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Award Number: DAMD17-00-1-0380

TITLE: The Use of Venezuelan Equine Encephalitis Replicons  
Encoding  
the Her-2/neu Tumor Associated Antigen for the Prevention and  
Treatment of Breast Cancer

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REPORT DATE: May 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE May 2002	3. REPORT TYPE AND DATES COVERED Annual (15 Apr 2001 - 14 Apr 2002)
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4. TITLE AND SUBTITLE THE USE OF VENEZUELAN EQUINE ENCEPHALITISREPLICONS ENCODING THE HER-2/NEU TUMOR ASSOCIATED ANTIGEN FOR THE PREVENTION AND TREATMENT OF BREAST CANCER	5. FUNDING NUMBERS DAMD17-00-1-0380
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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)  
U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

20020930 055

12a. DISTRIBUTION / AVAILABILITY STATEMENT  
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)  
Overexpression and amplification of the Her-2/neu proto-oncogene has been implicated in the development of aggressive human breast cancer. Consequently, Her-2/neu provides a potential target for immunotherapy. Indeed, Her-2/neu specific cytotoxic T lymphocytes (CTL) can be detected in patients with breast and ovarian cancer. Nevertheless, the observed response to Her-2/neu is inadequate to prevent tumor progression. Our overall goal is to determine whether genetic vaccination is a feasible strategy to enhance Her-2/neu specific T cell activity, and in turn prevent and/or treat breast cancer. Specifically, we will employ a novel *in vivo* gene transfer system based on the Venezuelan Equine Encephalitis (VEE) virus. VEE is an alphavirus which has a number of properties that make it especially well suited for tumor antigen vaccination. The most salient of these is the observation that VEE preferentially infects dendritic cells (DC) *in vivo*. DC are known to be potent antigen presenting cells capable of efficiently activating naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Our approach is to establish VEE based replicons encoding Her-2/neu as well as cytokines, such as IL-12 and IL-18 known to promote CD4<sup>+</sup> Th1 and CD8<sup>+</sup> CTL activity. To directly determine the therapeutic efficacy of the VEE-based replicons, mice transgenic for the rat Her-2/neu gene (FVB/neu) which develop spontaneous focal mammary tumors with subsequent pulmonary metastasis will be employed. Additionally, FVB/neu mice bred to mice transgenic for the human major histocompatibility complex (MHC) class I molecule HLA-A2.1 (FVB/neu/A2-K<sup>b</sup>) will be used to examine the immunogenicity of Her-2/neu derived peptides which may have clinical relevance. We believe establishing a vaccine protocol using VEE replicons encoding Her-2/neu and appropriate cytokines will provide an effective strategy for enhancing tumor antigen-specific CD4<sup>+</sup> Th1 and CD8<sup>+</sup> CTL reactivity for the purpose of preventing tumor progression and providing long-term protection from tumor recurrence.

14. SUBJECT TERMS  
breast cancer

15. NUMBER OF PAGES  
7  
16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT  
Unclassified

18. SECURITY CLASSIFICATION OF THIS PAGE  
Unclassified

19. SECURITY CLASSIFICATION OF ABSTRACT  
Unclassified

20. LIMITATION OF ABSTRACT  
Unlimited

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**Overview:**

During the past year, we have been focusing on the objectives outlined for Specific Aim 2 in our approved statement of work, and on completing the objectives for Specific Aim 1. In addition to having completed testing and packaging of VEE replicon constructs encoding Her-2/neu, IL-12, IL-18, IL-2 and mGM-CSF as reported previously, we have completed *in vitro* testing and packaging of a truncated Her-2/neu replicon that encodes only the extracellular and transmembrane domains of the Her-2/neu protein (Neu/ECD-TM). We have begun a series of *in vivo* experiments to evaluate the therapeutic efficacy of this replicon and have obtained our most encouraging results to date.

We have begun a series of experiments in both wild type FVB, and in FVB mice transgenic for the rat Her-2/neu gene (FVB/neu) in which both sets of mice are vaccinated with VEE replicons and subsequently challenged with Her-2/neu expressing tumors. Specifically, both wild type mice which reject the tumor cells, and transgenic mice, which are normally permissive for growth, were twice immunized with  $5.0 \times 10^5$  VEE replicons encoding Neu/ECD-TM ten days apart. Mice were subsequently challenged with  $2.0 \times 10^6$  Her-2/neu<sup>+</sup> F-H2N1 tumor cells injected subcutaneously in the rear flank. Immunized transgenic mice displayed a significant delay in the appearance of tumor formation with one mouse remaining tumor free throughout the course of the experiment (Figure 1). In contrast, unimmunized FVB/neu mice rapidly develop palpable tumors within 10 – 14 days following injection. As expected, both untreated and immunized wild type FVB mice rejected the tumors in all but one instance. In addition to repeating this experiment, we have immunized several groups of transgenic mice with the intention of monitoring spontaneous tumor formation compared to untreated controls.

The rat Her-2/neu gene encodes for an 185kDa transmembrane protein. In order that we may have protein to work with experimentally, we have spliced fragments of the gene into a bacterial expression plasmid that generates HIS tagged protein fragments which may be purified on nickel resin. One protein generated from the bacterial expression system, encompassing parts of the extracellular and transmembrane domains, has been used to stimulate CD4<sup>+</sup> T cell proliferation. Currently, we are working on a protocol in which the HIS tagged protein fragments are affixed to nickel chelate beads. Aggregation of the protein fragments in this manner allows for enhanced uptake by antigen presenting cells and subsequent peptide loading into the MHC Class I pathway for presentation to CD8<sup>+</sup> T cells<sup>1</sup>. We are hopeful that this system for loading class I restricted peptides on APCs will expand our ability to characterize the CD8<sup>+</sup> T cells response to Her-2/neu encoding replicons through the use of *in vivo* CTL and ELISPOT assays.

Recent reports have demonstrated the utility of *in vivo* CTL assays using Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeled target cells<sup>2</sup>. Briefly, splenocytes are pulsed with peptide (to which the subject has been immunized) and labeled with 5 $\mu$ m CFSE. As an internal control, non-pulsed splenocytes are labeled with 0.5  $\mu$ m CFSE, admixed and adoptively transferred to immunized mice. Upon analysis of recovered cells by flow cytometry, the disappearance of antigen pulsed cells (CFSE high peak) relative to internal control cells (CFSE low peak) is indicative of CTL activity. While we have established this system in the lab in another transgenic murine system, the lack of a H2-K<sup>d</sup> restricted Her-2/neu peptide has hampered our efforts in this system. Consequently, the above mentioned nickel bead chelation protocol will allow us to label target cells and more effectively evaluate CTL activity in Her-2/neu immunized mice.

As we have completed testing and packaging of several cytokine encoding replicons, we plan to expand our immunization protocol to include these constructs. We believe the expression of proinflammatory cytokines will further expand the antigen specific population and increase CTL activity. We are working toward determining the optimal combinations of antigen and cytokine encoding VEE replicons for efficient generation of CD4<sup>+</sup> Th and CD8<sup>+</sup> CTL reactivity. This will further allow us to establish an effective approach of immunotherapy targeting Her-2/*neu* for the treatment and prevention of breast adenocarcinoma in transgenic mice.

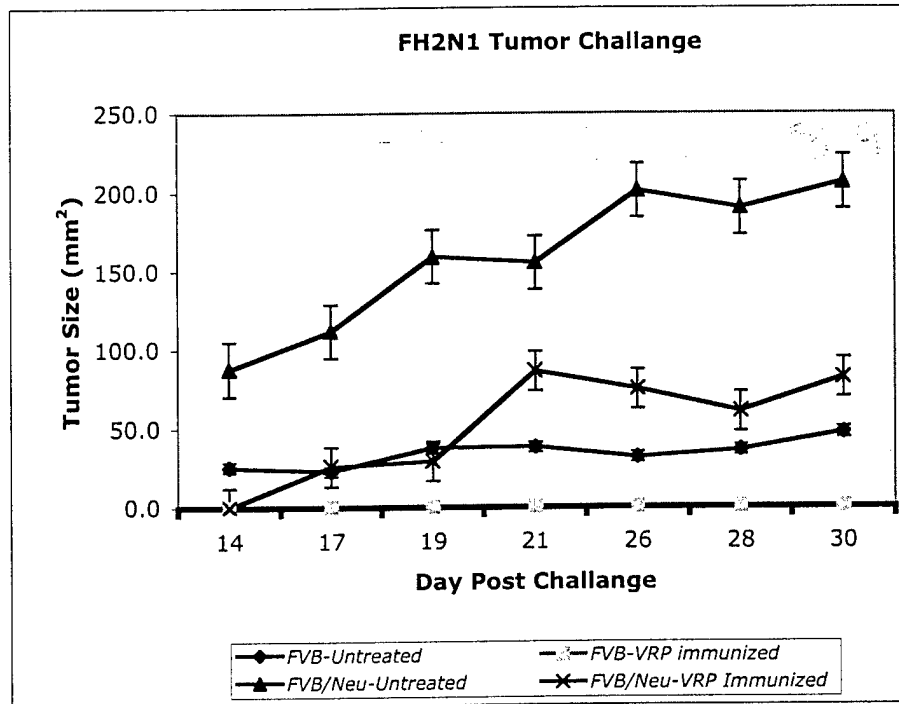
**Key Accomplishments:**

- Completed in vitro testing and packaging of the truncated Her-2/*neu* VEE replicon encoding the extracellular and transmembrane domains of the protein.
- Completed two tumor challenge experiments in VEE Her-2/*neu* (ECD-TM) replicon immunized FVB/*neu* mice demonstrating significantly delayed tumor development.
- We have begun experiments to evaluate the efficacy of VEE replicons encoding the extracellular and transmembrane domains of Her-2/*neu* in the prevention of spontaneous breast adenocarcinoma development in FVB/*neu* mice.
- We are in the process of modifying an in vivo CTL assay protocol for use in the Her-2/*neu* transgenic murine model.

**Reportable Outcomes:**

- None.

## Figures:



**Figure 1.** Delayed tumor growth in VEE replicon immunized mice. FVB and FVB/*neu* mice were immunized twice at 10-day intervals with  $5.0 \times 10^5$  IU VEE replicons encoding the extracellular and transmembrane domains of Her-2/*neu*. One week after the second vaccination, mice received  $2.0 \times 10^6$  F-H2N1 tumor cells injected subcutaneously in the left flank. Challenged mice were monitored for tumor growth over a 30-day period and palpable tumors were measured using a Vernier type caliper. Data is representative of two different experiments, four mice per group. One immunized FVB/*neu* mouse remained tumor free throughout the experiment.

**Summary:**

We have completed much of the work described for Specific Aim 1, and have made significant progress on the experiments required for Specific Aim 2. We have completed all in vitro testing and packaging of replicons including the truncated Her-2/*neu* replicon encoding the extracellular and transmembrane domains of the protein. We have made progress in evaluating the therapeutic efficacy of VEE replicons in preventing the development and progression of breast adenocarcinoma in mice challenged with F-H2N1 tumor cells. Specifically, we have demonstrated delayed onset and inhibited tumor growth in challenged mice immunized with the truncated Her-2/*neu* replicon. We have begun a series of experiments to evaluate the efficacy of VEE replicons in preventing the spontaneous development of breast adenocarcinoma in FVB/*neu* mice.

While we have been able to consistently demonstrate CTL activity against Her-2/*neu* in immunized FVB mice, obtaining these same results in FVB/*neu* mice has proved challenging. We have begun experimenting with a new, more sensitive system for evaluating CTL activity in vivo using antigen pulsed target cell labeled with CFSE. We are hopeful that this assay will allow us to correlate CTL activity with inhibited tumor progression in immunized FVB/*neu* mice. Additionally, we are working on a protocol for coupling HIS tagged Her-2/*neu* protein fragments to nickel beads. These beads should prove useful for labeling APCs with antigen for use with *in vivo* CTL assays as well as ELISPOT assays to further characterize the CD4<sup>+</sup> and CD8<sup>+</sup> T cell cytokine secretion profile.

**References:**

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2. **Hernandez J**, Aung S, Redmond WL, Sherman LA. **2001**. Phenotypic and functional analysis of CD8<sup>+</sup> T cells undergoing peripheral deletion in response to cross-presentation of self-antigen. *Journal of Experimental Medicine* 194(6):707-717.