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13. ABSTRACT *(Maximum 200 Words)*

HER2/*neu* over-expression is found in 30-40% of breast cancer biopsy and is indicative of metastasis and poor prognosis. Although significant advances have been made in the treatment of breast cancer, once metastasis has occurred the possibility of a complete cure is unlikely. A vaccine targeting HER2/*neu* could have significant therapeutic and preventative application by controlling the growth and spread of highly aggressive HER2/neu^+ cells. Gene vaccines, bacterial expression plasmids encoding the DNA sequence for antigens, represent a new approach to inducing strong anti-tumor immunity. Although gene vaccines have shown effectiveness in clinical trials, it is essential to demonstrate pre-clinical effectiveness for anti-tumor 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. We also found that vaccination was able to reduce metastasis when the tumor was injected intravenously, a model of tumor metastasis.

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PI - Signature

6/27/00

Date

Table of Contents

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). Gene vaccines, bacterial expression plasmids encoding the DNA sequence for antigens, represent a new approach to inducing strong anti-tumor immunity (3). Although gene vaccines have shown effectiveness in clinical trials (4, 5), it is essential to demonstrate pre-clinical effectiveness for anti-tumor 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. 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 (6).

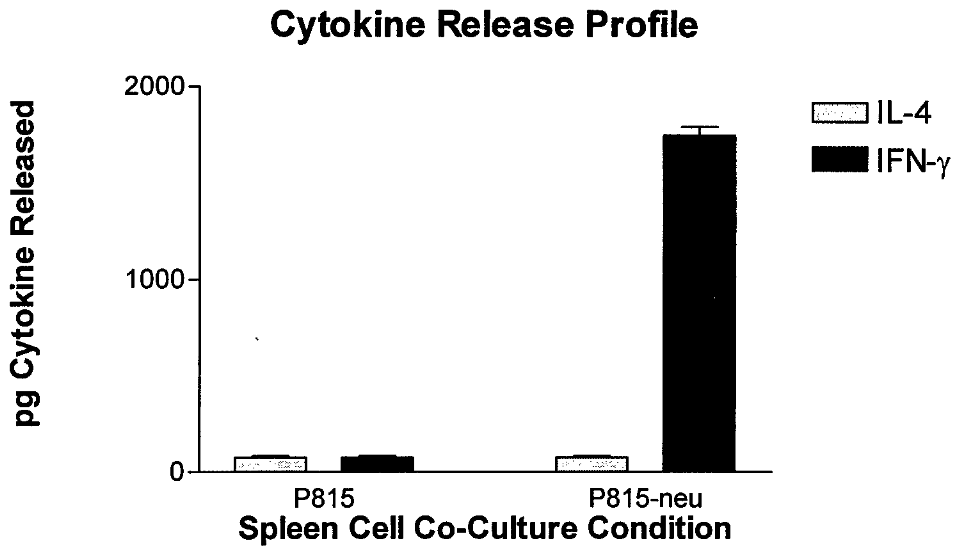
BODY:

The enclosed manuscript "DNA Vaccination Against HER2/*neu* Reduces Breast Cancer Incidence and Metastasis in Mice" 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 the manuscript in which the research is described.

Task 1: To demonstrate that mice vaccinated with ELVS-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 ELVS-neu, ELVS 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.26) and text page 11. We have immunized mice with increasing amounts of ELVS-*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 ELVS-*neu*. We did not evaluate results by ELISA since the flow cytometry was sufficient to conclude that a humoral immune response had been induced. We have not been able to perform a reproducible CTL assay. As an alternative to CTL assays we performed an Interferon gamma (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

ELVS-*neu* and not from control mice or mice vaccinated with only ELVS (see figure below). 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 ELVS-neu, ELVS 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 26) and text page 11.
    2. The result for the antitumor cellular response is shown in Figure 4a-c (page 28) and text page 12-14.
- Determine if co-administration of cytokine encoding plasmids increases immune responses, 120 mice (months 13-34).
    1. We have not performed this experiment yet.
- Determine whether liposomal-cytokine-peptide antigens can boost immune responses; 240 mice (months 13-34.)
    1. We have not performed this experiment yet.
- Challenge mice described above with 66.3-neo/*neu* and 66.3 neo 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 28) and Figure 5 (page 29) and text pages 13-15.
- Analyze results, prepare interim reports/publications (as appropriate).
    1. The enclosed manuscript is our first report on this subject.

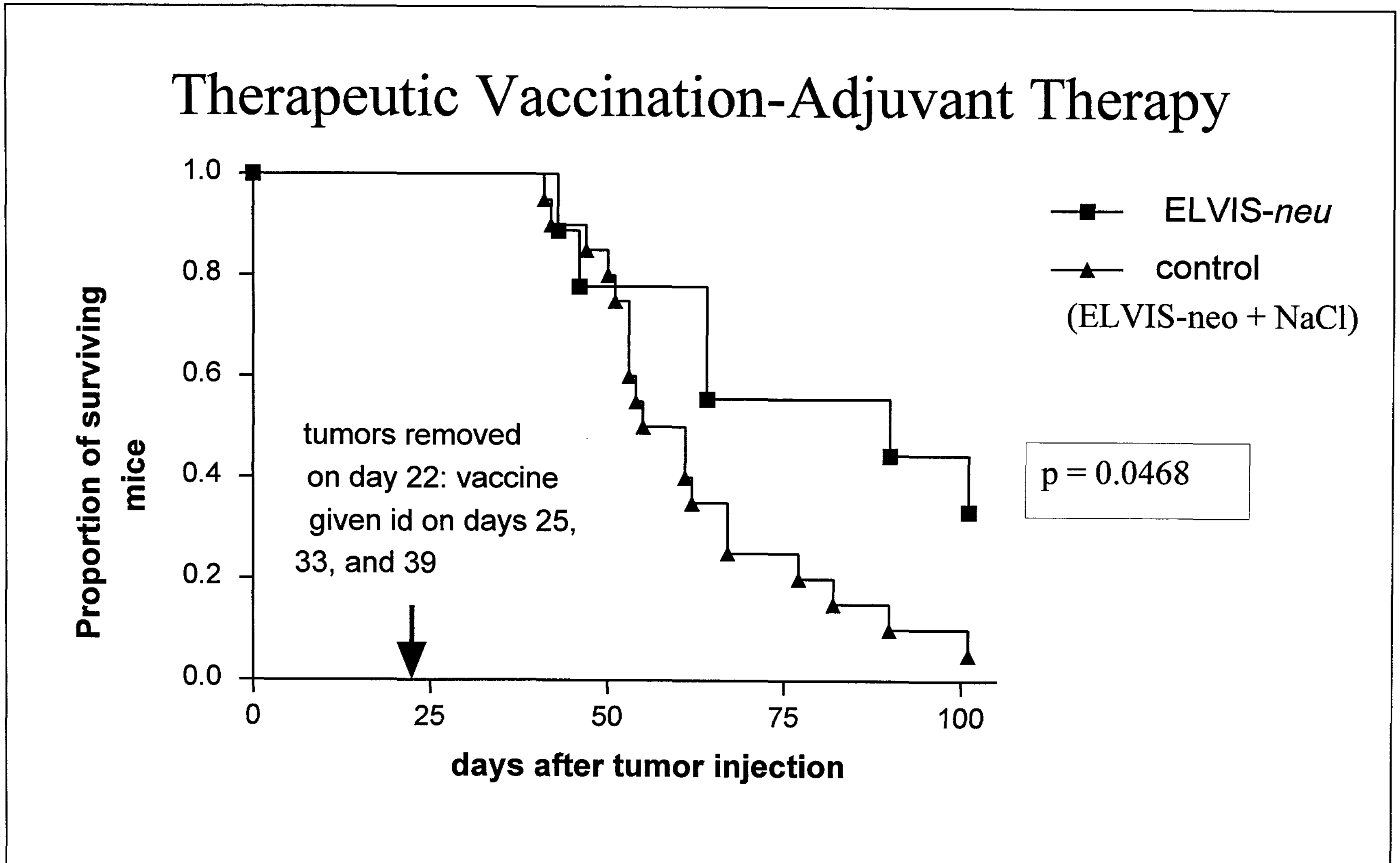
Task 2: To demonstrate that vaccination *after* tumor induction can reduce metastases and prolong survival (**therapeutic vaccination**). (months 18 - 36)

- Treat mice with different doses of 66.3-neo/neu and 66.3-neo cells injected into mammary fatpad. Remove primary tumor and vaccinate at different times with ELVS-neu and ELVS-control. 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 have performed one therapeutic vaccination (see figure on next page) in which mice a primary tumor of 66.3-neo/neu cells was removed on day 22 and the mice were vaccinated on days 25, 33 and 39 with ELVS-*neu*. The results indicate that therapeutic vaccination was able to increase the proportion of mice surviving for up to 100 days. The increase in survival was statistically significant (p=0.0468) if the data from the two control groups (ELVS-*neo*) and NaCl were pooled to increase the n value to 20 mice. This experiment is currently being repeated with 20 mice per group.
  2. Figure 4b (page 28) clearly demonstrated that the immunity induced by vaccination with ELVS-*neu* had no effect on the growth of 66.3-neo cells. In light of this result we see no reason to perform a therapeutic vaccination in mice challenged with 66.3-cells.

KEY RESEARCH ACCOMPLISHMENTS:

- Vaccination of mice with increasing amounts of ELVS-*neu* induces a proportionately greater level of humoral immunity.
- Vaccination of mice with ELVS-*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 ELVS-*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 ELVS-*neu* also provides protection to challenge when tumor cells are injected i.v., thus demonstrating reduced lung metastasis as a result of vaccination.
- Therapeutic vaccination, in which the mice were vaccinated after surgical removal of a primary breast tumor, resulted in increased survival compared to sham vaccinated mice.

Survival of Mice Vaccinated After Tumor Removal



REPORTABLE OUTCOMES:

1. One manuscript (enclosed) has been prepared.
2. This work has been presented at two 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 66.3-*neo/neu* 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 Department of Defense Breast Cancer Research Program Clinical Translational Research (CTR) award to perform a Phase I clinical trial of ELVS-*neu*. Our preliminary application has been selected for further consideration and we are preparing a full-length application for the August 2, 2000 deadline. The preliminary data from this application was used as justification for a clinical trial in stage IV breast cancer patients.

CONCLUSIONS:

To date, our work supports our hypothesis that vaccination with ELVS-*neu* can induce cellular and humoral immunity able to increase survival and in some instances, protect mice from challenge with a breast cancer cell line expressing *neu*. In our first therapeutic vaccine experiment there was increased survival of mice that had been vaccinated with ELVS-*neu* after surgical removal of a primary breast tumor. This work has clinical importance and we believe that sufficient pre-clinical efficacy of ELVS-*neu* has been demonstrated to justify a Phase I clinical trial in patients with stage IV breast cancer.

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APPENDICES:

The manuscript "DNA Vaccination Against HER2/*neu* Reduces Breast Cancer Incidence and Metastasis in Mice" is enclosed.

DNA Vaccination Against HER2/*neu* Reduces Breast Cancer Incidence and Metastasis

Short Title: DNA Vaccination Against HER2/*neu*

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ABSTRACT

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 HER2/*neu* into a novel plasmid called ELVIS that contains a Sindbis virus replicon that reproduces multiple copies of RNA. Mice vaccinated one time intramuscularly demonstrated a strong antibody response against A2L2, a murine breast cancer cell line transfected to express HER2/*neu*. Vaccinated mice challenged in the mammary fat pad with A2L2 had reduced tumor incidence and reduced tumor mass compared to mice challenged with tumor cells lacking the HER2/*neu* insert. Intradermal vaccination was also protective and required 80% less plasmid for a similar level of protection. Vaccination also reduced the incidence of lung metastasis and significantly reduced the numbers of lung metastases resulting from intravenous injection of A2L2 cells. Our data indicate that DNA vaccination against HER2/*neu* may reduce metastasis from breast and ovarian cancers.

INTRODUCTION

One third of women with breast cancer who have no detectable lymph node involvement at the time of diagnosis will eventually develop metastatic disease. Despite advances in clinical management, once breast cancer has metastasized the probability of a complete cure is greatly reduced (1). Vaccination against breast cancer could be used to attack active disease and as an adjunct to surgery to control the growth of metastases (2). One candidate for a vaccine target is the transmembrane protein p185 encoded by HER2/*neu*, since patients whose breast cancers express p185 have a poor prognosis (3). HER2/*neu*, a member of the tyrosine kinase receptor family, accelerates the growth and metastatic potential of breast cancer cells and reduces options for treatment. A vaccine that targets HER2/*neu*-expressing cells could have significant therapeutic and preventive application by controlling the growth and spread of the highly aggressive HER2/neu^+ cells (2). Indeed, Herceptin®, a recently approved therapeutic monoclonal antibody (4) has as its target HER2/*neu*.

Antitumor vaccines can be developed by immunizing vertebrates with bacterial expression plasmids that encode the DNA sequence for HER2/*neu* and other tumor antigens *(5)*. DNA vaccines have been shown to induce a strong, lasting immune response that includes the generation of cytotoxic T lymphocytes (CTL), the main mechanism for immunological control of tumor growth (5).

We report here that vaccination of mice with a novel plasmid expressing the DNA sequence for HER2/*neu* protected mice from challenge with a HER2/*neu*-expressing murine breast tumor cell line. The mice were protected from challenge with tumor cells injected intravenously and those injected directly into the mammary tissue.

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 that is tumorigenic and metastatic in syngeneic mice (6). The cell line was free of *Mycoplasma* and of the following murine viruses: reovirus type 3, pneumonia virus, K virus, Theiler's encaphalitis 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% FCS, sodium pyruvate, nonessential amino acids, L-glutamine, and vitamins (GIBCO-BRL, Grand Island, NY) incubated in a humidified 5% CO_2-95% air incubator at 37°C.

Transfection to generate *neu*-expressing cells. Samples of 1 x 10^5 66. 3 mammary tumor cells were plated in 35-mm culture dishes and twenty-fours later, when 50 to 60% confluence had been achieved, the cells were transfected using Lipofectin (GIBCO-BRL). The culture medium was removed 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 pSV2-*neo* or 4.5 μg pSV2-*neu* plus 0.5 μg pSV2-*neo* (plasmids provided by Dr. M.-C. Hung, University of Texas M. D. Anderson Cancer Center). After 24 h incubation, the plasmid-Lipofectin mixture was aspirated and replaced with culture medium containing 5% FBS. 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 non-transfected 66.3 cells.

Tumor cell injections. Tumor cells were harvested from subconfluent cultures by incubation with 0.25% trypsin and 0.02% EDTA solution for 1 min at 37° C. The cells were dislodged from the culture flasks, washed in medium, centrifuged, and resuspended in 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 HER2/*neu* 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 h 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 h 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%-BSA. The filters were washed in TBST, and then incubated for 1 h with donkey anti-rabbit-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 a 1:500 dilution of an antibody to actin (Sigma Chemical, St. Louis, MO) in TBST with 2.5% BSA.

Immunoprecipitation. Lysates of mammary tumor cell that were surface-labeled with NHSLC-biotin (sulfosuccinimidyl-6-(biotinamido)hexanoate (Pierce, Rockford, IL) were prepared as previously described (7). Aliquots of 100 μg of protein from 66.3-*neo* and 66.3-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 h. The precleared lysates were incubated for 16 h with either normal mouse IgG, monoclonal antibody to p185 (Ab-3, Oncogene Research Products), IgG prepared from sera collected from ELVIS-vaccinated mice, or IgG prepared from ELVIS-*neu* vaccinated mice. Immune complexes were immunoprecipitated by 2 h incubation at 4° C with protein A/protein G agarose. The bound complexes were washed and separated on 7.5% SDS-PGE gels under reducing conditions and transferred to nitrocellulose filters. Biotin-labeled proteins were detected by incubating the filters with horseradish peroxidase-linked streptavidin (Pierce) and ECL detection reagents.

Plasmids used for vaccination. The ELVIS plasmid was obtained from David Driver, Chiron Technologies, San Diego, CA, and has been previously described in detail (8). Rat HER2/*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. i.m. injections of 0.1 ml were administered to the quadriceps with a 24-guage needle. i.d. injections of 0.20 ml were administered on the shaved back also using a 24-guage needle. i.m.injections contained 100 µg of plasmid DNA and were formulated with 0.25% bupuvicaine (Sigma Chemical Co.). 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 h.

Flow cytometry. Either A2L2 or 66.3 cells were mixed with immune serum diluted 1:100 in PBS and incubated for 1 h 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 h 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 Corporation, Hialeah, FL).

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 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 HER2/*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 HER2/*neu* gene was eliminated from the mammary tumor cells. Loss of p185 and/or neomycin resistance was noted in from 24 to 36 % of the metastasis-derived clones.

The A2L2 clone was selected for the tumor challenge experiments since it retained a high level of p185 expression (Fig. 1). The level of p185 expression by the A2L2 cell line has remained consistently 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 Fig. 2 a-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 HER2/*neu* insert did not result in positive staining of the A2L2 cells (Fig. 2 e) . Immune sera obtained from mice vaccinated three times i.m. at 2 week intervals had the highest level of immunofluorescence (Fig. 2 f). There was no staining in the absence of immune serum (Fig. 2 g), and the pooled antisera from panel A did not stain 66.3-*neo* cells (Fig.2 h), 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 HER2/*neu*.

Immunoprecipitation of p185 from A2L2 cells with immune sera

When the protein product of HER2/*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 Fig. 3, the monoclonal *Neu*-Ab3 precipitated p185 from A2L2 cells while 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 HER2/*neu* transgene in A2L2 cells with transport of some of the translated p185 protein 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 1.0×10^4 A2L2 or 66.3-*neo* cells into the mammary fat pad. The mice were monitored daily for palpable tumors and the tumors were measured with calipers when sufficiently large. As shown in Fig. 4 a, when challenged with A2L2 cells, the mice vaccinated with a single injection of 100 μg 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. 4 b). 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 upon 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 1.0×10^4 A2L2 cells 14 days after the third vaccination. As shown in Fig. 4 c, 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 HER2/*neu* was protective against challenge with a HER2/*neu*-transfected tumor compared to challenge with the parental tumor not expressing HER2/*neu*. ELVIS-*neo* 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 ten mice intradermally with ELVIS-*neu* and ELVIS. Since 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, as compared to the previous i.m. experiment using 100 μg of plasmid and three vaccinations. As shown in Fig. 4 d, 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 ten mice vaccinated with ELVIS. Thus, vaccination with ELVIS-*neu* reduced the incidence of tumor induction and lowered the mass of tumors that did develop.

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 1×10^4 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 Fig. 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 2.5×10^4 A2L2 cells, resulting in greater lung tumor burden, but even so there were significantly fewer metastases in the ELVIS-*neu* vaccinated mice.

DISCUSSION

Nucleic acid immunization was introduced by Wolff et al. (9), 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 (10). Chen et al. (11) demonstrated that vaccination of mice with plasmids expressing either full-length HER2/*neu* or its extracellular domain induced substantial protective immunity against challenge with a HER2/*neu*-expressing mouse mammary tumor. Amici et al. (12), using HER2/*neu* transgenic mice, demonstrated that genetic vaccination reduced the outgrowth of mammary tumors. In this report, we are the first to report the inhibition of metastasis by DNA vaccination against HER2/*neu*.

We vaccinated mice against HER2/*neu* using a plasmid-based gene transfer vector derived from Sindbis virus (ELVIS) that utilizes a self-replicating virus vector RNA (replicon) for gene expression. 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 1,000-fold lower doses than would be required for a comparable response using constructs of conventional expression vectors (8). We found that a single i.m. injection with 1 μg of ELVIS-*neu* induced an Ab response to A2L2 cells expressing p185, the gene product of HER2/*neu*, 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 HER2/*neu* since there was no reaction with 66.3 cells that do not express p185 (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 HER2/*neu*-positive tumor A2L2, and this effect was specific for the A2L2 cells since 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., since i.d. vaccination with ELVIS-*neu* reduced tumor incidence to 6 of 10 mice compared with 10 of 10 in mice given ELVIS vaccination (Fig. 4). Furthermore, i.d. injection required only 20 μg of plasmid per 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).

It has been reported that ELVIS can induce CTL at very low doses (8), and experiments to test this in our system are on-going. We demonstrated that low doses of ELVIS-*neu* induced antibodies, but they were not as efficient as larger doses for inhibition of tumor incidence. Vaccination with ELVIS-*neu* had no apparent adverse effect on normal tissues that express p185 (skin, kidney, and bronchial epithelium). Similarly, Herceptin™, which also targets p185, has not produced autoimmune responses (4).

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). Intravenous 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* is a strong indicator that metastasis from a primary breast tumor could be reduced, although we have yet to test this. We also plan to test the ability of vaccination with ELVIS-*neu* to reduce metastasis from resected tumors. In this model, primary A2L2 tumors in the mammary fat are surgically removed after metastases have already been seeded in distant organs and then the mice are vaccinated. This model of therapeutic vaccination is more representative of the clinical situation in which patients with resected HER2/*neu*-positive tumors relapse due to distant metastasis (1).

Although others have reported that DNA vaccination can reduce spontaneous tumors arising in HER2/*neu* transgenic mice (12) or in a transplantable tumor model (11), we are the first to report the inhibition of metastasis by DNA vaccination against HER2/*neu*. Metastasis is the major cause of breast cancer deaths (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 the current findings justify further research and development of this potent new strategy to target metastatic breast cancer.

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FIGURE LEGENDS

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.

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* (panel 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 was tested using 66.3-*neo* cells.

Figure 3. Immunoprecipitation of a 185-kDa protein from A2L2 lysates with IgG from ELVIS-*neu* vaccinated mice. The products of immunoprecipitation reactions using lysates of biotin-labled tumors cells and IgG from vaccinated mice (ELVIS or ELVIS-*neu*), control mouse IgG, or monoclonal antibody to p185 (*Neu*-Ab3) were separated on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose. The biotin-labeled bands were detected following incubation with HRP-streptavidin and ECL reagents. A 185 kDa

band was precipitated by *Neu*-Ab3 and IgG from the sera of ELVIS-*neu* vaccinated mice from lysates of A2L2 cells, and not from lysates of the 66.3-*neo* cells.

Figure 4. Tumor protection from vaccination with ELVIS-*neu*

a) Time course of tumor incidence in mice vaccinated with ELVIS (■), ELVIS-*neu* (▲) or PBS (●). Groups of 10 mice received 100 μg of DNA or 0.1 ml PBS injected i.m. in the hind leg. Two weeks later the mice were challenged with injection of A2L2 cells in the mammary fatpad. The mice were examined daily and the incidence of palpable tumors ($\geq$ 1 mm) was recorded. The incidence of tumor in the ELVIS-*neu* vaccinated mice was reduced compared with the ELVIS vaccinated mice, but the difference was not significant (p=0.087, Fisher's Exact test).

b) Mice were vaccinated as in panel a) and challenged with 66.3-*neo* cells. The vaccination with ELVIS or ELVIS-*neu* gave no protection against the mammary tumor cells lacking high expression of p185.

c) Groups of 15 mice were injected three times at 2-week intervals with ELVIS (■), ELVIS-*neu* (▲), or PBS (●). Two weeks after the final vaccination the mice were challenged by injection of A2L2 cells in the mammary fatpad, and tumor incidence recorded. The incidence of tumors in the ELVIS-*neu* group was significantly reduced compared with the ELVIS-vaccinated group (p=0.0092, Fisher's Exact test).

d) Groups of 10 mice were vaccinated i.d. with 20 μg of ELVIS or ELVIS-*neu* DNA, three times at 2-week intervals, and then challenged with injection of A2L2 cells into the mammary fatpad. The tumor incidence was not significantly lower in the ELVIS-*neu* group, but the tumor weight (mice were killed 35 days after injection) was significantly lower in this group compared with weights of tumors in the ELVIS-vaccinated mice (p=0.02, unpaired t-test).

Figure 5. ELVIS-*neu* protection against experimental metastasis of A2L2 cells. Groups of 10 mice were vaccinated three times with 100 μg of ELVIS or ELVIS-*neu* DNA , or with 0.1 ml of PBS by i.m. injection at 2-weeek intervals. Fourteen days after the final vaccination the mice were injected i.v. with 1 x 10^4 A2L2 cells. The mice were killed 21 days later, and the lung metastases counted. There were significantly fewer metastases in the ELVIS-*neu*-vaccinated mice (p=0.031), but not in ELVIS-vaccinated mice (p=0.49), compared with the PBS injected mice (unpaired t-test).

The abbreviations used are: FITC, fluoresceine isothiocyanate , SDS-PAGE, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis; TBST, 150 mM NaCl, 10 mM Tris-HCl with 1% Triton X-100, pH 8.0; NHSLC-biotin, sulfosuccinimidyl-6-(biotinamido)hexanoate.

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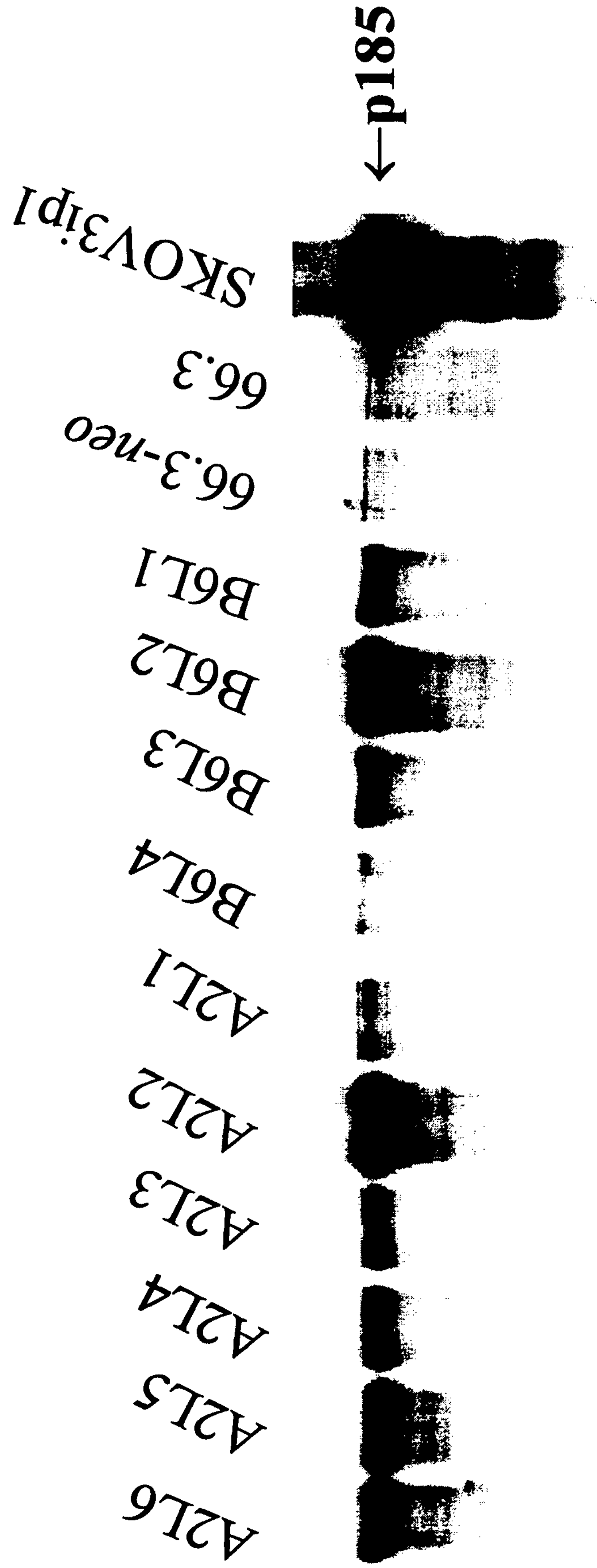
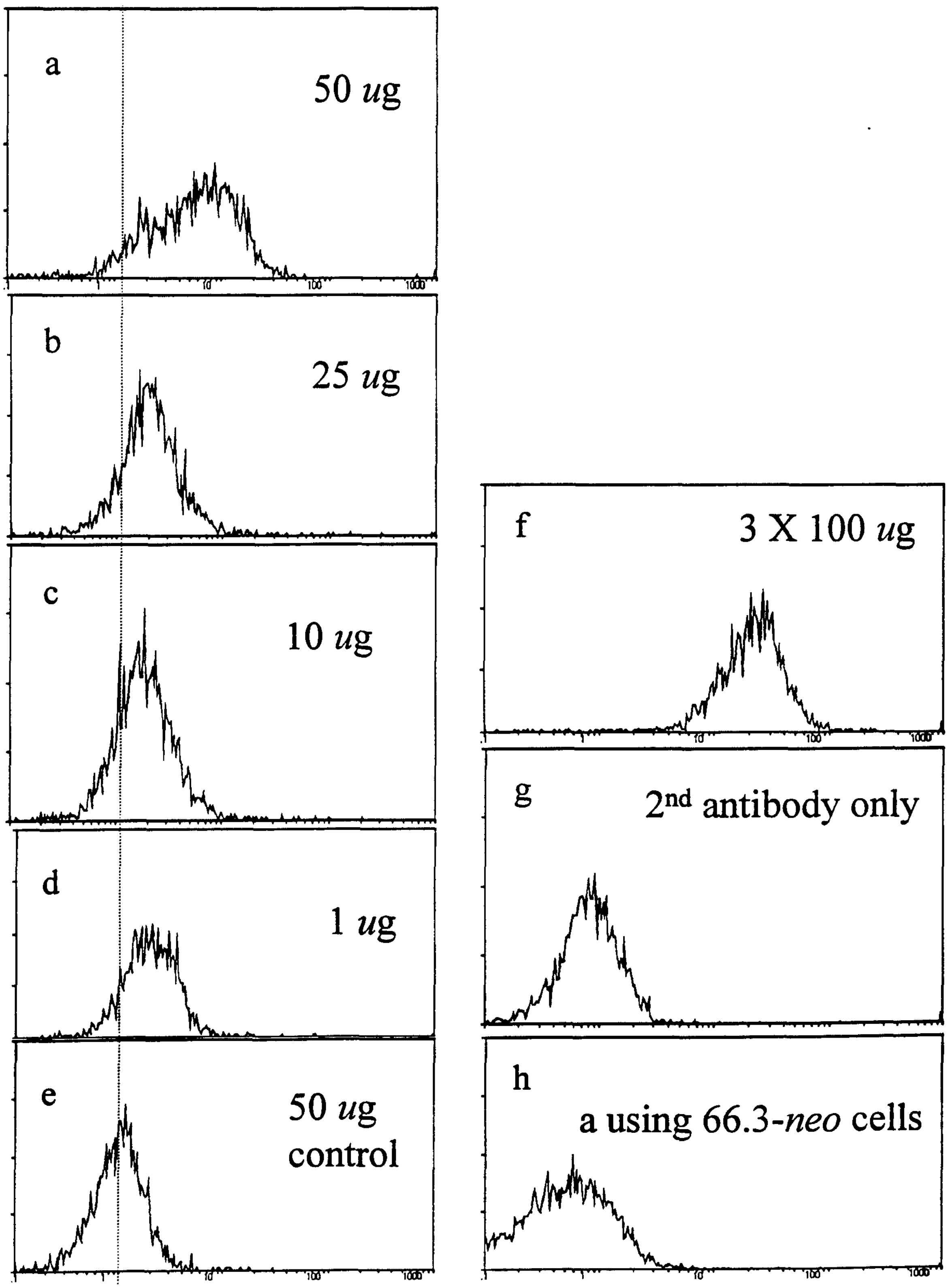


←p185
SKOV3ip1
66.3
66.3-neo
B6L1
B6L2
B6L3
B6L4
A2L1
A2L2
A2L3
A2L4
A2L5
A2L6

Figure 1

Figure 2



a
50 *u*g
b
25 *u*g
c
10 *u*g
d
1 *u*g
e
50 *u*g control
f
3 X 100 *u*g
g
2nd antibody only
h
a using 66.3-*neo* cells

Figure 3

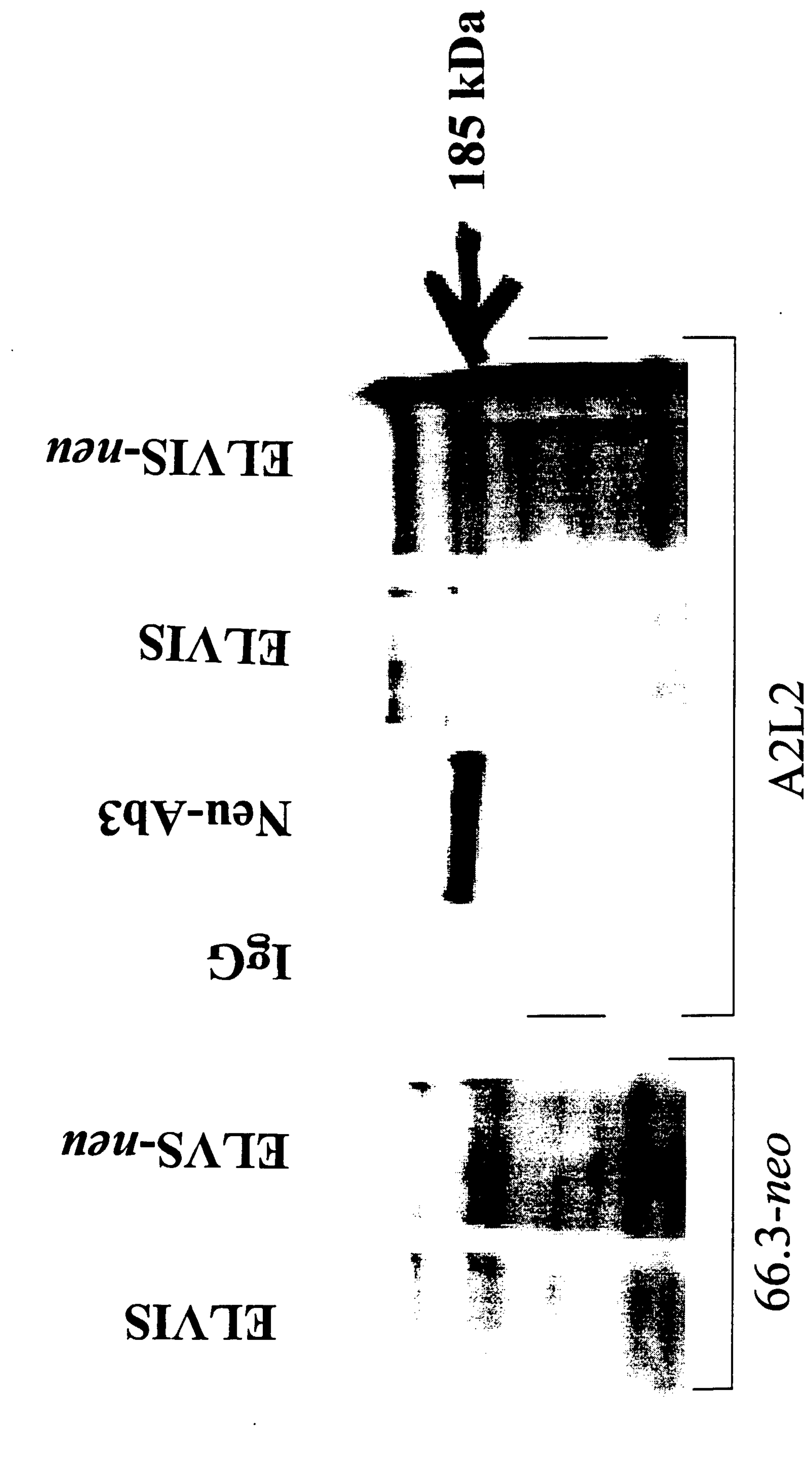
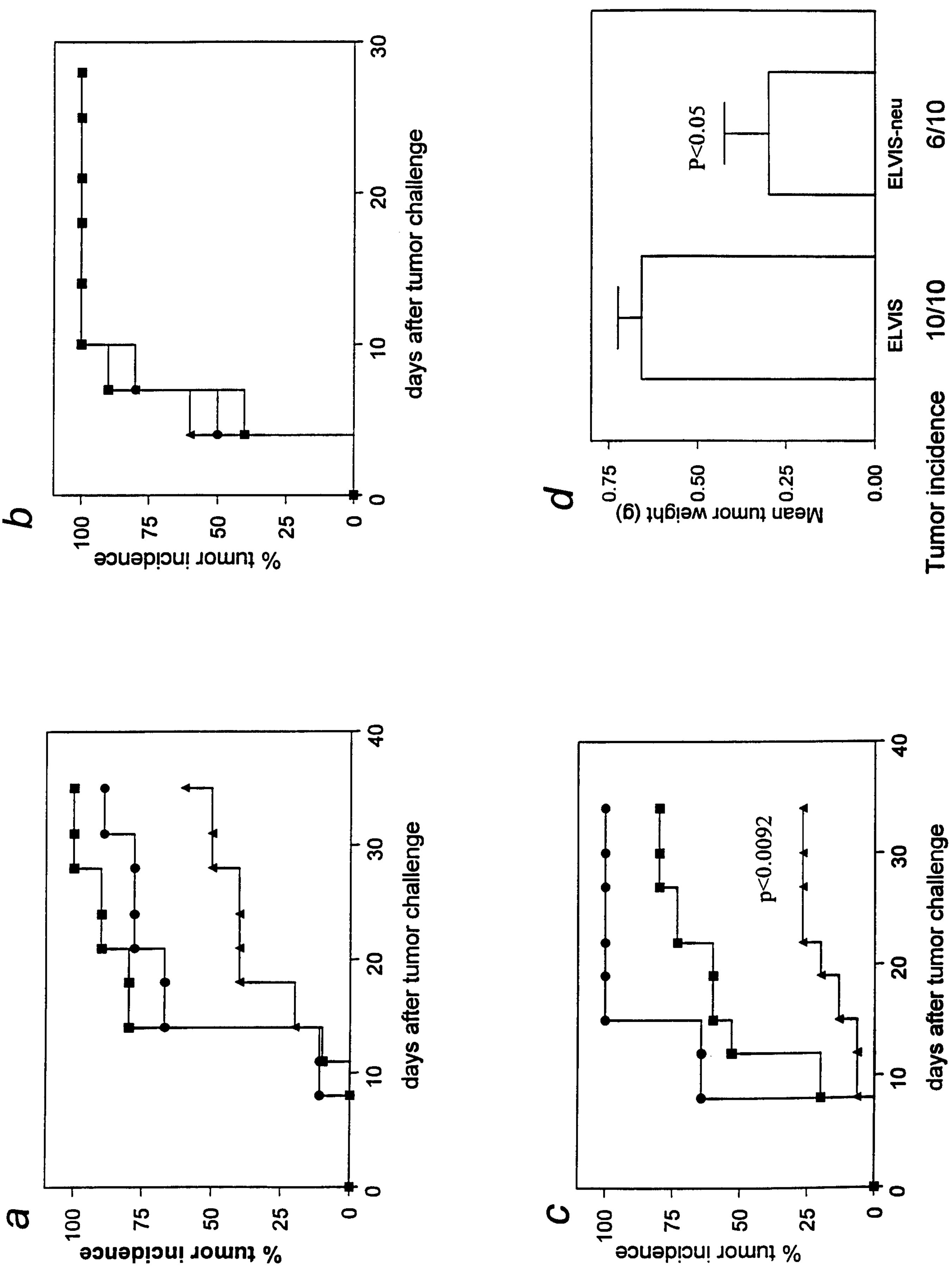
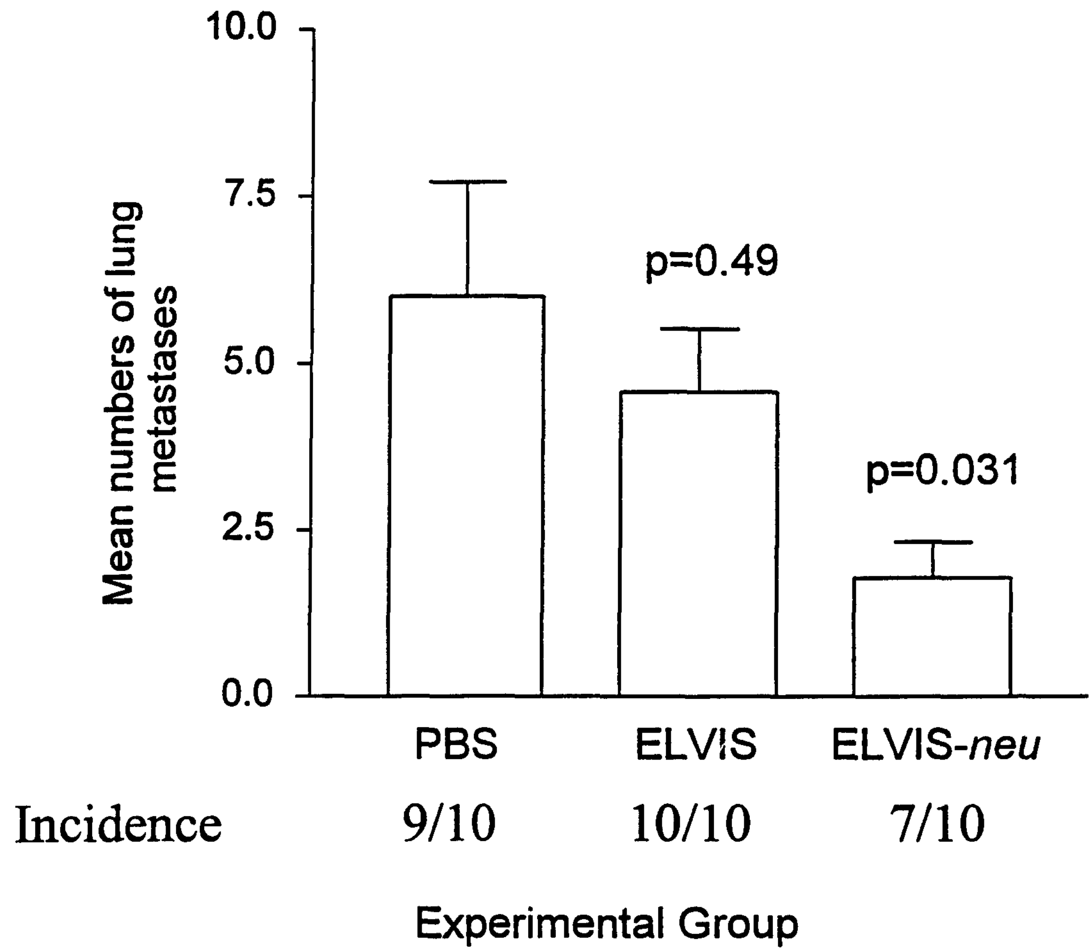

185 kDa
ELVIS-*neu*
ELVIS
Neu-Ab3
IgG
ELVS-*neu*
ELVIS
A2L2
66.3-*neo*

Figure 4


a
% tumor incidence
days after tumor challenge
b
% tumor incidence
days after tumor challenge
c
% tumor incidence
days after tumor challenge
p<0.0092
d
Mean tumor weight (g)
P<0.05
ELVIS
ELVIS-neu
Tumor incidence
10/10
6/10


10.0
7.5
5.0
2.5
0.0
Mean numbers of lung metastases
p=0.49
p=0.031
PBS
ELVIS
ELVIS-*neu*
Incidence
9/10
10/10
7/10
Experimental Group

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