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DNA Vaccines Encoding Full-Length or Truncated Neu Induce Protective Immunity against Neu-expressing Mammary Tumors

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ABSTRACT

We generated DNA expression vectors encoding the full-length neu cDNA (designated $pNeu_N$), the neu extracellular domain ($pNeu_E$), or the neu extracellular and transmembrane domains ($pNeu_{TM}$). The 293 cells transfected with $pNeu_N$ or $pNeu_{TM}$ expressed the neu extracellular domain on the surface membrane, whereas 293 cells transfected with $pNeu_E$ secreted the extracellular domain of neu into the culture supernatant. We examined whether i.m. injection of either of these plasmids could induce protective immunity in FVB/N mice against the adoptive transfer of Tg1-1 cells, a *neu*-expressing tumor cell line generated from a mouse mammary tumor that spontaneously arose in a FVB/N *neu*-transgenic mouse. The i.m. injection of $pNeu_{TM}$ or $pNeu_E$, and to a lesser extent $pNeu_N$, induced protective immunity against a subsequent challenge with Tg1-1 cells in FVB/N mice. In addition, the coinjection of a plasmid encoding interleukin-2 (designated pIL-2) augmented the efficacy of each of the pNeu plasmids for inducing protective immunity. The plasmid $pNeu_{TM}$ seemed to be the most effective for inducing anti-neu antibodies. However, the generation of detectable anti-neu antibodies in response to any one of these pNeu plasmids was not enhanced by coinjection of pIL-2 and was not required for protective immunity against Tg1-1 cells. These studies demonstrate that DNA expression vectors encoding soluble or membrane-bound forms of neu lacking the cytoplasmic kinase domain can be effective in inducing protective antitumor immunity.

INTRODUCTION

The *neu* oncogene was originally identified by its ability to transform NIH 3T3 cells *in vitro* (1). Mice immunized with *neu*-transformed NIH 3T3 cells developed Abs[2] that were reactive with a 185-kDa surface phosphoprotein and were subsequently found to react with a group I receptor tyrosine kinase encoded by the *neu* oncogene designated $p185^{neu}$ (2). Subsequently, *neu* was found to be highly homologous to a gene on human chromosome 17 (17q21) designated *erbB-2* (*HER-2/neu*; Ref. 3). erbB-2/neu is a member of the EGFR family that can form homo- and heterodimeric receptor complexes leading to *trans*-tyrosine phosphorylation and signal transduction (reviewed in Refs. 4 and 5).

Overexpression of *erbB-2/neu* can lead to neoplastic transformation. *erbB-2* is overexpressed in 15–40% of all human breast cancers (6, 7). Moreover, overexpression of *erbB-2* in breast neoplasms is associated with a poorer rate of survival and a higher risk for recurrent disease after primary therapy (6, 8–12). That this association may define a causal relationship is indicated by studies with mice transgenic for the activated or wild-type *neu* proto-oncogene under the control of the mouse mammary tumor virus promoter. Transgenic mice that express activated *neu* develop multiple mammary tumors at an early age (13, 14). Moreover, transgenic mice with the wild-type *neu* gene under the mouse mammary tumor virus promoter also develop focal mammary tumors, albeit with slower kinetics (15). The relative selectivity of *erbB-2* overexpression in human adenocarcinomas and the association of *erbB-2* and *neu* with a pathogenic mechanism responsible for neoplasia make the protein product of these genes an attractive target for immunotherapy of mammary tumors (reviewed in Ref. 16).

We examined whether the injection of plasmid DNA encoding neu could induce protective immunity against a mammary tumor that overexpresses this oncogene. Several studies have shown that naked plasmid DNA directing synthesis of a target antigen can induce humoral and/or cellular immune responses against the transgene product when injected into skin or muscle (reviewed in Refs. 17 and 18). Moreover, i.m. injection of DNA-encoding human CEA has been found to confer protective immunity against a challenge with murine tumor cells transfected to express human CEA (19).

However, unlike CEA, neu is a type I surface membrane tyrosine kinase that can associate with other members of the EGFR family, induce intracellular phosphorylation, and transduce a positive growth signal (20–22) and possible neoplastic transformation (23–25). As such, plasmid vectors encoding the full-length neu could conceivably adversely affect the physiology of the cells that take up plasmid DNA. For this reason, we developed *neu* plasmid DNA expression vectors encoding either the full-length neu or a truncated neu that lacked the cytoplasmic kinase domain. Furthermore, to compare plasmid-based vaccines that encoded a membrane-bound *versus* a secreted antigen, we also constructed a plasmid encoding a truncated neu that lacked both the cytoplasmic and transmembrane domains.

We examined whether each of these plasmid vectors could induce protective immunity against mammary tumors that express the neu protein when injected into the skeletal muscle of FVB/N mice. To do this, we established a tumor cell line, designated Tg1-1, derived from a *neu*-expressing mammary tumor that arose spontaneously in a FVB/N *neu*-transgenic mouse. FVB/N mice received i.m. injections of each of these plasmids before challenge with a sufficient number of Tg1-1 cells to establish tumors in nonimmunized mice. Also, because studies have indicated that the injection of a DNA vaccine along with a plasmid vector encoding an immune-stimulatory cytokine, such as IL-2, could induce a greater Ab or helper T-cell response against the target antigen than injection of plasmid that encoded the antigen alone (26–28), we examined whether coinjection of a plasmid vector that encoded IL-2 could enhance the ability of pNeu constructs to induce protective antitumor immunity.

MATERIALS AND METHODS

Abs, Peptides, and Antisera. We purchased Ab3, a mouse mAb that was specific for a peptide corresponding to a COOH-terminal region of the neu-encoded protein, and Ab4, a mouse mAb that was specific for the extracellular domain of neu, from Oncogene Science, Inc. (Uniondale, NY). To generate antisera reactive with the NH_2-terminal extracellular domain of neu, a peptide, HLDMLRHKYQGC, was synthesized by using a modification of the Merrifield solid-phase method by Biosynthesis (Louisville, TX). This peptide was conjugated to TT (Connaught Laboratories, Mount Pocono, PA) by reacting

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[2] The abbreviations used are: Ab, antibody; CEA, carcinoembryonic antigen; IL-2, interleukin 2; mAb, monoclonal Ab; CMV, human cytomegalovirus; FACS, fluorescence-activated cell-sorting; TT, tetanus toxoid; EGFR, epidermal growth factor receptor; APC, antigen-presenting cell.

the TT carrier protein with succinimidyl *m*-maleimidobenzoate (Sigma, St. Louis, MO), allowing it to couple with the COOH-terminal cysteine of the peptide as described previously (29). BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were immunized i.p. with the TT-peptide conjugate in complete Freund's adjuvant (Life Technologies, Inc., North Andover, MA) and boosted i.p. with the peptide-protein conjugate in incomplete Freund's adjuvant (Life Technologies, Inc.) to develop antipeptide antisera. Fluorescein-conjugated mAbs specific for mouse H-2 K^q or H-2 D^q were purchased from PharMingen (San Diego, CA).

DNA Expression Vectors. The cDNA encoding the entire rat *neu* gene (30) was inserted into the polylinker of pRc/CMV (Invitrogen, San Diego, CA) to produce $pNeu_N$. The plasmid $pNeu_E$, encoding the extracellular domain of neu without the cytoplasmic and transmembrane domain of neu, was generated from the PCR product of $pNeu_N$ using the primers CGCAAGCTTCATC-ATGGAGCTGGC and CGTCTAGAGGGCTGGCTCTCTGCTC. The PCR product of the expected size was isolated via agarose gel electrophoresis, digested with *Hin*dIII and *Xba*I, and cloned into the multiple cloning site of pRc/CMV. Similarly, the plasmid $pNeu_{TM}$, encoding the extracellular domain and the transmembrane domain of neu, was generated from the PCR product of $pNeu_N$ by using the primers CGCAAGCTTCATCATGGAGCTGGC and ATGCGGCCGCTTTCCGCATCGTGTACTTCTTCCGG. Again, the PCR product of the expected size was isolated via agarose gel electrophoresis, digested with *Hin*dIII and *Not*I, and cloned into pCMV. To produce an IL-2 expression vector designated pIL-2, a 680-bp *Hin*dIII-*Bam*HI fragment of pBC12/HIV/IL-2 (ATCC No. 67618; Ref. 31) was inserted into the pRc/CMV plasmid. Each insert was flanked by the CMV enhancer/promoter at the 5′ end and by the polyadenylation signal and transcription termination sequences from the bovine growth hormone gene at the 3′ end. As a control, the pRc/CMV plasmid without an insert, designated pCMV, was used.

Cell Lines and Animals. FVB/N activated *neu* transgenic mice (Ref. 13; OncoMouse) were purchased from Charles River (Hollister, CA) and maintained in our animal facilities. At 36 months, these animals developed spontaneous mammary tumors and were sacrificed. To generate mammary tumor cell lines, the tumors were minced into single cells, washed several times in isotonic saline, and cultured in DMEM (BioWhittaker, Walkersville, MD) containing 20% fetal bovine serum, L-glutamine (200 mM), nonessential amino acid (Irvine Scientific, Santa Ana, CA), sodium pyruvate (Irvine Scientific), and Fungi-bact (Irvine Scientific). Syngeneic nontransgenic FVB/N mice were purchased from The Jackson Laboratory.

Immunoblotting. To examine whether $pNeu_N$, $pNeu_E$, or $pNeu_{TM}$ could direct the synthesis of the desired protein product, we transfected each into human 293 cells via calcium phosphate, as described previously (32). Thirty-six h after transfection, each supernatant was collected and concentrated 10-fold using Aquacide II (Calbiochem, La Jolla, CA). The cells were removed from the culture flask and lysed in 300 μl of lysis buffer [50 mmol/liter Tris-HCl (pH 8.0) containing 0.1% SDS (Sigma) and 1% NP40 (Sigma)] containing the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), aprotinin (2 μg/ml), and leupeptin (2 μg/ml). For immunoprecipitation, each sample was incubated for 1 h at 4°C with 20 ng of anti-neu Ab4. After washing three times with lysis buffer, each sample was incubated for 1 h at 4°C with 100 μl of protein A-Sepharose slurry (Pierce, Rockford, IL). The protein A-Sepharose was pelleted by centrifugation and washed four times with lysis buffer. The pelleted Sepharose was boiled for 5 min in 2× SDS gel loading buffer [100 mM Tris-CL (pH 6.8), 200 mM DTT, 4% SDS, 0.2% bromphenol blue, and 20% glycerol]. Similarly, an aliquot of concentrated supernatant was mixed with an equal volume of 2× SDS gel loading buffer. After boiling, each sample mixture was loaded into separate wells of a 7.5% polyacrylamide gel containing 0.1% SDS. After 45 min of electrophoresis at 200 V, the size-separated proteins in the gel were electrotransferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA) for 1 h at 200 V. The membrane then was treated with blocking buffer [5% BSA in 25 mM Tris-HCl (pH 8.0) and 125 mM NaCl] overnight at 4°C and then incubated for 1 h at room temperature with antisera raised against peptide C (corresponding to the NH_2-terminal portion of neu) in blocking buffer. After washing, the membrane was incubated for 1 h with horseradish peroxidase-conjugated goat antimouse immunoglobulin (1:2000) in blocking buffer, developed in substrate buffer, and then exposed to Kodak XAR-5 film for approximately 5 min at room temperature.

Isolation of DNA Plasmids for i.m. Injection. *Escherichia coli* strain XL1-blue transformed with $pNeu_N$, $pNeu_E$, $pNeu_{TM}$, pIL-2, or the control plasmid, pCMV, was grown in L Broth (Bio101, Inc., Vista, CA). Large-scale preparation of the plasmid DNA was carried out by alkaline lysis using Qiagen Plasmid-Mega kits (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's instructions. Endotoxin was removed by extraction with Triton-X-114 (Sigma), as described previously (33). The DNA was precipitated and stored at −70°C. For experimental use, the DNA was suspended in sterile saline at a concentration of 2 mg/ml and stored in aliquots at −20°C for subsequent use in the immunization protocols.

Flow Cytometry. To examine cells for the expression of *neu*, transfected 293 cells were stripped from the culture flasks by incubating the adherent cells at 37°C for 5 min in PBS (pH 7.2) containing 10 mM EDTA. The removed cells were washed in FACS buffer consisting of RPMI 1640 (Life Technologies, Inc.) with 2% fetal bovine serum and 0.1% sodium azide. Cells were incubated with Ab4 or an isotype control immunoglobulin, MOPC-21, before they were washed and stained with a fluorescein-conjugated goat antimouse immunoglobulin Ab (Biosource Biologicals, Inc., Victoria, TX), as described previously (34).

Indirect flow cytometric analysis was used to examine sera for anti-neu binding activity. For this, we tested whether antisera could react specifically with cells transfected to express the neu surface protein (Fig. 1) but not mock-transfected cells. The cells were processed as described above. Approximately 3×10^5 cells/analysis were incubated with a 1:20 dilution of antiserum or control antiserum at 4°C for 30 min. Cells were washed three times with FACS buffer and then stained for 30 min at 4°C with the FITC-conjugated rat mAb specific for mouse immunoglobulin (PharMingen). The cells were washed again and then suspended in FACS buffer containing propidium iodide for analysis, as described previously (34).

ELISA for Anti-Neu Abs. Anti-neu Ab was measured by ELISA. Sera were collected from the mice before and 4 weeks after the last injection with plasmid DNA. An ELISA was performed using plates coated overnight with 1 μg/ml purified rabbit Ab specific for the cytoplasmic domain of neu (C18; Santa Cruz Biotechnology, Inc.) in PBS (pH 7.2). The plates were washed four times with washing buffer containing 0.05% Tween 20 in borate-buffered saline [0.1 M borate and 0.2 M NaCl (pH 8.2)] and incubated overnight at 4°C in blocking buffer containing 1% BSA and 0.05% sodium azide in borate-buffered saline. Sonicated membrane lysates were prepared from a neu-expressing mammary tumor cell line (#32-LU) by suspending cells in PBS containing protease inhibitors [50 mM phenylmethylsulfonyl fluoride, pepstatin A (1 μg/ml), aprotinin (2 μg/ml), and leupeptin (1 μg/ml; all from Sigma)] before sonication for 10 s × 3 at 4°C. The sonicate was spun at 15,000 × *g* for 15 min, and the pellet was suspended in lysis buffer consisting of PBS with the four protease inhibitors and 0.2% NP40 (Sigma) at a final concentration of 10^7 cell membranes/ml. This mixture was sonicated for 10 s × 3 at 4°C and aliquoted into tubes that were stored at −20°C until they were used in the ELISA. A 1:7 dilution of the membrane lysate in PBS was added to each well and allowed to incubate at room temperature for 2 h. Control wells were not treated with membrane lysate or were treated with lysates from cells that did not express neu. The plates were again washed four times with washing buffer

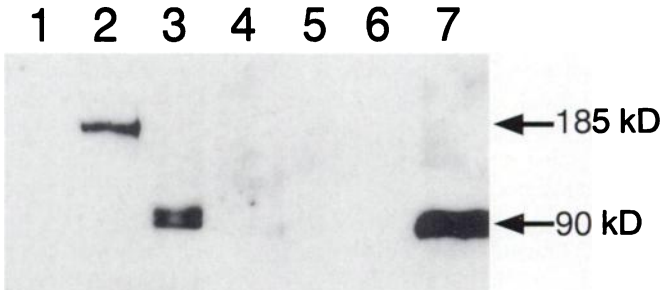

Fig. 1. Expression of neu by 293 cells transfected with $pNeu_N$, $pNeu_{TM}$, or $pNeu_E$. Immunoprecipitates with Ab4 and protein A-Sepharose of cell lysates (*Lanes 1–4*) or culture supernatants (*Lanes 5–7*) were analyzed by SDS-PAGE and immunoblot analysis using anti-neu peptide C antisera. *Top*, *numbers* indicate the different sample lanes. *Lane 1* is the immunoprecipitate of cell lysate from 293 cells. *Lanes 2–4* are immunoprecipitates of cell lysates from 293 cells transfected with $pNeu_N$, $pNeu_{TM}$, and $pNeu_E$, respectively. *Lanes 5–7* are immunoprecipitates of culture supernatants from 293 cells transfected with $pNeu_N$, $pNeu_{TM}$, and $pNeu_E$, respectively. *Right*, *arrows* indicate the approximate molecular sizes of the protein detected with the anti-neu peptide antisera.

and allowed to react with mouse sera diluted 1:20 or 1:100 in blocking buffer. After another 1 h of incubation at room temperature, the plates were washed four times with washing buffer. To each well we added biotinylated goat antimouse IgG Ab (Kirkegaard & Perry, Gaithersburg, MD) diluted 1:2000 in blocking buffer. After another 1-h incubation at room temperature, the plates were washed four times with washing buffer and then incubated with 45 μl of alkaline phosphate substrate (Sigma) for 3 h. The absorbance of each well at 405 nm was measured using an ELISA plate reader. The specific absorbance value for each sample was calculated by subtracting the absorbance of the sample tested on plates without membrane lysates of neu-expressing cells from the absorbance measured for the sample on plates coated with membrane lysates from neu-expressing cells. The absorbance values of plates without membrane lysates of neu-expressing cells generally did not exceed 0.1.

DNA Immunization Method. The 6–8-week-old FVB/N mice (The Jackson Laboratory) were anesthetized with methoxyflurane. Plasmid DNA (100 μg/injection) suspended in 100 μl of saline was injected into the right quadriceps muscles through a 28-gauge needle each week for 4 weeks. Mice were bled via the retro-orbital plexus at weekly intervals to assess anti-neu Abs.

Tumor Challenge. Mice were inoculated with Tg1-1 cells by s.c. injection in sterile PBS through a 20-gauge needle over the flank. Tumors were measured by caliper in three dimensions, and the volumes were calculated using the formula: tumor volume = (width × length × depth) $m^3 \times \pi/6$.

RESULTS

Expression of $pNeu_N$, $pNeu_E$, or $pNeu_{TM}$ in 293 Cells. We generated $pNeu_N$, $pNeu_{TM}$, and $pNeu_E$ plasmid vectors that encoded the full-length neu protein, the neu extracellular and transmembrane domains, or the neu extracellular domain, respectively. The latter two plasmids each should encode a truncated neu protein of 90 and 87 kDa, respectively. The 293 cells were transfected with each construct and examined for protein expression by immunoblotting. A mouse antiserum raised against a peptide corresponding to the extracellular domain of neu, designated peptide C, detected a protein with a molecular size of 185 kDa in lysates prepared from 293 cells transfected with $pNeu_N$ (Fig. 1). Similarly, the antipeptide C antiserum detected proteins of approximately 90 kDa in size from lysates prepared from 293 cells transfected with $pNeu_{TM}$. Lysates of 293 cells transfected with $pNeu_E$, on the other hand, did not have detectable amounts of neu (Fig. 1). However, in contrast to the concentrated supernatants of 293 cells transfected with either $pNeu_N$ or $pNeu_{TM}$, the supernatants of $pNeu_E$-transfected cells were found to contain a protein of approximately 90 kDa that reacted with the antipeptide C antiserum (Fig. 1).

We also examined 293 cells transfected with each construct for surface expression of neu via flow cytometry. As anticipated, 293 cells transfected with either $pNeu_N$ or $pNeu_{TM}$ expressed surface neu, whereas 293 cells transfected with $pNeu_E$ did not stain with the anti-neu Ab (Fig. 2).

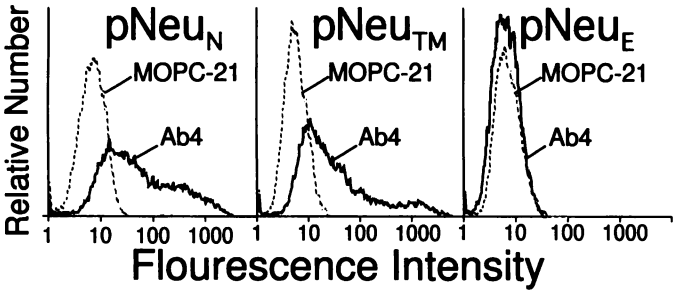

Fig. 2. Flow cytometric analysis of 293 cells transfected with $pNeu_N$ (*left panel*), $pNeu_{TM}$ (*center panel*), or $pNeu_E$ (*right panel*). The *dotted lines* represent the fluorescence histograms of the cells incubated with a nonspecific control Ab (*MOPC-21*), whereas the *dark solid lines* depict the histograms of cells incubated with the anti-neu mAb (*Ab4*). The cells were then stained with FITC-conjugated goat antimouse immunoglobulin and analyzed by flow cytometry. The histograms depict the relative cell number with respect to their logarithmic green fluorescence intensity.

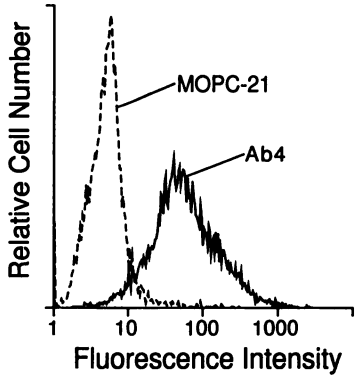

Fig. 3. Flow cytometric analysis of Tg1-1 cells. The *dotted lines* represent the fluorescence histograms of the cells preincubated with a nonspecific control Ab (*MOPC-21*), whereas the *dark solid lines* depict the histograms of cells preincubated with the anti-neu mAb (*Ab4*) before staining with the FITC-conjugated goat antimouse immunoglobulin. The histograms depict the relative cell number *versus* the logarithmic green fluorescence intensity.

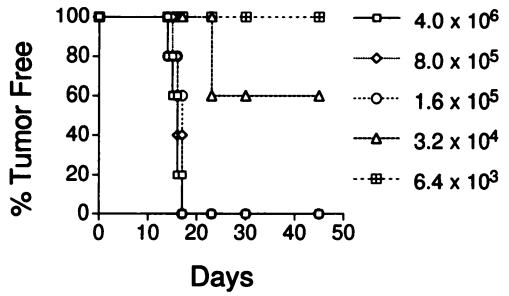

Fig. 4. Titration of the number of Tg1-1 cells required to form tumors in FVB/N mice. We injected five groups of five FVB/N mice with varying numbers of Tg1-1 cells in the flank and then monitored them for the development of tumor nodules at the site of injection. The numbers of Tg1-1 cells given to mice of each group ranged from 6.4×10^3 to 4.0×10^6 cells/mouse, as indicated by the following symbols: □, 4.0×10^6; ◇, 8.0×10^5; ○, 1.6×10^5; △, 3.2×10^4; ⊞, 6.4×10^3.

Development of the Tg1-1 Neu-expressing Cell Line. We excised mammary tumors that spontaneously developed in FVB/N mice that were transgenic for the activated *neu* oncogene. Cells were adapted to *in vitro* culture, cloned by limiting dilution, and then tested for the expression of neu via flow cytometry. One cell line, designated Tg1-1, was selected because of its vigorous growth and high-level expression of neu (Fig. 3). This cell line also was found to express H-2 K^q and D^q MHC class I surface antigens (data not shown). Titration studies demonstrated that these cells generated tumor nodules in FVB/N mice when $\geq 1.6 \times 10^5$ cells were injected s.c. (Fig. 4). Moreover, flow cytometric analyses demonstrated that these cells continued to express neu, even after they were excised from secondary s.c. tumor nodules (data not shown).

Anti-Neu Ab Response of FVB/N Mice Injected with pNeu Plasmid DNA. We examined whether the DNA of various pNeu plasmids could induce anti-neu Abs when injected into the skeletal muscle of FVB/N mice. We also examined whether a plasmid encoding IL-2 could enhance the anti-neu Ab response to any one of these plasmid vectors. Accordingly, FVB/N mice were injected with either pCMV (group 1), pIL-2 (group 2), $pNeu_N$ + pIL-2 (group 3), $pNeu_{TM}$ + pIL-2 (group 4), $pNeu_E$ + pIL-2 (group 5), $pNeu_N$ (group 6), $pNeu_{TM}$ (group 7), or $pNeu_E$ (group 8). Each mouse received i.m. injections of 100 μg of plasmid DNA at weekly intervals for 4 weeks. Before the first injection and 4 weeks after the last DNA injection,

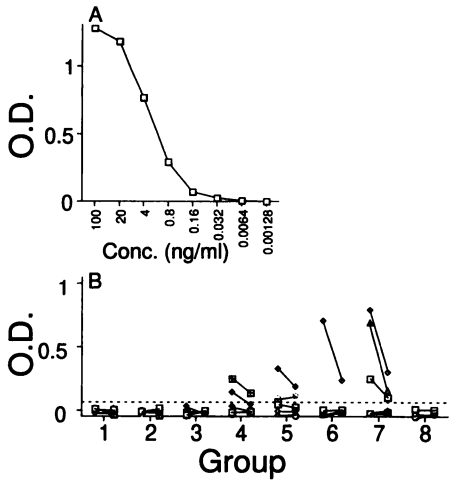

Fig. 5. ELISA for anti-neu Abs. *A*, the standard curve depicting the anti-neu binding activity of Ab4 at different concentrations, as indicated on the *abscissa* in ng/ml. The specific absorbance measured at 405 nm is indicated on the *ordinate*. This assay was run in parallel with the ELISA on serum samples of mice that were injected with plasmid DNA. *B*, anti-neu binding activity of sera from mice injected with pCMV (*Group 1*), pIL-2 (*Group 2*), $pNeu_N$ and pIL-2 (*Group 3*), $pNeu_{TM}$ and pIL-2 (*Group 4*), $pNeu_E$ and pIL-2 (*Group 5*), $pNeu_N$ (*Group 6*), $pNeu_{TM}$ (*Group 7*), or $pNeu_E$ (*Group 8*). The sera, collected 4 weeks after the final injection of plasmid DNA, were diluted 1:20 and 1:100 in blocking buffer before the assay. The specific absorbance values for the two dilutions of each serum sample are indicated by the *symbols connected by lines*: the *symbol to the left* represents the specific absorbance by ELISA of the 1:20 dilution; and the *symbol to the right* represents the specific absorbance by ELISA of the 1:100 dilution. Samples with specific absorbance *below* the *dashed lines* were scored as not having detectable anti-neu Ab.

the sera were collected and tested for anti-neu Abs by ELISA. Titration studies revealed that our ELISA could detect ≥0.8 ng/ml Ab4, an anti-neu mAb (Fig. 5*A*). In contrast, mouse Abs of irrelevant specificity (*e.g.*, MOPC-21) did not react with the ELISA plates, even at concentrations of ≥1 μg/ml (data not shown). Serum samples diluted 1:20 or 1:100 in sample buffer were analyzed in parallel with Ab4 (Fig. 5*B*). As expected, none of the sera collected from animals before the injection of plasmid DNA had detectable anti-neu binding activity (data not shown). Also, none of the animals injected with pCMV (group 1, $n = 6$) or pIL-2 (group 2, $n = 8$) developed anti-neu Abs (Fig. 5*B*). Half of the animals injected with $pNeu_{TM}$ (group 7, $n = 6$), one of the animals injected with $pNeu_N$ (group 6, $n = 8$), and none of the animals injected with $pNeu_E$ (group 8, $n = 8$) developed detectable anti-neu Abs (Fig. 5*B*). Consistent with the ELISA-detecting antisera specific for neu, only those sera with ELISA binding activity greater than 2 SDs above the mean value for control serum (Fig. 5*B*, *dotted line*) reacted with neu-transfected cells, as assessed by flow cytometry (data not shown).

Coinjection of pIL-2 did not enhance the anti-neu Ab response to any of the pNeu plasmids. None of the animals injected with $pNeu_N$ + pIL-2 (group 3, $n = 8$) made detectable anti-neu Abs. Moreover, only two of the $pNeu_{TM}$ + pIL-2 group (group 4, $n = 8$) and two of the $pNeu_E$ + pIL-2 group (group 5, $n = 8$) developed antisera with low-level anti-neu binding activity (Fig. 5*B*).

A repeat experiment yielded similar results. At 4 weeks after the final injection of plasmid DNA, only two of the animals injected with $pNeu_{TM}$ ($n = 8$) and three of the animals injected with $pNeu_{TM}$ + pIL-2 ($n = 8$) developed detectable anti-neu antisera by ELISA (data not shown). Moreover, none of the animals ($n = 8$ in each group) that were injected with $pNeu_N$ alone or $pNeu_E$ with or without pIL-2 developed detectable anti-neu antisera 4 weeks after the final injection. Only one animal developed low-level anti-neu antisera in response to coinjection of $pNeu_N$ + pIL-2. Collectively, for the two experiments, 6% (1 of 16) of the mice injected with $pNeu_N$, 36% (5 of 14) of the animals injected with $pNeu_{TM}$, and 0% (0 of 16) of the animals injected with $pNeu_E$ developed detectable anti-neu Abs before the tumor challenge (Fig. 6, groups 6–8, respectively; △). Moreover, 6% (1 of 16) of the animals injected with $pNeu_N$ + pIL-2, 31% (5 of 16) of the mice injected with $pNeu_{TM}$ + pIL-2, and 13% (2 of 16) of the animals injected with $pNeu_E$ + pIL-2 generated detectable anti-neu Abs (Fig. 6, groups 3–5, respectively; △).

Resistance of DNA Vaccine-treated FVB/N Mice to Challenge with Tg1-1. Four weeks after the final injection of plasmid DNA, each mouse was challenged with 3×10^5 Tg1-1 cells, approximately twice the number of cells required to form tumors in nonimmunized mice (Fig. 4). In experiment 1, all of the animals injected with either pCMV ($n = 6$) or pIL-2 ($n = 8$) developed palpable tumors 2 weeks after the challenge with Tg1-1 cells. However, tumors never developed in two of eight mice (25%) injected with $pNeu_N$, in five of six mice (84%) injected with $pNeu_{TM}$, and in four of eight mice (50%) injected with $pNeu_E$ (Fig. 6, groups 6–8, respectively). Animals coinjected with each of the pNeu plasmids and pIL-2 seemed to have a greater resistance to challenge with Tg1-1. Tumors never developed in six of eight mice (75%) coinjected with $pNeu_N$ + pIL-2, in six of eight mice (75%) coinjected with $pNeu_{TM}$ + pIL-2, or in eight of eight mice coinjected with $pNeu_E$ + pIL-2 (Fig. 6, groups 3–5, respectively).

Similar results were obtained in a subsequent experiment (Fig. 6, experiment 2). None of the animals injected with either pCMV ($n = 7$) or pIL-2 ($n = 7$) were free of palpable tumors 2 weeks after challenge with 3×10^5 Tg1-1 cells (Fig. 6, groups 1 and 2, respectively). On the other hand, three of eight mice (37%) injected with $pNeu_N$, five of eight mice (62%) injected with $pNeu_{TM}$, and four of eight mice (50%) injected with $pNeu_E$ remained tumor-free after challenge with Tg1-1 (Fig. 6, groups 6–8, respectively). Again, the coinjection of pIL-2 with any pNeu plasmid seemed to enhance the resistance to the tumor challenge, because tumors never developed in five of eight mice (62%) coinjected with $pNeu_N$ + pIL-2, in six of eight mice (75%) coinjected with $pNeu_{TM}$ + pIL-2, and in seven of eight mice (87%) coinjected with $pNeu_E$ + pIL-2 (Fig. 6, groups 3–5, respectively).

Combined, the proportions of animals in both experiments that resisted challenge with Tg1-1 were 0% (0 of 14) in group 1, 0% (0 of 16) in group 2, 69% (11 of 16) in group 3, 75% (12 of 16) in group 4, 94% (15 of 16) in group 5, 31% (5 of 16) in group 6, 71% (10 of 14) in group 7, and 50% (8 of 16) in group 8. As such, the mice

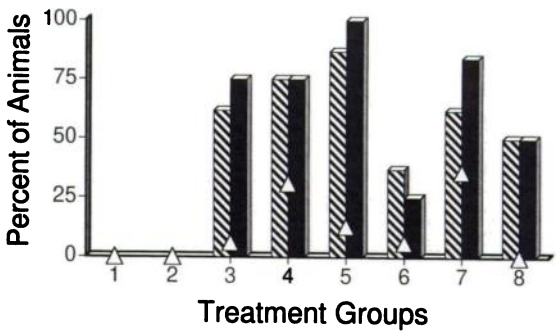

Fig. 6. The proportions of animals that resisted challenge with 3×10^5 Tg1-1 cells after injection with naked plasmid DNA. The results of two separate experiments are depicted. The height of each bar indicates the percentage of mice in each group of experiment 1 (▨) or experiment 2 (■) that did not develop tumors after s.c. injection of 3×10^5 Tg1-1 cells, 4 weeks after the last injection of plasmid DNA. The treatment group numbers are indicated at the *bottom* of the figure for mice injected with pCMV (*Group 1*), pIL-2 (*Group 2*), $pNeu_N$ and pIL-2 (*Group 3*), $pNeu_{TM}$ and pIL-2 (*Group 4*), $pNeu_E$ and pIL-2 (*Group 5*), $pNeu_N$ (*Group 6*), $pNeu_{TM}$ (*Group 7*), or $pNeu_E$ (*Group 8*). △, the overall percentage of animals in each group that developed detectable anti-neu Abs, as indicated on the *ordinate*.

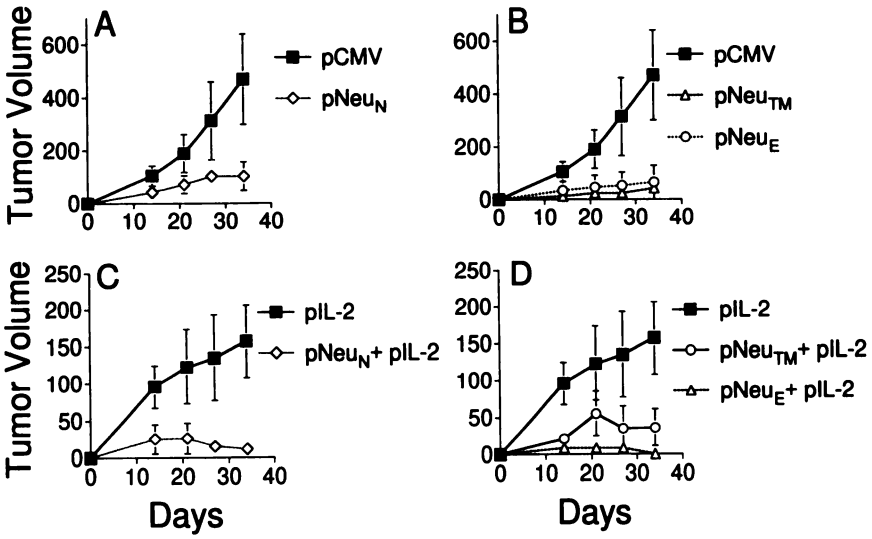

Fig. 7. Growth of Tg1-1 tumor nodules in FVB/N mice that previously had been injected with naked plasmid DNA. FVB/N mice received four weekly i.m. injections of: *A*, pCMV (■) or pNeu$_N$ (◇); *B*, pCMV (■), pNeu$_{TM}$ (△), or pNeu$_E$ (○); *C*, pIL-2 (■) or pNeu$_N$ and pIL-2 (◇); or *D*, pIL-2 (■) or pNeu$_{TM}$ and pIL-2 (○) or pNeu$_E$ and pIL-2 (△). On day 0, 4 weeks after the final DNA injection, 3×10^5 Tg1-1 cells were injected s.c. into the flank of each animal. The calculated mean volumes of tumor nodules are depicted only for those animals that developed tumors *versus* the number of days after tumor challenge. *Error bars*, the SD about the mean tumor volume for each group.

coinjected with pIL-2 and any one of the pNeu plasmids had a significantly lower incidence of tumors than mice injected with pCMV or pIL-2 ($P < 0.05$, the Bonferroni *t* test). Moreover, the mice injected with pNeu$_{TM}$ or pNeu$_E$ alone also had a significantly lower incidence of tumors than mice injected with pCMV or pIL-2 ($P < 0.05$, the Bonferroni *t* test). Although the proportion of animals that remained tumor-free in groups coinjected with pIL-2 and any one of the pNeu plasmids (groups 3–5) seemed greater than that of the group injected with the respective pNeu plasmid alone (groups 6–8), this difference did not reach statistical significance. Nevertheless, the combined proportion of tumor-free mice in groups 3–5 [79% (38 of 48)] with pIL-2 was significantly higher than that of the combined proportion in groups 6–8 [50% (23 of 46)] without pIL-2 ($P < 0.01$, Student's *t* test).

Among the animals that developed tumors, we noted significant differences in the kinetics of tumor growth between the various groups. On each day examined (*e.g.*, 15, 21, 27, and 34 days after challenge), the mean volumes of the tumors that developed in mice injected with pNeu$_N$, pNeu$_{TM}$, or pNeu$_E$ were significantly smaller than those of control mice injected with pCMV (Fig. 7, *A* and *B*; $P < 0.01$, the Bonferroni *t* test). Although the tumor volumes of mice injected with pIL-2 appeared smaller than those of animals injected with pCMV, the differences in mean tumor volumes of the two groups did not reach statistical significance. Nevertheless, the mean volumes of the tumors that developed in animals that received any one of the pNeu expression vectors with pIL-2 were significantly smaller than those of animals injected with pIL-2 alone (Fig. 7, *C* and *D*; $P < 0.01$, the Bonferroni *t* test).

DISCUSSION

We constructed DNA plasmids that encoded the full-length neu (pNeu$_N$), the neu extracellular and transmembrane domains (pNeu$_{TM}$), or the neu extracellular domain alone (pNeu$_E$). Whereas transfection of cells with pNeu$_N$ or pNeu$_{TM}$ generated cells that expressed surface membrane-bound neu, transfection of cells with pNeu$_E$ generated transfectants that instead secreted a truncated form of the neu protein (Figs. 1 and 2). These constructs allowed us to examine whether i.m. injections of plasmids encoding the full-length neu, a truncated surface-membrane-bound neu, or a truncated and secreted neu could induce protective immunity against Tg1-1, a syngeneic neu-expressing tumor that arose spontaneously in a neu-transgenic FVB/N mouse.

We found that i.m. injection of any one of the naked pNeu plasmids could induce protective immunity against Tg1-1. The proportions of animals that remained tumor-free in groups injected with pNeu$_{TM}$ [10 of 14 (71%)] or pNeu$_E$ [8 of 16 (50%)] were significantly greater than that of the control group injected with pCMV (Fig. 6). Furthermore, of the animals that did develop tumors, the mean tumor volumes of mice injected with any one of the pNeu plasmids were significantly smaller than those of mice injected with the control plasmid DNA (Fig. 7).

Concerns over the use of the full-length *neu* oncogene as a DNA vaccine include chance transformation of transfected cells or induction of autoimmunity against the neu intracellular domain, a domain that is highly conserved among the members of the EGFR family (20, 21, 23–25). We therefore examined whether plasmid DNA encoding a truncated neu that lacked the intracellular domain could induce protective immunity against *neu*-expressing mammary tumors. We found that plasmids encoding truncated neu proteins that lacked the neu cytoplasmic domain or the cytoplasmic and transmembrane domains of neu were at least as effective as pNeu$_N$. If anything, the proportion of mice that were protected in the groups injected with pNeu$_N$ [5 of 16 (31%)] seemed lower than that of mice injected with either pNeu$_{TM}$ or pNeu$_E$ [10 of 14 (71%) or 8 of 16 (50%), respectively]. Conceivably, the pNeu$_N$ plasmid may be taken up and expressed less efficiently than either pNeu$_{TM}$ or pNeu$_E$ after i.m. injection, perhaps because its cDNA is twice as large as that of the other plasmid vectors. Alternatively, expression of the full-length neu by transfected somatic cells may be less effective in inducing antitumor immunity than expression of a truncated neu protein. However, it should be noted that the differences between the proportion of animals protected from Tg1-1 after injection with pNeu$_N$ *versus* pNeu$_{TM}$ or pNeu$_E$ did not reach statistical significance ($P \geq 0.05$, the Bonferroni *t* test) and had a relatively low statistical power (*e.g.*, $\beta \leq 0.6$ or 0.2 for the differences between mice injected with pNeu$_N$ *versus* pNeu$_{TM}$ or pNeu$_E$, respectively). As such, it would take much larger groups of animals to verify that pNeu$_N$ was less effective than either pNeu$_{TM}$ or pNeu$_E$ in inducing protective immunity against the adoptive transfer of Tg1-1 cells. In any case, our studies demonstrate that it is not necessary for a plasmid to encode the full-length neu, with its large cytoplasmic kinase domain, to function as an effective DNA vaccine against neu-expressing tumors.

Although direct injection of plasmid DNA into muscle can result in transgene expression in myocytes, fibroblasts, and a few APCs, such

as tissue macrophages or dendritic cells, the mechanism(s) by which DNA vaccines induce immune responses is not known (35). At first, it was assumed that antigen presentation might be mediated by the transfected muscle cells themselves. However, muscle cells express only low levels of MHC class I antigens and lack expression of MHC class II antigens or costimulatory molecules, such as CD80 (B7.1) or CD86 (B7.2; Ref. 36). Another possibility is that the immune response to DNA vaccines injected into muscle results from a chance transfection of bystander APCs, such as macrophages or dendritic cells. However, such APCs are sparse within muscle tissue, although they may be recruited to muscle by the local inflammation after the DNA injection. A more likely possibility is that muscle cells serve as a reservoir of expressed antigen with subsequent transfer to bone marrow-derived APCs. A few recent studies have provided support for this model (37–39).

It is noteworthy, in this regard, that we did not discern a significant difference between $pNeu_{TM}$ and $pNeu_{E}$ in their ability to induce protective immunity against the challenge with Tg1-1 cells, despite the fact that $pNeu_{TM}$ encodes a protein that is confined primarily to the surface membrane of the transfected cell, whereas $pNeu_{E}$ directs the synthesis of a secreted protein. Conceivably, the antigen provided to APCs is not in the form of an intact polypeptide but is rather in the form of peptides and denatured polypeptides generated within the transfected cell and subsequently transferred to APCs via heat shock protein chaperones (40, 41). In this case, the bulk of the intact protein that is secreted or expressed on the surface of transfected cells may not be destined to undergo processing for antigen presentation. Alternatively, more subtle quantitative differences may exist between $pNeu_{TM}$ and $pNeu_{E}$ in their ability to induce protective antitumor immunity that would not be apparent in the studies described here.

$pNeu_{TM}$ did seem more effective than $pNeu_{E}$ in inducing anti-neu Abs. The proportion of mice that developed detectable anti-neu antisera after injection with $pNeu_{TM}$ [5 of 14 (6%)] was significantly greater than that of mice injected with $pNeu_{E}$ [0 of 16 (0%)] or $pNeu_{N}$ [1 of 16 (6%); $P < 0.05$, the Bonferroni t test]. Nevertheless, even $pNeu_{TM}$ was not highly effective, because most of the animals injected with this plasmid failed to generate detectable anti-neu Abs, despite our use of a highly sensitive ELISA capable of detecting as little as 1 ng/ml anti-neu mAb (Fig. 5). This could be due to the fact that the mouse neu, which shares approximately 97.3% amino acid sequence homology with the rat neu used in these studies, is expressed in the kidney, intestine, and testis of adult mice (42). As such, mice may be tolerant of many of the epitopes present on rat neu that are shared with the mouse neu protein, limiting the ability to generate neu-specific Abs. Alternatively, although not exclusive of the former possibility, there may be strain differences in the ability of mice to generate humoral immune responses to the rat neu protein.

In any case, the development of protective immunity against the adoptive transfer of Tg1-1 cells did not seem dependent on the generation of anti-neu Abs. Consistent with this, we noted that coinjection of pIL-2 did not significantly augment the Ab response to any one of the pNeu plasmids but did enhance the overall ability of the pNeu-injected mice to resist tumor challenge with neu-expressing cells (Fig. 6). As such, it seems that the protection against the adoptive transfer of Tg1-1 that is achieved by i.m. injection of any one of the pNeu plasmids depends on the generation of a cellular immune response against cells that express the neu protein. Such cellular immune responses could result in production of CTLs that recognize peptides presented in the context of MHC molecules of the neoplastic cell (43, 44). Alternatively, T-cell recognition of tumor-specific antigens may allow for the activation of monocytes, macrophages, and/or other cell types that then mediate antitumor cytotoxicity (45–47). The incorporation of strategies that enhance the generation of such cellular immune responses to DNA vaccines (48) may improve the efficacy of such plasmids for inducing protective immunity against tumors that overexpress the erbB-2/neu oncogene product.

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