# Therapy of Advanced Established Murine Breast Cancer with a Recombinant Adenoviral ErbB-2/*neu* Vaccine

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## Abstract

**ErbB-2 (HER-2/*neu*) is a transforming oncogene expressed by a substantial fraction of breast cancers, and monoclonal antibody therapy directed toward this antigen is an established treatment modality. However, not all tumors respond, and with a monoclonal antibody directed to a single epitope, there is always the risk of tumor escape. Furthermore, passive antibody therapy requires continual treatment. Whereas cancer vaccines have prevented the growth of tumors, it has been far more difficult to treat large established tumors. Here, we show that vaccination with a recombinant adenovirus expressing a truncated ErbB-2 antigen can cure large established subcutaneous ErbB-2–expressing breast cancers in mice, and can also cure extensive established lung metastatic disease. We also show that the mechanism of protection involves antibody-mediated blockade of ErbB-2 function, independent of Fc receptors. We conclude that a vaccine inducing antibodies to a functional oncogenic receptor could have tremendous therapeutic potential against cancers overexpressing such molecules.** [Cancer Res 2008; 68(6):1979–87]

## Introduction

The ErbB-2 (HER-2/*neu* in humans and *neu* in rodents) oncogene, a member of the epidermal growth factor receptor family, is overexpressed in 20% to 30% of breast cancers and is associated with treatment resistance and a poorer clinical outcome at every stage (1–3). Its importance as a therapeutic target is highlighted by the development of trastuzumab (Herceptin), a humanized monoclonal antibody targeting the HER-2/*neu* protein, which is approved for the treatment of patients with breast cancer (4–7). However, not all HER-2/*neu*–expressing tumors respond to trastuzumab, and with use of a monoclonal antibody directed to a single epitope, there is always the risk of tumor escape due to loss or mutation of the target. Furthermore, passive antibody therapy requires continual treatment over a prolonged period of time, incurring considerable inconvenience and expense. A vaccine inducing endogenous production of anti–HER-2/neu antibodies might provide a more prolonged therapeutic benefit without the need for repeated administrations. Also, such a vaccine might induce antibodies to multiple epitopes of the HER-2/neu protein, and thus, reduce the risk of tumor escape, as well as more effectively inhibit the function of the target molecule (8).

In previous studies, we found that a vaccine consisting of a recombinant adenovirus expressing the rat *neu* oncogene extracellular and transmembrane segments (Ad-*neu*ECTM) prevented or delayed the development of breast tumors in *neu* transgenic mice when given to the mice starting at 4 to 6 weeks of age, before tumors were detectable, and the vaccine could also prevent the growth of an adoptively transferred *neu*-expressing syngeneic TUBO mammary carcinoma when the vaccine was given prior to tumor inoculation (9, 10). The vaccine was effective whether given as free adenovirus (9) or given as syngeneic dendritic cells transduced with the recombinant vector (10). The latter approach, although more cumbersome, had the advantage of avoiding interference from preexisting antiadenoviral immunity. The mechanism of protection was shown to be antibody-mediated, requiring $CD4^+$ T helper cells only during the first 2 days after vaccination to provide help for antibody induction, and not requiring $CD8^+$ T cell immunity at all (9). In contrast to trastuzumab, which has been shown to require Fc receptors for activity (11), implying a role for antibody-dependent cellular cytotoxicity (ADCC), the antibodies induced by the vaccine worked just as well in FcR-null mice, and therefore, must protect against tumors by a different mechanism (9). This mechanism seems to be independent of complement activity or other immune cells, as the antibody inhibited the growth of TUBO cells in culture in the absence of other cells or complement. These results suggested that the antibodies might cause the death of tumor cells by directly inhibiting the signaling through the *ErbB-2* gene product, although the precise mechanism of the inhibition was not well elucidated. This finding suggests that a HER-2/*neu*–expressing adenoviral-based vaccine may be more effective because it can induce antibodies that could protect against tumor by multiple independent mechanisms—a third advantage of such an active vaccine over passive immunotherapy.

We have now applied this approach to therapeutic vaccination against established tumors. Although many cancer vaccine approaches have prevented the growth of autochthonous or transplanted tumors subsequently inoculated in mice in tumor challenge experiments, it has been far more difficult to treat large established tumors (12). Even for adoptively transferred tumors in mice, although small "established" tumors have been successfully treated (13), only rare examples have been published in which established tumors >1 cm in diameter have been successfully treated by vaccine therapy (14). Here, we show that vaccination with a recombinant adenovirus expressing a truncated *neu* antigen (Ad-*neu*ECTM) can effectively treat and cure large established subcutaneous *neu*-expressing breast cancers >2 cm in diameter in mice, as well as cure extensive lung metastatic disease. This protection is antibody-mediated, as we have now shown, through an effect on ErbB2 function. These results show the striking potential of vaccine-induced antibodies binding to a functional receptor encoded by an oncogene, such as HER-2/*neu*. This model

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seems to more closely mimic human HER-2–positive breast cancer, in which spontaneous immune responses to HER-2 are observed even without vaccines or immunotherapy (15, 16), compared with the *neu*-transgenic mouse, in which central tolerance occurs (17, 18), in contrast to what has been observed in humans. An additional observation is that with vaccine therapy, the tumors tend to progress for some weeks prior to showing regression, even when complete cure would eventually occur. This delay may reflect the time needed to induce an adequate immune response. Therefore, for vaccine therapy of cancer, it might be safer to design protocols that do not to remove patients from the study too quickly when they initially progress on treatment.

## Materials and Methods

**Cell lines.** TUBO cells were generated from a mammary gland carcinoma from a BALB-*neu*T (*neu*-transgenic) mouse (19). N202.1A and N202.1E cells were derived from a breast cancer from a FVB mouse (H-$2^q$) transgenic for the rat *neu* oncogene (20). These cell lines were kindly provided by Dr. P. Nanni (University of Bologna, Bologna, Italy). Neu protein is highly expressed on the cell surface of TUBO and N202.1A cells and is absent on N202.1E cells. Human embryonic kidney cells (HEK) 293 cells were obtained from American Type Culture Collection. All cell lines were maintained at 37°C in 5% $CO_2$ in DMEM medium supplemented with 10% fetal bovine serum (FBS), L-glutamine, sodium pyruvate, streptomycin, and penicillin.

**Mice.** Female BALB/c mice were obtained from the Cancer Research Facility, NIH (Frederick, MD). BALB-β2-microglobulin (β2m) knockout (KO) mice [C.129 P2(B6)-$\beta2m^{tm1Unc}$] lacking $CD8^+$ T cells, and BALB-IFN-γ KO mice [C.129 S7(B6)-$Ifn\gamma^{tm1Ts}$] were purchased from The Jackson Laboratory. BALB-IgHJ KO mice [C.129(B6)-IgH-$J^{tm1Dhu}$], null for the *IgH-J* gene and deficient in B cells, and BALB-FcεR KO mice [C.129 P2(B6)-$Fc\varepsilon r1g^{tm1N12}$] lacking activating IgFcεRs, and the high-affinity FcγRI/III, were obtained from Taconic Farms, Inc. All mice were used at 8 to 10 weeks of age. Animal experiments were conducted in accordance with protocols approved by the Animal Care and Use Committee of the National Cancer Institute.

**Reagents.** Rat anti-mouse CD4 (clone GK1.5) and anti-mouse CD8 (clone 2.43) monoclonal antibodies were obtained from the Frederick Cancer Research and Development Center, National Cancer Institute (Frederick, MD). As a control antibody, rat IgG was purchased from ICN Pharmaceuticals. FITC-conjugated anti-mouse immunoglobulin was purchased from BD PharMingen. Adenoviral vectors were prepared as previously described (10). Briefly, the E1, E3-deleted vectors were generated using the AdMax system (Microbix) and a cDNA encoding the rat *neu* oncogene lacking the intracellular signaling domain, but expressing the extracellular and transmembrane domains (Ad-*neu*ECTM), in HEK 293 cells by calcium phosphate precipitation and homologous recombination. Ad-Null, a control vector expressing no transgene, was similarly prepared. All adenoviral vectors were double plaque–isolated, expanded in HEK 293 cells, purified on a cesium chloride density gradient, titered as plaque-forming units (pfu) per milliliter, and stored at −70°C.

***In vivo* depletion of $CD4^+$ and $CD8^+$ T cells.** *In vivo* T cell depletions were previously described (9). Briefly, monoclonal antibodies GK1.5 or 2.43 were used to deplete $CD4^+$ T cells or $CD8^+$ T cells, respectively, by the i.p. injection of 0.5 mg of either antibody. Three days later, specific depletion of the respective T cell subset (>95%) was verified by fluorescence-activated cell sorting (FACS) analysis of peripheral blood mononuclear cells, using FITC-conjugated anti-CD4 or phycoerythrin-conjugated anti-CD8 antibodies (BD PharMingen).

**Vaccination against subcutaneously established tumors.** Tumors were established in mice by the s.c. injection of $10^6$ TUBO cells suspended in 0.2 mL of PBS in the flank. On the indicated day after injection of tumor, mice were immunized i.p. with a single dose of $10^8$ pfu of Ad-*neu*ECTM, or with $10^8$ pfu of Ad-Null as a control. Each mouse was monitored twice weekly for tumor growth measured in two perpendicular diameters using a caliper. Tumor volume ($mm^3$) was calculated using the formula $V = a \times b^2/2$, where $a$ is the largest and $b$ is the smallest diameter (21) and represented as mean volume ± SD.

**Vaccination against pulmonary metastases.** Pulmonary metastases were established by a single tail vein injection of $2 \times 10^5$ TUBO cells in 0.1 mL of PBS. Mice were randomly separated into groups on the day of the tumor injection and immunized i.p. with $10^8$ pfu of Ad-*neu*ECTM. Control groups were untreated or were immunized i.p. with $10^8$ pfu of Ad-Null. Pulmonary metastases in each group were evaluated by enumerating nodules in lungs perfused with a 15% solution of India ink and fixed with Fekete's solution at designated time points.

**Detection of serum anti-neu antibodies.** Serum anti-neu antigen antibodies were measured as previously reported (9). Serum was serially diluted 10-fold to $10^4$-fold in PBS, and the level of anti-neu antibodies was evaluated by FACS analysis using secondary FITC-conjugated anti-mouse immunoglobulin antibody (BD PharMingen) on neu-expressing N202.1A cells. N202.1A cells plated at $3 \times 10^5$ cells/well in U-bottomed 96-well microtiter plates were incubated with the serially diluted serum followed by secondary antibodies. After washing and suspension in FACS buffer containing 1 mg/mL of propidium iodide to gate out dead cells, anti-neu antibodies were measured using a FACSCalibur flow cytometer (BD Biosciences) versus N202.1E cells as negative controls. Anti-neu antibodies were expressed as mean fluorescence intensity (± SD).

**Adoptive transfer of immune sera.** Serum from BALB/c mice i.p. vaccinated with $10^8$ pfu of Ad-*neu*ECTM was obtained on day 14 after immunization, pooled and diluted 3-fold with PBS, sterile-filtered, and stored at 4°C until use. Neu-specific antibodies in the serum were verified as described. Tumor-bearing mice were injected i.p. with 0.3 mL of the serum from days 1 to 8 or days 12 to 22 after TUBO implantation. Animals treated with serum from Ad-Null–immunized mice served as controls.

**Inhibition of ErbB2 and phosphorylated ErbB2 expression.** Alteration of ErbB2 and ErbB2 phosphorylation by treatment with sera of vaccinated mice was directly measured on 96-well cultured cells using a cell-based ELISA assay as described previously (22). Cells were seeded at $1 \times 10^4$ cells per 96-well plastic plate on day 0 and allowed to grow in the presence of 10% FBS for 24 h. On day 1, the medium was removed and relevant cells were treated with medium containing 10% FBS + 5% sera from vaccinated mice, 10% FBS + 1% sera from vaccinated mice + 4% sera from unvaccinated mice, or 10% FBS + 5% sera from unvaccinated mice. The cells were incubated for 8, 4, 1, or 0 h and phosphorylated protein and total protein were quantitated by the Cellular Activation of Signaling ELISA (CASE Kit, SuperArray Bioscience) following the instructions of the manufacturer. Briefly, the treated cells were fixed in 4% formaldehyde and endogenous peroxidase quenched with quenching buffer, followed by blocking for 1 h. Primary antibody specific for phosphorylated ErbB2 or pan-ErbB2 was then added to two identical sets of each well and incubated for 1 h at room temperature. After washing, the wells were incubated with horseradish peroxidase–conjugated secondary antibody for 1 h, and the color was developed with 10 min of incubation at room temperature and measured at 450 nm absorbance on a plate reader. Thereafter, relative cell numbers per well was determined. Cells were washed and incubated in cell staining buffer for 30 min, and treated with 1% SDS (1 h at room temperature) and absorbance was measured at 595 nm. The results were calculated as antibody (450 nm) per cell concentration (595 nm).

**Statistical analysis.** Student's $t$ test was used to evaluate the significance of differences between experimental groups. $P < 0.05$ was considered significant. Statistical analysis was done using JMP statistical software (SAS Institute).

## Results

To examine the effects of the vaccination, in initial experiments, BALB/c mice ($n = 5$ per group) were injected s.c. with $10^6$ TUBO cells on day 0 and immunized with i.p. Ad-*neu*ECTM on days 2 or 5. In our previous study, a single dose of $10^7$ pfu of Ad-*neu*ECTM was sufficient to protect the mice from a subcutaneous challenge with $1 \times 10^6$ TUBO cells (9). Therefore, for the therapeutic study, we

used $10^7$ pfu and $10^8$ pfu, or the empty vector Ad-Null $10^8$ pfu and no treatment as controls. Mice in the control groups developed large tumors (mean volume, 1,851 ± 161 $mm^3$) by day 25 after injection with TUBO cells (Fig. 1*A*). However, mice immunized with $10^8$ pfu of Ad-*neu*ECTM on day 0 showed no tumor growth. After immunization on day 5, tumors that were 30 to 40 $mm^3$ were observed to regress after a short lag, and completely regressed by days 20 to 25 after TUBO implantation (Fig. 1*A*). Protection against tumor was consistently observed in mice immunized with $10^8$ pfu of Ad-*neu*ECTM, but not with $10^7$ pfu, although even with the $10^7$ dose, four out of five mice showed eventual regression of the tumor. Therefore, $10^8$ pfu of Ad-*neu*ECTM was used for further experiments.

To address whether vaccination with Ad-*neu*ECTM would be effective for more fully established tumors, we immunized mice with Ad-*neu*ECTM on days 7, 10, or 15 after TUBO injection, when mean tumor volumes for the groups were 60 ± 8, 130 ± 39, or 374 ± 112 $mm^3$, respectively. Tumor regression was observed beginning on day 7 after immunization and all tumors completely disappeared between days 25 and 45 after vaccination (Fig. 1*B*). The rate of tumor regression was dependent on the tumor volume. Mice having tumors >374 $mm^3$ needed 30 days to be cured of tumors, whereas 20 days was sufficient for mice with tumors of ≤60 $mm^3$. All immunized mice remained completely free of tumor through day 155 when the experiment was terminated.

To compare the effect of the vaccine on established tumors of different sizes, mice were s.c. injected with $1 \times 10^6$ TUBO cells. On day 17, the mice were divided into groups based on tumor size and were then immunized with $10^8$ pfu Ad-*neu*ECTM. To make the comparison more stringent, mice with smaller tumors were placed in the Ad-Null or nontreatment groups. Their average tumor sizes were 225 ± 33 and 134 ± 10 $mm^3$, respectively. The initial tumor size in each of the four groups immunized with Ad-*neu*ECTM was an average of 201 ± 29, 263 ± 66, 391 ± 37, or 589 ± 94 $mm^3$ at the time of immunization. All the tumors in the vaccinated groups grew to a maximum size on days 20 to 22 after tumor implantation, then regressed completely by day 35, and did not recur through day 155. In contrast, tumors in the groups of control mice given no vaccine or Ad-Null continued to grow (Fig. 2*A*), even though their initial tumor sizes were smaller than those in Ad-*neu*ECTM–immunized mice (the difference in the growth rates of control tumors between Figs. 1 and 2 was likely due to differences in the condition of the tumors at the time of inoculation; thus, comparisons can be made only within experiments done at the same time). None of the untreated mice or mice vaccinated with Ad-Null showed tumor regression. In contrast, immunization with $10^8$ pfu of Ad-*neu*ECTM cured all mice of established tumor, even when the initial burden was as large as 589 $mm^3$ (Fig. 2*B*).

To determine the efficacy of the Ad-*neu*ECTM vaccine for the treatment of substantially larger established tumors, mice bearing tumors >600 $mm^3$ (groups of mean 800, 2,200, 3,500 and 5,500 $mm^3$, respectively) were immunized i.p. with $10^8$ pfu of Ad-*neu*ECTM. Tumors <2,000 $mm^3$ continued to grow for 7 days after immunization, but then began to regress by 10 days, and completely disappeared around day 30 (Fig. 2*C*). This vaccine was effective against established tumors as large as 3,500 $mm^3$ (Fig. 2*C*). In that case, four of six mice (in two separate experiments) had complete regression of tumor by day 40 after immunization. However, vaccination with Ad-*neu*ECTM was not sufficient to cure mice of tumors >5,500 $mm^3$. With tumors of this size, mean tumor volume regressed to <4,000 $mm^3$ by 2 weeks after immunization, but then the tumors started to re-grow thereafter. Thus, a single dose of Ad-*neu*ECTM vaccine effectively cured mice of established TUBO tumors as large as 3,500 $mm^3$. The ability to treat large tumors is shown in Fig. 2*D*, in which an ulcerating 2,000 $cm^3$ subcutaneous tumor is seen to resolve and then disappear over 26 days following immunization.

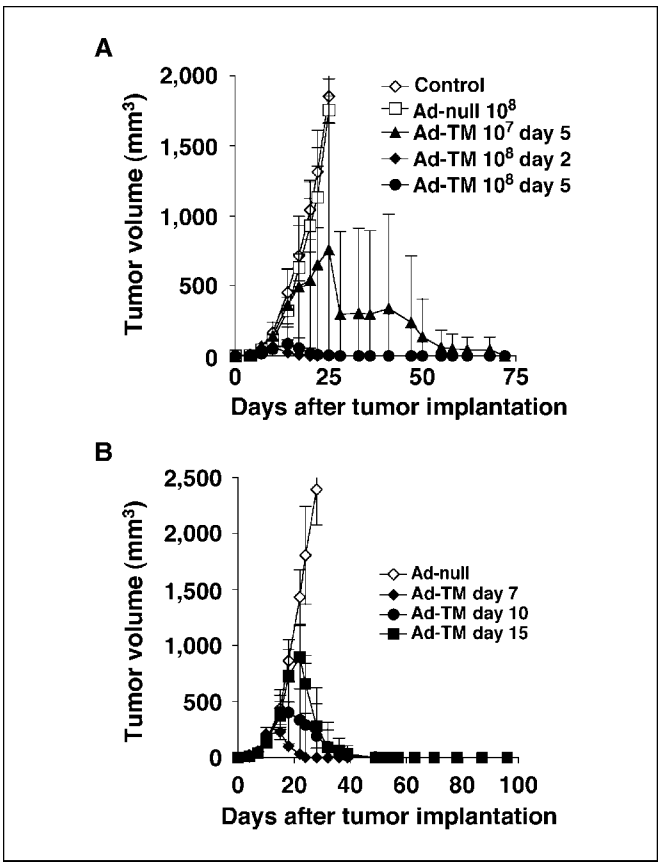


**Figure 1.** Protection of mice against tumor by one dose of therapeutic vaccine. *A,* BALB/c mice were injected s.c. with TUBO ($10^6$ cells) on day 0 and blindly divided among the groups ($n$ = 5). Mice were i.p. immunized with one dose of Ad-*neu*ECTM (either $10^7$ or $10^8$ pfu) or with Ad-Null ($10^8$ pfu) on days 2, 5 (*A*), 7, 10, or 15 (*B*) after tumor implantation. Untreated mice were used as a negative control. Each mouse was monitored twice weekly for tumor growth by measuring two perpendicular diameters using a caliper. Similar results were observed in three independent experiments, and a representative experiment is shown.

To examine the role of $CD4^+$ and $CD8^+$ T cells and antibodies in the therapeutic effects of the Ad-*neu*ECTM vaccine, we depleted mice of $CD4^+$ or $CD8^+$ T cells 1 day prior to immunization. The mice were vaccinated i.p. with $10^8$ pfu of Ad-*neu*ECTM, 14 days after s.c. inoculation with $10^6$ TUBO cells when they had established tumors of a mean volume of 295 ± 51 $mm^3$. Although the $CD8^+$ T-cell–depleted mice were cured of tumor similar to the nondepleted Ad-*neu*ECTM-immunized control group, the $CD4^+$ T-cell–depleted mice did not show tumor regression after vaccination (Fig. 3*A*). However, depletion of $CD4^+$ or $CD8^+$ T cells 4 days postvaccination had no effect on vaccine efficacy. These results indicate that neither $CD4^+$ nor $CD8^+$ T cells acted as effector cells, but that $CD4^+$ T cell help was needed at the time of vaccination to facilitate antitumor humoral immunity. The induction of anti-neu antigen antibodies was examined in serum

collected 35 days after TUBO implantation. By FACS analysis using FITC-labeled anti-mouse antibody and ErbB-2/*neu*–expressing N202.1A cells (9), anti–ErbB-2 antibodies were detected in all mice vaccinated with Ad-*neu*ECTM, except for mice depleted of $CD4^+$ cells using the anti-CD4 antibody prior to vaccination (Fig. 3*B*). Moreover, similar levels of anti-neu antibodies were present in sera from mice that were treated with anti-CD4 or anti-CD8 antibody 4 days after vaccination (Fig. 3*B*). To further confirm the role of antibody in this therapeutic vaccine approach, we adoptively transferred immune sera from mice vaccinated with Ad-*neu*ECTM into mice with established TUBO tumors. Mice receiving serum from Ad-*neu*ECTM vaccinated mice showed significantly delayed tumor growth compared with mice injected with serum from animals treated with Ad-Null ($P < 0.05$; Fig. 3*C*). Tumor growth was completely blocked in mice by every other day treatment with anti-neu sera from days 1 to 8 after tumor injection. However, the amount of anti-neu antigen sera that could be transferred was not sufficient to cure mice of tumor (initial mean volume of 200 $mm^3$) that was established for 12 days, even if it was effective for delaying tumor growth beyond day 40 (Fig. 3*C*).

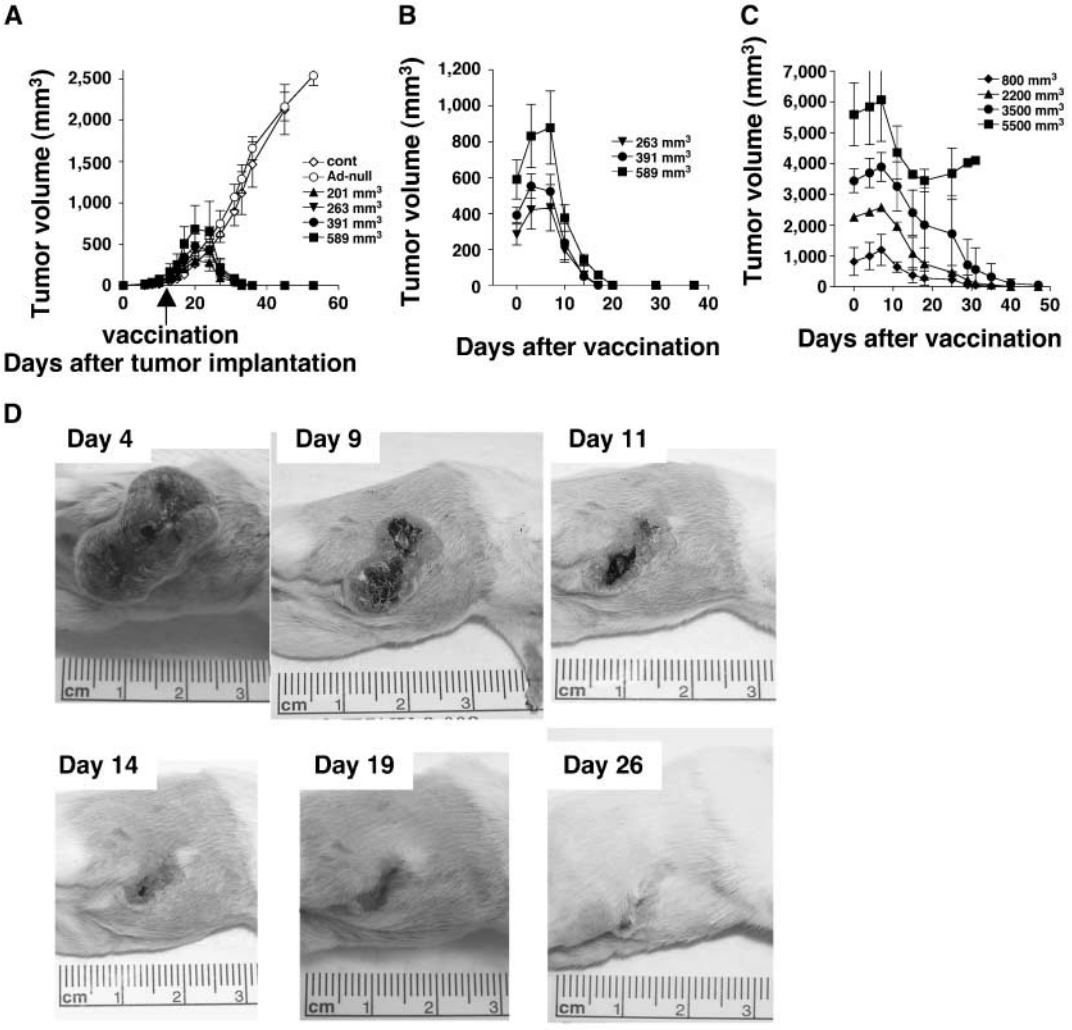


**Figure 2.** Effect of vaccine immunotherapy against established tumors. *A,* tumors were established in BALB/c mice by s.c. injection of $10^6$ TUBO cells. On day 17 after tumor injection, the mice ($n = 5$) were divided according to the size of tumor. The mean tumor volumes of the groups were 589 ± 94, 391 ± 37, 263 ± 66, and 201 ± 29 $mm^3$. They were i.p. immunized with $10^8$ pfu of Ad-*neu*ECTM on day 17. Mice with smaller tumors were used for Ad-Null ($10^8$ pfu) or for the nontreated group to make the comparison more stringent; their average tumor volumes were 225 ± 33 and 134 ± 10 $mm^3$, respectively. The effect of immunotherapeutic vaccination was monitored by measuring tumor volume twice weekly. *B,* data from *A* was plotted on an expanded scale from day 0 until day 40 of vaccination in order to clearly show the therapeutic effects against the larger three tumor groups of 589, 391, and 263 $mm^3$. Similar results were observed in three repeated experiments. *C,* for the development of very large tumors in mice, BALB/c mice were injected s.c. with $10^6$ TUBO cells at several different time points; that is, 4, 10, 12, and 15 wk before immunization. As observed over the next several weeks thereafter, mice were selected for each group ($n = 3$) based on their health status. The mean volumes of established tumor of the groups were 800 ± 373, 2,200 ± 31, 3,500 ± 312, and 5,500 ± 838 $mm^3$, respectively. The effect of immunotherapeutic vaccination was monitored by measuring tumor volume twice weekly. Similar results were observed in two repeated experiments. *D,* immunotherapeutic effects resolving an ulcerating subcutaneous tumor within 4 wk of vaccination with Ad-*neu*ECTM. A TUBO tumor as large as 1,000 $mm^3$ was established in a BALB/c mouse 4 wk after subcutaneous injection ($10^6$ cells). The tumor-bearing mouse was vaccinated i.p. with Ad-*neu*ECTM ($10^8$ pfu) and monitored by photographing the tumor twice weekly. Similar results were observed in three other mice, which resolved the tumor mass.

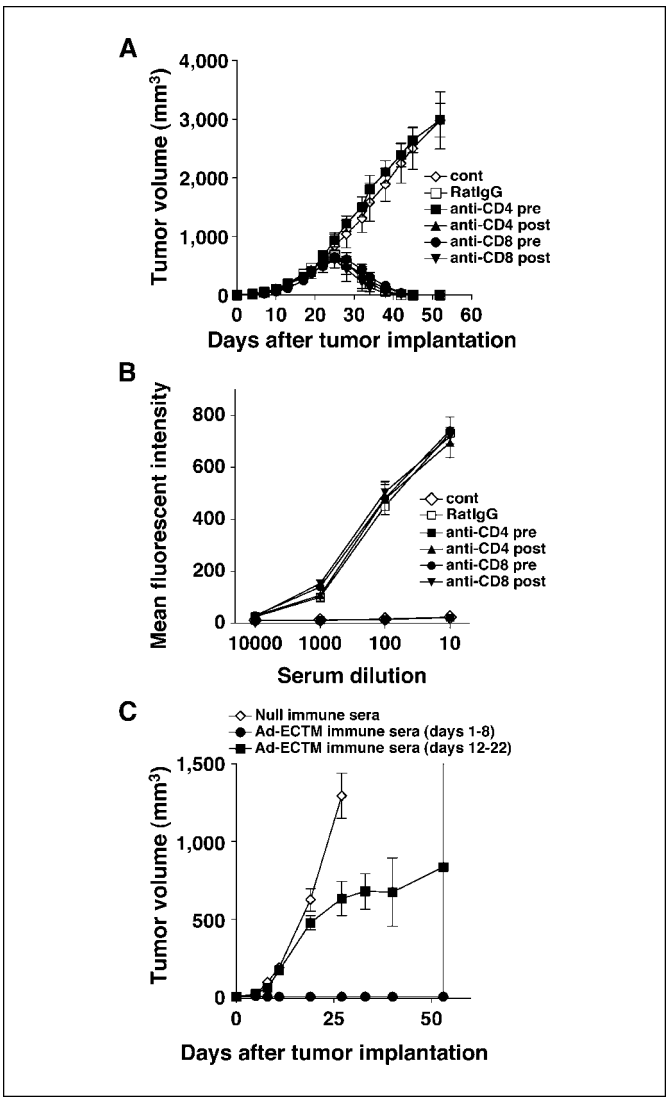


**Figure 3.** CD4$^+$ T cells are required at the time of Ad-*neu*ECTM vaccination for immunotherapeutic effect, but not thereafter. *A,* antitumor therapeutic immune responses require CD4$^+$ T cell help during the immunization. Tumors were established in BALB/c mice injected s.c. with $10^6$ TUBO cells. On day 14 after tumor implantation, mice were therapeutically immunized i.p. with Ad-*neu*ECTM ($10^8$ pfu). One day prior to the vaccination, two groups ($n$ = 3) were injected i.p. with rat anti-CD4 antibody (clone GK1.5) or anti-CD8 antibody (clone 2.43) to deplete CD4$^+$ T cells or CD8$^+$ T cells, respectively. The other two groups ($n$ = 3) were similarly treated on day 4 after vaccination to deplete each subset of T cells. A single injection of antibody (0.5 mg) was sufficient to remove virtually all CD4$^+$ cells or CD8$^+$ T cells over the next 3 to 4 wk as confirmed by flow cytometry. As a control antibody, rat IgG was used 1 day before the vaccination. TUBO-injected, but nonimmunized, mice were included as a negative control. The effect of vaccination was monitored by measuring tumor volume twice weekly. *B,* detection of anti-neu antibody in serum of mice in *A*. On day 35 after TUBO implantation, serum was collected from each mouse and diluted 10-fold to $10^4$-fold in PBS. Serum from JhD mice immunized with Ad-*neu*ECTM was included as a negative control. Anti-neu antibody levels were evaluated by FACS analysis using FITC-conjugated anti-mouse immunoglobulin and neu-expressing N202.1A cells; *points,* mean fluorescence intensity; *bars,* SD. *C,* adoptive transfers of anti-ErbB2 immune sera into mice establishing tumor. Tumor was established in BALB/c mice by s.c. injection of $10^6$ TUBO cells on day 0. Anti-neu immune serum was collected on day 14 from Ad-*neu*ECTM–immunized mice and pooled after 3-fold dilution with PBS followed by sterile filtration, and stored at 4°C until used. Established tumor-bearing mice ($n$ = 3–5) were treated with 0.3 mL of the diluted sera i.p. every other day from day 12 to 22, or from day 1 to 8 after TUBO implantation. Ad-Null immunized mouse sera were used as a control and i.p. injected every other day from days 12 to 22. The mice were monitored by measuring tumor volume twice weekly. Similar results were observed in two repeated experiments.

From the above data, the therapeutic effects of vaccination seemed to be mediated by humoral immunity, were dependent on the presence of CD4$^+$ T cells only at the time of vaccination to provide help, and were completely independent of CD8$^+$ T cell activity. To further investigate the mechanism, we vaccinated mice deficient for the high-affinity Fc receptor (FcγR-KO), B cells (JhD), or CD8$^+$ T cells (β2m-KO). The different knockout mice bearing 200 to 300 mm$^3$ of TUBO tumors were immunized i.p. with $10^8$ pfu of Ad-*neu*ECTM on day 14 after tumor implantation. FcγR-KO mice and β2m-KO mice underwent complete tumor regression by days 30 to 35 (Fig. 4*B* and *C*). However, vaccination with Ad-*neu*ECTM had no effect on tumor growth in JhD mice lacking B cells and antibody responses (Fig. 4*D*). This clearly shows a requirement for an antibody response for the antitumor effects elicited by Ad-*neu*ECTM vaccination, and confirms the lack of any role of CD8$^+$ T cells, which is absent in β2m-KO mice. It also indicates the lack of a requirement for Fc receptors for antibody-mediated protection, implying that ADCC is not necessary, in contrast with the case of trastuzumab (11).

If the protection is antibody-mediated but does not require Fc receptors as would be the case if ADCC were the mechanism, could the antibody be working by blocking the functional activity of the ErbB2/*neu* oncogene product? To address this question, we examined the ability of the antisera to block phosphorylation or modulate levels of ErbB2/neu on the TUBO cells. Cells were incubated in 5% sera, made up of either 5% control sera, 1% specific serum from immunized mice and 4% control serum, or 5% specific serum from immunized mice, thus keeping the total amount of serum added constant (Fig. 5). We found a progressive dose-dependent inhibition of ErbB2 phosphorylation as well as total ErbB2 levels over 8 hours in two independent experiments (Fig. 5). We cannot compare absolute amounts of phosphorylated ErbB2 and total ErbB2 directly because they are measured with different antibodies. Nevertheless, even with the decrease in total ErbB2 per cell as an indication of down-modulation of ErbB2 by the antibody (Fig. 5*B*), the level of phosphorylated ErbB2 was reduced even more compared with control serum than was the total ErbB2 (Fig. 5*A*), an indication of inhibition of phosphorylation as well. Thus, at 8 hours, the phosphorylated ErbB2 was inhibited 62% by the 5% specific serum compared with the control serum, whereas the total ErbB2 was inhibited 50%. Also, comparing the kinetics, the total ErbB2 decreased to a steady-state plateau from 1 to 8 hours, whereas the phosphorylated ErbB2 was still decreasing at 8 hours. Thus, at 5% specific serum, the total ErbB was reduced at 8 hours to 55% of its starting value at 0 hours, whereas the phosphorylated ErbB2 was reduced at 8 hours to 38% of its value at 0 hours. Therefore, the adenovirus vaccine induces the production of antibodies that can inhibit phosphorylation of the ErbB2 oncogene product as well as down-modulate its overall level. Either of these antibody effects on ErbB2 should affect its oncogenic activity, therefore, these results provide a plausible explanation for the ability of the antibodies to act autonomously of other effector cells.

Metastatic tumors are much more difficult to treat than primary tumors for a variety of reasons, and it is critically important for therapeutic cancer vaccines to have an effect on all sites of disease. To examine the systemic effect of Ad-*neu*ECTM vaccination in a TUBO pulmonary metastasis model, we injected BALB/c mice i.v. with TUBO cells. The lungs were removed at various times after vaccination and the number of metastases counted. Mice injected i.v. on day 0 with TUBO and immunized

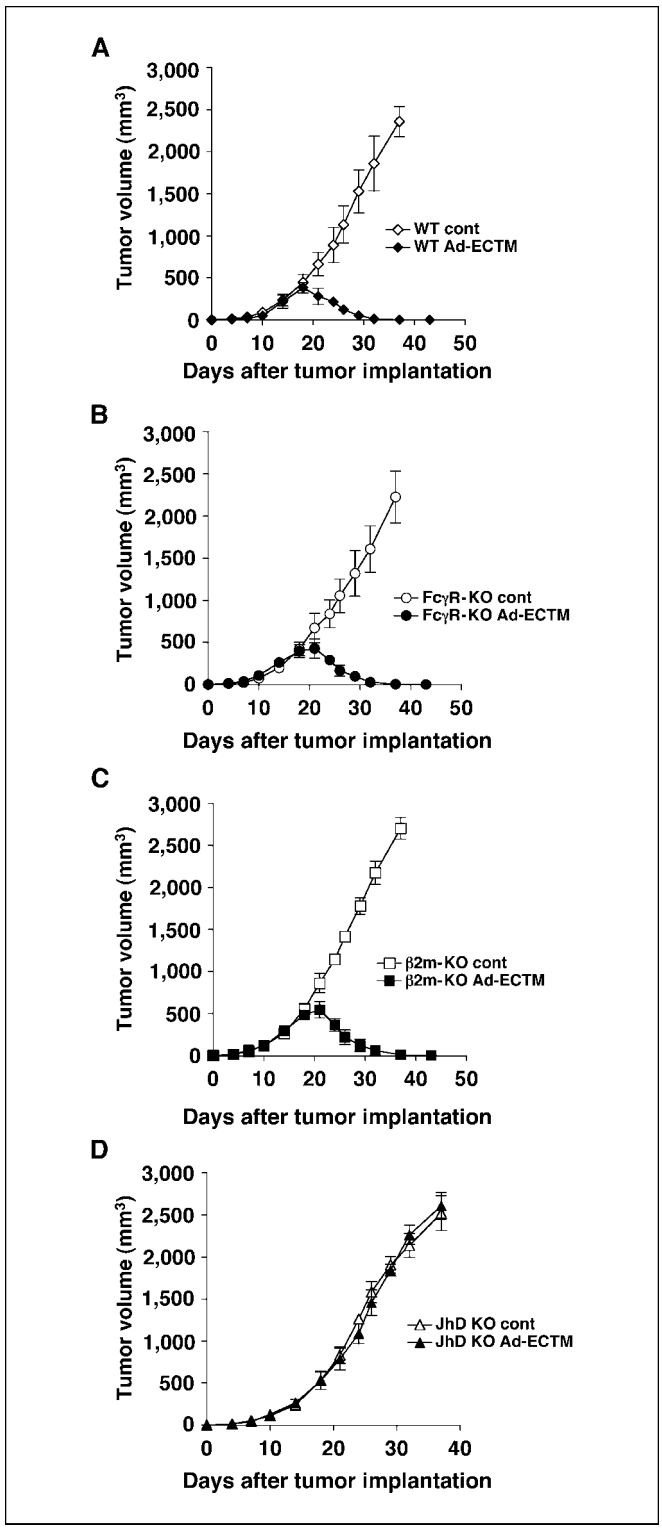


**Figure 4.** Antitumor immunotherapeutic effects of Ad-*neu*ECTM vaccination are independent of $CD8^+$ T cells and Fc receptor–mediated responses. Subcutaneous tumors (200–300 $mm^3$) were established in wild-type (*A*), high-affinity Fc receptor gene knockout (*Fcγr-KO*; *B*), $CD8^+$ T-cell–deficient β2m gene knockout (*β2m-KO*; *C*) or B cell–deficient (*JhD*; *D*) mice ($n$ = 5) by injection of TUBO ($10^6$ cells). On day 14 after tumor implantation, mice were vaccinated with $10^8$ pfu of Ad-*neu*ECTM i.p. Wild-type BALB/c mice ($n$ = 5) were included as a control group and treated in the same way. Each mouse was monitored by measuring tumor volumes twice weekly. Similar results were observed in two repeated experiments.

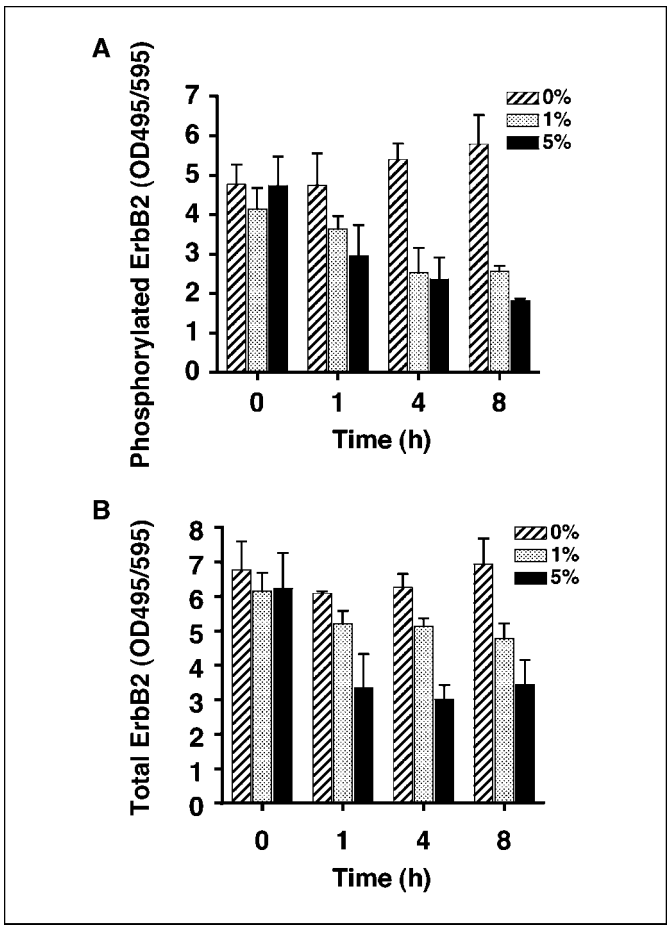


**Figure 5.** Inhibition of ErbB2/*neu* oncogene product phosphorylation by antisera from Ad-*neu*ECTM–immunized mice. Effects of serum from vaccinated mice on ErbB2 expression and phosphorylation in TUBO cells. Inhibition of phosphorylated ErbB2 (*A*) and total ErbB2 (*B*) expression levels in TUBO cells by sera of vaccinated mice was determined. TUBO cells were plated at $1 \times 10^4$ cells per 96-well dish in the presence of 10% FBS and 1 d after seeding, vaccinated sera was added at respective concentrations of 0% (*hatched columns*), 1% (*stippled columns*), 5% (*solid columns*) were added and cultured for 8, 4, 1, or 0 h. ErbB2 and phosphorylated ErbB2 were measured as ErbB2 or pErbB2/relative cell number (OD 450/595) by cellular ELISA as described in Materials and Methods. *Bars,* SD. Similar results were observed in repeated experiments.

i.p. with $10^8$ pfu of Ad-*neu*ECTM on the same day showed no tumor nodules on day 21 (Fig. 6*A*). In contrast, the lungs from the untreated and Ad-Null vaccinated control groups showed numerous (>250) metastases. Next, we assessed the therapeutic effects of immunization with Ad-*neu*ECTM initiated on day 6 after TUBO cell injection on the number of tumor nodules on days 21 or 35 in the lungs. Whereas earlier established subcutaneous tumors (185 ± 62) regressed within 2 weeks of Ad-*neu*ECTM immunization, 6-day established lung metastases required >2 weeks to undergo complete regression (Fig. 6*A*). To further investigate lung metastatic tumor burden on the efficacy of the Ad-*neu*ECTM vaccination, we immunized mice 9, 15, or 20 days after the i.v. injection of TUBO cells, and lung metastases were evaluated on days 23 to 58 (Fig. 6*C*). Additional mice given tumor at the same time as controls had mean numbers of 23 ± 9, 144 ± 25, or 237 ± 29 tumor nodules detected on days 9, 15, or 20 after injection of tumor, respectively (Fig. 6*B*). The therapeutic

efficacy against lung metastases was dependent on the number of tumor nodules in the lung and the time the vaccine was given (Fig. 6*C*). It took at least 21 days with the initial burden of 25 tumor nodules, 33 days with the initial burden of 150 nodules, and 38 days with the initial burden of 240 metastases for these tumors to be eradicated (Fig. 6*C*). These mice would have died of their disease by this time if not treated, but instead, were completely cured by a single dose of Ad-*neu*ECTM. These results dramatically show the power of this vaccine to treat established lung metastases of a breast carcinoma, illustrated by representative lungs in Fig. 6*D*.

The results also make an additional important point. In contrast to classical chemotherapeutic agents, which may quickly initiate regression of a tumor if effective at all, vaccine immunotherapy exhibits a delayed effect because of the time required for the vaccine to induce an immune response. Therefore, tumors may continue to grow for some weeks after therapy. Such progression on treatment should therefore not be interpreted as a failure of the vaccine immunotherapy and cause for removal of the patient from the clinical trial because, as observed in these mice, initial continued tumor growth followed by delayed regression is the expected outcome even when the vaccine will ultimately completely cure the tumor.

## Discussion

ErbB-2 (HER-2/*neu*) is a member of the ErbB family of growth factor receptors that is often constitutively overexpressed and functions as an oncogene product in a substantial fraction of human breast cancers (1–3). Passive administration of the recombinant monoclonal antibody trastuzumab (Herceptin) directed against the HER-2/*neu* receptor has proven to be an

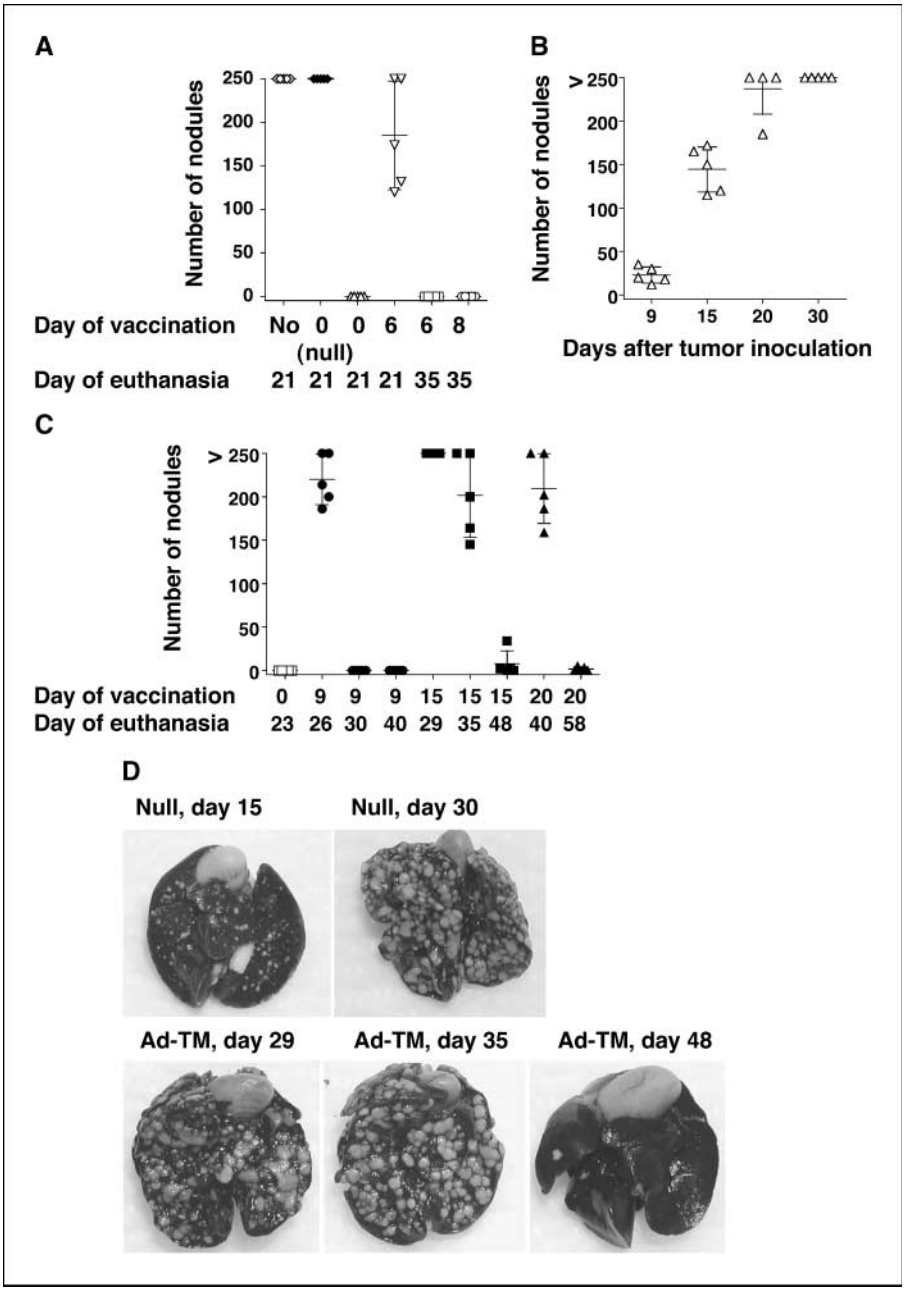


**Figure 6.** Immunotherapy of lung metastases with Ad-*neu*ECTM vaccination. BALB/c mice ($n = 5$) were injected i.v. with TUBO ($2 \times 10^5$ cells on day 0) and lung metastases were enumerated by counting tumor nodules after perfusion of the lungs with India ink to make the metastases visible against the black background of the lung. *A,* immunotherapeutic effects of Ad-*neu*ECTM vaccination against TUBO pulmonary metastases. Mice were immunized with Ad-*neu*ECTM $10^8$ pfu i.p. on days 0 or 6, and sacrificed for metastases evaluation on days 21 or 35 after TUBO injection. Control mice were nonimmunized or immunized with Ad-Null $10^8$ pfu i.p. on day 0. *B,* lung metastatic tumors in untreated mice. Tumor nodules in the lung were counted on days 9, 15, and 20 after TUBO injection. *C,* mice were vaccinated with Ad-ECTM. Mice were immunized with $10^8$ pfu of Ad-*neu*ECTM i.p. on days 0, 9, 15, or 20 after i.v. tumor injection and sacrificed for metastasis evaluation on days 23 to 58 after TUBO injection, as indicated. Metastases of TUBO cells were monitored by counting tumor nodules in the lung; *bar,* mean; *columns,* SD. Similar results were observed in three repeated experiments. *D,* immunotherapeutic effects resolving pulmonary metastases of TUBO cells (*C*) by vaccination with Ad-*neu*ECTM. Tumor metastases in lung were established in BALB/c mice by i.v. injection of $2 \times 10^5$ TUBO cells. Mice were immunized on day 15 with $10^8$ pfu of Ad-Null (*Null*) or Ad-Neu-ECTM (*Ad-TM*). The mean number of tumor nodules was 144 at the time of vaccination. Lungs were harvested on the day indicated. Mice in Ad-Null group did not survive beyond day 35. Similar results were observed in other mice in the same group.

effective therapy in breast cancer (4–7). Despite its importance as an immunotherapeutic target, a number of antitumor vaccine approaches targeting HER-2/*neu* have not met with success. Here, we show that an adenoviral vector expressing a nonsignaling *neu* antigen can cure large established *neu*-expressing subcutaneous mammary tumors and pulmonary metastases in mice. The *neu*-expressing TUBO tumor cell line was derived from a spontaneous breast cancer of a BALB-*neu*T transgenic mouse. This tumor can be transferred into wild-type BALB/c mice and exhibits rapid growth and will ultimately kill the mice. However, a single intraperitoneal injection of Ad-*neu*ECTM, expressing the extracellular and transmembrane domains of the *neu* oncogene, results in long-term tumor regression even in mice with large tumor burdens. Although tumor challenge models and small established tumors have been successfully treated with vaccines (13), it has been notoriously difficult to cure large established tumors, even when foreign tumor antigens are overexpressed (12, 14). The rat *neu* is closely homologous to the mouse molecule, and not nearly as foreign as other model tumor antigens such as ovalbumin or influenza hemagglutinin against which cancer vaccines have been less effective against large established tumors. Although it has been more difficult to see protection once spontaneous autochthonous tumors are growing in BALB-*neu*T transgenic mice (9), these transgenic mice greatly overexpress the *neu* oncogene from birth, not just after sexual maturity, and show mammary gland histologic changes before 3 weeks of age (23, 24). Therefore, these transgenic mice display a central tolerance to neu oncogene dominant peptides characterized by the deletion of T cell clones reacting with high affinity (17, 18). A comparable massive neonatal deletion of T cells does not seem to take place in human breast cancer patients, whose tumors overexpressing HER-2 (ErbB-2) do not arise until much later in adulthood. As evidence for lack of a central tolerance in humans, it is not uncommon for these patients to display a spontaneous anti–ErbB-2 T-cell and B-cell reactivity during tumor progression (15, 16). Therefore, the model of TUBO cells in wild-type BALB/c mice is a better model of human breast cancer than the model of ErbB-2/neu transgenic mice with central tolerance (18). If such patients with HER-2/neu–positive breast cancer display a spontaneous immune response to their cancers, which we do not see in the TUBO-injected mice, then it should be even easier to boost that response with a vaccine to induce a therapeutically effective response. As the goal of tumor immunotherapy is to treat established tumors that have reached a clinically detectable size, as well as to prevent recurrence and the development of metastases, this vaccine strategy holds promise for the treatment of HER-2–positive breast cancer.

The use of small quantities of recombinant adenovirus for immunization locally does not have the same risk of complications that could be seen with the delivery of large amounts of adenovirus systemically for gene therapy. Furthermore, any interference of preexisting antibody to adenovirus on vaccine efficacy clinically can be overcome by the use of adenovirus-pulsed dendritic cells (10) or by the use of a less common serotype of adenovirus.

The protective mechanism seems to be purely antibody-dependent, requiring $CD4^+$ T cells only at the time of vaccination to provide a helper function for antibody induction. $CD4^+$ T cells are not required later as effector cells, and $CD8^+$ T cells were not required at any time point studied. Antibodies are both necessary, as shown by the lack of effect seen in antibody-deficient mice, and sufficient, as shown by the antitumor effect of passive serum transfer to naïve mice bearing tumors. Passive serum transfer, however, was not as effective against large tumors as active immunization with Ad-*neu*ECTM, likely because the amount of antibody that could be passively transferred was limiting. Unlike trastuzumab, which requires functional Fc receptors for antitumor activity (11), the antibodies induced by Ad-*neu*ECTM vaccination do not require Fc receptors for protective activity. This finding suggests that these antibodies function more like the 2C4 (25) monoclonal antibody that blocks HER-2 dimerization and subsequent receptor function, rather than like trastuzumab, that acts more through ADCC (11). This conclusion is also supported by the finding that antibodies elicited by the vaccine can inhibit growth of TUBO cells *in vitro*, in the absence of other cells (9). It was confirmed here by studies showing inhibition of phosphorylation of the neu oncogene product as well as its down-modulation by sera from immunized mice (Fig. 5). Thus, the current study shows that the approach which was successful for prophylactic vaccination (9) can be applied for the immunotherapy of established tumors, and further identifies the molecular mechanism. This ability to affect oncogene product levels and function may be advantageous because such vaccine-induced antibodies could potentially employ both mechanisms and thus be more effective at eradicating tumors. Also, polyclonal antibodies elicited by a vaccine, by targeting multiple epitopes on the ErbB-2/Her-2/neu receptor, may be more effective at inhibiting receptor dimerization or modulating the receptor from the cell surface than could a monoclonal antibody directed to a single epitope (8). Moreover, continuous antibody production by the patient's own immune system avoids the need for repeated, inconvenient, and expensive infusions of antibody. Thus, this approach has the potential to be more cost-effective as well as more efficacious. In addition, it is possible that if the antibodies induced by the vaccine work through a mechanism different from that of trastuzumab, they may synergize with trastuzumab or circumvent trastuzumab resistance. Our results show the clinical potential for a vaccine that induces antibodies to an overexpressed functional cell surface receptor encoded by an oncogene such as HER-2/*neu*. Plans for a clinical trial of antitumor vaccination using an adenoviral vector expressing nonsignaling human HER-2/*neu* in breast cancer patients that overexpress the oncogene are in progress.

This study also shows a qualitative difference between vaccine immunotherapy and conventional drug-based antineoplastic chemotherapy. Chemotherapeutic agents are usually immediately active against tumor on administration, and the expectation has been developed that therapeutic benefit should be seen in a fairly short time frame. Yet, here, we see that with vaccine-based immunotherapy, the tumors continued to grow for several weeks after vaccination. Nevertheless, once the immune response was established, the tumors were controlled and eradicated within 4 to 5 weeks. Thus, vaccine immunotherapy may require a different set of expectations, and a much longer period of observation may be required before a patient need be considered a treatment failure in the clinical setting.

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