu* transgenic mice) NT cells into the right upper mammary fat pad. FVB/N mice were vaccinated two weeks later and *neu* transgenic mice were vaccinated 1 day later with 3 simultaneous subcutaneous injections of 10^6 3T3-*neu*/GM into the right and left hind limbs and left upper limb. All mice were monitored twice a week for a change in tumor growth. Plotted is the mean +/- SEM of the products of the 2 perpendicular diameters (mm^2) for 5-8 mice/group as a function of days post-tumor implantation. Control mice in each study received similar injections with the 3T3/GM mock vaccine. Similar results were obtained in four independent experiments.

♦= controls 3T3/GM (mock vaccine); Δ= Vaccination 3T3-*neu*/GM.

* $p< 0.05$ as determined by unpaired Student's t-test.

Figure 2. Doxorubicin, Paclitaxel, and Cyclophosphamide can enhance the antitumor effect of the neu-targeted vaccine in *neu* transgenic mice when given in proper sequence.

Between 5 and 8 mice per group received either: 1) mock vaccination (3T3/GM) as control; 2) 3T3-*neu*/GM vaccine alone; 3) chemotherapy and a mock vaccine (3T3/GM); or 4) chemotherapy and 3T3-*neu*/GM vaccine. In all experiments 5 x 10^4 NT cells were implanted in the right mammary fat pad on day 0 and mice were vaccinated on day 3. Vaccination consisted of either 3T3-*neu*/GM or 3T3/GM given subcutaneously at three sites (10^6 cells per site), the left and right hind limbs and left upper limb. All mice were monitored twice a week for a change in tumor growth. Plotted is the mean +/- SEM of the products of the 2 perpendicular diameters (mm^2) for 5-8 mice/group as a function of days post-tumor implantation. Similar results were obtained in two independent experiments.

(A) Doxorubicin 5 mg/kg was given i.v. on day 2, one day before vaccination.

(B) Doxorubicin 5 mg/kg was given i.v. on day 10, one week after vaccination.

(C) Paclitaxel 20 mg/kg was given i.p., on day 2 one day before vaccination.

(D) Paclitaxel 20 mg/kg was given i.p. on day 10, one week after vaccination.

(E) Cyclophosphamide 100 mg/kg was given i.p. on day 2, one day before vaccination.

(F) Cyclophosphamide 100 mg/kg was given i.p. on day 10, one week after vaccination.

♦ = controls-3T3/GM (mock vaccine); Δ = Vaccination 3T3-*neu*/GM; • = 3T3/GM (mock vaccine) + chemotherapy; x = Vaccination 3T3-*neu*/GM vaccine + chemotherapy

* $p < 0.05$ as determined by unpaired Student's t-test between chemotherapy group alone and chemotherapy and vaccine group

Figure 3. Polychemotherapy can enhance the antitumor effect of the neu-targeted vaccine in *neu* transgenic mice.

Between 10 and 14 mice per group (pooled from two independent experiments) received either: 1) controls 3T3/GM (mock vaccine); 2) 3T3-*neu*/GM vaccine alone; 3)

polychemotherapy and 3T3/GM (mock vaccine); or 4) polychemotherapy and 3T3-*neu*/GM vaccine. 5 x 10^4 NT cells were implanted in the right mammary fat pad on day 0. Vaccination (3T3-*neu*/GM or 3T3/GM) was given at three sites (10^6 cells per site), the left and right hind limbs and left upper limb, on day 3. The chemotherapy consisted of i.p. CTX 100 mg on day 2 (one day prior to the vaccine) and i.v. DOX 5 mg/kg on day 10 (7 days after the vaccine). All mice were monitored twice a week for tumor occurrence. ♦= controls 3T3/GM (mock vaccine); Δ = Vaccination 3T3-*neu*/GM; • = 3T3/GM (mock vaccine) + Cyclophosphamide 100 mg/kg + Doxorubicin 5mg/kg; X = Vaccination 3T3-*neu*/GM vaccine + Cyclophosphamide 100 mg/kg + Doxorubicin 5mg/kg.

Figure 4. Chemotherapy enhances the potency of neu-specific vaccine through a mechanism distinct from direct tumor lysis.

Between 5 and 8 *neu* transgenic mice were vaccinated subcutaneously with 3T3-*neu*/GM cells given at three sites (10^6 cells per site), the left and right hind limbs and left upper limb, with and without chemotherapy. Two weeks after vaccination, mice were challenged into the mammary fat pad with 5 x 10^4 NT cells. Mice in the control group received a mock vaccination (3 X10^6 3T3/GM). Mice were observed three times a week for tumor occurrence. Results are shown as tumor free probability (y-axis) on days post-tumor challenge (x-axis). Similar results were obtained in two independent experiments. Mice in the chemotherapy/vaccine group received:

(A) Doxorubicin 5mg/kg was given i.v. 7 days after the vaccine.

(B) Paclitaxel 20 mg/kg was given i.p. one day prior to the vaccination.

(C) Cyclophosphamide 100 mg/kg was given i.p. one day prior to the vaccination.

♦ = controls-3T3/GM (mock vaccine); Δ = 3T3-*neu*/GM vaccine only; • = chemotherapy and 3T3/GM (mock vaccine); X = chemotherapy and 3T3-*neu*/GM vaccine

Figure 5. Cyclophosphamide and Paclitaxel but not Doxorubicin given in sequence with the neu-targeted vaccine results in an increase in neu-specific T cells in *neu* transgenic mice.

ELISPOT analysis was used to determine the number of neu-specific T cells induced in *neu* transgenic mice with a 3T3-*neu*/GM vaccine with or without chemotherapy. 5 $x10^4$ NT tumor cells were implanted in the right mammary upper fat pad on day 0 and mice were vaccinated on day 3. Vaccination (3T3-*neu*/GM or 3T3/GM) was given subcutaneously at three sites (10^6 cells per site), the left and right hind limbs and left upper limb. Mice were sacrificed 12 days after the administration of the vaccine and the T cells were isolated from spleen as described in materials and methods. ELISPOT analysis was performed as described in materials and methods.

3 mice per group received either: 1) controls 3T3/GM (mock vaccine); 2) 3T3-*neu*/GM vaccine alone; 3) chemotherapy and 3T3/GM (mock vaccine); 4) chemotherapy and 3T3-*neu*/GM vaccine. Plotted are the mean (3 wells per condition) +/- standard deviation of the number of spots counted in the wells containing the T cells and the stimulators cells minus the number of spots counted in the well containing the T cell alone. NT-B7 stimulator cells do not give any background (data not shown). p value was determined by unpaired Student's t-test between vaccine group and chemotherapy + vaccine group.

Figure 6. Paclitaxel and Cyclophosphamide appear to enhance the T helper 1 response of a 3T3-*neu*/GM vaccine in *neu* transgenic mice.

To study the Th1 and Th2 balance in *neu* transgenic mice given chemotherapy in sequence with the vaccine, γ-IFN (A & C) and IL-4 (B & D) ELISPOT analyses were performed on $CD4^+$ T cell. 5 $x10^4$ NT tumor cells were implanted in the right upper mammary fat pad on day 0 and mice were vaccinated on day 3. Vaccination (3T3-*neu*/GM or 3T3/GM) was given subcutaneously at three sites (10^6 cells per site), the left and right hind limbs and left upper limb. Mice were sacrificed 12 days after the administration of the vaccine and the $CD4^+$ T cells were isolated from spleen as described in materials and methods. ELISPOT analysis was performed as described in materials and methods. 4 mice per group received either: 1) controls-3T3/GM (mock vaccine); 2) 3T3-*neu*/GM vaccine alone; 3) chemotherapy and 3T3/GM (mock vaccine); 4) chemotherapy and 3T3-*neu*/GM vaccine. Plotted are the mean (3 wells per condition) +/- standard deviation of the number of spots counted in the wells containing the T cells and the stimulators cells minus the number of spots counted in the well containing the T cell alone. NT-B7 stimulator cells do not give any background (data not shown).

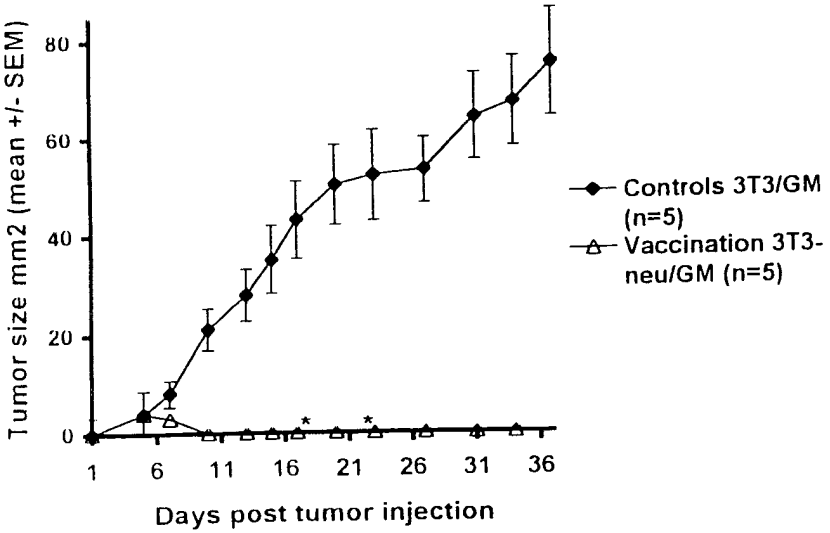
p value was determined by unpaired Student's t-test between vaccine group and chemotherapy + vaccine group.

Figure 7. Chemotherapy does not impair neu-specific Cytolytic T Lymphocytes (CTL) in *neu* transgenic mice.

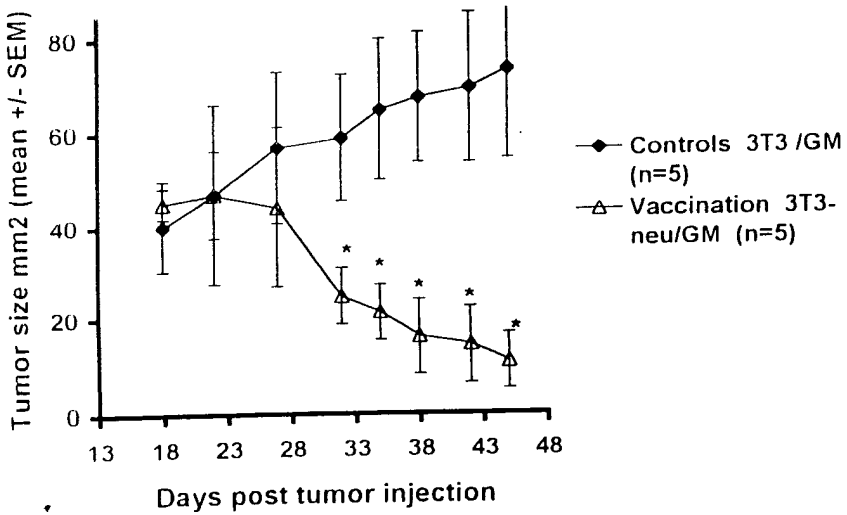
5 x 10^4 NT cells were implanted in the right mammary gland on day 0 and mice were vaccinated on day 3 with 3T3-*neu*/GM cells given at three subcutaneous sites (10^6 cells per site), the left and right hind limbs and left upper limb. The chemotherapy regimen consisted of the administration of CTX 100 mg on day 2 and DOX 5 mg/kg on day 10. A 4-hour chromium-release assay was performed using splenocytes isolated from *neu*

transgenic mice (3 treated mice per group). Splenocytes were isolated 4 weeks after treatment, were incubated for two weeks with Mitomycin C- and γ-IFN treated NT-B7 cells, and tested in a 4-hour chromium release assay for neu-specific lysis. Both 3T3 and 3T3-*neu* cells were used as targets. The neu-specific lysis was calculated by subtracting the percent lysis against 3T3 from the percent lysis of 3T3-*neu* cells. ♦= controls-3T3/GM (mock vaccine); Δ = 3T3-*neu*/GM vaccine only; • = chemotherapy and 3T3/GM (mock vaccine); x = chemotherapy and 3T3-*neu*/GM vaccine (group 1), mice developing tumor after tumor challenge; □ = chemotherapy and 3T3-*neu*/GM vaccine (group 2), mice remaining free of tumor after tumor challenge.

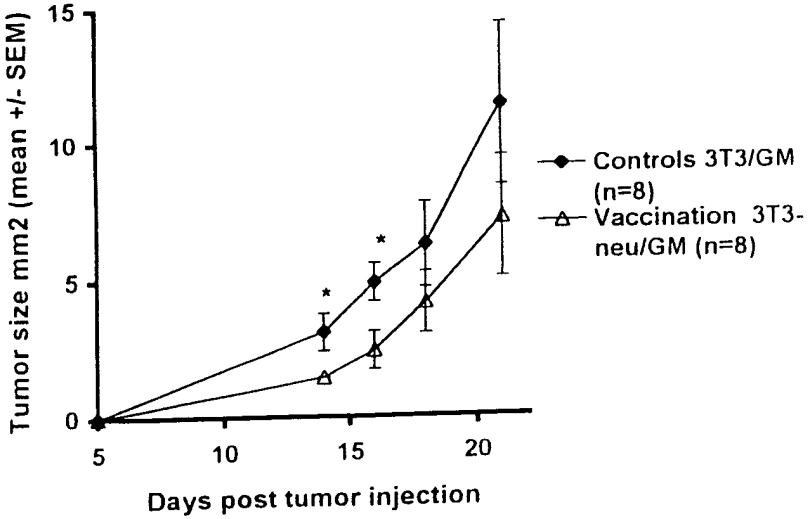
A Prevention: FVB/N mice



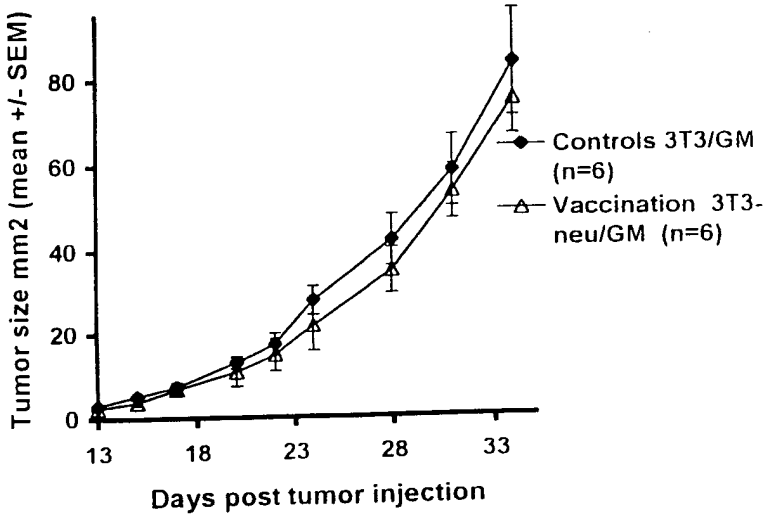
B Treatment: FVB/N mice



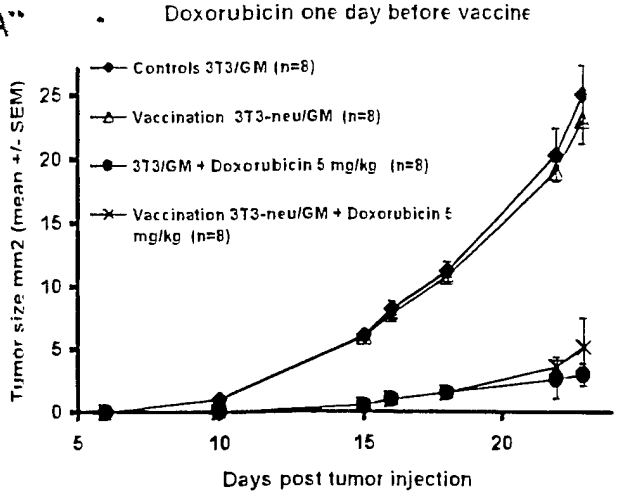
C Prevention: *neu* transgenic mice



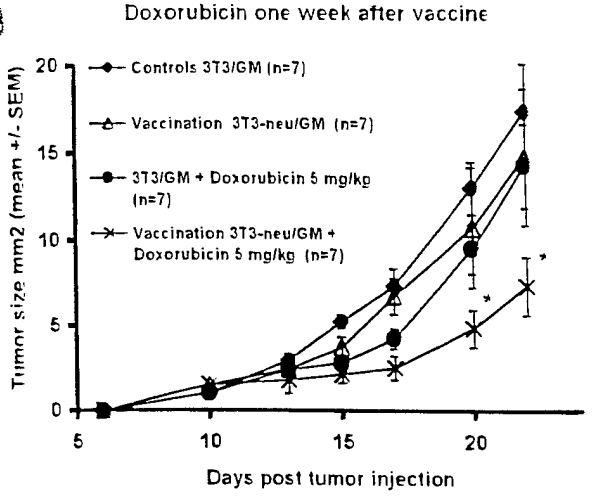
D Treatment: *neu* transgenic mice



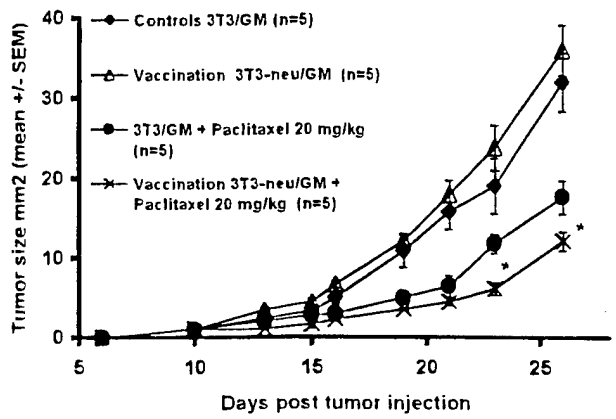
A


Doxorubicin one day before vaccine
Controls 3T3/GM (n=8)
Vaccination 3T3-neu/GM (n=8)
3T3/GM + Doxorubicin 5 mg/kg (n=8)
Vaccination 3T3-neu/GM + Doxorubicin 5 mg/kg (n=8)
Tumor size mm2 (mean +/- SEM)
Days post tumor injection

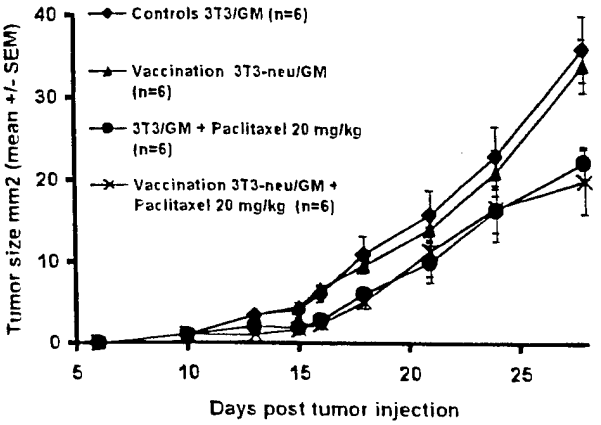
B


Doxorubicin one week after vaccine
Controls 3T3/GM (n=7)
Vaccination 3T3-neu/GM (n=7)
3T3/GM + Doxorubicin 5 mg/kg (n=7)
Vaccination 3T3-neu/GM + Doxorubicin 5 mg/kg (n=7)
Tumor size mm2 (mean +/- SEM)
Days post tumor injection

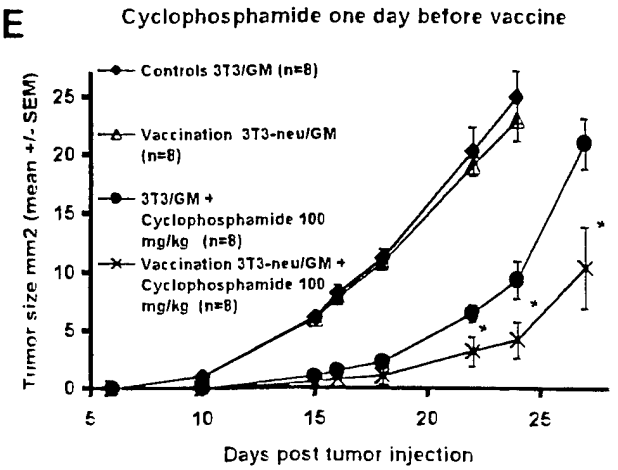
C


Paclitaxel one day before vaccine
Controls 3T3/GM (n=5)
Vaccination 3T3-neu/GM (n=5)
3T3/GM + Paclitaxel 20 mg/kg (n=5)
Vaccination 3T3-neu/GM + Paclitaxel 20 mg/kg (n=5)
Tumor size mm2 (mean +/- SEM)
Days post tumor injection

D


Paclitaxel one week after vaccine
Controls 3T3/GM (n=6)
Vaccination 3T3-neu/GM (n=6)
3T3/GM + Paclitaxel 20 mg/kg (n=6)
Vaccination 3T3-neu/GM + Paclitaxel 20 mg/kg (n=6)
Tumor size mm2 (mean +/- SEM)
Days post tumor injection

E


Cyclophosphamide one day before vaccine
Controls 3T3/GM (n=8)
Vaccination 3T3-neu/GM (n=8)
3T3/GM + Cyclophosphamide 100 mg/kg (n=8)
Vaccination 3T3-neu/GM + Cyclophosphamide 100 mg/kg (n=8)
Tumor size mm2 (mean +/- SEM)
Days post tumor injection

F

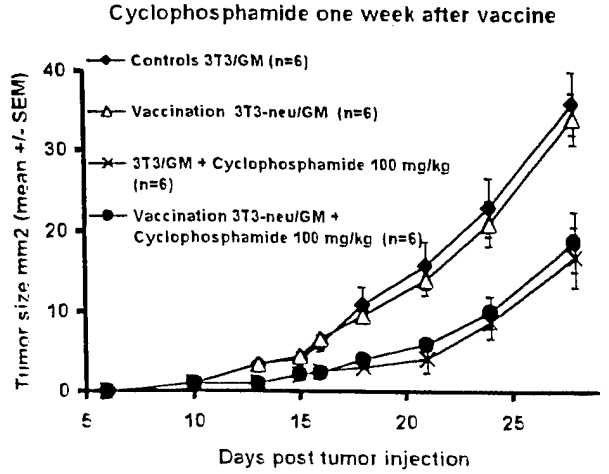
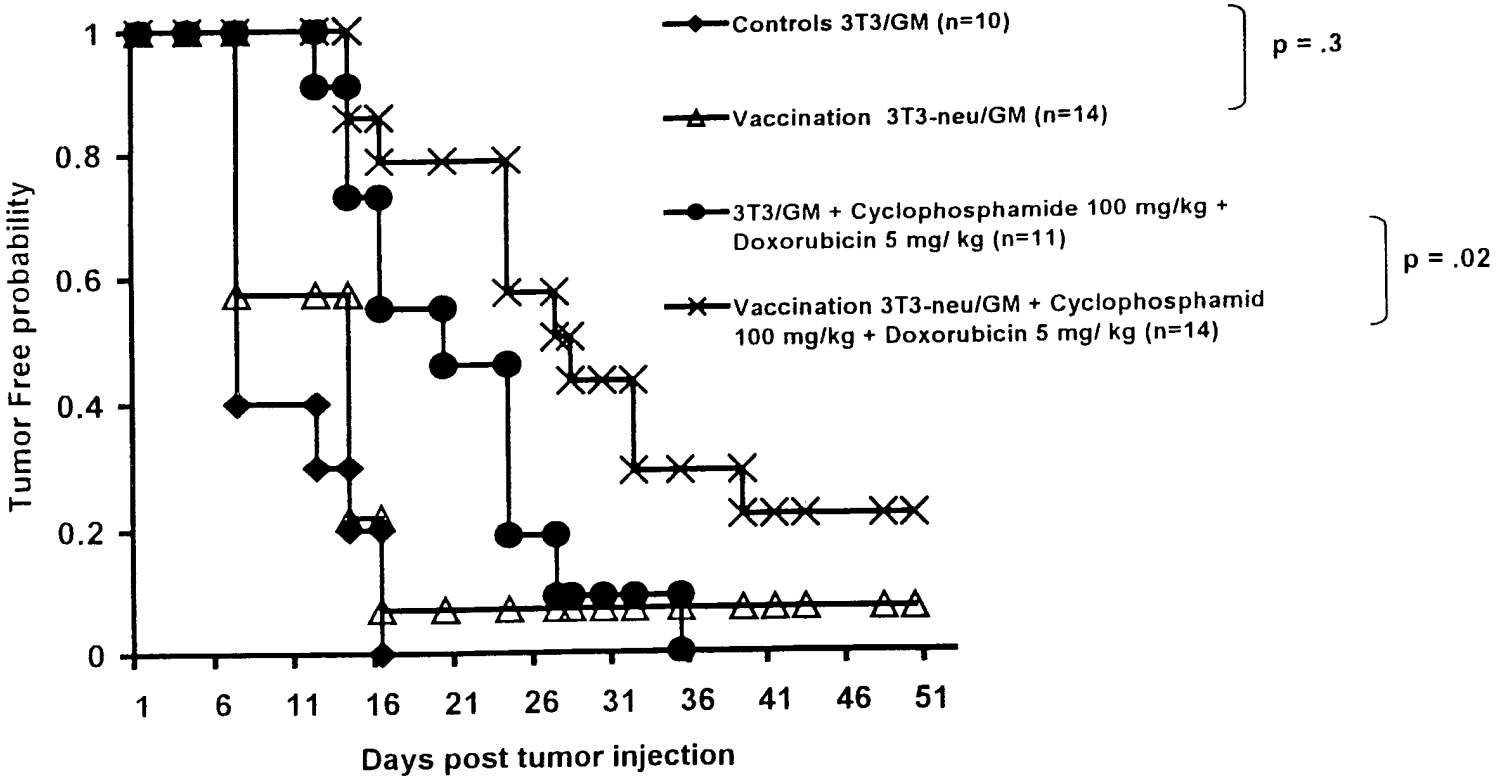
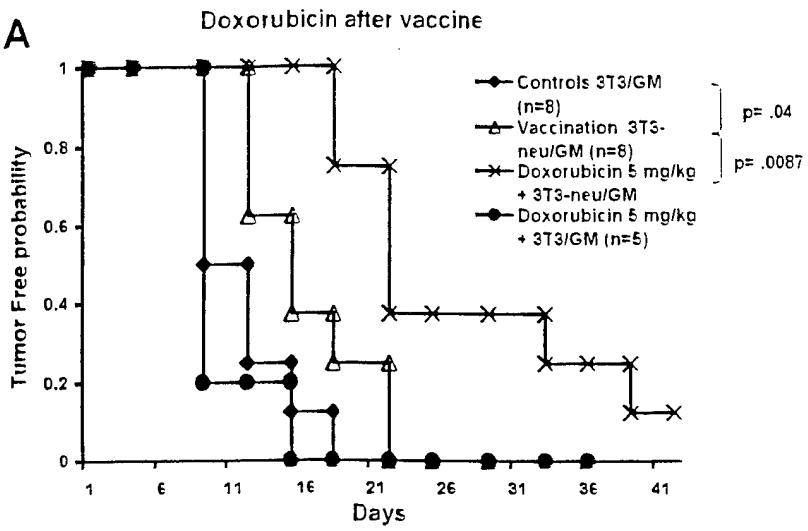

Cyclophosphamide one week after vaccine
Controls 3T3/GM (n=6)
Vaccination 3T3-neu/GM (n=6)
3T3/GM + Cyclophosphamide 100 mg/kg (n=6)
Vaccination 3T3-neu/GM + Cyclophosphamide 100 mg/kg (n=6)
Tumor size mm2 (mean +/- SEM)
Days post tumor injection

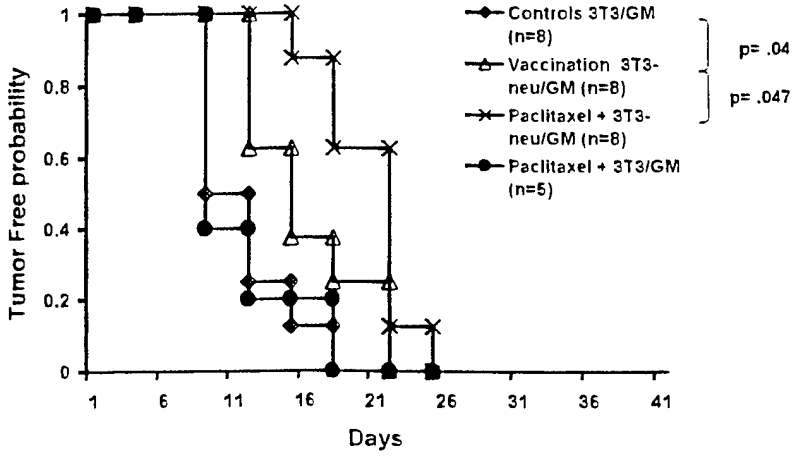
Fig 7


Tumor Free probability
1
0.8
0.6
0.4
0.2
0
1
6
11
16
21
26
31
36
41
46
51
Days post tumor injection
Controls 3T3/GM (n=10)
Vaccination 3T3-neu/GM (n=14)
p = .3
3T3/GM + Cyclophosphamide 100 mg/kg + Doxorubicin 5 mg/ kg (n=11)
Vaccination 3T3-neu/GM + Cyclophosphamid 100 mg/kg + Doxorubicin 5 mg/ kg (n=14)
p = .02

A


Doxorubicin after vaccine
Tumor Free probability
Days
Controls 3T3/GM (n=8)
Vaccination 3T3-neu/GM (n=8)
Doxorubicin 5 mg/kg + 3T3-neu/GM
Doxorubicin 5 mg/kg + 3T3/GM (n=5)
p= .04
p= .0087

B


Paclitaxel before vaccine
Tumor Free probability
Days
Controls 3T3/GM (n=8)
Vaccination 3T3-neu/GM (n=8)
Paclitaxel + 3T3-neu/GM (n=8)
Paclitaxel + 3T3/GM (n=5)
p= .04
p= .047

C

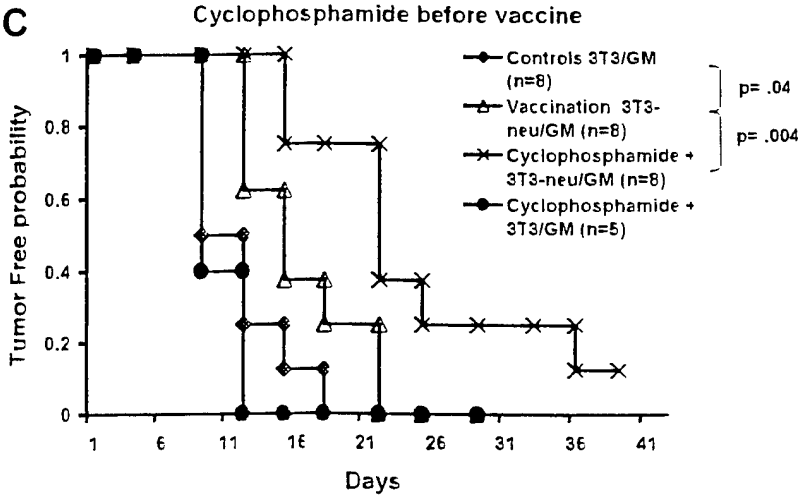
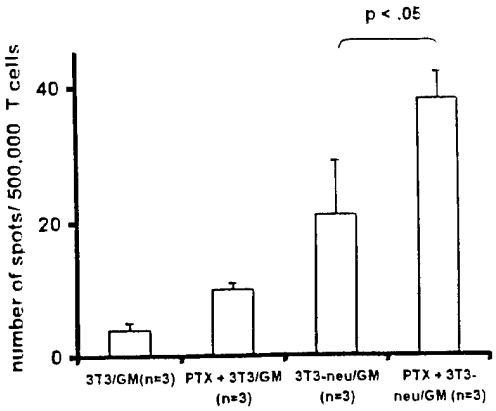
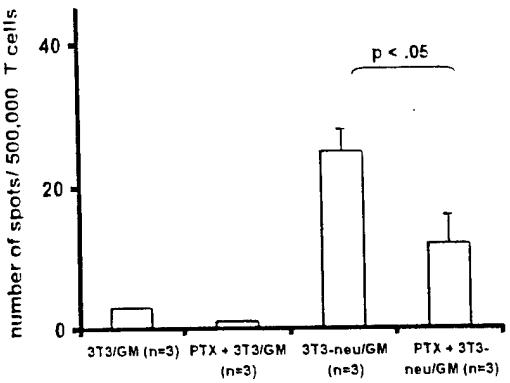

Cyclophosphamide before vaccine
Tumor Free probability
Days
Controls 3T3/GM (n=8)
Vaccination 3T3-neu/GM (n=8)
Cyclophosphamide + 3T3-neu/GM (n=8)
Cyclophosphamide + 3T3/GM (n=5)
p= .04
p= .004

Fig 4

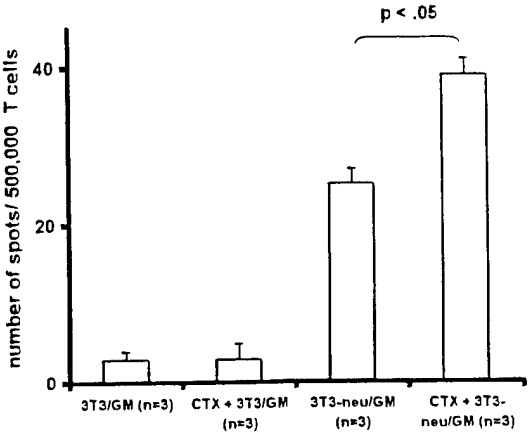
A


Paclitaxel before vaccine
p < .05
number of spots/ 500,000 T cells
40
20
0
3T3/GM(n=3)
PTX + 3T3/GM (n=3)
3T3-neu/GM (n=3)
PTX + 3T3-neu/GM (n=3)

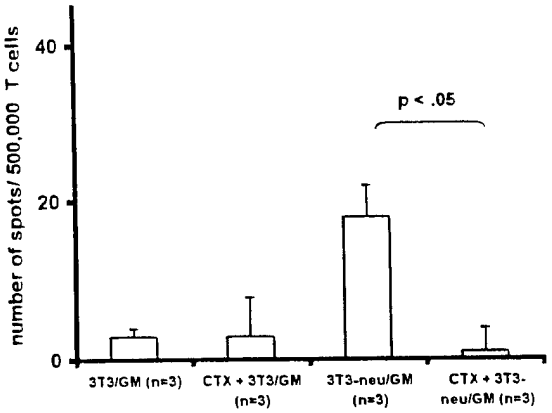
B


Paclitaxel after vaccine
p < .05
number of spots/ 500,000 T cells
40
20
0
3T3/GM (n=3)
PTX + 3T3/GM (n=3)
3T3-neu/GM (n=3)
PTX + 3T3-neu/GM (n=3)

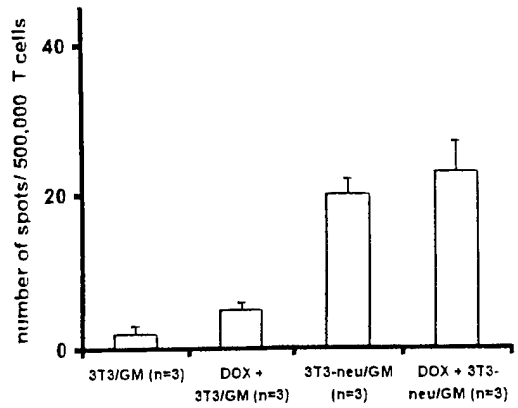
C


Cyclophosphamide before vaccine
p < .05
number of spots/ 500,000 T cells
40
20
0
3T3/GM (n=3)
CTX + 3T3/GM (n=3)
3T3-neu/GM (n=3)
CTX + 3T3-neu/GM (n=3)

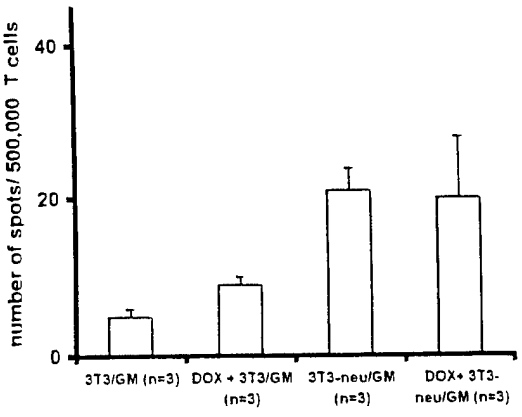
D


Cyclophosphamide after vaccine
p < .05
number of spots/ 500,000 T cells
40
20
0
3T3/GM (n=3)
CTX + 3T3/GM (n=3)
3T3-neu/GM (n=3)
CTX + 3T3-neu/GM (n=3)

E


Doxorubicin before vaccine
number of spots/ 500,000 T cells
40
20
0
3T3/GM (n=3)
DOX + 3T3/GM (n=3)
3T3-neu/GM (n=3)
DOX + 3T3-neu/GM (n=3)

F

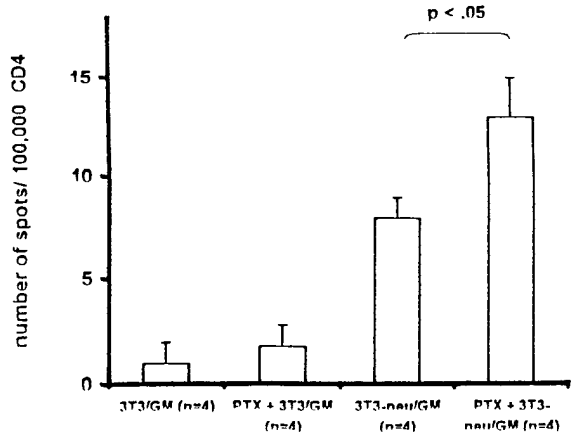

Doxorubicin after vaccine
number of spots/ 500,000 T cells
40
20
0
3T3/GM (n=3)
DOX + 3T3/GM (n=3)
3T3-neu/GM (n=3)
DOX+ 3T3-neu/GM (n=3)

Figs

IFN gamma ELISPOT--CD4+

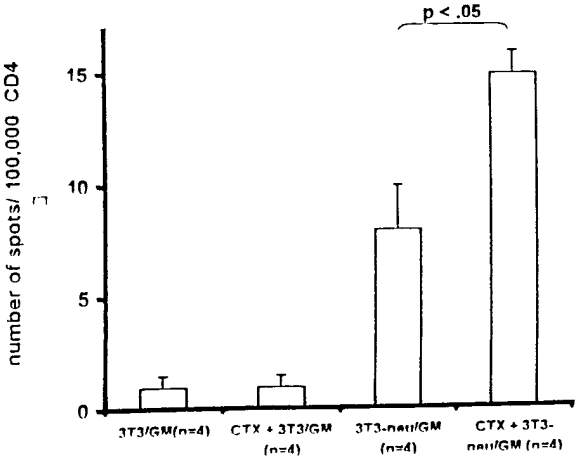
A

Paclitaxel before vaccine



C

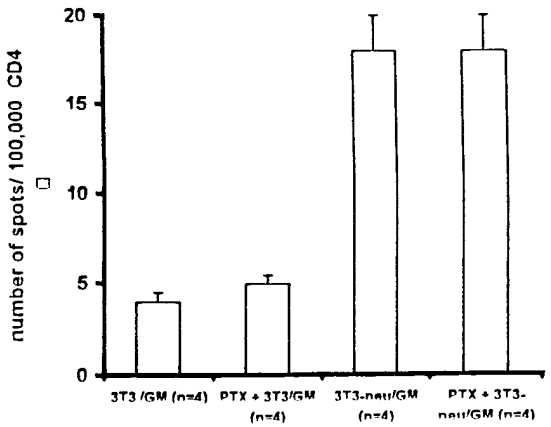
Cyclophosphamide before vaccine



IL-4 ELISPOT--CD4+

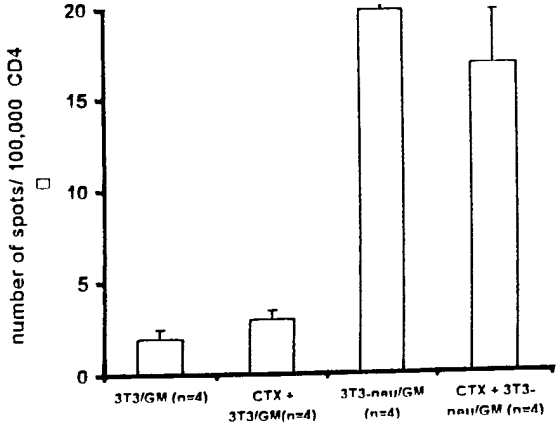
B

Paclitaxel before vaccine



D

Cyclophosphamide before vaccine



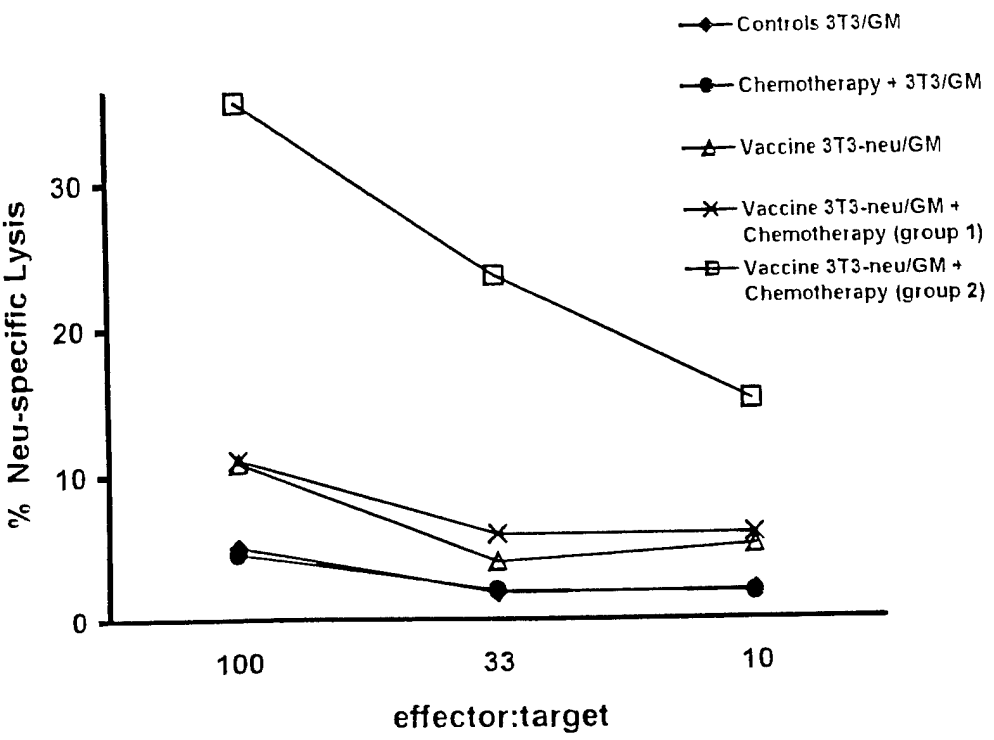

Controls 3T3/GM
Chemotherapy + 3T3/GM
Vaccine 3T3-neu/GM
Vaccine 3T3-neu/GM + Chemotherapy (group 1)
Vaccine 3T3-neu/GM + Chemotherapy (group 2)
% Neu-specific Lysis
30
20
10
0
100
33
10
effector:target

Fig 1

The collaboration of both humoral and cellular HER-2/neu-targeted immune responses is required for the complete eradication of HER-2/*neu*-expressing tumors.[1]

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Running Title: Both B and T cell responses are required to reject neu tumor.

Key Words: Her-2/*neu*, cancer vaccines, cellular immunity, humoral immunity, breast cancer

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[3] The abbreviations used are: neu, HER-2/neu; CTL, cytolytic T lymphocyte; IgG, immunoglobulin G; IL-2, interleukin-2; NK cell, natural killer cell; rVV, recombinant vaccinia virus; mGM-CSF, murine granulocyte-macrophage colony-stimulating factor; HBSS, Hank's balanced salt solution; TA; tumor antigen; HA, hemagglutinin; 3T3wt, NIH-3T3; 3T3/GM, 3T3wt cells producing mGM-CSF; 3T3-*neu*, NIH-3T3 cells expressing rat *neu*; 3T3-HA, NIH-3T3 cells expressing HA; 3T3-*neu*/GM, 3T3-*neu* cells producing mGM-CSF, NT, *neu*-expressing mouse mammary tumor line; ADCC, antibody-dependent cell cytotoxicity.

[4] Jaffee, E.M., Reilly, R.T., and Ercolini, A.M. unpublished data.

Abstract

HER-2/*neu* (*neu*) transgenic mice (*neu-N* mice), which express the non-transforming rat proto-oncogene, demonstrate immunological tolerance to neu that is similar to what is encountered in patients with *neu*-expressing breast cancer. We have previously shown that a significant increase in neu-specific T cells, but no induction of neu-specific antibody, is seen after neu-specific vaccination in *neu-N* mice. In contrast, a significant induction of both neu-specific T cell and antibody responses is found in non-toleragenic FVB/N mice after vaccination. These mice are fully protected from a subcutaneous challenge with NT cells, a mammary tumor cell line derived from a spontaneous tumor that arose in a *neu-N* mouse, while *neu-N* mice are not. In this study, we demonstrate that $CD4^+$ T cell-depleted FVB/N mice show no induction of neu-specific IgG after vaccination and are unable to reject an NT challenge (0 of 10 mice tumor-free). Conversely, the depletion of natural killer cells has no effect on vaccine-mediated tumor rejection (100% of mice tumor-free). In $CD8^+$ T cell-depleted animals, where vaccine-induced neu-specific IgG titers were normal, NT growth was delayed but only 10% of mice remained tumor free, demonstrating that neu-specific IgG, alone, is insufficient for protection from NT challenge. To directly assess the necessity for the combination of neu-specific cellular and humoral immune responses, SCID mice were given an adoptive transfer of CTL plus IgG derived from FVB/N mice. Animals that were given CTL that recognize an irrelevant antigen plus neu-specific IgG developed tumors at a rate similar to $CD8^+$ T cell-depleted FVB/N mice.

Animals receiving an adoptive transfer of neu-specific CTL plus control IgG derived from naive FVB/N mice were only partially protected from NT challenge (50% of animals tumor-free). However, only animals receiving the combination of neu-specific CTL and neu-specific IgG were fully protected from NT challenge (100% of animals tumor-free). These studies specifically define the immunological requirements for the eradication of neu-expressing tumors in this model system, demonstrating that both cellular and humoral neu-specific responses are necessary for protection from an NT challenge. These data suggest that vaccines optimized to induce maximal T- *and* B-cell immunity to neu, and possibly to similar putative tumor-rejection antigens, may lead to more potent *in vivo* anti-tumor immunity.

Introduction

Current vaccine strategies for the treatment of solid tumors tend to focus on the cellular arm of the immune response. However, the success of passive immunotherapy through the administration of monoclonal antibodies that target neu[3] (1), CD19 (2), or the epidermal growth factor receptor (3) has generated renewed interest in the application of humoral immunity in tumor eradication. Trastuzumab, a recombinant humanized monoclonal antibody to neu, administered as a single agent or in combination with chemotherapy, produces durable objective responses in women with *neu*-overexpressing breast cancer (4). Similarly, passive immunotherapy with monoclonal antibodies against neu was shown to have a dramatic effect on spontaneous tumor development in transgenic mice expressing rat *neu* (5). We have documented the existence of immunological tolerance to neu in these mice similar to what is observed in patients with *neu*-expressing breast cancers (6). While *neu-N* mice are capable of generating cytotoxic T lymphocytes (CTL) against neu after neu-specific vaccination, there is little or no vaccine-mediated induction of neu-specific IgG in mice vaccinated after 8 weeks of age (6). Furthermore, while the growth of *neu*-expressing transplantable tumors in vaccinated *neu-N* mice is significantly delayed relative to control animals, tumor growth is not completely prevented (6). This is in stark contrast to what is seen in the absence of tolerance. In non-transgenic FVB/N mice, a significant induction of both neu-specific CTL and neu-specific IgG is seen and mice are completely protected from a transplantable tumor challenge (6). In this study, data are presented demonstrating the induction of neu-specific humoral and cellular immunity by active

immunization. Furthermore, the combination of neu-specific humoral and cellular immune responses fully protects from a *neu*-expressing tumor challenge, where the absence of either the cellular or humoral arm leads to incomplete protection. These data have important implications for the development of vaccines that induce immunity against antigens that are targets of both B and T cell responses.

Materials and Methods

Mice. Eight- to ten-week old *neu-N* mice (line N#202, (7)), FVB/N (National Cancer Institute, Bethesda, MD), and Balb/c SCID (NCI) mice were used. All experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Cell Lines and Media. The *neu*-expressing NT mammary tumor cell lines were previously described (6). The 3T3wt (ATCC, Rockville, MD) and the 3T3-*neu* cell line (CRL-1915, ATCC, Rockville, MD) which expresses rat *neu* (8), were maintained as described (6). NIH-3T3 cells transfected with a plasmid encoding HA were maintained under selection with 1 mg/ml geneticin (Life Technologies, Grand Island, NY). HA expression was verified by FACS analysis (data not shown). The 3T3wt and 3T3-*neu* lines were genetically modified to express mGM-CSF by retroviral transduction as described (6).

T cell Lines. The neu-specific $CD8^+$ T cell line Fneu-CTL was established from an FVB/N mouse after neu-specific plasmid DNA vaccination followed by NT challenge. This animal was tumor-free for greater than 100 days, after which time the animal was sacrificed and

splenectomized. Splenocytes were maintained in culture in RPMI (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 0.5% L-glutamate and 1% penicillin/streptomycin (JRH Biosciences, Lenexa, KS), and 0.1% 2-mercaptoethanol (Sigma, St. Louis , MO) at 37°C and 5% CO_2. Fneu-CTL cells have been in culture for greater than 24 months and are stimulated every 9 days by co-culture with mitomycin-treated 3T3-*neu* cells and irradiated syngeneic splenocytes. The Fneu-CTL line is 100% $CD8^+$, expresses a single TCR β-chain variable region (Vβ4) as determined by flow cytometry, and shows a high degree of specificity for *neu*-expressing cell lines as determined by chromium release assay (data not shown). Fneu-CTL cells were suspended in sterile hanks balanced salt solution (Life Technologies, Grand Island, NY) at a concentration of $4x10^7$ cells per ml for adoptive transfer. Control $CD8^+$ T cells specific for HA (FHA-CTL) were similarly obtained from FVB/N mice after vaccination with $3x10^7$ plaque forming units of HA rVV. The FHA-CTL line has been maintained *in vitro* for approximately one year and is stimulated as described above for Fneu-CTL except with NIH-3T3 cells transfected with a plasmid encoding HA. These cells are specific for the HA protein and do not lyse *neu*-expressing cell lines (data not shown).

Isolation of Neu-specific IgG. Eight week old female FVB/N mice were primed with a s.c. injection of $5x10^6$ neu-expressing NT cells. After 14 days, animals were sacrificed and blood was obtained by cardiac puncture, pooled, and allowed to coagulate. The serum was then pooled and the total IgG was obtained by ammonium sulfate precipitation and dialyzed against

phosphate buffered saline (Life Technologies, Grand Island, NY). Neu-specific immunoreactivity of the total IgG was verified by flow cytometry as described previously (6). Control IgG was obtained from naive FVB/N mice. Protein concentrations were determined using the Lowry Assay (Sigma, St. Louis, MO)) according to the manufacturer's directions. Total IgG samples were adjusted to a final concentration of 10 mg/ ml using HBSS prior to injection.

For quantitation of neu-specific serum IgG, serum samples were obtained by tail bleed one day prior to tumor challenge (*neu-N* and FVB/N mice) or one week after the transfer of neu-specific serum (SCID mice) and neu-specific IgG titers determined as described (6).

Whole-cell Vaccinations. Vaccinations with 3T3/GM or 3T3-*neu*/GM cells were performed as described (6).

Chromium-Release Assays. Animals were vaccinated with 3T3-*neu*/GM or 3T3/GM and CTL prepared as described (6). Lytic function was determined against 3T3wt, 3T3-*neu*, and NT cells in a 4-hour ^{51}Cr-release assay. Percent neu-specific lysis was determined by the following formula: % neu-specific lysis = (% lysis against 3T3-*neu* targets) – (% lysis against 3T3wt targets).

Depletion Studies. The depletion of $CD4^+$ and $CD8^+$ T cell subsets and NK cells was accomplished by i.p. injection of 500 μg GK1.5 (anti-CD4) or 2.43 (anti-CD8) antibody or pk136 (anti-NK) antibody, respectively, as described (6).

Tumor Challenge Experiments. Tumor challenge consisted of $5x10^4$ (for *neu-N* mice), $1x10^6$ (for SCID mice), or $5x10^6$ (for FVB/N mice) NT cells s.c. in the right hind limb 14 days after receiving a whole-cell vaccine (*neu-N* and FVB/N mice) or one day prior to adoptive transfer (SCID mice). Animals were monitored twice weekly for the development of palpable (>5mm in diameter) tumors. Animals were sacrificed before tumors reached a diameter greater than 12mm.

Adoptive Transfer Experiments. One day following s.c. tumor challenge, eight week old female SCID mice received $2x10^7$ T cells via tail vein injection and/or passive infusion of 1 mg IgG given i.p. T cells were maintained *in vivo* by daily i.p. injections of 20,000 international units of recombinant human IL-2 (Chiron, Emeryville, CA). IgG injections were given weekly throughout the experiment. Animals were monitored as described above.

Statistical Analyses. Statistical analyses were performed using the Statview software program (SAS Institute Incorporated, Cary, NC). Kaplan-Meier non-parametric regression analyses for tumor challenge experiments were performed and significance determined using the Mantel-Cox log-rank test.

Results and Discussion

We previously described the existence of tolerance to neu in *neu-N* mice (6). One measure of neu-specific tolerance in *neu-N* mice is the absence of an inducible neu-specific humoral response above low baseline levels of neu-specific IgG (6). In addition, splenocytes from *neu-N* mice taken 14 days after neu-specific vaccination, while capable of neu-specific lysis

of 3T3-*neu* cell targets (6), cannot lyse NT cells in a 4-hour ^{51}Cr-release assay (data not shown). The net result of this is incomplete protection from tumor challenge and partial prevention of spontaneous tumor formation in vaccinated animals. In contrast, FVB/N mice, in which tolerance to the rat neu protein does not exist, generate robust neu-specific antibody and CTL responses to vaccination and are fully protected from NT challenge (6). In this model of *neu*-expressing tumor eradication, successful anti-tumor immunity appears to be associated with the induction of both neu-specific humoral and cellular immunity in non-tolerized mice. Therefore, we used FVB/N mice to define the immunological requirements for the successful rejection of *neu*-expressing tumors.

In order to determine the relative importance of the various T cell subsets in the anti-tumor response, FVB/N mice were depleted of $CD4^+$ T cells, $CD8^+$ T cells, or NK cells by antibody injection (GK1.5, 2.43, and pk136, respectively). The animals were then vaccinated, challenged two weeks later with NT cells, and monitored for tumor development. In addition, serum samples were obtained prior to tumor challenge and neu-specific IgG titers determined. These data, summarized in **Table I**, demonstrate an absolute requirement for both $CD4^+$ T cells as well as $CD8^+$ T cells in mediating tumor-free survival. The results confirm recently reported data demonstrating a similar $CD4^+$- and $CD8^+$-T cell dependence for protection from an NT challenge in *neu-N* mice (6). The tumor-free survival of CD4-depleted mice is essentially indistinguishable from that of immunocompetent mice receiving a mock vaccination ($p = 0.53$ vs. no deplete, 3T3/GM). In the absence of $CD4^+$ T cell help, these animals would be incapable

of generating either a T cell or B cell effector response; the lack of neu-specific B cell effectors is confirmed by the absence of a neu-specific IgG response in these animals. Mice depleted of $CD8^+$ T cells, where $CD4^+$ T cell help is still in place, develop neu-specific IgG at titers that are identical to that obtained in undepleted animals receiving a neu-specific vaccination (no deplete, 3T3-*neu*/GM). Additionally, these mice demonstrate a significant delay in the appearance of palpable tumors relative to the mock vaccine controls ($p<0.001$ vs. no deplete, 3T3/GM). However, only 10% of the $CD8^+$ depleted animals remained tumor-free beyond day 60 post-challenge, whereas all undepleted animals receiving a neu-specific vaccination were tumor-free.

The fact that FVB/N mice depleted of $CD8^+$ T cells were not fully protected from NT challenge despite the presence of normal neu-specific IgG titers suggested that a neu-specific antibody response, while growth inhibitory, was not sufficient for tumor eradication. To more directly assess the necessity for neu-specific antibody in tumor rejection, we sought to reconstitute the neu-specific immune response of FVB/N mice in a SCID model system. SCID mice were given a subcutaneous tumor challenge on day 0. Animals were then divided into groups receiving either 1) Fneu-CTL cells plus daily IL-2, 2) total IgG derived from FVB/N mice primed against rat *neu*, 3) Fneu-CTL cells plus neu-specific IgG plus IL-2, or 4) total IgG derived from naive FVB/N mice plus FHA-CTL plus IL-2. An *in vitro* analysis of the lytic ability of the Fneu-CTL line in a 4-hour ^{51}Cr-release assay demonstrated excellent specificity for neu (**Figure 1A**). A similar study of the lytic ability of the FHA-CTL line showed excellent specificity for HA-expressing cells with no recognition of neu-expressing targets (**Figure 1B**).

Six days after the first injection of neu-specific IgG, SCID mice had serum antibody titers of approximately 150 (data not shown), similar to what we have reported in FVB/N mice after 3T3-*neu*/GM vaccination (**Table 1**). The tumor-free survival of these mice is shown in **Figure 2**. Consistent with our observations in the FVB/N depletion studies, SCID mice given neu-specific IgG alone showed a significant delay in the appearance of palpable tumors ($p < 0.001$ relative to IL-2 alone), however no animals remained tumor-free. This study also confirmed the importance of a potent neu-specific CTL response as 50% of animals given neu-specific $CD8^+$ T cells were tumor-free beyond the 50-day endpoint of the experiment. Additional control groups in which animals received either FHA-CTL cells or total IgG derived from naive FVB/N mice were indistinguishable from animals receiving IL-2 alone (data not shown). Furthermore, animals receiving neu-specific T cells plus IgG from naive mice were indistinguishable from animals receiving only neu-specific T cells (data not shown). Most striking, however, was the finding that the combination of neu-specific IgG and neu-specific CTL gave significantly better protection from tumor challenge than either treatment alone; all animals in the CTL/IgG combination group were tumor-free beyond the endpoint of the experiment. These data demonstrate that, while neu-specific CTL play a dominant role in tumor rejection, the greatest level of protection is seen when both neu-specific $CD8^+$ T cells and antibodies are present.

There is a large body of evidence in the literature to support the idea that antibodies directed against neu can inhibit the growth of *neu*-expressing tumors (9-11) through several mechanisms, including ADCC and the inhibition of signal transduction through neu. Thus, it is

likely that the neu-specific antibody response in CD8-depleted mice is mediating the growth inhibitory effects in these studies. The fact that NK-depleted mice are fully protected from tumor challenge suggests that NK-mediated mechanisms of immunity do not play a significant role in the delayed tumor growth seen in the CD8-depleted group. This does not, however, exclude a role for macrophage- or monocyte-mediated ADCC in addition to complement fixation and/or the direct growth inhibition of NT cells by antibody-mediated blockade of signaling through neu. Clynes et al. (10) recently reported that the *in vivo* growth inhibitory activity of trastuzumab, an FDA-approved monoclonal antibody against the human *neu* gene product, is only partially mediated by the direct interaction of the antibody with surface neu on a *neu*-overexpressing target tumor cell line. In addition to the antibody-mediated tumor growth inhibition, the data demonstrated a major role for Fc-receptor-dependent mechanisms in tumor eradication (10). While the data did not exclude a specific role for NK cells in tumor rejection, a dependence on cells expressing both FcγRIIb and FcγRIII (i.e. monocytes and macrophages) was demonstrated. This is consistent with our observation that NK cells are not required for protection from subcutaneous tumor challenge in vaccinated FVB/N mice (Table I).

The Fneu-CTL line used in the adoptive transfer experiments described above was derived from an FVB/N mouse and demonstrates significant lysis of *neu*-expressing targets. However, the neu-specific T cell repertoire of *neu-N* mice is functionally distinct from that of FVB/N mice in its lytic ability[4]. Similarly, patients with neu-positive tumors would be expected to express a neu-specific T cell repertoire that reflects tolerance to this "self" protein. It is

possible that under these conditions of more limited neu-specific CTL lytic ability, the presence of significant neu-specific IgG titers is even more important. Whether the effect of the antibody and CTL interaction is synergistic or simply additive remains to be determined.

Neu-specific CTL and antibody responses have been found in patients with neu-expressing tumors (12-15). In addition, several groups have demonstrated vaccine-induced neu-specific CTL and antibody responses in animal model systems with vaccine-mediated protection from neu-expressing tumor challenge as well as spontaneous tumor formation (16-20). However, there has been no evidence to date correlating protective anti-tumor immunity with both antigen-specific CTL and antibody responses. Here we show that the induction of neu-specific CTL and IgG responses in non-tolerized mice after neu-specific vaccination is potent enough to fully protect these animals from challenge with a *neu*-expressing tumor line. Significantly, we have also demonstrated that optimal anti-tumor immunity is achieved only when *both* neu-specific CTL and neu-specific IgG are present. Together, these studies suggest that vaccines optimized to induce maximal T- *and* B-cell immunity to neu, and possibly to similar putative tumor-rejection antigens, may lead to more potent *in vivo* anti-tumor immunity.

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Table I. The Effects of Lymphocyte Subset Depletion on Tumor-free Survival in FVB/N Mice.

Group	Neu-specific IgG (Titer ± SD)	Mean Tumor-free Survival (Days ± SD)	# Tumor-free/ Total Animals
No deplete, 3T3/GM	none detected	16 ± 6	0/10
CD4 Deplete, 3T3-*neu*/GM	none detected	16 ± 5	0/20
CD8 Deplete, 3T3-*neu*/GM	160 ± 21	33 ± 10*	2/20
NK Deplete, 3T3-*neu*/GM	157 ± 24	60*	20/20
No Deplete, 3T3-*neu*/GM	163 ± 27	60*	20/20

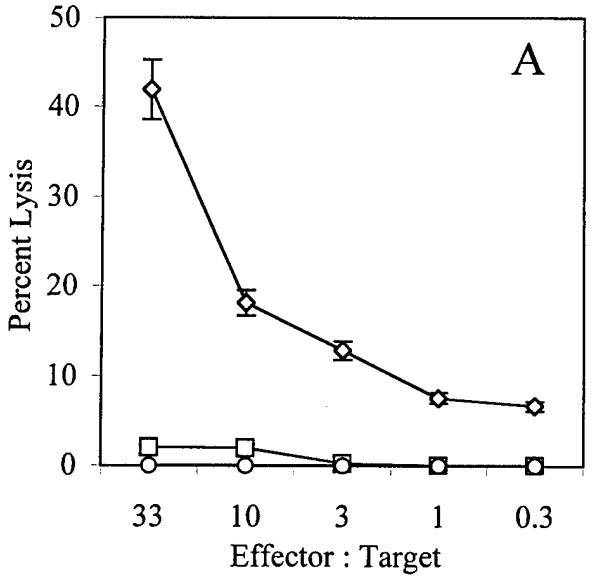
FVB/N mice were depleted of $CD4^+$ T cells, $CD8^+$ T cells, or NK cells as described in Methods. Depletion was verified by flow cytometric analysis of splenocytes (data not shown) and depletion maintained by twice-weekly injections of the depleting antibody. Animals were then vaccinated with 3T3/GM (control vaccine) or 3T3-*neu*/GM (neu-specific vaccine) followed 14 days later by s.c. NT challenge. The mice were monitored for the development of palpable tumors (> 5 mm diameter). The average tumor-free survival in days ± standard deviation as well as the number of animals tumor-free at the completion of the experiment (day 60 post-challenge) are reported for each group. In addition, serum samples were taken from each animal one day prior to tumor challenge and analyzed for neu-specific IgG as described in Methods. The titer reported is the greatest dilution of serum for which a shift in mean fluorescence intensity of binding to 3T3-*neu* cells is seen relative to an irrelevant control antibody. These data are combined from two separate experiments.

* Estimate of the mean tumor-free survival is biased because animals that were tumor-free at the conclusion of the experiment were assigned a tumor-free survival of 60 days.

Legends.

Figure 1. The Fneu-CTL and FHA-CTL T cell Lines are Specific for Neu and HA, Respectively. Fneu-CTL (**A**) and FHA-CTL (**B**) $CD8^+$ T cells were used in a 4-hour ^{51}Cr-release assay at the effector-to-target ratios indicated. The percent lysis is indicated for incubation with 3T3-wt (□), 3T3-neu (◇) and 3T3-HA (○). Data were obtained in triplicate. The mean value is shown with SD indicated by error bars.

Figure 2. SCID Mice Require both Neu-specific CTL and Neu-specific IgG for Tumor Eradication. SCID mice were given a s.c. NT challenge followed by $2x10^7$ neu-specific $CD8^+$ T cells (○), 1 mg/week IgG from neu-primed mice (□) or both (●). Control animals received HA-specific T cells, 1 mg/week IgG from naive mice, and IL-2 (◆). This experiment was repeated twice ($n \geq 5$ animals per group per experiment) with similar results. A representative experiment is shown.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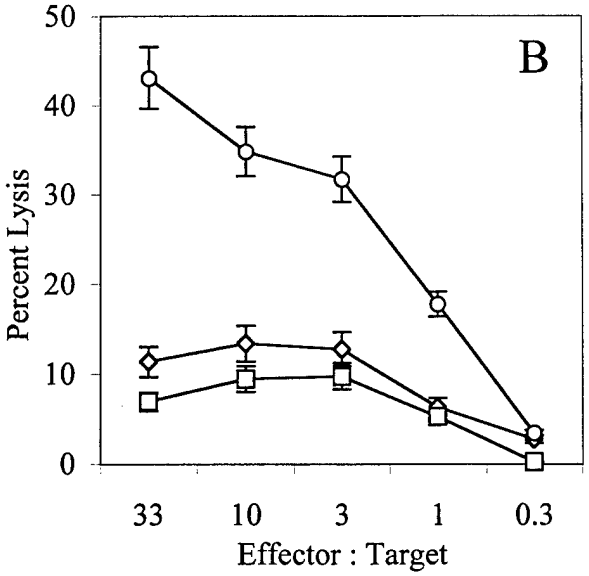
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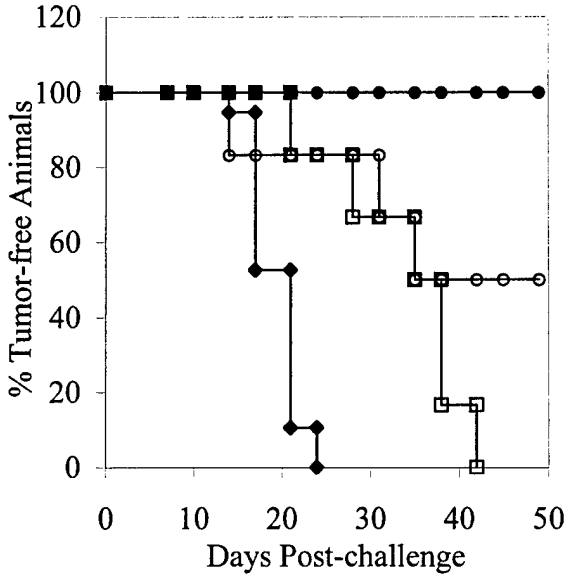
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