AD_________________

Award Number: W81XWH-04-1-0678

TITLE: Sensitivity of Breast Tumors to Oncolytic Viruses

PRINCIPAL INVESTIGATOR: Maryam Ahmed, Ph.D.

CONTRACTING ORGANIZATION: Wake Forest University Health Sciences
Winston Salem, NC 27157

REPORT DATE: August 2005

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

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 Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-08-2005	Annual	15 JUL 2004 - 14 JUL 2005

4. TITLE AND SUBTITLE
Sensitivity of Breast Tumors to Oncolytic Viruses

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0678

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Maryam Ahmed, Ph.D.

E-mail: mahmed@wfubmc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Wake Forest University Health Sciences
Winston Salem, NC 27157

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

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

13. SUPPLEMENTARY NOTES
Report contains color plates: All DTIC reproductions will be in black and white.

14. ABSTRACT
The goal of this project is to develop novel therapies for breast cancer based on the oncolytic virus, vesicular stomatitis virus (VSV). Studies have shown that matrix (M) protein mutants of VSV, such as rM51R-M virus, are excellent candidates for anti-tumor therapies due to the ability of these viruses to target and kill tumor cells, while sparing normal cells. However, not all tumors are amenable to VSV treatments in vivo. In data presented here, we determined that normal mammary cells are more resistant to VSV-induced cytopathic effect than breast cancer cells. However, in syngeneic breast cancer system in vivo, rM51R-M virus is only partially effective at killing breast tumors derived from 4T1 cells. Our results indicate that the immune response may be attenuating the replication and spread of this virus at the tumor site. To enhance the ability of rM51R-M virus to selectively target and kill tumor cells, we carried out a combination treatment together with the anti-tumor cytokine, IL-12. Our results indicated that the combination therapy was more effective than individual therapies at preventing the growth of the tumor. Therefore, using a virus that induces the immune response, together with an anti-tumor cytokine, IL-12, enhances the killing potential in an aggressive breast tumor model. The information obtained from these studies will allow us to develop better viral or combination therapies to treat aggressive tumors that are resistant to more conventional therapies.

15. SUBJECT TERMS
Vesicular stomatitis virus, interleukin 12, breast cancer, interferon

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	11	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Cover……………………………………………………………………………………1

SF 298……………………………………………………………………………..……2

Introduction…………………………………………………………..…………....4

Body……………………………………………………………………………………..4

Key Research Accomplishments………………………………………….………10

Reportable Outcomes…………………………………………………………….10

Conclusions……………………………………………………………………….....10

References……………………………………………………………………………..11

Appendices…………………………………………………………………………..

INTRODUCTION:

The goal of this project is to develop novel therapies for breast cancer based on the oncolytic virus, vesicular stomatitis virus (VSV). VSV is currently being developed as an oncolytic virus for the selective treatment of several types of cancers (1-3, 5, 7, 9, 10). Previous studies have shown that VSV kills tumor cells more effectively than many normal cell types due to defects in the antiviral response (including IFN) in tumor cells (3, 9). However, our preliminary studies indicate that several tumor cell lines, including human breast cancer cells, are differentially susceptible to killing by VSV. Experiments proposed here seek to determine whether human breast cancer cells and murine breast cancer cell lines that produce metastatic tumors *in vivo* show differential susceptibility to VSV-induced cell killing. Furthermore, we plan to investigate whether the efficacy of VSV therapies can be enhanced by co-treatment with an anti-tumor cytokine, IL-12. Our hypothesis is that we will enhance killing of tumors that are more resistant to virus infection, as well as those that are highly sensitive to infection.

BODY:

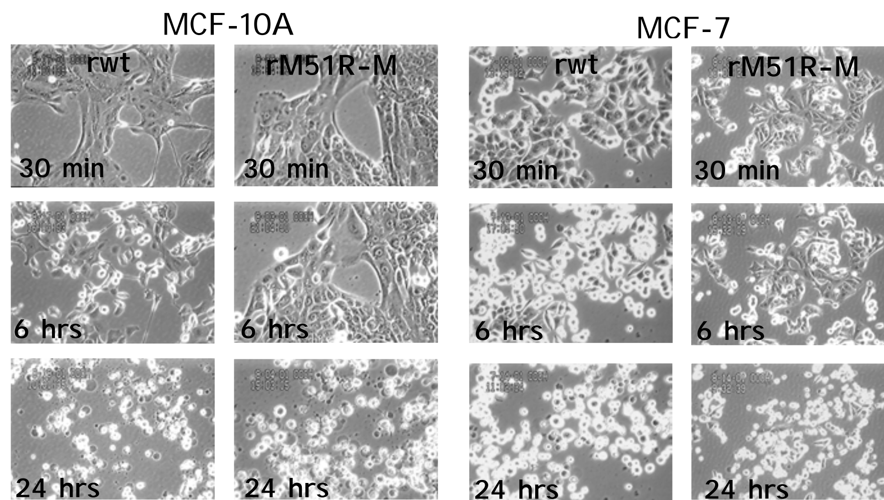
The previous goal of this proposal was to engineer a virus expressing IL-12 to treat breast cancer cells in vivo. However, we modified this goal and received a no-cost extension due to several difficulties that were encountered. First of all, we had a difficult time cloning the IL-12 gene within the VSV genome. Although this has been done with other cytokines, it proved to be more problematic with IL-12. During this work, we also found that another group published a paper in which they used a virus very similar to our M51R-M mutant virus to treat 4T1 cells in vitro (4). This group found that the M51R-M mutant was only partially effective at treating this particular tumor type. 4T1 cells are poorly immunogenic and thus, induce weak tumor-specific immune responses. Therefore, it is possible that these tumor cells grow more aggressively and are thus more difficult to treat with VSV. Therefore, we wanted to ask whether the immune response was aiding, or hindering treatment with VSV. This is a critical question in the oncolytic virus field but few investigators have investigated the role of the immune response during tumor therapies. Furthermore, we wanted to determine whether addition of IL-12 naked DNA would enhance tumor treatment. Thus, we obtained a plasmid from David Mahvi at University of Wisconsin-Madison that expresses IL-12 in vitro and in vivo and has been used to treat tumors in vivo (8). We reasoned that we could better control levels of IL-12 expression from plasmid DNA than that expressed from the virus. This would provide an advantage for therapies in which there exists a fine balance in the ability of the immune response to aid in therapies without eliciting a deleterious inflammatory response. In addition to these studies, we first wanted to ask how individual breast cancer cells versus normal cells respond to infection with VSV.

Aim1: To determine whether human breast cancer cells and a murine breast cancer cell line that produces metastatic tumors *in vivo* (4T1) show differential susceptibility to VSV-induced cell killing (month 1-6).

Task1: Determine the ability of a wt strain of VSV (rwt virus) and an M protein mutant virus (rM51R-M) to kill nontumorigenic cells versus breast cancer cells in vitro.

M protein mutants of VSV are being designed as safer, and effective, vectors for tumor therapies due to their inability to suppress the type I IFN response (1, 9). Therefore, the idea is that these viruses enhance IFN activity in normal cells which serves to attenuate virus replication and spread in normal tissues, while retaining the ability to target and kill tumor cells. To test this hypothesis, we determined the ability of a wt virus (rwt) and an M protein mutant of VSV (rM51R-M) to kill MCF7 breast cancer cells as compared to MCF10A nontumorigenic mammary cells. Cells were infected with rwt and rM51R-M viruses at a multiplicity of 10pfu/cell. At various times post infection, phase contrast images were taken illustrating the cytopathic effects of VSV in these cells

Figure 1: Nontumorigenic MCF-10A cells are more resistant to the cytopathic effects induced by VSV than MCF-7 cells.



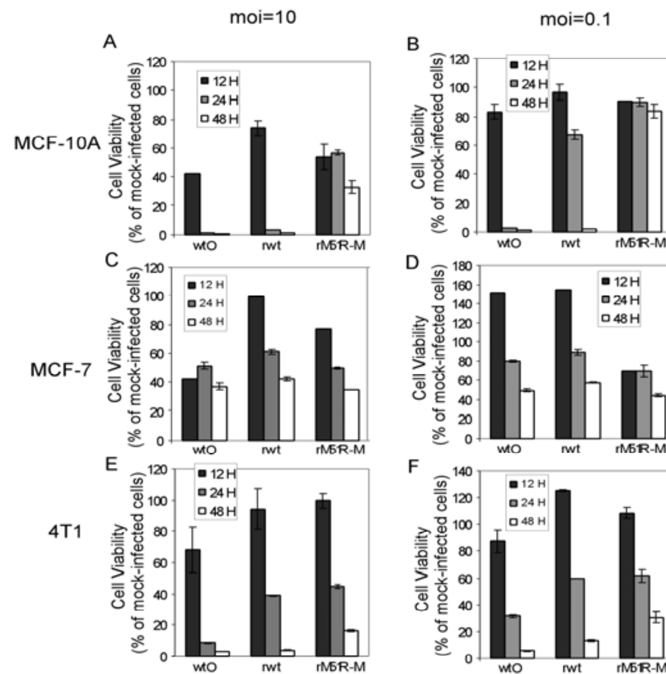
As shown in figure 1, both rwt and rM51R-M virus effectively killed MCF7 breast cancer cells starting as early as 30 minutes post-infection, as indicated by the rounding of cells characteristic of VSV infection. By 6 hours post-infection, most cells were rounded. In contrast, MCF10A nontumorigenic cells were more resistant to VSV-induced cytopathic effect, especially that of rM51R-M virus.

To quantitate the ability of VSV to kill these cells, we carried out an MTT assay to measure the metabolic activity in infected cells (Figure 2). MCF10A, MCF7 and a mouse breast cancer cell line, 4T1, were infected with rwt and rM51R-M viruses at MOIs of 10 and 0.1 pfu/cell. As a control, cells were infected with a naturally occurring wt strain of VSV (wtO) that exhibits cytopathic effect both in vivo and in vitro. The lower MOI (0.1) measures the ability of virus to spread to surrounding cells and exert it cytopathic effect, whereas the higher MOI determines the ability of cells to die in a synchronous virus infection.

At both MOIs (10 and 0.1pfu/cell), MCF10A cells were more resistant to infection with rM51R-M virus than the wt strains of VSV, wtO and rwt viruses (Figure 2). For example at 24-48 hours post-infection, while most of cells infected with rwt and wtO viruses died, 40-80% of rM51R-M virus-infected cells remained viable. This is presumably due to the ability of these cells to mount an effective IFN response to the virus infection. In contrast, rwt and rM51R-M virus were equally effective at killing MCF7 breast cancer cell lines. In addition, the mouse breast cancer

cell line, 4T1, was also susceptible to killing by VSV since by 48hours post-infection, approximately 5-20% of cells infected with each of the viruses remained viable.

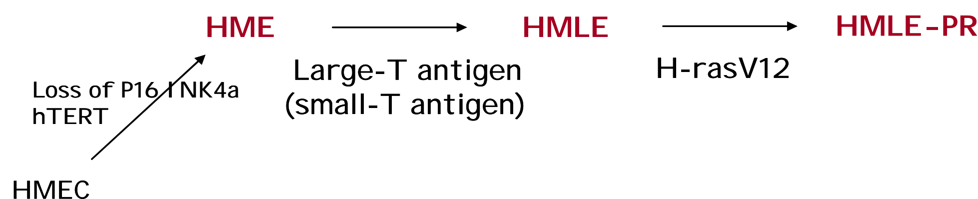
Figure 2: Nontumorigenic MCF-10A cells are more resistant to killing by rM51R-M virus than human and mouse breast cancer cells.



Task 2: Determine the susceptibility of breast cancer cells at different stages of tumorigenesis to infection with VSV.

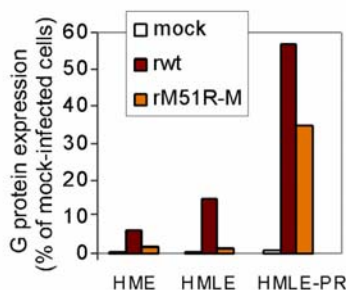
Our hypothesis is that during tumorigenesis, cells acquire defects in their antiviral responses that render them susceptible to infection with VSV. Therefore, as shown in figures 1 and 2, whereas normal cells are resistant to VSV, tumor cells are more sensitive. To further elucidate this point, we utilized mammary cells that are in different stages of tumorigenesis (6). Weinberg's group developed several transformed cell lines by the introduction of specific cancer-associated genes into human mammary epithelial cells (HMECs) as shown in Figure 3. Studies showed that the tumorigenicity of these cells was dependent on the level of ras oncogene expression. Therefore, these cells represent a powerful tool to determine how cancer cells at different stages of tumorigenesis respond to infection with VSV.

Figure 3: Human Breast Cancer Cells Generated by Oncogenic Transformation of HMEC



For these experiments, HME, HMLE (large T antigen) and HMLE-PR (large T antigen and H-Ras) cells were infected with rwt or rM51R-M viruses for 6 hours at a multiplicity of 10 pfu/cell. Cells were incubated with antibodies to the viral G protein and surface expression of G protein was determined by flow cytometry (Figure 4). We can see that HMLE-PR cells are more sensitive to VSV infection than the HME and HMLE cells as indicated by greater surface expression of G protein. This result suggests that as cancer cells acquire greater mutations for growth advantage, they also acquire mutations rendering them more susceptible to infection with VSV.

Figure 4: Human mammary cells transformed with SV40 large-T antigen and H-Ras oncoprotein are more susceptible to VSV infection than normal mammary cells.



In summary, these initial studies show that while breast cancer cells are sensitive to VSV-induced cytopathic effect, normal mammary cells are more resistant to the virus. Furthermore, they show that as cells become more tumorigenic, they may acquire additional mutations in antiviral responses rendering them more susceptible to VSV. These results are similar to those obtained in the prostate system, where normal prostatic epithelial cells obtained from prostatectomy patients are more resistant to infection and killing by VSV than the prostate cancer cell line, LNCaP (1). However, we have shown that prostate cancer cells are differentially susceptible to VSV infection. Therefore, it is important to note that not all cancer cells contain defects in antiviral pathways rendering them sensitive to VSV infection. Therefore, cancer cells may evolve to be inherently resistant to treatment with a variety of therapeutic agents, including VSV. In addition, it is possible that the immune response may also alter the ability of VSV to target and kill tumor cells in vivo. Therefore, Aim 2 of this proposal addresses these concerns and seeks to determine whether the effectiveness of VSV treatment can be enhanced by co-treatment with the anti-tumor cytokine, IL-12.

Aim 2: Determine the ability of VSV, together with IL-12, to treat breast cancer in vivo.

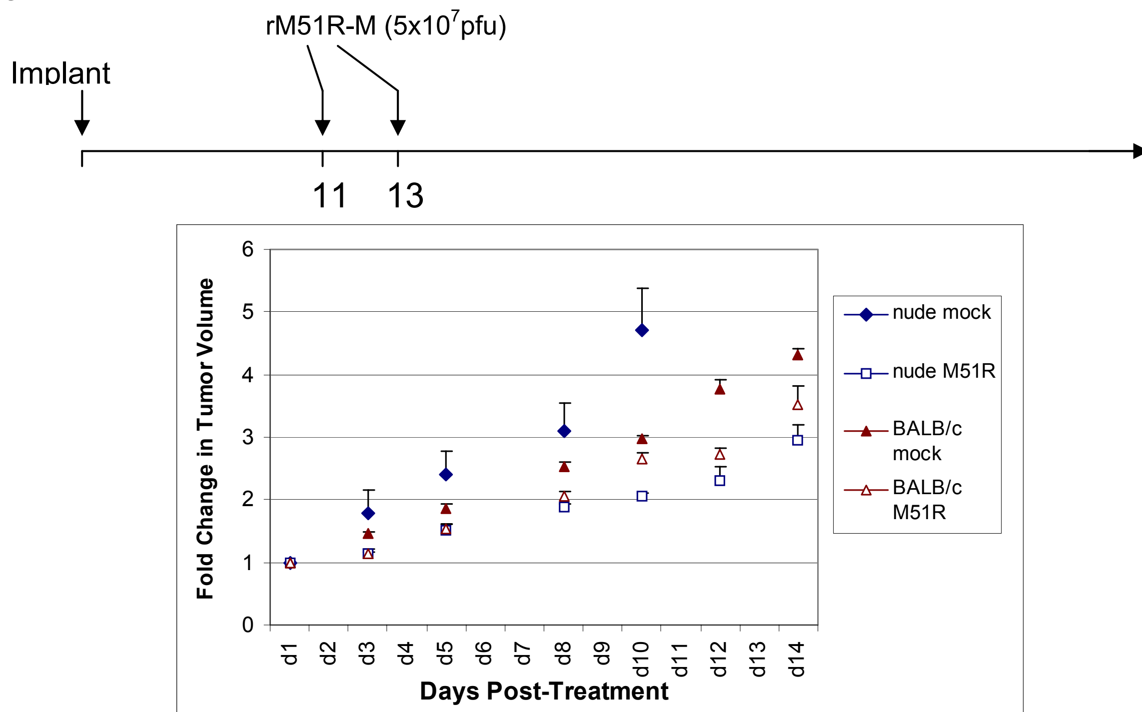
Task 1: Determine the role of the immune response during anti-tumor therapies with VSV. This will be done by implanting 4T1 cell in the flanks of athymic nude mice and immuno-competent mice and treating them intratumorally with rM51R-M virus.

For these pilot experiments, $5x10^5$ 4T1 cells were implanted in the flanks of BALB/c nu/nu and BALB/c wt mice. When palpable tumors had formed, they were treated with rM51R-M virus intratumorally at day 11 and 13 post-implantation or mock-treated. 5 mice were included in each group. Each treatment provided $5x10^7$ pfu of virus at the tumor site. The tumor volume and weight of mice was measured 3 times/week. The health status of the mice was monitored daily.

Animals exhibiting signs of lethal VSV infection, such as hind limb paralysis, excessive tumor burden, or other signs of illness were euthanized as indicated by ACUC guidelines of Wake Forest University Health Sciences.

Figure 5 shows the fold change in tumor volume (as measured by the formula w^2 x L/2, where w=width of tumor and L=length of tumor) from the time of the initial treatment. The tumors of mock-treated nude animals showed uncontrolled growth over time and had to be euthanized by day 10 post-treatment due to excessive tumor burden. In contrast, nude mice treated with VSV showed decrease in rate of tumor growth indicating that rM51R-M virus was effective at delaying rate of tumor growth. BALB/c wt animals that were mock-treated also exhibited uncontrolled growth of tumor, but did not reach levels seen in the mock-treated nude animals. These data illustrate that the immune response does play some role in decreasing growth of the tumor. In addition, although the tumor volumes of BALB/c wt animals treated with rM51R-M virus decreased to the same levels as that seen with rM51R-M treated nude animals, the difference from that of mock-treated wt animals was not very great. These pilot experiments suggest that the immune response does control growth of the tumor and may play a role in decreasing the effectiveness of rM51R-M virus therapies in these animals, perhaps by attenuating virus infection and spread. However, we plan to carry out further experiments in additional animals to obtain statistical significance and complete these studies.

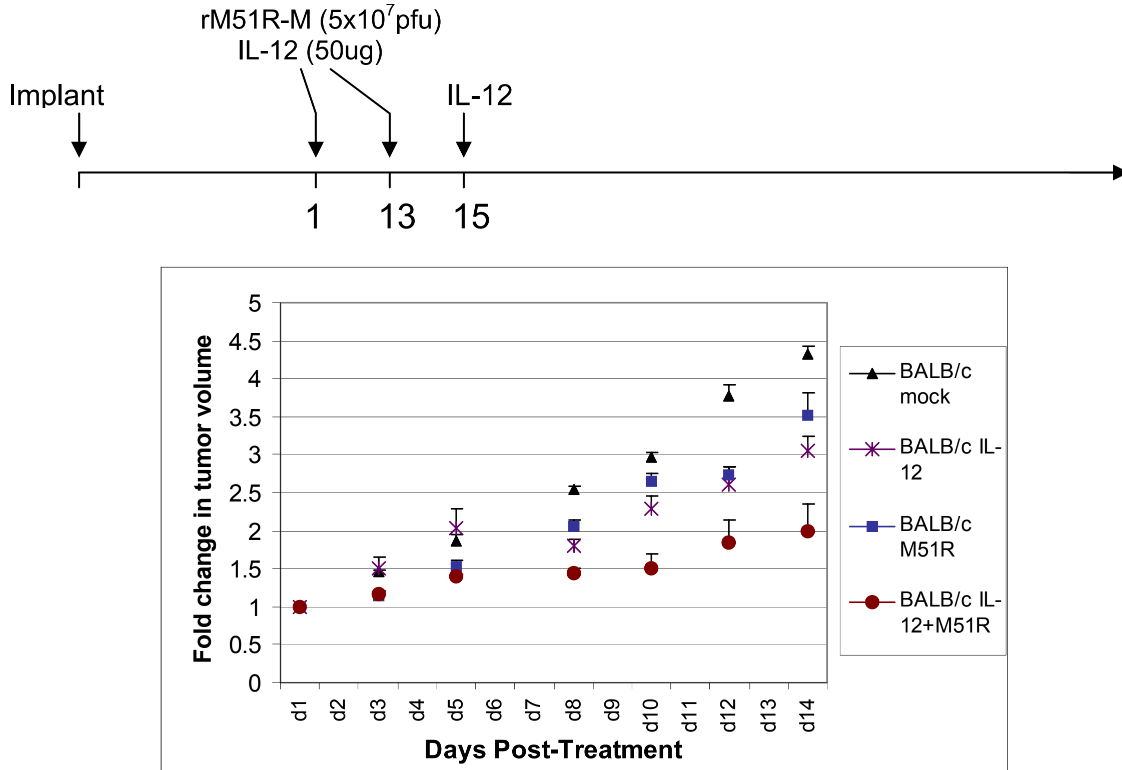
Figure 5: Treatment of 4T1 tumors in BALB/c wt and nude animals



Task 2: Determine the effectiveness of rM51R-M virus therapies combined with IL-12 for the treatment of breast cancer in vivo.

For these experiments, we implanted 4T1 cells into the flanks of BALB/c mice as before. When palpable tumors had formed, we divided animals randomly into 4 groups of 5 mice. Group 1 was mock-treated, Group 2 was treated with rM51R-M virus at day 11 and 13 post-implantation. Group 2 was treated with 50ug of IL-12 naked DNA at days 11, 13 an 15 post-implantation. Group 4 received treatment with both rM51R-M and IL-12 according to the respective schedules. As before, tumor volumes, weights of animals, and signs of illness were monitored consistently. Figure 6 shows the changes in tumor volumes over the course of the treatment.

Figure 6: Treatment of breast tumors with rM51R-M virus and IL-12.



Data indicates that while rM51R-M virus and IL-12 therapies alone had a minimal effect on tumor killing, the combination of rM51R-M virus together with IL-12 produced a more dramatic result. This is a very exciting result that suggests that boosting the immune response with both an oncolytic virus that activates the immune response as well as a cytokine that produces tumor specific T cell responses provides the best therapy for a tumor type that is poorly immunogenic and may have mechanisms to suppress immune responses. The combination therapy may be able to overcome some of those suppressive mechanisms. Although this is a very interesting result, these data must be repeated with additional animals. Furthermore, we are in the process of carrying out studies to determine the nature of the immune response by determining IL-12 levels in tumor tissues as well and IFN gamma levels in the spleens of treated animals. Upon completion of these studies, we will be able to better understand the role of the immune response in treatment of this particular type of aggressive breast cancer model and perhaps use this information to design more effective viruses and combination therapies for treatments.

KEY RESEARCH ACCOMPLISHMENTS:

- VSV, specifically rM51R-M virus, kills tumor cells while sparing normal cells
- Cells at later stages of tumorigenesis acquire mutations that render them more susceptible to infection with VSV.
- The immune response serves to limit growth of aggressive 4T1 tumors.
- rM51R-M virus decreases tumor volume in BALB/c nude mice, but does not do so in wt immunocompetent animals.
- The combination of rM51R-M virus and IL-12 therapy is most effective at decreasing tumor burden in vivo.

By boosting the immune response by treatment with factors that enhance IL-12 production (and subsequent anti-tumor effects), we may be able to more effectively treat aggressive tumors that are resistant to more conventional therapies.

REPORTABLE OUTCOMES:

Abstract: Department of Defense Breast Cancer Research Program Meeting, 2005. Sensitivity of Breast Tumors to Oncolytic Viruses.

Seminar: Breast Cancer Center of Excellence, Wake Forest University Health Sciences, 2006. Treatment of Breast Cancer with Oncolytic VSV.

CONCLUSIONS:

The data from these studies throw light on several key questions in the oncolytic virus field that we seek to study in more detail in future proposals. The first key point our results make is that normal mammary cells are more resistant to VSV infection than are breast cancer cells. In addition, as tumor cells evolve greater mutations for growth advantages, they are also more susceptible to virus infection. This may not be the case for all tumors, but is a concept that holds true to many types. The second key point is that the immune response may both hinder and aid in tumor therapies. It may hinder oncolytic therapy by attenuating virus growth and spread. However, it may aid in therapies by promoting tumor specific immune responses. In these studies, using a virus that induces the immune response, together with an anti-tumor cytokine, IL-12, enhances the killing potential in an aggressive breast tumor model.

This project has been changed from the original format, but we think the changes have greatly improved the project and given us valuable insights into oncolytic tumor therapies. Very few people have evaluated the role of the immune response during virus therapies. Therefore, these studies provide the basis for more extensive studies to determine the role of the immune response during treatments with VSV. The information obtained from these studies will allow us to develop better viral or combination therapies to treat aggressive tumors that are resistant to more conventional therapies.

REFERENCES:

1. **Ahmed, M., S. D. Cramer, and D. S. Lyles.** 2004. Sensitivity of prostate tumors to wild type and M protein mutant vesicular stomatitis viruses. Virology **330:**34-49.
2. **Balachandran, S., and G. N. Barber.** 2001. Oncloytic activity of vesicular stomatitis virus is effective against tumors exhibiting aberrant p53, Ras, or Myc function and involves the induction of apoptosis. J. Virol **75:**3474-3479.
3. **Balachandran, S., and G. N. Barber.** 2000. Vesicular stomatitis virus (VSV) therapy of tumors. IUBMB Life **50:**135-138.
4. **Ebert, O., S. Harbaran, K. Shinozaki, and S. L. C. Woo.** 2004. Systemic therapy of experimental breast cancer metastases by mutant vesicular stomatitis virus in immune-competent mice. **12:**350-358.
5. **Ebert, O., K. Shinozaki, T. G. Huang, M. J. Savontaus, A. Garcia-Sastre, and S. L. Woo.** 2003. Oncolytic vesicular stomatitis virus for treatment of orthotopic hepatocellular carcinoma in immune-competent rats. Cancer Res. **63:**3605-11.
6. **Elenbaas, B., L. Spirio, F. Koerner, M. D. Fleming, D. B. Zimonjic, J. L. Donaher, N. C. Popescu, W. C. Hahn, and R. A. Weinberg.** 2001. Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. Genes Dev. **15:**50-65.
7. **Huang, T. G., O. Ebert, K. Shinozaki, A. Garcia-Sastre, and S. L. Woo.** 2003. Oncolysis of hepatic metastasis of colorectal cancer by recombinant vesicular stomatitis virus in immune-competent mice. Mol. Ther. **8:**434-40.
8. **Shi, F., A. L. Rakhmilevich, C. P. Heise, K. Oshikawa, P. M. Sondel, N.-S. Yang, and D. M. Mahvi.** 2002. Intratumoral Injection of Interleukin-12 Plasmid DNA, Either Naked or in Complex with Cationic Lipid, Results in Similar Tumor Regression in a Murine Model. Mol Cancer Ther **1:**949-957.
9. **Stojdl, D. F., B. Lichty, S. Knowles, R. Marius, H. Atkins, N. Sonenberg, and J. C. Bell.** 2000. Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. Nat. Med. **6:**821-825.
10. **Stojdl, D. F., B. D. Lichty, B. R. tenOever, J. M. Paterson, A. T. Power, S. Knowles, R. Marius, J. Reynard, L. Poliquin, H. Atkins, E. G. Brown, R. K. Durbin, J. E. Durbin, J. Hiscott, and J. C. Bell.** 2003. VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. Cancer Cell. **4:**263-75.