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## Table of Contents

Front Cover	1
SF 298 Report Documentation Page	2
Foreword	3
Table of Contents	4
Abstract	5
Introduction	6
Body of Report	
Methods	7
Results	10
Discussion	12
Conclusions	16
References	17
Figure legends	21
Figures	23
Appendices	31

## ABSTRACT

**Introduction:** Detection of Ductal Carcinoma In Situ (DCIS) increased dramatically with the advent of mammographic screening. Vaccines targeted against the HER-2/neu extracellular domain(ECD) protein might reduce the likelihood of progression to invasive disease for patients with high grade DCIS lesions. The vaccine strategy was tested in MMTV/c-neu transgenic mice that develop DCIS and tumors that overexpress rat neu ECD.

**Results:** Sixteen animals immunized with gD-neu and ten animals immunized with chicken albumin. Transgenic and non-transgenic developed high serum anti-neu antibody titers after vaccination. Transgenic animals immunized with CFA/neu-ECD had a marked statistically significant decrease in the rate and number of tumors developed by 52 weeks of age when compared to transgenics that were immunized with controls ( $p>0.005$ ). Lymphocytes from nodes of transgenic and non-transgenic animals immunized with neu-ECD proliferated four fold in response to in vitro culture with neu-ECD but not in response to Her-2(human).

**Conclusions:** MMTV neu transgenic mice develop an immune response and decreased tumor growth after vaccination with a "self" protein. Results demonstrate the potential of a vaccine strategy for the prevention of tumor formation in patients at risk of forming HER-2/neu based tumors.

## INTRODUCTION

A diagnosis of breast cancer was given to approximately 183,400 new women in 1994.[1] profoundly affecting their lives as well as those of their families. Approximately 15% to 20% will be diagnosed with ductal carcinoma in situ (DCIS), which is considered to be a premalignant or stage 0 lesion, destined in 10-30 % of cases to develop into an invasive cancer.[2-10] The problem created by detection of DCIS is by no means trivial. Once patients are aware of the potential for progression, most elect for aggressive treatment which has led to an overall increase in the rates of mastectomy.[11] Early detection of a lesion prior to its becoming an invasive cancer is a true opportunity for prevention of disease but not if the treatment carries with it the sequelae of treatment for invasive breast cancer. If indeed we are going to detect more early lesions, we need a rational treatment plan that entails therapy that is less aggressive than mastectomy or radiation therapy.

We received a DOD idea grant in late 1994 to develop a vaccine to prevent the progression of ductal carcinoma in situ (DCIS) to invasive breast cancer, based on knowledge of the expression of oncogene protein products in in situ (DCIS) and invasive breast cancer. The MMTV/c-neu transgenic (Transgenic) mouse model was chosen to test a vaccine strategy because these Transgenic animals develop spontaneous mammary tumors that overexpress c-neu (rat) protein and mirror human breast tumors that overexpress HER-2 on both a gross and histological level. This mouse is fully immunocompetent which permits the testing of immunologically based intervention. Our first aim was to get the rat protein purified for testing and demonstrate that rats could be immunized with a "self" protein. Our second aim was to establish the transgenic colony and prove that these animals would generate an immune response to neu, which could be considered a self protein in the transgenic animals. Lastly, we hoped to use the neu protein with an adjuvant when animals were 9-12 weeks of age and determine if creating an immune response was sufficient to delay or decrease tumor development in the transgenic animals.

## METHODS

### Cloning of neu

Specific oligonucleotides were synthesized on the basis of the published NEU DNA sequence. Total cellular RNA was extracted from DHFR/G8 cells (NIH3T3 cells transfected with Rat neu[12] and used as a template in RT PCR to generate the rat neu extracellular domain coding sequence.

### Expression of gDNEU Extracellular Domain (ECD)

A gD-neu ECD fusion protein was constructed by ligation of the coding sequences for amino acids 1-53 of the herpes simplex virus type 1 glycoprotein D [13] to the sequences encoding amino acids 57-687 of neu. The gD-neu ECD cDNA was inserted into the cytomegalovirus-based expression vector pRK5 [14]. This construct was transiently transfected into human embryonic kidney 293 cells using a calcium phosphate precipitation protocol.

### Preparation of neu protein and Pooled Mouse Sera Against neu

Purified rat neu protein was provided by Genentech (South San Francisco, CA): approximately 15L of S/N were concentrated by Filtron Ultrasette 30K MWCO ultrafiltration unit to about 1.0L. This material was passed through a 40 mL anti gD column (1766) in the cold room. The column was then washed to a baseline O.D. 280 with PBS and again washed with PBS + 1M NaCl to remove any non-specific binding. The column was eluted with 0.1M acetic acid + 0.5M NaCl pH 2.9. Eluted fractions were neutralized with 1M tris pH 8.0 and dialyzed vs PBS with 3 changes of 1L. Final sample was 0.22  $\mu$ m filtered and an O.D. 280 of 0.320 taken with a volume of 11.2 mL. A non-reduced, 10-20% tricine, gives a molecular weight of 81,850, and a purity of about 94%. This material was blotted onto PVDF and probed with an anti-mouse horseradish peroxidase. No murine IgG from the 1766 column was detected.

Pooled sera from Balb/c mice immunized with either gD neu ECD in RIBI SQ or  $1 \times 10^7$  3T3 cells transfected with neu were used as positive control for the ELISA.

### Immunization of Rats with neu ECD protein

Rats were immunized with gD-neu ECD to demonstrate whether an immune response can be induced against a normal self protein. Twelve rats were immunized subcutaneously, six with gD-neu ECD and IFA, six with gD-neu ECD and IFA (gD-neu ECD at 17  $\mu$ g per



immunization). Three immunizations were administered during the initial 35 days of the experiment. Six bleeds were performed over a period of 150 days on each mouse to collect sera for ELISA analysis.

### Mouse Colony

MMTV/c-neu transgenic mice obtained from William Muller, PhD at McMaster University were bred and genotyped by PCR (see below) at weaning.

### DNA Isolation & PCR Protocol for Genotyping of Mice

DNA was prepared from tail or ear punch tissue from mice. Actin primers were used for negative control, DNA prepared from a known transgenic mouse with tumor was used as a positive control. A standard PCR cocktail containing: 1x of 10x PCR Buffer (Boehringer Mannheim), 2.5 mM MgCl<sub>2</sub>, 2.1 pM neu F' (F' denotes the forward primer, R' the reverse primer), 2.1 pM neu R', 2.1 pM actin F', 2.1 pM actin R', 200 μM dNTP mix, and 0.6 U/μL Taq polymerase Buffer (Boehringer Mannheim). Reactions were then run out on a 1.5% agarose gel containing ethidium bromide and visualized under UV light.

### Immunization Study

Groups of transgenic and non-transgenic mice were immunized with 6.7 μg of rat erbB-2/c-neu or an irrelevant control antigen of chicken serum albumin (CSA) in complete Freund's adjuvant at 8-12 weeks of age. A boost of 6.7 μg of rat erbB-2/c-neu or a control antigen (chicken albumin) in Incomplete Freund's Adjuvant (IFA) was given 14 weeks after the initial immunization. Palpable mammary tumors were measured on a weekly basis and serum immunoglobulin was measured by ELISA at 2, 4, 8, 16, 22 and 31 weeks after the original immunization.

Palpable mammary tumors were measured with calipers and the tumor volume calculated according to the formula:  $4(p/3) \times (\text{length}/2) \times (\text{width}/2) \times (\text{depth}/2)$ . Total tumor volume is calculated as the total tumor volume divided by the number of animals in the group. When an animal dies, the data on the animal is no longer included in the total and the number of animals in the group is decreased by one.

### Serum ELISA protocol

96 well plates (Maxisorb, Nunc) were coated with a dilution of appropriate protein (0.75 μg/mL for gD-neu ECD or 1.00 μg/mL for chicken albumin) at 50 μL/well. Incubate 2 hours at 25°C or overnight at 4°C. Plates were blocked, incubated for 1 hour at 25°C and

washed. 50  $\mu$ L/well of diluted serum (50%) was added to first well of titration series (total 12 dilutions in triplicate for each sera). Incubate for 1 hour at 25°C. Plates were washed with buffer and (0.05% Tween20 in 1xPBS).

and dilute horseradish peroxidase (HRP) conjugated sheep anti-mouse Ig antibody was added. After incubation for 1 hour at 25°C, plates were washed, 50  $\mu$ L/well of HRP substrate (TMB from Kirkegaard & Perry) was added, and allow to develop for at least 15-20 minutes. Reactions were stopped with 50  $\mu$ L/well of 1M phosphoric acid and plates were read at 450 nm (OD) on a plate reader (Vmax from Molecular Devices).

Data Analysis - Average of triplicates were graphed for each point in titration series and a sigmoid plot was created. Curve fit equations were used to determine the titer for each serum. (Data analysis software: DeltaGraph Pro from DeltaPoint).

#### **Lymphocyte Proliferation:**

The lymph node proliferative response was measured by culturing a single cell suspension of primed lymph node cells at a density of  $5 \times 10^5/150$  ul/well in a 96 well plate. Lymphocytes were incubated with neu protein (courtesy of Genentech) or ovalbumin. After 72 hours of culture, the cells were pulsed for 18 hours with 1 uCi/well of  $^3$ H-thymidine. The cultures were harvested and  $^3$ H-thymidine incorporation determined in a scintillation counter.

## RESULTS

### Preliminary Rat Immunizations

We have demonstrated the ability to immunize rats with the *neu* protein and elicit an immune response in spite of the presence of low levels of *neu* expression in the rat (Figure 1). We did not detect any evidence of toxicity as judged by weight loss, hair loss, or failure to thrive. The vaccine failed to demonstrate any major toxicities after 3 1/2 months in these preliminary experiments.

### Development of *neu* Colony

Founder mice were obtained from Dr. William Muller as described above. The T50 in our colony was greater than described by Dr. Muller. Our T50 for tumor development was 38-40 weeks whereas his was 24 weeks. Our colony T50 is shown in Figure 4.

### Development of Vaccine Components

We developed a construct of the c-*neu* protein which was incorporated into the GM-CSF-Idiotype construct that was obtained from Dr. Ronald Levy. The idiotype was cut out and replaced with the c-*neu* sequence. While we were able to isolate the construct of the anticipated size which contained *neu*, we were unable to express it and purify significant quantities. This was also a significant problem in Dr. Levy's laboratory. For this reason, we decided to prove the concept using CFA which we knew would elicit a strong response and thus help us to determine if the strategy of vaccination would be worthwhile pursuing.

### Immunization Experiment

Serum response in *neu* transgenics after vaccination with *neu* is shown in Figure 2. Serial serum titers of immunized animals demonstrates a marked response to vaccination with the *neu* ECD protein in CFA. Transgenic animals developed average serum titers of 1:25,000 8 weeks after injection which were sustained over the course of the experiment with the addition of one boost 13 weeks after the initial immunization. The non-transgenic controls immunized with *neu*-ECD developed a higher response ( $p < 0.05$ ), with average *neu* Ab titers of 1:100,000 by 8 weeks post-injection as did the control Transgenic animals immunized with chicken albumin. Mean titers at 39-43 weeks of age (31 weeks post injection) were 1:9000, 1:90,000, 1:110,000 for the *neu* immunized Transgenic, non-Transgenic littermates, and chicken albumin immunized Transgenic animals, respectively.

### *Lymphocyte proliferation*

Results of the lymphocyte proliferation response to incubation with the immunizing protein are shown in Figure 3A and 3B. Transgenic and non-Transgenic animals immunized with neu-ECD proliferated four fold in response to in vitro culture with neu-ECD but not in response to Human Her-2 (Figure 3A). Animals immunized with neu or CSA had the same degree of lymphocyte proliferation.

### *Tumor Development in response to neu*

The overall tumor volume in the erbB-2 immunized Tg group was initially statistically significantly less than the volume of tumor in the control immunized and non-immunized Tg ( $p < 0.05$ ), as shown in Figure 5. Tumor volumes in the colony and the animals immunized with CFA and control antigen were equivalent, suggesting that CFA alone had no significant impact on delaying the onset of tumors by itself.

Transgenic animals that were immunized with CFA/neu-ECD had a marked and statistically significant decrease in the rate and number of tumors developed by 52 weeks of age when compared to Transgenic animals that were immunized with CFA /chicken albumin ( $p < 0.005$ ). Kaplan Meier plots comparing animals immunized with CSA and neu are shown in Figure 6.

## DISCUSSION

Immunotherapy as a therapeutic strategy has perhaps best been studied in B cell lymphoma. Although patients with low grade lymphoma often go into remission following radiation therapy and chemotherapy, these standard therapies rarely result in cure. B cell lymphomas have unique antigenic determinants on their cell surface, within variable regions of immunoglobulin heavy and light chains (idiotype), and thus have a target for immune modulation. Ronald Levy and his associates over two decades, have pioneered the use of immunotherapy for this disease in both patients and the 38CI3 animal model.

Passive antibody therapy was shown to be capable of both partial and complete remission. Of a total of 45% patients treated with passive antibody in several trials, 20% sustained a complete remission that has lasted longer than 9 years[15]. An additional 50% had partial responses. In order to overcome the arduous task of manufacturing unique antibodies, and to develop a sustained immune response, Dr. Levy changed strategies and began to develop a vaccine approach. Vaccination with tumor Ig protein in the 38CI3 model can protect against tumor challenge and cure animals with established lymphomas [16, 17]. In 41 patients vaccinated with tumor idiotype Ig 8 key hole limpet hemocyanin emulsified in an adjuvant, 20 (49%) generated an immune response. Those with response demonstrated a significant improvement in clinical outcome as measured by first remission and freedom from progression, 7.9 years, 1/3 years  $p=0.001$ . [18]

The experimental and clinical results in low grade lymphoma are extremely encouraging and suggested to us that a similar strategy might be very successful in breast cancer. Perhaps the most exciting opportunity for intervention is at the time of detection of DCIS, when there is minimal disease burden and the cancer is in a pre malignant or stage 0 stage. There has been over a 500% increase in the detection of DCIS since the advent of mammography. Not all of these lesions progress, but the reason for aggressive intervention is because once invasive breast cancer is detected, all patients have some risk of metastatic recurrence and death. Standard therapies such as chemotherapy and hormone therapy are not useful in DCIS, and for invasive breast cancer, they do not prevent the majority of systemic recurrence (they reduce the likelihood of recurrence by 20-30%). DCIS presents an ideal opportunity for manipulation because patients have minimal residual disease and do not have a risk of metastatic spread. What would be required is a cell surface target. One of several different histologic types of breast cancer is associated with a known cell surface antigen c-erbB-2, also known as Her-2/neu.

As has been discovered in other cancers such as lymphoma, cancers that occur in a particular organ are not all the same, and choosing one of the subtypes of cancer may lead to the development of successful targets, as it has in lymphoma. Not surprisingly, breast cancer is not a single disease, but a heterogeneous collection of tumors with different biologic behavior, each with their own pathway to progression. One pathway may be through the overexpression of HER-2/neu protein. High grade DCIS is the precursor lesion to invasive tumors that overexpress HER-2/neu. Frequently, invasive cancer is found in association with DCIS. In those invasive cancers that overexpress Her-2/neu that have associated DCIS lesions, both overexpress the oncogene protein[19]. High grade DCIS lesions in women (30-50% of all DCIS lesions detected) are thought to be the most likely to progress to invasive cancer, or at least progress over the shortest time line. [2-10] Although only 20-25% of all invasive breast cancers overexpress HER-2/neu, well over 85% of high grade DCIS lesions express HER-2/neu.

One of the genetic alterations thought to play a role in the development of human breast cancer is the HER-2/neu proto-oncogene[20, 21]. Amplification of HER-2/neu has been found in 20-25% of human breast cancers and is thought to be associated with a poor prognosis.[22-24] The HER-2 protein is a member of the tyrosine kinase family and the extracellular domain of the protein, p185HER2 has been identified and cloned, and used as a target for immunotherapy in experimental models[25, 26]. It has also been observed that HER-2/neu is expressed more consistently in high grade DCIS than in invasive cancer[19, 27]. Expression in high grade DCIS has been reported to be as high as 85-100%. A recent report of eighty-six in situ cancers showed that all large cell (high grade) DCIS lesions showed c-erb B-2 (HER-2/neu ) staining on paraffin sections[28].

There is evidence that targeting the extracellular domain of the HER-2/neu protein may have promising therapeutic potential. Monoclonal antibodies (mAbs) directed toward this cell surface antigen have been shown to inhibit tumor cell growth[29]. Combinations of Ab and exotoxin A have been shown to inhibit tumor growth in vitro[30]. A phase II trial, initiated by Genentech, has demonstrated clinical activity in patients with advanced Her2/neu expressing breast cancer with minimal toxicity[31]. It has been shown that passive antibody therapy inhibits the development of tumors in HER-2/neu transgenic mice[32]. A vaccine targeted against the HER-2/neu ECD protein might be an ideal treatment to reduce the likelihood of progression to invasive disease. The potential of such a vaccine is supported by the evidence that the human HER-2/neu protein, when used as a

vaccine preparation in guinea pigs and rhesus monkeys, induces both cellular and humoral immunity, and sera from these animals inhibits the growth of human breast carcinoma cell lines that overexpress HER-2[25, 26].

The MMTV/c-neu transgenic mouse develops spontaneous mammary tumors which mirror human comedocarcinoma of the breast on both a gross and histological level. In comparison to xenograft transplant models, the MMTV/c-neu mouse has several characteristics which facilitate the development and assessment of novel therapeutics. Because neoplastic lesions develop spontaneously, the transgenic mouse permits study throughout neoplastic progression and within a fully homologous system. This mouse is also fully immunocompetent which permits the testing of immunologically based intervention. In the MMTV/c-neu transgenic (Transgenic) mouse, the erbB-2/neu gene is expressed under the control of the mouse mammary tumor virus (MMTV) promoter and the protein is found in significantly elevated levels in transgenic mammary tumors, as well as at low levels in normal mammary tissue, spleen and thymus. To determine whether expression of the transgene results in immunological tolerance, the response to erbB-2/neu was measured by serum ELISA and lymph node proliferation in primed transgenic and non-transgenic littermates.

One of the potential pitfalls of a vaccine directed against Her-2/neu is the fact that this antigen is expressed at low levels in normal cells. In mouse models, c-erb-B2 has been shown to be expressed at higher levels in development and again in mammary tissue during lactation. Thus, the idea of directing the immune response against such an antigen either as treatment for or prevention of cancer would require a response against a "self" antigen. We have shown that we can immunize rats with the rat neu protein and generate serum anti-neu antibodies. c-neu Transgenic mice also develop an immune response, both demonstrated by serum anti-neu antibody production and lymphocyte proliferation.

However, tolerance to such a response may be a problem. In women with progressing Her-2/neu over expressing tumors measurable levels of circulating antibodies can be detected[33]. There is growing evidence however that using Her-2/neu as a target may be successful. We have demonstrated that tumor formation is substantially delayed in Transgenic mice. Others are also beginning to demonstrate some effect. Kipps has shown protective immunity in FVB/N neu Transgenic mice against adoptive transfer[34]. Passive Ab therapy shows some effect in trials and has been shown to be effective in Transgenic mice as well. It is not clear which immunization strategy will present the "antigen" of

interest in the most effective manner. Successful vaccination of patients with some of these other vaccine approaches, such as dendritic cells is very encouraging and demonstrates the "translatibility" from the animals model to the clinic.

Our preliminary experiments demonstrate that vaccination has the potential to delay the onset of tumor formation even in animals genetically altered and programmed to develop neu tumors, a very stringent model for proof of effect. Others have shown a similar effect with passive antibody in the mutated neu transgenics, where 50% of animals did not develop tumors when treated with passive antibody weekly[32]. We focused on the proof of concept and used a powerful adjuvant that elicits a strong humoral response. Other strategies that are geared more toward eliciting a cellular response may be more effective. Much more work needs to be done. A number of approaches are being studied in the lymphoma animal model systems including GM-CSF-idiotype fusion proteins produced in mammalian cells[35] and bacteria[36], idiotype pulsed dendritic cells[37], naked DNA encoding the idiotype protein[38] and adenovirus encoding the idiotype protein. Some of these strategies have been extended to patients with exciting protein results[37]. Because of the difficulty in producing sufficient quantities of protein from the GM-CSF protein fusion constructs, both Dr. Levy and our group have decided not to pursue this avenue. In order to speed the translatibility of our research efforts, we will be joining forces with Dr. Levy and taking advantage of his clinical and animal experience with vaccine adjuvants and his demonstrated ability to translate techniques from the laboratory to the clinic. Our clinical group and Dr. Levy will be submitting a Clinical Translational Research (CTR) proposal to the DOD in June of this year to refine a vaccine strategy in animals and test in women with newly diagnosed DCIS.



## CONCLUSIONS

The MMTV neu transgenic mice develop an immune response to vaccination with one immunization and only one boost of gD-neu and CFA/IFA as evidenced both by the development of high serum antibodies to neu and lymphocyte proliferation in response to neu. This response markedly decreased the rate of tumor development in the neu immunized transgenics when compared to controls ( $p < 0.005$ ). These results demonstrate the potential of a vaccine strategy for the prevention of tumor formation in patients at risk to erbB-2/c-neu based tumors.

In order to determine whether a vaccine intervention at the DCIS stage is possible, preclinical animal work will need to be done to prove that intervention with the vaccine once premalignant lesions had developed can still block the progression to invasive cancer. In order for a vaccine strategy to succeed, a vaccine that is powerful, non-toxic in humans must be used. Work is now underway to test a variety of immunotherapeutic vaccine strategies that appear to be clinically effective in the setting of other malignancies.

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## FIGURE LEGENDS

**Figure 1:** Rat Serum Titers. Sera from four rats immunized with CFA and neu at 4 weeks of age are shown. Times of immunization boosts are shown on the x axis. Error bars represent the standard deviation of the four animals. Each sera was analyzed in triplicate.

**Figure 2:** Mouse Serum Titers. Error bars represent one standard deviation above and below the mean value. At each time point, the serum titers for each group showed statistical significance ( $p \leq 0.05$ ) when compared to the serum titer of each of the other groups with the exception of the following nine points: transgenic mice immunized with CSA + CFA compared to non-transgenic mice immunized with erbB-2 + CFA (at the first 5 time points), transgenic mice immunized with CSA + CFA compared to transgenic mice immunized with erbB-2 + CFA (8 weeks, 31 weeks), and transgenic mice immunized with erbB-2 + CFA compared to non-transgenic mice immunized with erbB-2 + CFA (8 weeks, 22 weeks).

**Figure 3A and 3B:** Lymphocyte Proliferation. Transgenic (Tg) and nontransgenic (nt) mice were immunized with neu or chicken serum albumin (CSA) and CFA. Lymph nodes were harvested after 10 days and then cultured in the presence of in vitro immunogen as described in the methods. Figure 3A shows animals immunized with neu and CSA. In vitro immunogen is either neu or HER-2 (listed last on figure legend). The graph shows the increase in proliferation with rising concentrations of in vitro antigen. Figure 3B shows a comparison of neu immunized animals (top graph) and CSA(lower graph) immunized animals. In vitro antigen concentration is 10  $\mu\text{g/ml}$  for all experiments and Tg and nonTg animals are compared. Maximum tritiated thymidine incorporation is shown on the y-axis in all figures.

**Figure 4:** Kaplan Meier plot of the entire colony showing the pattern of tumor onset. The entire colony of transgenic mice was checked for tumors at three times during the experiment. The % of tumor free mice at the appropriate age is plotted on the graph to determine to T50 of the colony. The T50 is 41 weeks for this colony (greater than previously published data). The colony represents experience with over 400 animals.

**Figure 5:** Mean tumor volumes for each group of animals are plotted with respect to age. Groups include the entire colony, Tg mice immunized with neu and CSA. Total tumor

volume, which often includes more than one mammary tumor is measured in each animal. The total tumor volume for the group is divided by the total number of animals in the group. The immunization with CSA appears to have no effect on tumor volume when compared to unimmunized, where the immunization with gD-neu ECD appears to have delayed the onset of the tumors. More data is required to determine if the tumor growth rate is also decreased, but slopes of the curve appear to be similar for each group.

***Figure 6:*** Kaplan Meier plot of experimental mice. The % tumor free animals is plotted with respect to age. This plot clearly shows that the T50 for the CSA/CFA immunized animals is parallel to that of the unimmunized animals in Figure 4 and that the T50 gD-neu ECD/CFA immunized animals has not been reached by the 48th week of the experiment. There is a significant delay in the tumor onset as well as the total number of animals that develop tumors following immunization with gD-neu.

## FIGURES

Figure #	Description	Page #
Figure 1	Rat Serum Titers	24
Figure 2	Mouse Serum Titers	25
Figure 3A	Lymphocyte Proliferation	26
Figure 3B	Lymphocyte Proliferation	27
Figure 4	Colony T50	28
Figure 5	Tumor volume	29
Figure 6	Kaplan Meier plot	30



Rats 18-21: Ave. titer vs. days post immunization

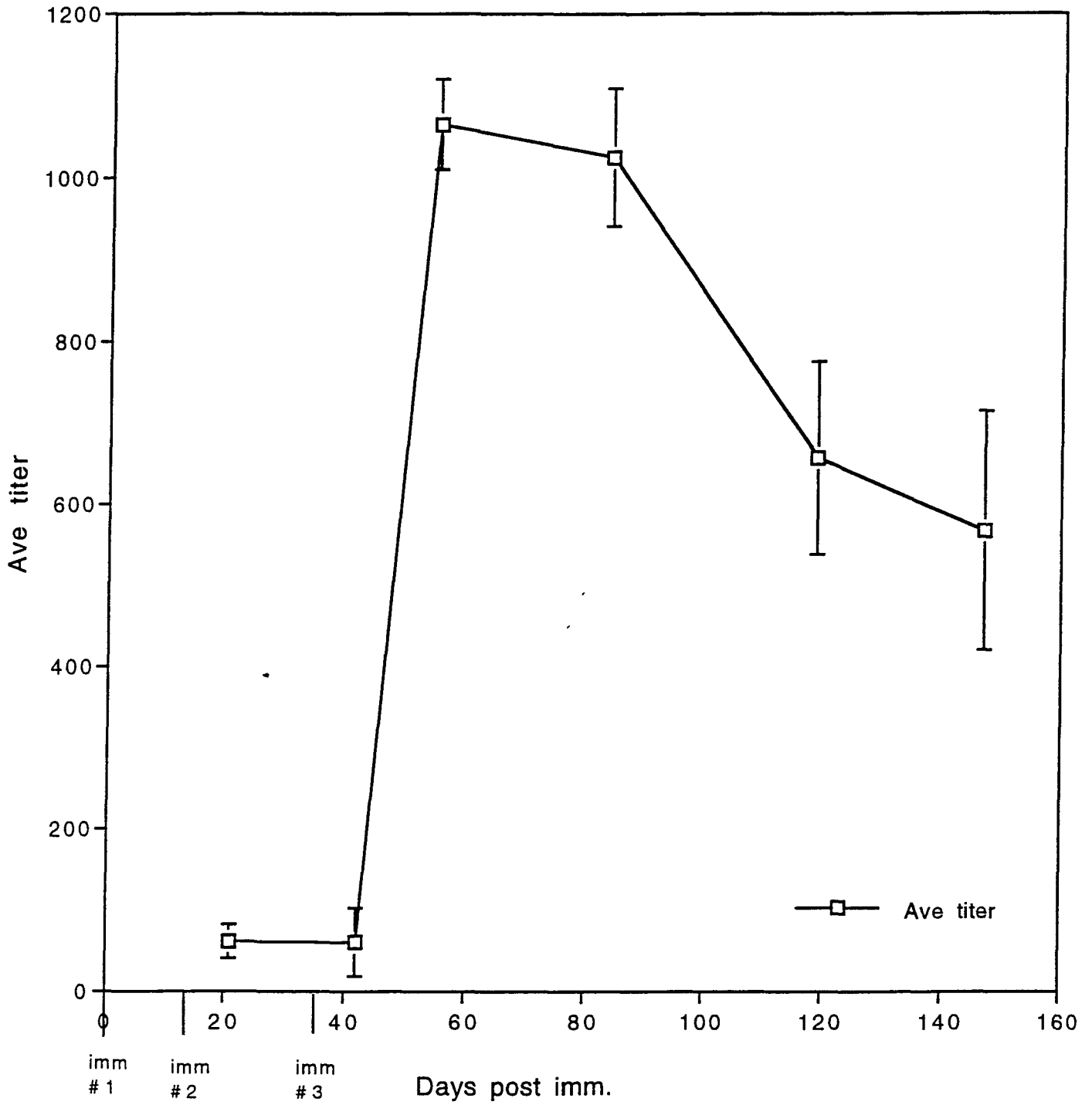


FIGURE 1

## Serum Titers of Experimental Animals

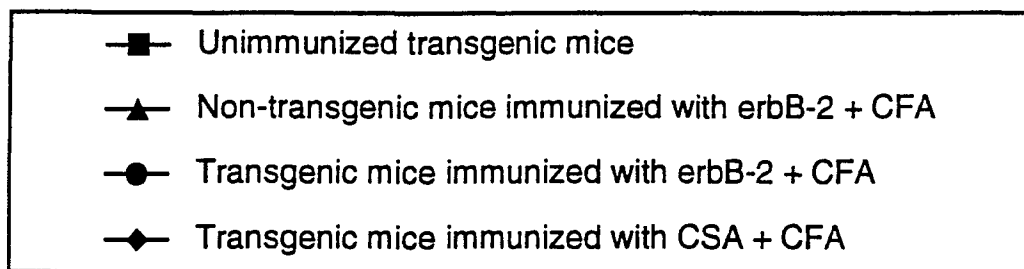
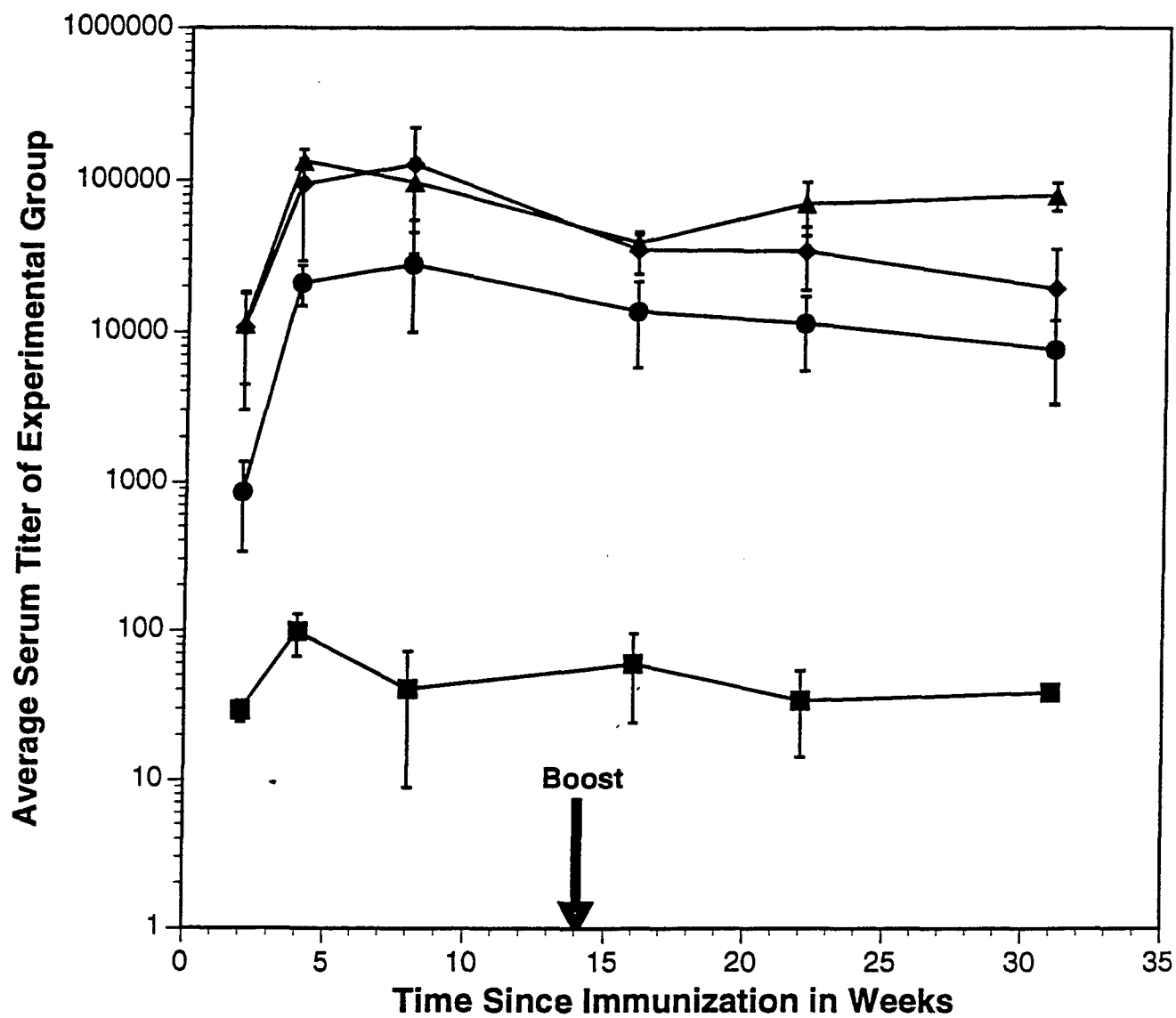


FIGURE 2

proliferation of neu ECD-primed lymph node cells

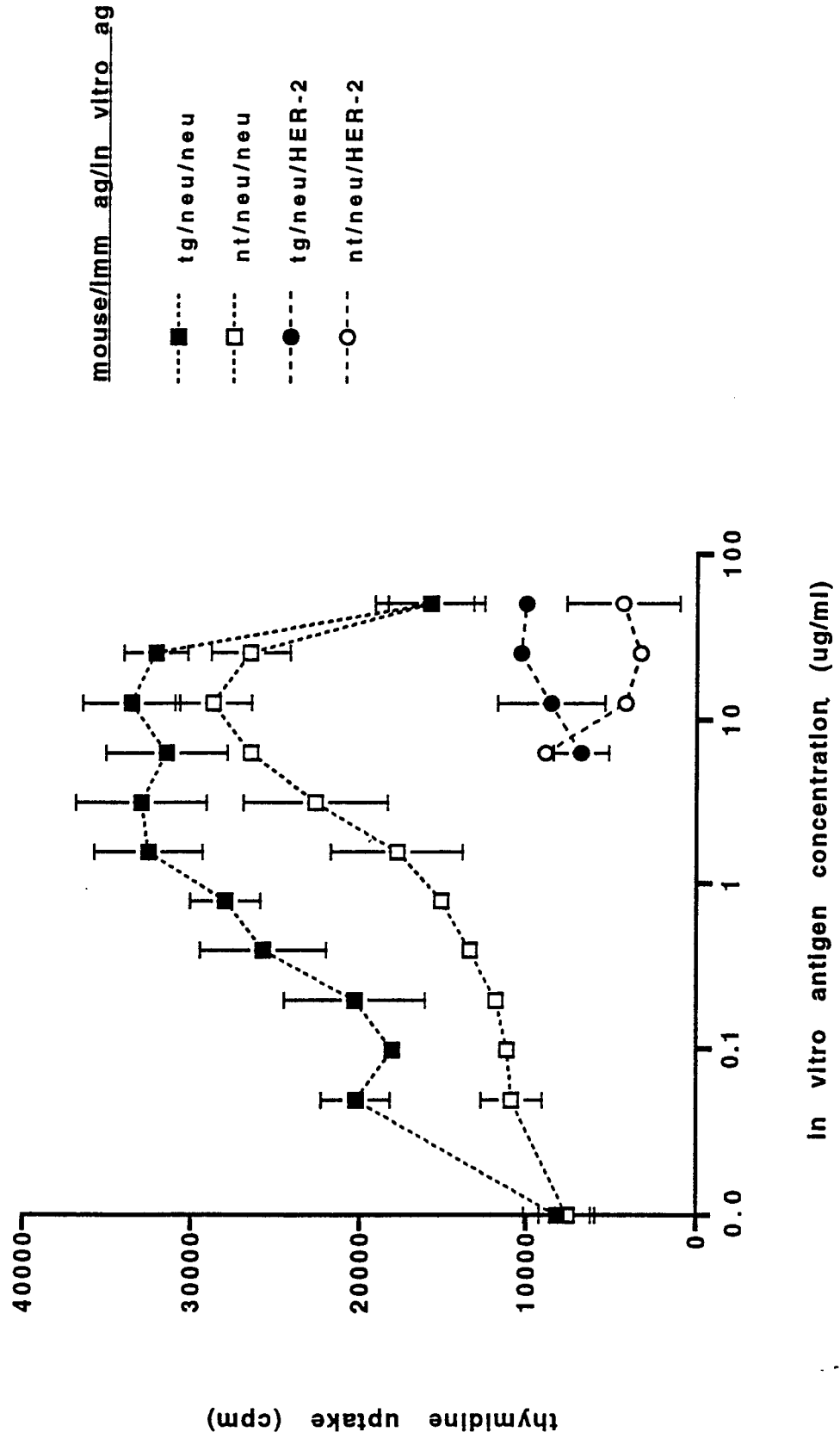
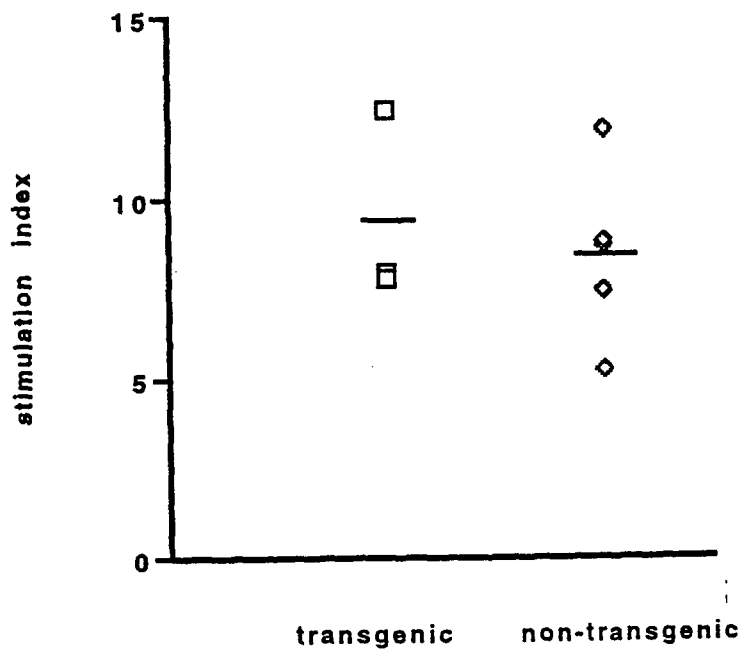


FIGURE 3A

transgenic and non-transgenic mice  
Immunized with CSA



transgenic and nontransgenic mice  
Immunized with CSA

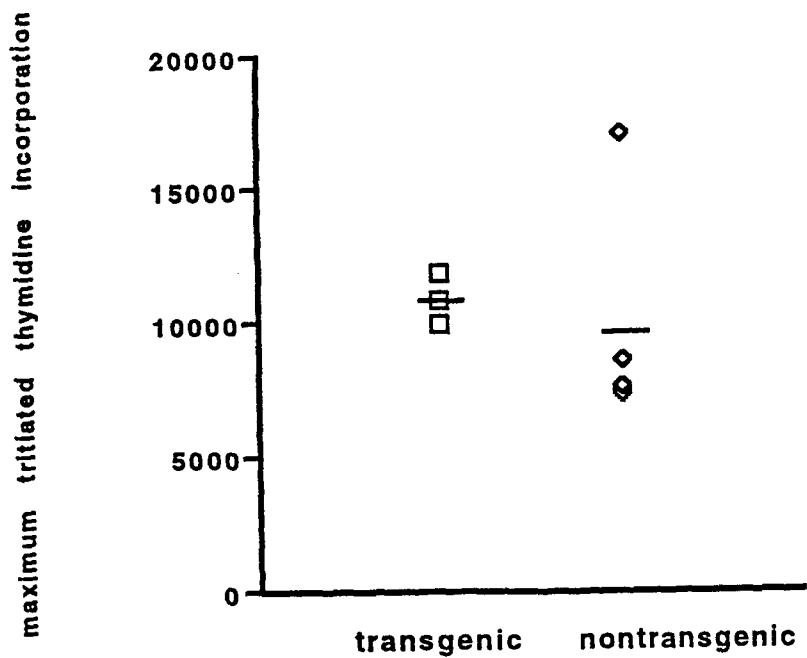


FIGURE 3B

## Kaplan-Meier Plot of Tumor Onset in MMTV-c neu transgenic colony

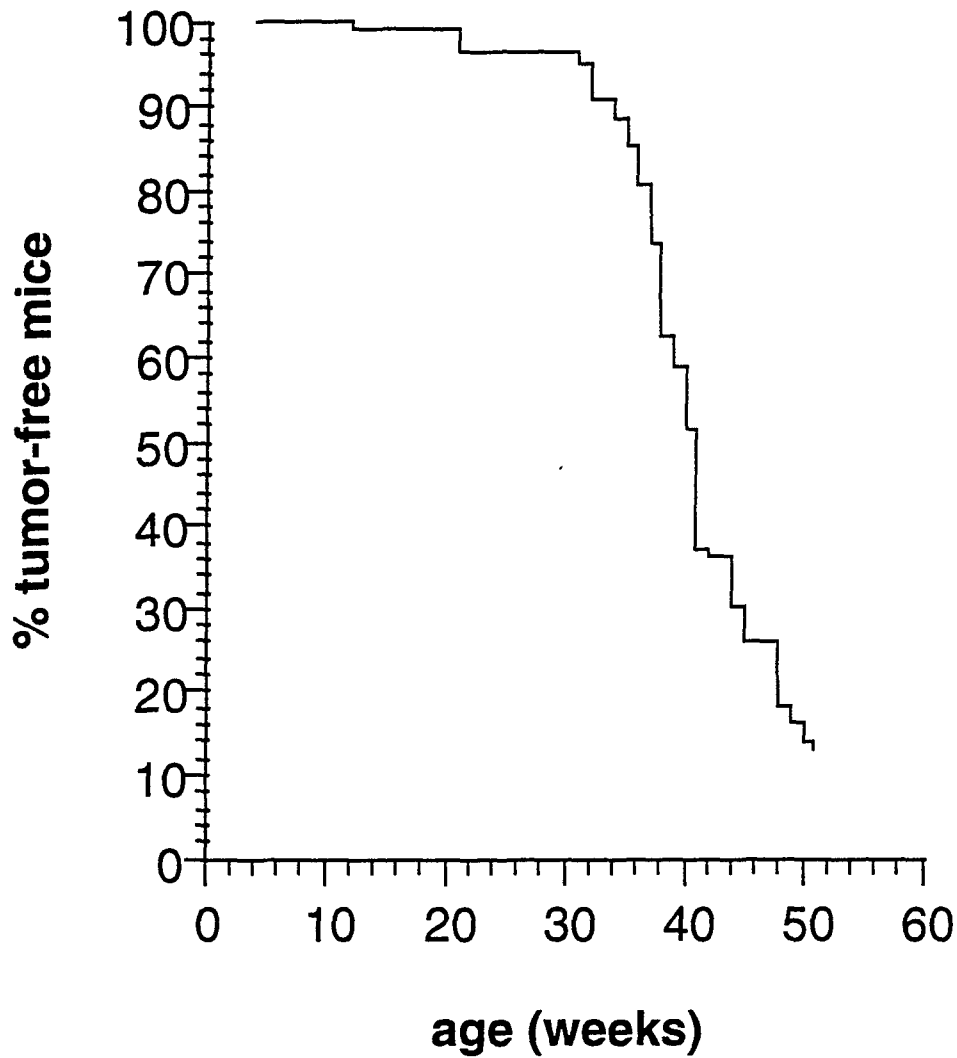
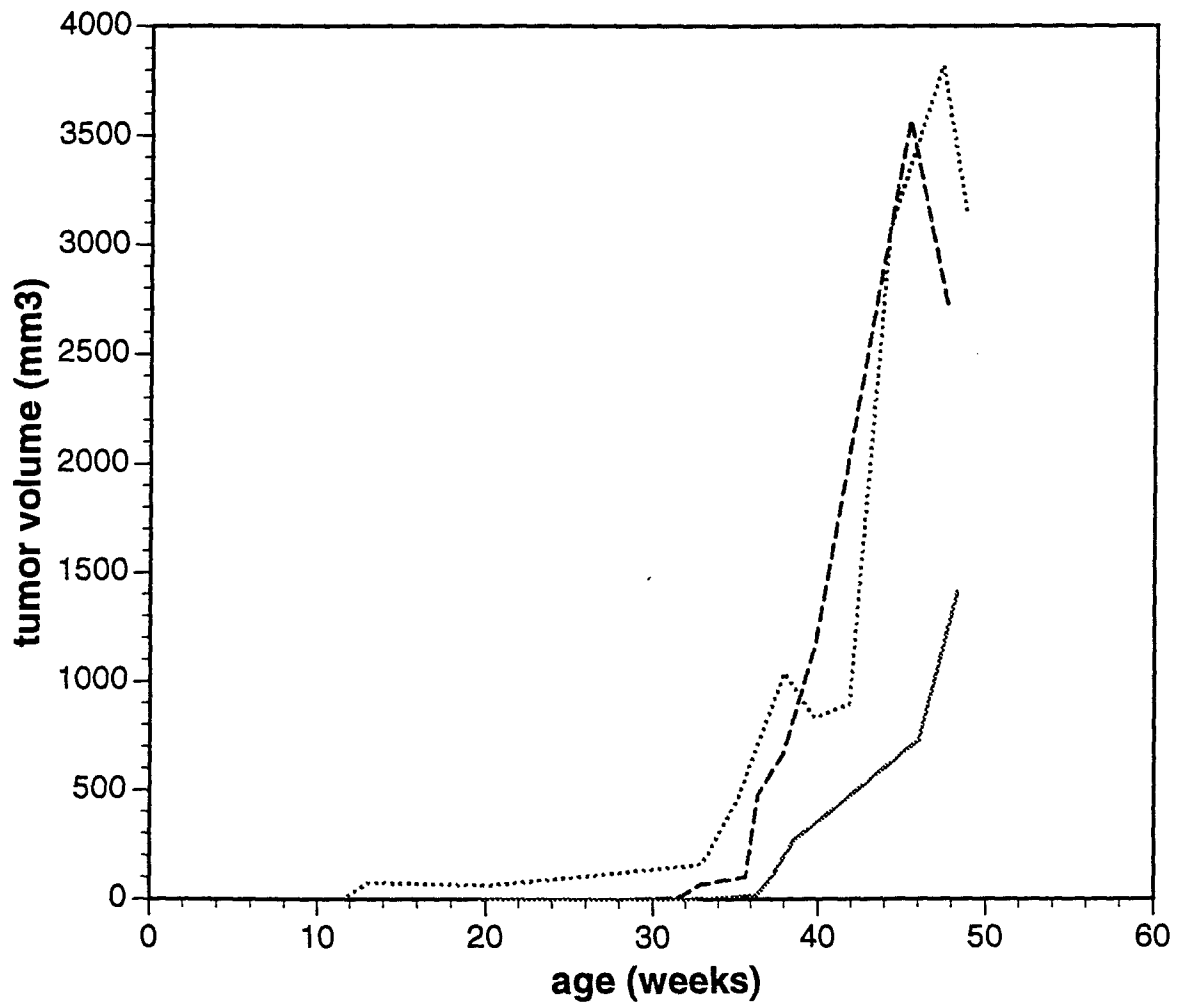


FIGURE 4

## Tumor Burden of MMTV-erbB-2/neu Transgenic Mice



- Transgenic mice immunized with CSA + CFA
- Transgenic mice immunized with erbB-2 + CFA
- ..... Unimmunized mice

**FIGURE 5**

## Kaplan-Meier Plot of Tumor Onset in Immunized Animals

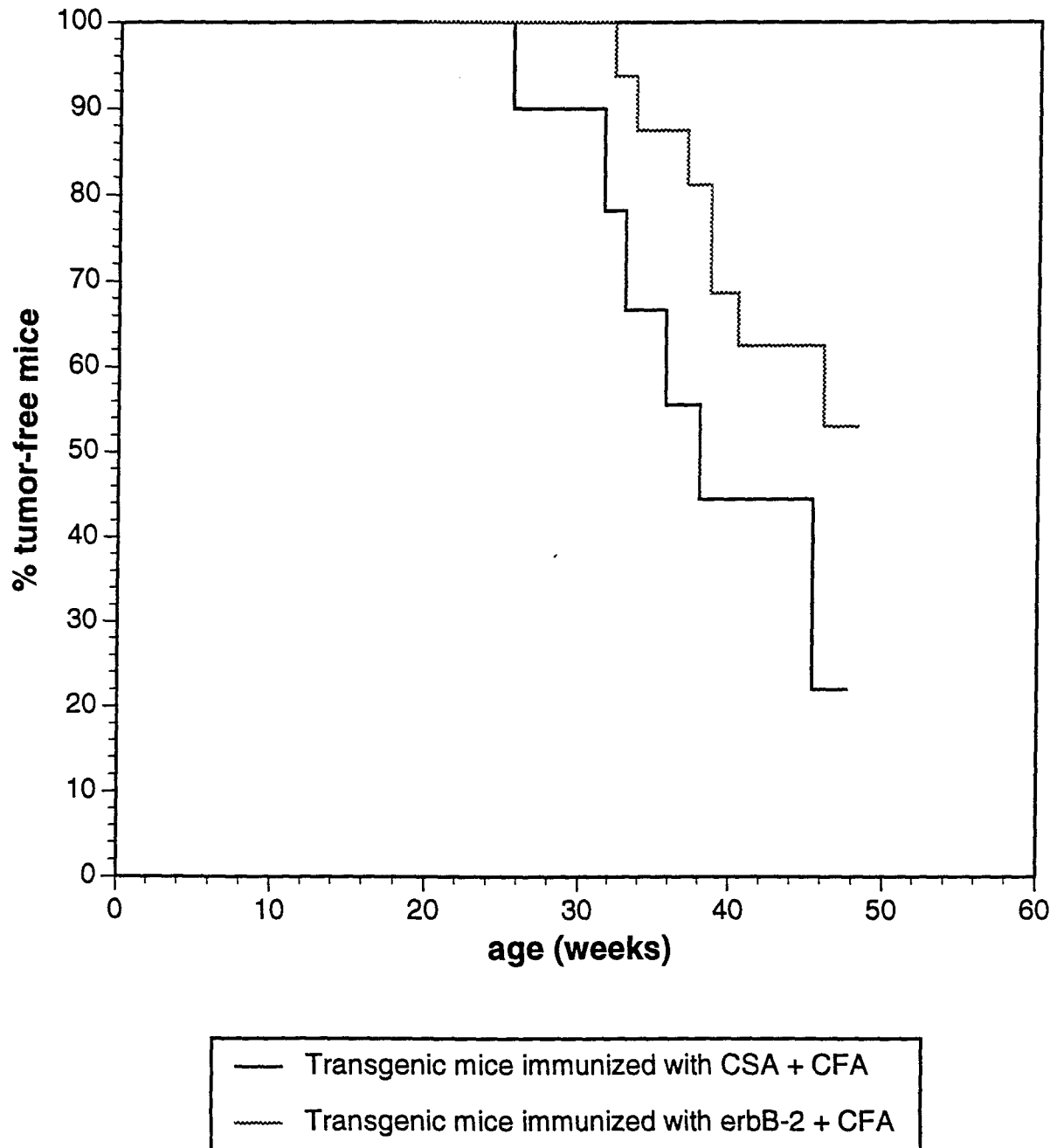


FIGURE 6

## Bibliography of Related Abstracts

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REPLY TO  
ATTENTION OF:

MCMR-RMI-S (70-1y)

10 Aug 98

MEMORANDUM FOR Administrator, Defense Technical Information  
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DAMD17-94-C-4027	ADB196161 †✓
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