HER-2/neu Is a Tumor Rejection Target in Tolerized HER-2/neu Transgenic Mice

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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 nontolerogenic 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 target an immune response against a protein that is artificially overexpressed by the model tumor and not normally expressed in the host, cancer. 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 overexpression alone is sufficient for mammary carcinogenesis. These mice do not seem to exhibit tolerance to neu (33, 34).
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 (RH Biosciences, Lenexa, KS), and 0.2% insulin (Eli Lilly and Co., Indianapolis, IN), and maintained at 37°C in 5% CO2. NIH-3T3 cells were grown in 3T3 media: DMEM (Life Technologies) with 10% bovine calf serum, 1% L-glutamine, 1% Na pyruvate, 1% nonessential amino acids, 1% Na pyruvate, 0.5% penicillin/streptomycin, 0.02% gentamicin (RH Biosciences, Lenexa, KS), and 0.2% insulin (Eli Lilly and Co., Indianapolis, IN), and maintained at 37°C in 5% CO2. NIH-3T3 cells (ATCC, Manassas, VA) were grown in 3T3-media: DMEM (Life Technologies, Inc.) with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 1% l-glutamine, 1% Na pyruvate, 1% nonessential amino acids, 1% Na pyruvate, 0.5% penicillin/streptomycin, 0.02% gentamicin (RH Biosciences, Lenexa, KS), and 0.2% insulin (Eli Lilly and Co., Indianapolis, IN), and maintained at 37°C in 5% CO2. NIH-3T3 cells were assayed by flow cytometry in vitro 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 gene transfer was assessed by 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 avoiding extensive subpassage and to insure the reproducibility of each passage and to insure the reproducibility of each line NSF-60 (39). The NT2 and NT5 tumor lines were assayed by flow cytometry in vitro 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 gene transfer was assessed by 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 ensure 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 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (Sigma Chemical Co., St. Louis, MO), and 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (Sigma Chemical Co.). neu-specific IFN-γ (PharMingen, San Diego, CA), 2 μg/ml biotinylated rat antineu 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 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 × 10^6 NT2 target cells/well with serial dilutions of T cells (1 × 10^3 cells/well to 1 × 10^4 cells/well). Reagents used in the assay were: 10 μg/ml rat antineu IFN-γ (PharMingen, San Diego, CA), 2 μg/ml biotinylated rat antineu 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 mononal 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.

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were given a series of five weekly injections of $1 \times 10^7$ 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, naive 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 $5 \times 10^6$ 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.

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.
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.).

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.

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 × 10^5 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. 5A, 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. 5B, 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.

**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. 6A, 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. 6B). 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-
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.

**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 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.
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-transgenic mammary tumor model, in which mice are not tolerant to the transgene-encoded neu 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 tolized 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. It is also possible that the lower-avidity T cells undergo peripheral anergy induction in the transgenic mice.

We have shown that two neu-specific vaccines can generate anti-tumor 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

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<tr>
<th>Group (number of animals)</th>
<th>% Tumor-free animals (day 24 after challenge)</th>
<th>( P )</th>
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<tbody>
<tr>
<td>No depletion, no vaccine</td>
<td>0</td>
<td>—</td>
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<tr>
<td>(( n = 30))</td>
<td></td>
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<tr>
<td>Mock depletion, vaccine</td>
<td>75 (( n = 31))</td>
<td>0.007</td>
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<tr>
<td>( CD4 ) depletion, vaccine</td>
<td>0 (( n = 22))</td>
<td>0.31</td>
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<tr>
<td>( CD8 ) depletion, vaccine</td>
<td>40 (( n = 33))</td>
<td>0.02</td>
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<tr>
<td>NK depletion, vaccine</td>
<td>68 (( n = 30))</td>
<td>0.004</td>
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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 and postlactational mice. Third, these studies use whole-cell and rVV detected in fetal or virgin females, is clearly up-regulated in nursing cell-mediated cytotoxicity against simian virus 40-transformed cells. An antibody-based mechanism for tumor immunity. J. Immunol., 153: 2064–2071, 1994.


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