Vaccination by Genetically Modified Dendritic Cells Expressing a Truncated neu Oncogene Prevents Development of Breast Cancer in Transgenic Mice

Yoshio Sakai,1 Brian J. Morrison,1 J. Douglas Burke,1 Jong-Myun Park,2 Masaki Terabe,2 John E. Janik,3 Guido Forni,4 Jay A. Berzofsky,2 and John C. Morris1,3

1Cancer Gene Therapy Section, Metabolism Branch, 2Molecular Immunogenetics and Vaccine Research Section, Vaccine Branch, and 3Clinical Trials, Metabolism Branch, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland; and 4Department of Clinical and Biological Sciences, University of Turin, Orbassano, Italy

ABSTRACT

Dendritic cells (DCs) are powerful antigen-presenting cells that process antigens and present peptide epitopes in the context of the major histocompatibility complex molecules to generate immune responses. DCs are being studied as potential anticancer vaccines because of their ability to present antigens to naive T cells and to stimulate the expansion of antigen-specific T-cell populations. We investigated an antitumor vaccination using DCs modified by transfer of a nonsignaling neu oncogene, a homologue of human HER-2/neu, in a transgenic model of breast cancer. BALB-neuT mice develop breast cancers as a consequence of mammary gland-specific expression of an activated neu oncogene. We vaccinated BALB-neuT mice with bone marrow-derived DCs transduced with Ad.Neu, a recombinant adenovirus expressing a truncated neu oncoprotein. The vaccine stimulated the production of specific anti-neu antibodies, enhanced interferon-γ expression by T cells, and prevented or delayed the onset of mammary carcinomas in the mice. Over 65% of vaccinated mice remained tumor free at 28 weeks of age, whereas all of the mice in the control groups developed tumors. When challenged with a neu-expressing breast cancer cell line, vaccinated tumor-free animals had delayed tumor growth compared with controls. The antitumor effect of the vaccine was specific for expression of neu. Studies showed that CD4+ T cells were required in order to generate antitumor immunity. Importantly, the effectiveness of the vaccine was not diminished by preexisting immunity to adenovirus, whereas the protection afforded by vaccination that used direct injection of Ad.Neu was markedly reduced in mice with anti-adenovirus antibody titers. DCs modified by recombinant adenoviruses expressing tumor-associated antigens may provide an effective antitumor vaccination strategy.

INTRODUCTION

Cancer cells express tumor-associated antigens (1, 2) that can be potentially targeted by the immune system. Dendritic cells (DCs) are powerful antigen-presenting cells that play a central role in generating and directing immune responses through the processing of antigens and presentation of epitopes in the context of surface MHC molecules to interact with T cells. DCs also express a full complement of costimulatory molecules and cytokines that are required to sustain and direct the immune response (3, 4). Major efforts at developing antitumor vaccines have focused on harnessing DCs to effectively present tumor antigens to the immune system. A number of clinical trials have examined the efficacy of epitope-modified DCs, most often by “pulsing” DCs ex vivo with synthetic peptides based on antigens (5–10), mutant onco genes (11), or immunoglobulin idiotypes (12) expressed by tumors. Peptide loading has a number of potential drawbacks including a need for detailed knowledge of the antigen sequence, identification of the recipient MHC molecule, and a requirement for a favorable binding affinity of the peptide for the MHC molecule. Peptide binding half-lives may be brief (13, 14), and the ability of a peptide to induce an antigen-specific CTL response correlates with the stability of this complex (15). An alternative strategy is to load DCs by incubating them with protein lysates manufactured from tumors. Tumor lysates, however, contain many antigens, most of which are not tumor specific.

The introduction of a gene encoding a tumor-associated antigen into DCs may overcome many of these problems. Gene expression and the physiologic processing of intact antigens will likely generate greater numbers of epitopes and more favorable antigen presentation. In addition, constitutive gene expression provides for continuous replenishment of low affinity epitopes bound on MHC molecules. Among gene delivery systems, recombinant adenoviruses (rAds) can efficiently infect DCs and offers the advantage of accepting up to 8-kb cDNA inserts. In addition, viral-associated proteins may provide the “danger signals” required for the activation of DCs and up-regulation of other costimulatory molecules, which results in stronger immune responses (16, 17).

The HER-2/neu (ErbB2) oncoprotein, a member of the epidermal growth factor receptor tyrosine kinase family (18, 19), is frequently overexpressed in cancers of the breast (20, 21), ovary (22), uterus (23), lung (24), and gastrointestinal tract (25). Its overexpression in breast cancer is associated with a poorer prognosis (20). Trastuzumab, a humanized antibody targeting HER-2/neu is approved for the treatment of advanced breast cancer, indicating the ability of this antigen to serve as a target for immunotherapy (26). Vaccination of patients with advanced breast cancer using HER-2/neu peptides or peptide-pulsed autologous DCs has been examined in clinical trials (8). Few responses, however, have been reported, although increases in antibody to HER-2/neu CTLs were seen in some patients (9).

To examine the efficacy of antitumor vaccination in a clinically relevant model, we studied the ability of DCs modified by an rAd expressing a truncated neu antigen to suppress the development of mammary carcinomas in a transgenic model of breast cancer in which the neu oncogene is expressed in tissue-specific fashion. We demonstrated that vaccination prevented or delayed the onset of cancers in this aggressive tumor model. Importantly, the efficacy of antitumor vaccination with rAd-infected DCs was unaffected by preexisting immunity to adenovirus.

MATERIALS AND METHODS

Cell Lines. TUBO cells derived from a breast cancer of a BALB-neuT mouse (27), and N202.1A cells isolated from a breast cancer of a FVB-neuN mouse, were the kind gift of Dr. Patrizia Nanni (University of Bologna, Bologna, Italy; refs. 28, 29) and were grown in Dulbecco’s modified Eagle’s medium (DMEM; BioSource, Rockville, MD) supplemented with 10% fetal bovine serum (Gemini, Woodland, CA). Both TUBO and N202.1A cells constitutively express neu. The neu-negative cell line, TS/A (also a gift of Dr. Patrizia Nanni), established from a spontaneous breast cancer from a BALB/c mouse (30) was grown in RPMI 1640 (BioSource) and 10% fetal bovine serum. Human 293 embryonic kidney and A549 lung cancer cells were grown in DMEM with 10% fetal bovine serum. Human 293 embryonic kidney and A549 lung cancer cells were grown in DMEM with 10% fetal bovine serum.

Received 11/4/03; revised 7/6/04; accepted 8/30/04.

Grant support: Supported by National Cancer Institute Project Z-01SC-010294, and, in part, by a grant from the Italian Association for Cancer Research (G. Forni).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C., Section 1734 solely to indicate this fact.

Note: Y. Sakai is a research fellow of Japan Society for the Promotion of Science.

Requests for reprints: John C. Morris, Metabolism Branch, Center for Cancer Research, National Cancer Institute, NIH, Building 10, Room 4N115, 10 Center Drive, Bethesda, MD 20892-1374. Phone: (301) 402-2912; Fax: (301) 402-1001; E-mail: jmorris@mail.nih.gov.

©2004 American Association for Cancer Research.
Adenoviral Vectors. The cDNA encoding the extracellular and transmembrane domains of the rat neu oncogene was provided by Dr. Augusto Amici (University of Camerino, Camerino, Italy; ref. 27). Ad.Neu, an E1-, E3-deleted rAd expressing **neu**, and Ad.null, an "empty" control vector were generated by homologous recombination with the AdMax system (Microbix, Toronto, Canada; ref. 31). Ad.GFP, an adenovirus expressing green fluorescent protein (GFP), was obtained from Quantum Biotechnologies (Quebec, Canada). All viruses were double plaque-isolated, expanded on 293 cells, purified on a cesium chloride gradient, titered by serial dilution as plaque forming units (pfu)/mL and stored at –70°C.

**Animals.** All animal studies were approved by the Animal Care and Use Committee of the National Cancer Institute. BALB-neuT mice expressing the rat **neu** oncogene under the control of a chimeric mouse mammary tumor virus (MMTV) promoter provide an aggressive model of mammary carcinogenesis as the transgene is overexpressed in the rudimentary mammary glands of 3-week-old mice (32–34). By 6 weeks, atypical lobular hyperplasia is seen that progresses to multiple **in situ** carcinomas that enlarge and converge to form rapidly growing, invasive, and metastasizing tumors in all 10 glands by 25 weeks of age (35). Female BALB/c mice were obtained from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD).

**Generation of Dendritic Cells.** DCs were generated with the method of Lutz et al. (36). Briefly, bone marrow was harvested from femurs of 8- to 10-week-old BALB/c mice. Erythroid cells were lysed with ammonium-chloride-potassium buffer (BioWhittaker, Walkersville, MD) and the nucleated cells were plated in plastic bacteriologic dishes in RPMI 1640 with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.-Invitrogen, Grand Island, NY) and 20 ng/mL murine granulocyte/macrophage-colony stimulating factor (GM-CSF; PeproTec, Rocky Hill, NJ). The cultures were refreshed with 10 mL of medium containing 20 ng/mL GM-CSF on days 3, 6, and 8. On day 8, the nonadherent DCs were collected and infected with rAds. The DCs were grown for 2 days and then followed for development of tumor.

Flow Cytometry. DCs were incubated with FITC- or phycoerytherin-labeled antimonu CD11c, CD11b, CD40, CD80, CD86, H-2Kd, I-Aα, CD8a (BD Pharmingen, San Diego, CA) and analyzed on a FACSort (Becton Dickinson, San Jose, CA). Forty-eight hours after infection with Ad.Neu or Ad.null, the DCs were incubated with antirat **neu** monoclonal antibody (OncoGene Research, La Jolla, CA) followed by incubation with secondary FITC-labeled rabbit antimouse immunoglobulin and analyzed by FACSsort. To detect cytoplasmic **neu**, the cells were permeabilized with Cytotox/Cytoperm (BD Pharmingen) and similarly stained.

Mixed Lymphocyte Proliferation and CTL Assay. Lymphocytes obtained from the spleens of 8- to 10-week-old naïve female BALB-neuT mice were incubated with irradiated unmodified DCs or DCs infected with either of the rAds. Four days later, the incorporation of [3H]thymidine was measured as described previously (37).

Vaccination of Mice with Ad.Neu-Modified Dendritic Cells. Groups of 5- to 6-week-old female BALB-neuT mice received three weekly subcutaneous injections of 1 × 10^7 DCs infected with Ad.Neu (DC-Ad.Neu) or Ad.null (DC-Ad.null) at a MOI 30, or of unmodified DCs. The mice were examined twice weekly for the development of tumors. Tumor volumes were calculated with the estimated volume of a rotational ellipse (39). Mice free of tumor at 28 weeks were challenged with a subcutaneous injection of 1 × 10^5 TUBO cells. Another group of BALB-neuT mice received Ad.null 1 × 10^6 pfu subcutaneously before **anti-neu** vaccination and was followed for the development of tumors after undergoing vaccination as described above. This group was compared with mice similarly pre Vaccinated with Ad.null and then vaccinated with **new** by direct subcutaneous injections of Ad.Neu 1 × 10^6 pfu, weekly for 3 weeks.

In another set of experiments, groups of 5- to 6-week-old BALB/c mice were given injections subcutaneously of Ad.null 1 × 10^6 pfu weekly for 4 weeks. Two weeks after the last injection of Ad.null, groups of mice were vaccinated either with 1 × 10^6 DC-Ad.Neu subcutaneously or directly with 1 × 10^6 pfu Ad.Neu subcutaneously, weekly for two weeks. One week after the final vaccination, the mice were challenged with 5 × 10^6 TUBO cells subcutaneously and were monitored for tumor growth.

In another experiment, BALB/c mice vaccinated with DC-Ad.Neu were challenged with 5 × 10^6 **new** TUBO cells subcutaneously. All of the mice remained tumor free, and at 37 days, the mice were divided into two groups and rechallenged with an injection of 5 × 10^6 **new** TUBO cells or **new** TSA cells, and were followed for development of tumors.

Antibody Depletion of Lymphocyte Populations. Groups of BALB/c mice were given intraperitoneal injections of 200 μg of anti-CD4- or anti-CD8 purified from the supernatants of hybridomas GK1.5 (ATCC) and 2.43 (ATCC), respectively, for 3 consecutive days, then every 3 days, or with anti-asialo-GM1 (WAKO, Richmond, VA). Five days after initiation of antibody treatment, the mice were vaccinated as described above. One week after the last vaccination, the mice were challenged with 5 × 10^6 TUBO cells and then were monitored for tumor growth.

Detection of serum Anti-**neu** Antibodies. Blood was obtained by retro-orbital venipuncture from BALB-neuT mice before vaccination and one week after the third vaccination. The blood was allowed to clot, and the sera from each treatment group were pooled. N20.1A cells (new) were used to detect and quantify anti-**neu** antibodies as described previously with slight modification (29). Briefly, 2 × 10^5 N20.1A cells were incubated with the sera diluted 1:10 in 2% bovine serum albumin in phosphate buffered saline at 4°C for 1 hour. The cells were washed and incubated with FITC-labeled rabbit antimouse immunoglobulin antibody (DAKO, Carpinteria, CA) and were submitted to flow cytometry. Mean fluorescence intensity was quantified with CellQuest Software (Becton Dickinson).

Detection of Anti-Adenovirus Antibodies. Sera were obtained pre- and postvaccination from the mice. Anti-adenovirus antibody titers were measured as described previously (40). Briefly, 5 × 10^5 A549 cells were seeded in a 24-well plate. Sixteen hours later, 10 μL of 2-fold serially diluted heat-inactivated test sera were incubated with 10 μL of Ad.GFP (2.5 × 10^6 pfu) at room temperature for 1 hour. The mixture was added to the A549 monolayer (MOI 30). Thirty-six hours later, the A549 cells were trypsinized, washed, and analyzed for GFP expression by flow cytometry. Antibody titers were assessed as the serum dilution that resulted in 50% inhibition of GFP expression compared with controls.

**Enzyme-Linked Immunosorbent Assay.** Spleens were obtained from BALB-neuT mice 1 week after the last vaccination, and T cells were isolated with the Pan-T Cell Isolation Kit (Miltenyi, Auburn, CA) in accordance with the manufacturer’s protocol. Thirty thousand spleen cells were plated in a 96-well dish coated with antimouse interferon γ (IFNγ) antibody (R&D Systems, Minneapolis, MN) and were incubated for 48 hours at 37°C. The plates were developed in accordance with the manufacturer’s protocol, and the spots were counted.

**Cytokine Secretion Assay.** One million spleen cells from DC-Ad.Neu-vaccinated BALB-neuT mice were incubated for 4 hours with an equal number of irradiated DC-Ad.Neu, DC-Ad-null, or unmodified DCs. IFNγ-secreting CD4+ and CD8+ T cells were detected by FACS analysis. The splenocytes were harvested and stained with phycocerythrin-labeled anti-IFNγ antibody together with peridinin chlorophyll protein–labeled CD45RA/B220 and FITC-labeled anti-CD4 or FITC-labeled antiCD8. Cells were incubated with 7-αmino-actinomycin D for 10 minutes and then analyzed on the FACSSort gating out dead and CD45R/B220-positive cells. The fraction of IFNγ-positive CD4 or CD8 cells were calculated and reported as a percentage.

**Immunohistochemical Analysis.** Mammary glands, primary breast cancers, and TUBO tumors were removed from BALB-neuT mice, frozen in OCT (optimum cutting temperature) compound (SAKUIRA-Finetek U.S.A. Inc., Torrance, CA), and were cryostat sectioned. Endogenous peroxidase activity was blocked followed by blocking of avidin and biotin with the Avidin/Biotin Blocking kit (Zymed Laboratories Inc., South San Francisco, CA). After washing with phosphate buffered saline, the tissues were stained with rabbit antimonu CD4 (BD Pharmingen) or antimonu CD8 (BD Pharmingen). Tumor sections were stained with **anti-neu** monoclonal antibody (OncoGene Research). The slides were washed and then incubated with biotinylated anti-rabbit antibody (Vector Laboratory, Burlingame, CA), stained with streptavidin-horseradish peroxidase (DAKO), and counterstained with hematoxylin.

**Statistical Analysis.** STATView version 5.01 software (SAS Institute, Cary, NC) was used for log-rank survival analysis and comparisons of tumor multiplicity with the Mann–Whitney U test. Enzyme-linked immunosorbent (ELISPOT) assay was analyzed with Student’s t test.
RESULTS

Effect of Adenovirus on Dendritic Cell Phenotype. DCs generated from 10-day bone marrow cultures expressed CD11c and CD11b, and lacked CD8α, indicating myeloid differentiation (Fig. 1A). They also expressed CD80, CD86, CD40, and MHC class I and class II. Compared with unmodified DCs, those infected with Ad.Neu (DC_{Ad.Neu}) or Ad.null (DC_{Ad.null}) expressed higher levels of CD80, CD86, CD40, and both MHC classes, indicating maturation (Fig. 1B).

Dendritic Cells Infected with Ad.Neu-Expressed Truncated neu mRNA and Oncoprotein. Thirty-six hours after infection with Ad.Neu or Ad.null, Northern analysis of RNA isolated from DC_{Ad.Neu} showed the expected 2.1-kb truncated neu mRNA transcript (Fig. 2A), which was not detected in DC_{Ad.null}. The transcript was absent in noninfected BALB-neuT bone marrow-derived DCs, even when analyzed by reverse transcription-PCR, indicating tissue-specific neu expression in the transgenic animals (data not shown). DCs, stained with antirat neu antibody 48 hours after infection with Ad.Neu or Ad.null and examined by flow cytometry, showed neu expression on the cell surface and in the cytoplasm (Fig. 2B). No expression was seen in DC_{Ad.null}.

Ad.Neu-Transduced Dendritic Cell-Stimulated Proliferation of Naïve BALB-neuT Lymphocytes. The function of the DCs was examined in mixed lymphocyte culture. Irradiated DC_{Ad.Neu}, DC_{Ad.null}, or unmodified DCs were cultured in various ratios with lymphocytes from the spleens of nonimmunized BALB-neuT mice and were pulsed with [3H]thymidine. Three to 4-fold greater stimulation of splenocyte labeling was seen with DC_{Ad.Neu} compared with DC_{Ad.null} or unmodified DCs (Fig. 3).

Vaccination of BALB-neuT Mice with DC_{Ad.Neu} Prevented Autochthonous Breast Cancers. Vaccination of BALB-neuT mice with DC_{Ad.Neu} significantly improved tumor-free survival compared with mice treated with DC_{Ad.null} or unmodified DCs (Fig. 4A). The median tumor-free survival of the mice treated with unmodified DCs compared with those receiving DC_{Ad.null} was not different (19.5 versus 20 weeks, P = 0.438). In contrast, the median tumor-free survival was not reached by 28 weeks in mice receiving DC_{Ad.Neu} (P < 0.0001). Furthermore, in the groups of mice treated with the unmodified DCs or DC_{Ad.null}, the onset of the first tumor occurred at 14.5 weeks, and all of the mice had developed at least one breast cancer by 23.5 weeks. In mice vaccinated with DC_{Ad.Neu}, the first breast lesion occurred at 20 weeks, and 14 (66.7%) of 21 mice were still free of tumor at 28 weeks. In view of the mean tumor multiplicity, DC_{Ad.Neu} also conferred significant protection (Fig. 4B). At 28 weeks, tumor was palpable in all 10 mammary glands of all of the mice treated with unmodified DCs or with DC_{Ad.null}. In the group of mice vaccinated with DC_{Ad.Neu}, the mean tumor multiplicity was only 2.9.
expressing tumor cell line, mice vaccinated at 5 to 7 weeks of age with Breast Carcinoma. To test whether DC Ad.Neu-vaccinated DCAd.Neu Inhibited Growth of a Transplantable tumors (data not shown).

various ratios, and the incorporation of \[3H\]thymidine was measured. In BALB-T mice vaccinated with DC Ad.Neu, tumors occurred at a /H11003 with 1\(\times\)10\(^5\) TUBO cells. A small number (n = 3) of surviving similar-age mice that had been vaccinated with unmodified DCs or DCAd.null were also challenged. At a mean of 10 days after injection with 1 \(\times\) 10\(^5\) TUBO cells, tumors were evident in the mice receiving unmodified DCs or DCAd.null. The time to the appearance of palpable TUBO tumors was similar (10 ± 1 day) to that of unvaccinated BALB/c or BALB-neuT mice (data not shown). In contrast, in the BALB-neuT mice vaccinated with DCAd.Neu tumors occurred at a mean of 56 days. Notably, in the DCAd.Neu-vaccinated mice, TUBO tumor growth occurred contemporaneous or subsequent to the development of an autochthonous breast cancer, suggesting a general loss of immunity to neu (Table 1).

To examine the specificity of protection derived from vaccination due to selection and outgrowth of neu-positive tumor cells. With DCAd.Neu BALB/c mice (n = 8) were vaccinated with 1 \(\times\) 10\(^6\) DCAd.Neu weekly for 2 weeks, and were then subcutaneously challenged with 5 \(\times\) 10\(^5\) TUBO cells a week later. All of the mice remained free of tumor at 37 days. These mice were rechallenged with 5 \(\times\) 10\(^5\) of either TUBO cells (n = 4) or neu-negative TS/A cells (n = 4). All of the vaccinated mice that received injections of TS/A cells developed tumors by day 10, whereas those receiving TUBO cells remained free of disease up to 121 days later (Table 2).

DCAd.Neu-Vaccination Induced Serum Anti-neu Antibodies and IFNγ Secretion by T Cells. We measured the induction of anti-neu antibodies in groups of BALB-neuT mice pre-vaccination and one week after the final vaccination. Major increases of anti-neu antibody titers were detected in the sera of mice vaccinated with DCAd.Neu-Minor changes were seen in the mice receiving DCAd.null, and no titers were detected in mice vaccinated with the unmodified DCs (Fig. 5A).

Tumor-specific CTLs could not be demonstrated by chromium-release assay in the vaccinated mice; however, IFNγ production by splenocytes was seen on ELISPOT (Table 3). The numbers of IFNγ-expressing T cells were increased in DCAd.Neu-vaccinated mice compared with the mice vaccinated with DCAd.null (P < 0.0001), or unmodified DCs (P < 0.0001). These results were confirmed by cytokine secretion assay. A more than 3.5-fold increase in the num-

(P < 0.0001). Vaccination of older groups of mice was less effective, and the vaccine had little effect in mice that had developed palpable tumors (data not shown).

Vaccination of BALB-neuT Transgenic and BALB/c Mice with DCAd.Neu Inhibited Growth of a Transplantable neu-Expressing Breast Carcinoma. To test whether DCAd.Neu-vaccinated BALB-neuT mice could successfully reject a challenge with a neu-expressing tumor cell line, mice vaccinated at 5 to 7 weeks of age with DCAd.Neu that were tumor free at 28 weeks (n = 6) were challenged with the neu\(^+\) TUBO cells. A small number (n = 3) of surviving similar-age mice that had been vaccinated with unmodified DCs or DCAd.null were also challenged. At a mean of 10 days after injection with 1 \(\times\) 10\(^5\) TUBO cells, tumors were evident in the mice receiving unmodified DCs or DCAd.null. The time to the appearance of palpable TUBO tumors was similar (10 ± 1 day) to that of unvaccinated BALB/c or BALB-neuT mice (data not shown). In contrast, in the BALB-neuT mice vaccinated with DCAd.Neu tumors occurred at a mean of 56 days. Notably, in the DCAd.Neu-vaccinated mice, TUBO tumor growth occurred contemporaneous or subsequent to the development of an autochthonous breast cancer, suggesting a general loss of immunity to neu (Table 1). Immunohistochemical staining of the TUBO tumors and late-occurring autochthonous breast cancers were positive for neu expression, which indicated that tumor escape was not due to selection and outgrowth of neu-negative tumor cells.

To examine the specificity of protection derived from vaccination

with DCAd.Neu BALB/c mice (n = 8) were vaccinated with 1 \(\times\) 10\(^6\) DCAd.Neu weekly for 2 weeks, and were then subcutaneously challenged with 5 \(\times\) 10\(^5\) TUBO cells a week later. All of the mice remained free of tumor at 37 days. These mice were rechallenged with 5 \(\times\) 10\(^5\) of either TUBO cells (n = 4) or neu-negative TS/A cells (n = 4). All of the vaccinated mice that received injections of TS/A cells developed tumors by day 10, whereas those receiving TUBO cells remained free of disease up to 121 days later (Table 2).

DCAd.Neu-Vaccination Induced Serum Anti-neu Antibodies and IFNγ Secretion by T Cells. We measured the induction of anti-neu antibodies in groups of BALB-neuT mice pre-vaccination and one week after the final vaccination. Major increases of anti-neu antibody titers were detected in the sera of mice vaccinated with DCAd.Neu-Minor changes were seen in the mice receiving DCAd.null, and no titers were detected in mice vaccinated with the unmodified DCs (Fig. 5A).

Tumor-specific CTLs could not be demonstrated by chromium-release assay in the vaccinated mice; however, IFNγ production by splenocytes was seen on ELISPOT (Table 3). The numbers of IFNγ-expressing T cells were increased in DCAd.Neu-vaccinated mice compared with the mice vaccinated with DCAd.null (P < 0.0001), or unmodified DCs (P < 0.0001). These results were confirmed by cytokine secretion assay. A more than 3.5-fold increase in the num-


\(\text{Table 1 DC}_{\text{Ad.Neu}}\) vaccination protects BALB-neuT mice from challenge with neu-expressing TUBO breast cancer cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TUBO tumor (days(*))</th>
<th>Breast cancer (days(*))</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC(_{Ad.Neu})†</td>
<td>108</td>
<td>80</td>
</tr>
<tr>
<td>DCnull</td>
<td>56</td>
<td>50</td>
</tr>
<tr>
<td>DCnull</td>
<td>56</td>
<td>25</td>
</tr>
<tr>
<td>DCnull</td>
<td>28</td>
<td>7</td>
</tr>
<tr>
<td>DCnull</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>DCnull</td>
<td>11</td>
<td>NA</td>
</tr>
<tr>
<td>DCnull</td>
<td>9</td>
<td>NA</td>
</tr>
<tr>
<td>DCnull</td>
<td>10</td>
<td>NA</td>
</tr>
</tbody>
</table>

Abbreviations: NA, not applicable.
† After injection of 1 \(\times\) 10\(^5\) TUBO cells.

Table 2 Vaccination induced specific immunity to neu-expressing tumors

<table>
<thead>
<tr>
<th>Tumor-free</th>
<th>TUBO</th>
<th>TS/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>4/4</td>
<td>4/4</td>
<td>0/4</td>
</tr>
</tbody>
</table>

Note. DCAd.Neu vaccinated BALB/c mice, tumor-free 37 days after subcutaneous injection of 5 \(\times\) 10\(^5\) neu\(^+\) TUBO cells, were rechallenged with 5 \(\times\) 10\(^5\) TUBO cells, or 5 \(\times\) 10\(^6\) neu\(^+\) TS/A cells. All mice receiving TS/A cells developed tumors by 10 days after injection. Mice re-challenged with TUBO cells remained tumor-free 121 days later.

Fig. 3. DCs infected with Ad.Neu stimulated the proliferation of naïve BALB-neuT lymphocytes. DCs infected with Ad.Neu (DC\(_{Ad.Neu}\)) or Ad.null (DC\(_{Ad.null}\)) or with unmodified DCs (DC) were mixed with naïve BALB-neuT mouse splenic lymphocytes in various ratios, and the incorporation of \[^{3}H\]thymidine was measured. Error bars, ± SEM.

Fig. 4. Effect of vaccination on tumors in BALB-neuT transgenic mice. In A, Kaplan–Meier plot of tumor-free survival. BALB-neuT mice (5–6 weeks old) were given injections in the flank with 1 \(\times\) 10\(^5\) DC\(_{Ad.Neu}\) (\(\square, n = 21\)), DC\(_{Ad.null}\) (\(\bullet, n = 11\)) or unmodified DCs (\(\bigcirc, n = 10\)), weekly for 3 weeks. Mice were followed for the development of breast tumors. In B, mean tumor multiplicity (mean number of tumors per mouse) in the treatment groups over time. Error bars, ± SEM. * * P < 0.0001.
Fig. 5. Antibody and cellular responses to vaccination. In A, vaccination with DC_{Ad.Neu} induced the production of anti-neu antibodies. Serum collected from BALB-neuT mice before vaccination and 1 week after the last (third) vaccination were analyzed for anti-neu antibodies, as described in Materials and Methods. The results represent the mean titers of five mice in each group. Error bars, ± SEM. In B, DC_{Ad.Neu} Vaccination increased the secretion of IFNγ from CD4+ and CD8+ T cells. Splenic T cells isolated from BALB-neuT mice 1 week after completion of the DC_{Ad.Neu} vaccination were assayed for IFNγ secretion after restimulation by DC_{Ad.Neu}, DC_{Ad.null}, or unmodified DCs by fluorescent antibody capture flow cytometry.

Table 3 Interferon γ ELISPOT

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spots/3 x 10^4 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC</td>
<td>106 ± 35.0</td>
</tr>
<tr>
<td>DC_{Ad.null}</td>
<td>214 ± 81.2</td>
</tr>
<tr>
<td>DC_{Ad.Neu}</td>
<td>461 ± 36.8*</td>
</tr>
</tbody>
</table>

Note: T cells isolated from spleens of BALB-neuT mice 1 week after completion of vaccination were assessed for IFNγ secretion by ELISPOT. The ELISPOT was performed in triplicate and repeated in two independent mice from each treatment group. Data are presented as a mean ± SD.

* The numbers of IFNγ-expressing T cells were increased in DC_{Ad.Neu}-vaccinated mice compared with the mice vaccinated with DC_{Ad.null} (P < 0.0001) or unmodified DCs (P < 0.0001).

Bars of IFNγ-expressing CD4+ lymphocytes was detected in the spleens of DC_{Ad.Neu}-Vaccinated BALB-neuT mice that were restimulated with DC_{Ad.Neu} compared with restimulation with unmodified DCs; and a greater than 2-fold increase was noted compared with restimulation with DC_{Ad.null}, indicating a neu-specific response (Fig. 5B). Thus, IFNγ-secreting CD8+ T lymphocytes was increased by 4-fold compared with stimulation with DC_{Ad.null} or with unmodified DCs.

Infiltration of Mammary Glands with CD4+ and CD8+ T Cells. Immunohistochemical staining of mammary tissue from BALB-neuT mice vaccinated with DC_{Ad.Neu} showed modestly increased numbers of CD4+ and CD8+ cells infiltrating the periphery of hyperplastic and dysplastic mammary glands (data not shown). The breast tissue of mice receiving the control vaccines showed little cellular infiltrate.

CD4+ T Cells Are Required for Generation of Antitumor Immunity with DC_{Ad.Neu}. To explore the mechanism of DC_{Ad.Neu} antitumor vaccination, we administered anti-CD4, anti-CD8, or anti-asialo-GM1 antibodies to groups of BALB/c mice before and during DC_{Ad.Neu} vaccination to deplete these lineages. One week after the last vaccination, the mice were challenged with 5 x 10^5 TUBO cells. The control animals, vaccinated with DC_{Ad.Neu} but not treated with antibodies, were protected from tumor growth (Fig. 6). Mice treated with anti-CD8 or anti-asialo GM1 antibodies also failed to form tumors; however, mice depleted of CD4+ cells developed tumors, indicating that CD4+ cells are required for the generation of antitumor immunity after vaccination with DC_{Ad.Neu}. Treatment with anti-CD4 after vaccination had no effect (data not shown).

Efficacy of DC_{Ad.Neu} Vaccination Is Unaffected by Preexisting Immunity to Adenovirus. To assess the effect of preexisting immunity to adenovirus on the efficacy of DC_{Ad.Neu} vaccination, 4-week-old BALB-neuT mice were given subcutaneous injections of Ad.null, 1 x 10^6 pfu, to generate anti-adenovirus immunity. Three weeks later, mice were divided into two groups and were vaccinated subcutaneously directly with Ad.Neu 1 x 10^6 pfu or with 1 x 10^6 DC_{Ad.Neu} Direct injection of Ad.Neu is protective in this model. At 28 weeks, four of seven mice receiving DC_{Ad.Neu} remained tumor-free. This is similar to mice treated with DC_{Ad.Neu} and not undergoing prevaccination with Ad.null (Fig. 7A; Fig. 4A). All of the mice vaccinated with Ad.null and then treated with Ad.Neu developed tumors by 24.5 weeks (Fig. 7A).

To confirm these results, BALB/c mice were given subcutaneous injections weekly for 4 weeks with Ad.null 1 x 10^6 pfu. All of the mice receiving Ad.null showed significant increases in serum anti-adenovirus antibody titers, from a mean of less than 1:2 to a mean greater than 1:256, 1 week after the last injection. Untreated mice had a mean baseline titer of less than 1:2 that remained unchanged over 6 weeks (data not shown). Two weeks later, the mice were vaccinated with either 1 x 10^6 pfu Ad.Neu, or 1 x 10^6 DC_{Ad.Neu}. One week later, the mice were challenged with 5 x 10^5 TUBO cells. Twenty-one days later, all of the mice treated directly with Ad.Neu had developed tumors, whereas six of seven mice receiving DC_{Ad.Neu} remained tumor-free (Fig. 7B), which suggested that immunity to adenovirus abrogated the protection afforded by direct injection of Ad.Neu.

DISCUSSION

We showed that vaccination of BALB neuT-transgenic mice with bone marrow-derived DCs modified by a rAd-expressing truncated neu oncoprotein prevented the development, or significantly delayed the onset, of breast cancer in the mice. Antitumor vaccination with DCs modified by adenoviral-mediated transfer of genes encoding tumor-associated antigens offers potential advantages over antigen-loading strategies that use peptides or tumor lysates. These include long-term antigen expression, activation of the DCs by the vector, and induction of DC maturation with enhanced expression of MHC and costimulatory molecules. DCs modified by adenovirus are able to stimulate naïve splenocytes to a greater degree than are unmodified DCs, possibly because of activation by viral proteins (41). This may enhance the vaccine’s efficacy, because mature DCs are more effective at generating immune responses (42).

and given injections of Ad.Neu 10^8 pfu subcutaneously, served as controls. In R, DCAd.Neu vaccination was effective in BALB/c mice with preexisting immunity to adenovirus in protecting against TUBO breast cancer, whereas the protection from direct vaccination with Ad.Neu was lost. BALB/c mice were injected weekly for 4 weeks with Ad.null 10^8 pfu s.c. Twenty-one days after injection of the TUBO cells, the tumor volumes were compared.

Fig. 7. Effect of preexisting immunity to adenovirus on the efficacy of DCAd.Neu vaccination. In A, the protective effect of vaccination with DCAd.Neu in BALB-neuT mice was not affected by preexisting immunity to adenovirus, and the benefit of direct vaccination using Ad.Neu was lost. BALB-neuT mice were injected with Ad.null 1 × 10^8 pfu s.c. Two weeks later, mice were vaccinated with either 1 × 10^9 DCAd.Neu s.c. (n = 7), or directly with 1 × 10^8 pfu Ad.Neu s.c. (n = 7). BALB-neuT mice (n = 7), not previously treated with Ad.null and given injections of Ad.Neu 1 × 10^8 pfu subcutaneously, served as controls. In R, DCAd.Neu vaccination was effective in BALB/c mice with preexisting immunity to adenovirus in protecting against TUBO breast cancer, whereas the protection from direct vaccination with Ad.Neu was lost. BALB/c mice were injected weekly for 4 weeks with Ad.null 1 × 10^8 pfu s.c. Twenty-one days after injection of the TUBO cells, the tumor volumes were compared.
man. The fact that these lesions were controlled by vaccination with gene-engineered DCs in a transgenic animal model that displays an early and diffuse overexpression of the neu antigen and relentlessly develops cancels, suggests that a new and rational approach exists to secure immunologic control of HER-2/neu-expressing lesions. Vaccination with DCs that are modified by rAd encoding nonfunctional tumor antigens, such as nonsignaling HER-2/neu, may offer a preventive strategy for patients at risk of metastases after primary treatment of a tumor, or for those at high risk of developing cancer.

ACKNOWLEDGMENTS

We wish to thank Dr. Leon van den Broeke for his help developing dendritic cell culture in our laboratory, Dr. Raya Mandler and Dr. Thomas A. Waldmann for their many helpful discussions, and Poonam Mannan for her tissue immunochemistry expertise.

REFERENCES


Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 2004 American Association for Cancer Research.
Vaccination by Genetically Modified Dendritic Cells Expressing a Truncated \textit{neu} Oncogene Prevents Development of Breast Cancer in Transgenic Mice

Yoshio Sakai, Brian J. Morrison, J. Douglas Burke, et al.


Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/21/8022

Cited articles This article cites 50 articles, 17 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/21/8022.full.html#ref-list-1

Citing articles This article has been cited by 14 HighWire-hosted articles. Access the articles at:
/content/64/21/8022.full.html#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.