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THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.
Her2/neu over-expression is found in 30-40% of breast cancer biopsy and is indicative of metastasis and poor prognosis. A DNA vaccine targeting Her2/neu could have significant therapeutic and preventative application by controlling the growth and spread of highly aggressive Her2/neu+ cells. Although DNA vaccines have shown effectiveness in clinical trials, it is essential to demonstrate pre-clinical effectiveness for anti-tumor DNA vaccines before clinical testing can begin. We have shown that vaccination of mice with a novel plasmid expressing the DNA sequence for Her2/neu protected mice from tumor incidence when challenged with a Her2/neu+ murine breast tumor cell line injected directly into mammary tissue or injected intravenously. In the previous year we switched to an improved version of our DNA vaccine and the results have been even better. We have also confirmed that vaccination can reduce tumor incidence and prolong survival in mice containing neu as a transgene under the control of a breast-specific promoter, demonstrating that our vaccine works with both naturally occurring and transplanted neu+ breast tumors. We have concluded our work to determine if vaccination following surgical-removal of the initial tumor will prolong survival. Unfortunately, our vaccine is not powerful enough to be used after a tumor has been established—even if the tumor is removed by surgery before vaccination.
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INTRODUCTION:

HER2/neu over-expression is found in 30-40% of breast cancer biopsy and is indicative of metastasis and poor prognosis (1). Although significant advances have been made in the treatment of breast cancer, once metastasis has occurred the possibility of a complete cure is unlikely (1). A vaccine targeting HER2/neu could have significant therapeutic and preventative application by controlling the growth and spread of highly aggressive HER2/neu+ cells (2). The newest type of tumor vaccines, gene vaccines, encode the DNA sequence for tumor antigens. Bacterial expression plasmids containing tumor gene sequences have been shown to induce strong anti-tumor immunity in mice (3). Although gene vaccines have shown effectiveness in clinical trials for infectious diseases (4), it is essential to demonstrate preclinical effectiveness for anti-tumor vaccines before clinical testing can begin (5-7). We have shown that vaccination of mice with a novel plasmid expressing the DNA sequence for HER2/neu protected mice from tumor incidence when challenged with a HER2/neu+ murine breast tumor cell line injected directly into mammary tissue. We also found that vaccination was able to reduce metastasis when the tumor was injected intravenously, a model of tumor metastasis. The plasmid we used for vaccination, called ELVIS, was created by Chiron Corp. (Emeryville, CA) and incorporates unique properties of Sindbis virus, a non-pathogenic alphavirus (8). Recently, Chiron Corp. has provided us with a more advanced version of their plasmid called ELVIS2, and we have shown it to be even more powerful than the original ELVIS when injected before mice a challenged with a tumor (protective vaccination). However, ELVIS2-neu was not able to prolong the life of mice when vaccinated followed surgical removal of a primary neu+ breast tumor (surgery model) or when vaccination was performed on a tumor bearing mouse (race model). Thus, different strategies are required in our two therapeutic vaccination models (surgery and race). The first strategy we are testing is to increase the effectiveness of the vaccine is to combine vaccination and chemotherapy. Several publications have indicated that combined chemotherapy and vaccine therapy is superior to either treatment used alone (9,10).

BODY:

The enclosed reprint “DNA Vaccination Against neu Reduces Breast Cancer Incidence and Metastasis in Mice” (11) contains a complete description of our accomplishments related to Task 1 of the Statement of Work. Below is shown the bulleted points from the Statement of Work for Task 1 with corresponding reference to the figure and page number of our article in which the research is described.

Task 1: To demonstrate that mice vaccinated with ELVIS-neu are protected from the growth and metastasis of mammary tumor cells expressing HER2/neu (protective vaccination). (months 1-24)

➢ Immunize mice with 1, 10 and 25 ug of ELVIS-neu, ELVIS control and PBS by i.m. route. Evaluate humoral and cellular responses by flow cytometry, ELISA and CTL assays; 360 mice (months 1-9).
1. Figure 2 (p. 262) and text page 263. We have immunized mice with increasing amounts of ELVIS-neu as shown in Figure 2 and the results were evaluated by flow cytometry. As shown, an antibody response resulted from the immunization and the response increased with increasing dose of ELVIS-neu. We did not evaluate results by ELISA since the flow cytometry was sufficient to conclude that a humoral immune response had been induced. As an alternative to CTL assays we performed an Interferon-γ (IFN-γ) release assay in which spleen cells from immunized mice were co-cultured for 5 days with P815 cells expressing neu or control P815 cells. The results clearly demonstrated IFN-γ only from spleen cells of mice vaccinated with ELVIS-neu and not from control mice or mice vaccinated with only ELVIS (Figure 6, p. 265). The IFN-γ release assay is widely used as an alternative to the CTL assay which has poor reproducibility and a very low signal to noise ratio.

Immunize mice with single dosage of ELVIS-neu, ELVIS control and PBS using 1, 2, or 3 repeat i.m. injections. Evaluate humoral and cellular responses; 360 mice (month 3-12).

1. The result for the humoral immune response comparing one immunization and three immunization is shown in Figure 2a and Figure 2f (page 262) and text page 263.
2. The result for the antitumor cellular response is shown in Figure 4a-c (page 264) and text page 264-266.

- Determine if co-administration of cytokine encoding plasmids increases immune responses; 120 mice (months 13-34).
  1. The ELVIS2-neu plasmid has proven to be so powerful that I do not feel it is necessary to use a cytokine plasmid to attempt to increase the biological activity (see figure immediately below showing the effectiveness of ELVIS2-neu and ELVIS-neu on lung metastasis).

- Determine whether liposomal-cytokine-peptide antigens can boost immune responses; 240 mice (months 13-34.)
  1. As above, the ELVIS2-neu plasmid has proven to be so powerful that I do not feel it is necessary to attempt to boost the immune response.

- Challenge mice described above with the neu+ A2L2 cells and control 66.3 cells by injection in the mammary fatpad or i.v. Determine tumor growth rate, survival and level of pulmonary metastasis in vaccinated and control mice (months 6-30).
  1. The results for this experiment are shown in Figure 4 (page 264) and Figure 5 (page 265) and text pages 264-266.

- Analyze results, prepare interim reports/publications (as appropriate).
  1. The enclosed publication (11) is our first report resulting from the research supported by this grant.

Task 2: To demonstrate that vaccination after tumor induction can reduce metastases and prolong survival (therapeutic vaccination) (months 18 - 36).

- Treat mice with different doses of A2L2 and 66.3 cells injected into mammary fatpad. Remove primary tumor and vaccinate at different times with ELVIS-neu and ELVIS. Score for incidence and extent of metastatic disease in different treatment groups; measure increased survival time in treated groups; 360 mice (month 18 - 36).
  1. We performed two therapeutic vaccinations in which a primary breast tumor of A2L2 cells was surgically removed on day 22 and the mice were vaccinated on days 25, 33 and 39 with ELVIS-neu (surgery model). The results from these experiments showed that therapeutic vaccination was not effective. The vaccine was not powerful enough to prolong the lives of the mice. We repeated this experiment twice using ELVIS2-neu rather than ELVIS-neu. Our conclusion is that neither plasmid, ELVIS-neu or ELVIS2-neu is sufficiently strong to rescue mice that have had a primary tumor surgically removed. We also tested both ELVIS-neu and ELVIS2-neu in a second therapeutic model in which the vaccine is given to mice with an actively growing tumor (race model) As with the surgery model, vaccination was ineffective at prolonging the life of the mice. We are now testing whether ELVIS-neu and ELVIS2-neu will be more potent in combination with chemotherapy. We are also currently testing if we
can use fewer cells in the race model in order to give the vaccination more of a chance to be effective before the tumor becomes overwhelming.

2. Figure 4b (page 264) clearly demonstrated that the immunity induced by vaccination with ELVIS-neu had no effect on the growth of 66.3 cells. Also, vaccination using ELVIS2-neu in mice challenged with 66.3 cells showed no effect. In a related experiment, mice that survived challenge with A2L2 following vaccinated with ELVIS-neu or ELVIS2-neu were challenged with 66.3 cells to determine if “cross priming” may have occurred to a “tumor antigen” present on 66.3 cells. All of the surviving mice died following challenge with 66.3 cells, indicating a lack of “cross priming” to the parental tumor.

KEY RESEARCH ACCOMPLISHMENTS:

- Vaccination of mice with increasing amounts of ELVIS-neu induces a proportionately greater level of humoral immunity.
- Vaccination of mice with ELVIS-neu results in the generation of neu-specific T cells in the spleen that release large amounts of IFN-γ in response to restimulation in vitro with neu-expressing cells.
- Vaccination with ELVIS-neu induces significant levels of anti-tumor immunity to breast cancer cells expressing neu. This is demonstrated by increased survival in vaccinated mice challenged in the mammary fat pad with a neu+ breast cancer cell line.
- The immunity to neu is antigen-specific and does not provide protection against a breast cancer cell line that does not express neu.
- Vaccination with ELVIS-neu also provides protection to challenge when tumor cells are injected i.v., thus demonstrating reduced lung metastasis as a result of vaccination.
- Neither ELVIS-neu nor ELVIS2-neu were sufficiently powerful to provide protection when mice were vaccinated after surgical removal of a primary breast tumor (surgery model). Also, neither ELVIS-neu nor ELVIS2-neu could protect mice when an active tumor was growing in the breast (race model).

REPORTABLE OUTCOMES:

1. One research report has been published (11).
2. This work has been presented at three scientific meetings:
   1) “DNA Vaccines”, Keystone Symposia, Snowbird, UT, April, 1999 by Lawrence B. Lachman, Ph.D.
   2) American Association of Cancer Research, annual meeting in New Orleans, LA, April, 2000 by Janet Price, D.Phil.
3. The 66.3-neo and A2L2 cell lines were prepared before this application was funded. However, P815-neu cells were prepared for the IFN-γ release assay and these are valuable cells to have for future experimentation.

4. We have applied for a 2003 Department of Defense Breast Cancer Research Program Idea Award to perform combined vaccine therapy and chemotherapy in the therapeutic vaccine models.

CONCLUSIONS:

Our findings supported our hypothesis that vaccination with ELVIS-neu could induce cellular and humoral immunity able to increase survival and protect mice from challenge with a breast cancer cell line expressing neu. Unfortunately, this vaccine was not sufficiently powerful to protect mice from an actively growing tumor or from a surgically removed primary tumor that had already seeded metastases. Our work has clinical importance at this point. However, we believe that increased effectiveness of the vaccine by combination with chemotherapy would be much stronger justification for a clinical trial.

REFERENCES:


CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. *Vaccine* 19:764-778.


**APPENDIX**

Reprint

DNA vaccination against neu reduces breast cancer incidence and metastasis in mice

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The gene for HER2/neu is overexpressed in 30–40% of breast and ovarian cancers, and this overexpression correlates with increased metastasis and poor prognosis. The HER2/neu gene product, a transmembrane protein kinase member of the EGF receptor family, has significant potential as a tumor antigen for vaccination. We inserted the sequence for neu into a novel plasmid called ELVIS containing a Sindbis virus replicon that reproduces multiple copies of mRNA. Mice vaccinated one time intramuscularly demonstrated a strong antibody response against A2L2, a murine breast cancer cell line transfected to express neu. Vaccinated mice challenged in the mammary fatpad with A2L2 had reduced tumor incidence and reduced tumor mass compared to mice challenged with tumor cells lacking the neu insert. Intradermal vaccination was also protective and required 80% less plasmid for a similar level of protection. Vaccination reduced the incidence of lung metastasis from mammary fatpad tumors and reduced the number of lung metastases resulting from intravenous injection of A2L2 cells. Cytotoxic T lymphocytes cultures of immune spleen cells with P815-neu cells produced high levels of interferon-γ indicating an antigen-specific Th1-type immune response resulting from the vaccination. In a spontaneous breast tumor model using neu transgenic mice, vaccination with ELVIS-neu protected against development of spontaneous breast tumors. Our preclinical data indicate that therapeutic vaccination of patients with ELVIS-neu may reduce metastasis from HER2/neu-expressing breast and ovarian tumors. Cancer Gene Therapy (2001) 8, 259–268

Key words: Antitumor; erbB-2; gene vaccine.
indicating that ELVIS-neu induced protective immunity against neu-expressing breast tumors and thus broke tolerance to neu. We anticipate testing ELVIS-neu in a phase 1b clinical trial with the goal of breaking tolerance to HER2/neu by vaccinating with the gene for neu a related, but xenoantigenic, form of HER2/neu.

MATERIALS AND METHODS

Cell lines and culture conditions

The mouse mammary tumor cell line designated 66.3 was obtained from Dr. F. R. Miller (Karmanos Institute, Detroit, MI). This variant of a mammary tumor in a BALB/c mouse is tumorigenic and metastatic in syngeneic mice.11 The cell line was free of Mycoplasma and of the following murine viruses: reovirus type 3, pneumonia virus, K virus, Thielers encephalitis virus, Sendai virus, minute virus, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by M.A. Bioproducts, Walkersville, MD). The cells were maintained in monolayer culture in Eagle's minimum essential medium supplemented with 5% fetal calf serum, sodium pyruvate, nonessential amino acids, l-glutamine, and vitamins (GIBCO-BRL, Grand Island, NY) incubated in a humidified 5% CO2–95% air incubator at 37°C. P815 mastocytoma cells were purchased from American Type Culture Collection (TIB-64, Manassas, VA) and cultured in suspension in same medium as the 66.3 cells described above.

Transfection to generate neu-expressing cells

Samples of 1 × 10⁵ 66.3 mammary tumor cells were plated in 35-mm culture dishes and 24 hours later, when 50–60% confluence had been achieved, the cells were transfected using Lipofectin (GIBCO-BRL). The culture medium was removed and a mixture of Lipofectin (20 μL) and plasmid DNA in 2 mL of serum-free culture medium was prepared following the manufacturer’s recommended procedure. The cells were incubated with either 5 μg of pSV2-neo or 4.5 μg of pSV2-neu plus 0.5 μg of pSV2-neo (plasmids provided by Dr. M. -C. Hung, University of Texas M. D. Anderson Cancer Center). After 24-hour incubation, the plasmid–Lipofectin mixture was aspirated and replaced with culture medium containing 5% fetal bovine serum. After 2 days in culture, the cells were replated in 100-mm diameter plates, and 400 μg/mL G418 was added to the medium. G418-resistant colonies were collected after 12–15 days growth, and the new clones expanded in culture. The transfected cells were maintained in culture in the presence of 400 μg/mL G418, a concentration that killed all nontransfected 66.3 cells. P815 cells were transfected using the same plasmids. Aliquots of 2 × 10⁴ cells were plated in 35-mm tissue culture plates and incubated for 24 hours with a mixture of plasmid DNA and Fugene (6 μL; Boehringer Mannheim, Indianapolis, IN). The cells from each plate were recovered and divided between individual wells of 24-well culture plates in 1 mL of medium containing 600 μg/mL G418. Wells with G418-resistant cells were identified after 10 days of culture, and these cells expanded in the culture.

Tumor cell injections

Tumor cells were harvested from subconfluent cultures by incubation with 0.25% trypsin and 0.02% EDTA solution for 1 minute at 37°C. The cells were dislodged from the culture flasks, washed in medium, centrifuged, and resuspended in phosphate-buffered saline (PBS). Female BALB/c mice were obtained from the Frederick Cancer Research Facility (Frederick, MD). To determine the experimental metastatic potential, tumor cell suspensions were injected into the lateral tail vein. Twenty-one days after i.v. injection, the mice were killed and the numbers of tumor colonies in the lungs recorded. To assess local tumor growth, cells were injected into the mammary fatpad. The mice were anesthetized by Metofane inhalation, the fur shaved over the lateral thorax, and a 5-mm skin incision made to reveal a mammary fatpad. The cells were injected in a volume of 0.1 mL into the fatty tissue, and the incision closed with a wound clip. Tumor growth was monitored by twice-weekly measurements using calipers.

Detection of p185 by immunoblotting

Protein extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 7.5% acrylamide) and transferred to Hybond nitrocellulose filters (Amersham, Arlington Heights, IL). The filters were incubated for 16 hours at 4°C with 5% powdered milk in TBST (150 mM NaCl, 10 mM Tris–HCl, 1% Triton X-100, pH 8.0), washed in TBST, and then incubated for 1 hour at room temperature with a 1:1000 dilution of a polyclonal antibody to p185 (sc-284; Santa Cruz Biotechnology, Santa Cruz, CA) in TBST with 1% bovine serum albumin (BSA). The filters were washed in TBST, and then incubated for 1 hour with donkey antirabbit–horseradish peroxidase–conjugated antibody (Amersham) diluted 1:1000 in TBST with 1% BSA. The filters were washed and developed with ECL detection reagents and exposed to Hyperfilm (Amersham). Equal loading and transfer of proteins were confirmed by stripping the filters and reprobing with an antibody to actin (Sigma, St. Louis, MO) in TBST with 2.5% BSA.

Immunoprecipitation

Lysates of mammary tumor cells that were surface-labeled with NHSLC-biotin (sulfosuccinimidyl-6-(biotinamido)-hexanoate; Pierce, Rockford, IL) were prepared as previously described.12 Aliquots of 100 μg of protein from 66.3 and A2L2 (p185-expressing) were precleared by incubation with mouse IgG and 30 μL of protein A/protein G agarose (Oncogene Research Products, Cambridge, MA) for 1 hour. The precleared lysates were incubated for 16 hours with either normal mouse IgG, monoclonal antibody to p185 (Ab-3, Oncogene Research Products), IgG prepared using protein A agarose (BioRad, Hercules, CA) from sera collected from ELVIS-vaccinated mice, or IgG prepared from ELVIS-neu–vaccinated mice. Immune complexes were immunoprecipitated by 2-hour incubation at 4°C with protein A/protein G agarose (BioRad). The bound complexes were washed and separated on 7.5% SDS-PAGE gels under reducing conditions and transferred to nitrocellulose.
filters. Biotin-labeled proteins were detected by incubating the filters with horseradish peroxidase-linked streptavidin (Pierce, St. Louis, MO) and ECL detection reagents (Amersham, Piscataway, NJ).

**Plasmids used for vaccination**

The ELVIS plasmid was obtained from John Polo (Chiron Technologies, San Diego, CA) and has been previously described in detail. Rat neu sequence was excised from pSV2-neu (described above) and inserted into ELVIS by standard techniques. The correct insertion of the complete gene was confirmed by sequence analysis.

**Vaccination of mice**

Intramuscular (i.m.) injections of 0.1 mL were administered to the quadriceps with a 24-gauge needle. Intradermal (i.d.) injections of 0.20 mL were administered on the shaved back also using a 24-gauge needle. The i.m. injections contained 100 μg of plasmid DNA and were formulated with 0.25% bupivacaine (Sigma). The i.d. injections were similarly formulated, but contained only 20 μg of DNA. At indicated times, blood was collected from the tail vein and serum separated by centrifugation after incubation at 37°C for 1 hour.

**Flow cytometry**

Either A2L2 or 66.3 cells were mixed with immune serum diluted 1:100 in PBS and incubated for 1 hour at 37°C. Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (PharMingen, San Diego, CA) diluted 1:1000 in PBS was added to the cell suspension, and the mixture incubated for 1 hour at 37°C. The cells were washed by centrifugation three times in PBS and then analyzed by flow cytometry using an EPICS Profile Analyzer (Coulter, Hialeah, FL).

**CTL cultures and cytokine release assays**

Spleens from mice vaccinated one time i.m. with either 100 μg of ELVIS-neu or 100 μg of ELVIS were harvested 9 weeks postvaccination. The spleens were homogenized by forcing through a stainless steel mesh using a syringe plunger into a Petri dish containing 10 mL of RS10 media (RPMI 1640, 10% heat-inactivated fetal calf serum, 1% nonessential amino acids, 100 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM 2-mercaptoethanol). Debris was removed from the cell suspension by filtering through nylon mesh into a 10-mL centrifuge tube. The cell suspension was centrifuged for 10 minutes at 700×g and the pellet was resuspended in 5 mL of AKC solution (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM NaEDTA, pH 7.4) for 5 minutes with gentle rocking to lyse the red blood cells. An additional 5 mL of RS10 media was added to the cell suspension and the cells were washed one time with RS10 media. P815-neu and P815 parental cells were γ-irradiated (37 Gy for 1.25 minutes) and 2×10⁵ spleen cells were cultured with either 1×10⁵ irradiated P815-neu or P815 cells in 10 mL of RPMI 1640 in six-well plates for 5 days at 37°C. Control cultures of only spleen and only P815 or P815-neu cells were cultured under identical conditions. Supernatants from the mixed leukocyte-tumor and the control cultures were collected following centrifugation and assayed for the presence of IL-4 and INF-γ using the mouse IL-4 and mouse INF-γ OptEIA sets from Pharmingen.

**Vaccination of MMTV-c-neu transgenic mice**

Female MMTV-c-neu transgenic mice were purchased from Charles Rivers Laboratories (Wilmington, MA). Groups of 10 seven-week-old mice were injected i.m. with 100 μg of either ELVIS-neu or control ELVIS plasmid DNA, prepared as described above. Each mouse received three injections at 2-week intervals. The date of detection of palpable mammary tumors on each mouse was recorded.

**RESULTS**

**Transfection of 66.3 mammary tumor cells with pSV2-neu**

The 66.3 mammary tumor cells did not express elevated levels of p185 (Fig 1). To create a tumor target with high expression of this potential tumor antigen, we transfected 66.3 cells with pSV2-neu and pSV2-neo. After cell lines were established in culture, the cells were injected i.v. into Balb/c mice and individual lung metastases were established as continuous cell lines. Protein lysates prepared from these cell lines or supernatants were analyzed by Western blotting. The blot was incubated with anti-p185 antibodies and horseradish peroxidase-linked secondary antibodies. The bands were visualized using chemiluminescence reagents. The results showed that the 66.3-neo cell line expressed higher levels of p185 than the 66.3 line.

**Figure 1.** Immunoblot analysis of p185 expression in 66.3 mammary tumor cells transfected with pSV2-neu. A protein lysate of the human ovarian cancer cell line SKOV3ip1 was used as a positive control, expressing abundant 185-kDa protein. The 66.3 and 66.3-neo cells expressed minimal amounts of p185. The 10 lysates prepared from lung metastases in mice injected with 66.3 cells transfected with pSV2-neu showed variable levels of p185 expression. The cell line designated A2L2 was used for the subsequent tumor challenge experiments.
in vivo-selected clones were analyzed by immunoblotting to detect p185 expression. Several clones showed significantly more p185 expression than the 66.3 parental cells or the control transfected cells (66.3-neo) (Fig 1). When the neu-transfected cells were injected into BALB/c mice, either i.v. or into the mammary fatpad, the rates of tumor take, tumor growth, and numbers of lung metastases that developed were indistinguishable from the parental 66.3 mammary tumor cells or the control 66.3-neo cells (data not shown). Thus, transfection with pSV2-neu did not alter the tumorigenic or metastatic potential of these cells.

Not all of the lung metastasis-derived lines retained high expression of p185 (Fig 1), suggesting that the injected cells were a heterogeneous mixture of p185-positive and negative cells, or alternatively, that in the absence of G418 selection pressure in vivo, the inserted neu gene was eliminated from the mammary tumor cells. Loss of p185 and/or neomycin resistance was noted in 24–36% of the metastasis-derived clones. The A2L2 clone was selected for the tumor challenge experiments because it retained a high level of p185 expression (Fig 1). The level of p185 expression by the A2L2 cell line has remained consistently

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**Figure 2.** Flow cytometric analysis of A2L2 cells with pooled antisera resulting from vaccination of mice with increasing amounts of ELVIS-neu or ELVIS. Mice were vaccinated one time i.m. with 1, 10, 25, or 50 µg of ELVIS-neu (panels a–d). Control mice were vaccinated once with 50 µg of ELVIS (panel e). Panel f shows the increase in antibody response resulting from vaccination of mice three times with 100 µg of ELVIS-neu. Panel g shows the lack of immunofluorescence with A2L2 cells in the absence of immune sera, and panel h shows the lack of immunofluorescence when the antisera in panel A were tested using 66.3-neo cells.

_Cancer Gene Therapy, Vol 8, No 4, 2001_
high relative to the control 66.3-neo cells for more than 1.5 years as measured by immunoblotting and flow cytometry. Immunohistochemistry of lung metastases in mice injected with A2L2 cells revealed p185 expression in the tumor cells (data not shown). Thus, the tumor challenge target cell line, A2L2, was shown to express p185 in vitro and in vivo and to be tumorigenic and metastatic in syngeneic mice.

Induction of antibody to A2L2 cells by vaccination with ELVIS-neu

Groups of five Balb/c mice were vaccinated one time i.m. with increasing doses of ELVIS-neu (1–50 μg), and 2 weeks later, the presence of IgG in the pooled sera was evaluated by flow cytometry using A2L2 cells. As shown in Figure 2a–d, increasing the vaccination dose of ELVIS-neu increased immunofluorescence. Sera obtained by vaccination with a single dose of the backbone ELVIS plasmid lacking the neu insert did not result in positive staining of the A2L2 cells (Fig 2e). Immune sera obtained from mice vaccinated three times i.m. at 2-week intervals had the highest level of immunofluorescence (Fig 2f).

There was no staining in the absence of immune serum (Fig 2g), and the pooled antisera from panel A did not stain 66.3-neo cells (Fig 2h), demonstrating that the immunofluorescence was specific for A2L2 cells and did not detect an epitope also expressed on 66.3-neo cells. These results demonstrated that ELVIS-neu was able to induce a humoral immune response specific for an expressed antigen of neu.

Immunoprecipitation of p185 from A2L2 cells with immune sera

When the protein product of neu, p185, was expressed in the cytosol of A2L2 cells, part of the intact molecule could be directed to the plasma membrane for presentation as a receptor and another part processed for presentation on Class I molecules. We performed immunoprecipitation on surface-radiolabeled A2L2 and 66.3 cells with a commercial monoclonal Ab to p185 and with immune sera described above. As shown in Figure 3, the monoclonal Neu-Ab3 precipitated p185 from A2L2 cells, whereas control IgG did not. Purified IgG from mice injected three times with 100 μg of ELVIS-neu also immunoprecipitated a band at 185 kDa from the A2L2 cells, but not from the 66.3-neo cells. Immune sera from mice injected three times with 100 μg of the backbone plasmid ELVIS did not precipitate an equivalent band in either 66.3-neo or A2L2 cells. These results demonstrate expression of the neu transgene in A2L2 cells with transport of some of the translated p185 proteins to the plasma membrane. In addition, the immunoprecipitation of p185 with the antisera resulting from vaccination with ELVIS-neu demonstrated an immune response to p185, the transgene product.

Protection from tumor induction following i.m. vaccination with ELVIS-neu

Groups of five mice were vaccinated one time i.m. with 100 μg of either ELVIS-neu, ELVIS, or PBS and challenged 14 days later by injection of 1.0×10⁴ A2L2 or 66.3-neo cells into the mammary fatpad. The mice were monitored daily for palpable tumors and the tumors were measured with calipers when sufficiently large. As shown in Figure 4a, when challenged with A2L2 cells, the mice vaccinated with a single injection of 100 μg of ELVIS-neu had a 60% tumor incidence compared to 100% for mice injected with ELVIS and 80% for mice injected with PBS. In contrast, the same vaccinations offered no protection from tumor incidence when mice were challenged with 66.3-neo (Fig 4b). There was no significant difference in the size of tumors in any
group. These results indicated that vaccination with ELVIS-neu resulted in some protection from tumor challenge with A2L2, but no protection against 66.3-neo cells.

Based on these results, a subsequent experiment was performed using groups of 10 mice each, and the vaccination regimen was increased to three i.m. injections of 100 μg given at 14-day intervals. The mice were challenged as above, with 1.0×10⁴ A2L2 cells 14 days after the third vaccination. As shown in Figure 4c, only 20% of mice vaccinated with ELVIS-neu developed tumors compared to 80% in mice vaccinated with ELVIS and 100% for PBS. This significant difference (P<0.0092) in tumor incidence clearly demonstrated that vaccination with an expression plasmid containing the cDNA for neu was protective against challenge with a neu-transfected tumor compared to challenge with the parental tumor not expressing neu. ELVIS-neu injection provided 20% protection compared to PBS, a result commonly referred to as a plasmid effect.

Protection from tumor induction following i.d. vaccination with ELVIS-neu

To determine if we could verify the protective effect of the ELVIS-neu vaccine using a route other than i.m., we vaccinated groups of 10 mice i.d. with ELVIS-neu and
Table 1. Comparison of i.m. and i.d. Injection Route

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>Injection route</th>
<th>Tumor incidence</th>
<th>Mean tumor weight (g) ± SEM</th>
<th>Metastasis incidence</th>
<th>Median number of metastases (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELVIS-neu</td>
<td>i.m.</td>
<td>9/10</td>
<td>0.62 ± 0.15t</td>
<td>4/10</td>
<td>0 (0–5)</td>
</tr>
<tr>
<td>ELVIS</td>
<td>i.m.</td>
<td>10/10</td>
<td>1.10 ± 0.12</td>
<td>9/10</td>
<td>3 (0–6)</td>
</tr>
<tr>
<td>ELVIS-neu</td>
<td>i.d.</td>
<td>8/10</td>
<td>0.44 ± 0.14t</td>
<td>4/10</td>
<td>0 (0–4)</td>
</tr>
<tr>
<td>ELVIS</td>
<td>i.m.</td>
<td>10/10</td>
<td>1.40 ± 0.34</td>
<td>7/10</td>
<td>1.5 (0–3)</td>
</tr>
</tbody>
</table>

*Groups of 10 mice were vaccinated three times at 2-week intervals with 20 µg of either ELVIS-neu or ELVIS. Two weeks following the final vaccination, 1 x 10⁶ A2L2 cells were injected into the mammary fatpad and 36 days later, the mice were killed.

tP = .0365 ELVIS-neu i.m. versus ELVIS i.m.

tP = .0193 ELVIS-neu i.d. versus ELVIS i.d.

ELVIS. Because it has been frequently demonstrated that i.d. vaccination requires lower doses of plasmid than i.m. vaccination, we reduced the dose to 20 µg of plasmid and vaccinated three times, compared to the previous i.m. experiment using 100 µg of plasmid and three vaccinations. As shown in Figure 4d, the tumor incidence for A2L2 was 60% for ELVIS-neu-vaccinated mice compared to 100% for mice vaccinated with ELVIS. When the mice were sacrificed 35 days after tumor challenge, the mean tumor weight for the six mice that developed tumors following ELVIS-neu injection was significantly lower than the mean tumor mass for the 10 mice vaccinated with ELVIS. Thus, vaccination with ELVIS-neu reduced the incidence of tumor induction and lowered the mass of tumors that did develop. Similar results were obtained when this exact experiment was repeated using three, rather than one, vaccination (Table 1). In addition to a decrease in the mean weight of the tumors that did develop, 0.44 g for ELVIS-neu–vaccinated versus 1.40 g for ELVIS-vaccinated, the incidence of metastasis was lower in the ELVIS-neu–vaccinated group. Serum samples from the vaccinated mice were analyzed by flow cytometry exactly as described in Figure 2. Mice vaccinated with ELVIS-neu by either i.m. or i.d. had an equivalent antibody response to A2L2 cells that was not present in mice vaccinated with ELVIS (data not shown because identical to Figure 2).

**Protection from experimental metastasis following i.m. vaccination with ELVIS-neu**

In addition to vaccination’s inhibition of primary-site tumor induction, we wanted to determine if vaccination could reduce metastasis to the lungs. The A2L2 cells can metastasize from the mammary fatpad tumors, but because the time period for this to occur varied between experiments, there was no way to determine the level of metastasis prior to sacrificing the mice. An alternative model, referred to as experimental metastasis, is to inject A2L2 cells i.v. and measure the number of lung metastases at a time previously determined for A2L2 cells. Groups of 10 mice vaccinated three times i.m. with 100 µg of ELVIS, ELVIS-neu, or PBS were injected i.v. with 1 x 10⁶ A2L2 cells 14 days following the last vaccination. On day 21, the mice were sacrificed and the lung metastases counted by eye. As shown in Figure 5, 3
of 10 mice vaccinated with ELVIS-neu did not have lung metastases compared to 1 of 10 for PBS-injected mice and 0 of 10 for ELVIS-injected mice. In addition, the number of metastases in the ELVIS-neu–vaccinated mice was significantly lower than in the ELVIS-vaccinated mice. In a repeat of this experiment we injected 2.5×10⁴ A2L2 cells, resulting in greater lung tumor burden, but even so there were significantly fewer metastases in the ELVIS-neu–vaccinated mice.

**CTL cultures and cytokine release assays**

Spleen cells from mice vaccinated once i.m. 9 weeks earlier with either 100 µg of ELVIS-neu or ELVIS were cultured with irradiated P815-neu or P815 cells for 5 days and the amount of INF-γ and IL-4 in the supernatant was determined. As shown in Figure 6, immune spleen cells co-cultured with P815-neu cell released an average of 1700 pg of INF-γ, whereas the same spleen cells co-cultured with P815 cells not expressing neu released background levels of INF-γ. There was negligible IL-4 production from either co-culture condition. These results clearly demonstrate that vaccination has induced a Th1 response and that the response is antigen-specific for p185, the gene product of neu, because co-culture with P815 cells did not stimulate the release of INF-γ.

**Vaccination of MMTV-c-neu transgenic mice**

Because the neu gene is of rat origin, the possibility exists that mice could recognize the gene product as a xenoantigen. Fortunately, a rat neu transgenic mouse is available in which neu gene expression is under the control of an MMTV promoter and neu breast tumors spontaneously develop at about 25 weeks of age. Two groups of ten FVB/N mice 8 weeks of age were vaccinated three times at 2-week intervals with 100 µg of ELVIS-neu or ELVIS. The mice received no further treatment. As shown in Figure 7, at 150 days after the first vaccination, palpable breast tumors developed in 9 of 10 mice vaccinated with ELVIS and only 6 of 10 mice vaccinated with ELVIS-neu. The onset of first breast tumors was delayed by approximately 40 days in the ELVIS-neu–vaccinated mice.

**DISCUSSION**

Nucleic acid immunization was introduced by Wolff et al, who showed that injection of plasmid DNA into skeletal muscle led to protein expression. Subsequent studies showed that plasmid DNA injection could evoke long-lasting cellular and humoral responses against the products of the injected genes. Chen et al demonstrated that vaccination of mice with plasmids expressing either full-length neu or its extracellular domain induced substantial protective immunity against challenge with a neu-expressing mouse mammary tumor. Amici et al, using neu transgenic mice, demonstrated that genetic vaccination with neu reduced the outgrowth of mammary tumors. In this report, we are also vaccinating with neu; however, we are using an expression plasmid containing a Sindbis virus replicon. The expression plasmid ELVIS utilizes a self-replicating virus vector RNA for gene expression. ELVIS is the first part of a two-part vaccine strategy. In the first part, ELVIS is used to induce a primary immune response and in the second part, VPRs are used to boost the immune response. VPRs have been demonstrated to induce protective immunity against several infectious diseases.

ELVIS, a strong expression vector, is reported to induce antibody and CTL to the encoded protein when injected i.m. into mice at a 100- to 1000-fold lower doses than would be required for a comparable response using constructs of conventional expression vectors. We found that a single i.m. injection with 1 µg of ELVIS-neu induced an antibody response to A2L2 cells expressing p185, a 50-µg injection increased the Ab response, and three 100-µg injections increased it still further (Fig 2). The humoral immune response resulting from ELVIS-neu vaccination appeared to be specific for cells transfected with neu because there was no reaction with 66.3 cells that do not express p185, the protein product of the neu gene (Fig 2). In addition, immunoprecipitation using purified IgG from mice vaccinated with ELVIS-neu demonstrated specificity for p185 (Fig 3).

A single i.m. vaccination with 100 µg of ELVIS-neu protected mice from developing tumors when challenged in the mammary fatpad with the A2L2, and this effect was specific for the A2L2 cells because it did not occur in 66.3-neo cells (Fig 4). Increasing the number of vaccinations with ELVIS-neu from one to three greatly improved this result with only 2 of 10 mice developing tumor compared to 8 of 10 vaccinated with ELVIS (Fig 4). The effective route of vaccination was not restricted to i.m., because i.d. vaccination with ELVIS-neu reduced tumor incidence in 6 of 10 mice (Fig 4) and 8 of 10 (Table 1) compared with 10 of 10 in mice given ELVIS vaccination. Furthermore, i.d. injection required only 20 µg of plasmid for each injection compared with the 100 µg required for i.m. vaccination. We did not test fewer than three i.d. injections. An interesting effect from i.d. injections that we did not observe with i.m. injections was reduced tumor mass in mice vaccinated with ELVIS-neu compared to ELVIS (Fig 4 and Table 1). Thus, even though a tumor developed, it is possible that the growth may be retarded by an immune mechanism.
It has been reported that ELVIS can induce CTL at very low doses\textsuperscript{15} and to confirm this, we prepared CTL cultures of immune spleen cells and P815 cells transfected with neu.\textsuperscript{7,15} As shown in Figure 6, spleen cells from mice vaccinated with ELVIS-neu and co-cultured with P815-neu cells produced high levels of INF-\(\gamma\) and negligible IL-4. Cultures of immune spleen cells with control P815 cells lacking neu did not secrete either cytokine. Other control cultures of ELVIS-vaccinated spleen cells with either P815 or P815-neu also produced negligible levels of INF-\(\gamma\) and IL-4. These results confirm that vaccination with ELVIS-neu is inducing a Th1 response that is specific for gene product of neu and indicates that T-cell immunity is the most likely explanation for reduced tumor incidence and reduced tumor mass in ELVIS-neu-vaccinated mice.

Vaccination with ELVIS-neu protected 3 of 10 mice from developing lung metastases following i.v. injection of A2L2 cells and reduced the mean number of lung metastases that did develop compared to mice injected with ELVIS (Fig 5). The i.v. injection of tumor cells is an experimental model of tumor metastasis in which tumor cells arrest and grow predominantly in the lungs, which is the first capillary bed they encounter. Reduction of lung metastases in this model by vaccination with ELVIS-neu indicates that metastasis from a primary breast tumor can be reduced. It is possible that a more robust immune response induced by prime-boost vaccination with the combination of ELVIS-neu followed by VPR-neu could reduce or even eliminate metastasis from a primary tumor. Vaccination with ELVIS-neu was also able to reduce spontaneous metastasis resulting from a tumor in the mammary fatpad. As shown in Table 1, both i.m. and i.d. vaccinations with ELVIS-neu resulted in lower incidence of lung metastasis from a primary tumor. Leitner et al\textsuperscript{17} have also shown reduced metastasis from a primary tumor following vaccination with a replicon containing DNA vaccine.

The neu gene that we have used for transfection of the 66.3 and P815 cells and for the creation of ELVIS-neu is of rat origin. Thus, a possibility exists that mice vaccinated with neu could be responding to p185 as a xenoantigen, even though the primary sequence of neu is highly conserved between rat and mice. A way to eliminate the xenoantigen possibility is to use neu transgenic mice that are tolerant to neu. In FVB/N mice, expression of the neu transgene is regulated by the mouse mammary tumor virus promoter and the mice spontaneously develop breast tumors at about 25 weeks of age.\textsuperscript{14} The neu transgene used to develop this strain of mice is "activated" neu which contains a single amino acid substitution.\textsuperscript{14} Using FVB/N mice, we found that vaccination with ELVIS-neu significantly retarded the development of spontaneous breast tumors (Fig 7). In addition, at 150 days postvaccination, 4 of 10 mice vaccinated with ELVIS-neu had not developed tumors compared with 1 of 10 mice vaccinated with ELVIS. Therefore, vaccination with ELVIS-neu both delayed tumor development and, in some mice, eliminated tumor development. Thus, tolerance to neu is not absolute in FVB/N mice and can be broken by vaccination with ELVIS-neu.

Once we have established the prime-boost protocol for ELVIS-neu and VPR-neu, we plan to test therapeutic vaccination. In this model, primary A2L2 tumors in the mammary fat will be surgically removed after metastases have already been seeded in distant organs and then the mice will be vaccinated. This model of therapeutic vaccination is more representative of the clinical situation in which patients with resected neu-positive tumors relapse due to distant metastasis.\textsuperscript{1} Metastasis is the major cause of breast cancer deaths.\textsuperscript{1} Surgery can cure breast cancer, but not if metastasis has occurred before detection and local tumor therapy. A vaccine targeting HER2/neu could potentially target established metastases, or prevent new metastases from disseminating from other lesions. Although DNA vaccines utilizing ELVIS have not yet been tested in clinical trials, we believe that the current findings justify further research and development of this potent new strategy to target metastatic breast cancer. We anticipate vaccinating patients with ELVIS-neu containing the gene for rat neu based on our findings shown in this report that the gene product, p185, from a highly conserved but foreign species can be used successfully for vaccination and will break tolerance to the native gene product. We are hopeful that the immunity which develops in vaccinated patients will be directed at the rapidly dividing breast cancer cells that greatly overexpress p185 and not to normal cells that express very low levels of the same protein.

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REFERENCES


MCMR-RMI-S (70-ly) 15 May 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for Information Management