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TITLE: The Preclinical Evaluation of Fever-Range, Whole Body
Hyperthermia as an Adjuvant to Chemotherapy and Cytokine
Immunotherapy for the Treatment of Breast Cancer

PRINCIPAL INVESTIGATOR: Michele T. Pritchard

CONTRACTING ORGANIZATION: Health Research, Incorporated
Buffalo, New York 14263

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6. AUTHOR(S)
Michele T. Pritchard

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Health Research, Incorporated
Buffalo, New York 14263

E-Mail:
mtp@acsu.buffalo.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

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U.S. Army Medical Research and Materiel Command
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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)
This grant was written to examine the effect of combining fever-range heat treatments (WBH) with cytokine immunotherapy and chemotherapy in a mouse model of human breast cancer. Progress in year number three of this grant has revealed that the 4T1 mouse model is sensitive to WBH, resulting in a tumor growth delay and illustrative of similar effects of heat in other mouse models in the laboratory of the PI's mentor. In addition, it has been found that the combination of IL-12 to whole body heat treatment enhances the anti-tumor effect of WBH alone. Mechanisms behind the observed enhancement in anti-tumor response remain to be clearly identified although work using another mouse model for human colon cancer suggests that the down stream effector molecule, IFN- γ , may play an important role. Unfortunately, many questions remain to be answered with regards to whether or not the anti-tumor effect of a chemotherapeutic drug, Doxorubicin, in both free and liposome-encapsulated form, can be enhanced using WBH. Finally, an examination of the effect of microsphere encapsulated IL-12 remains to be completed. However, the training value of this grant has still been exceptional over all three years and is viewed by the PI to have been the greatest opportunity and achievement of her graduate career.

14. SUBJECT TERMS
breast cancer, whole body hyperthermia, cytokine immunotherapy, chemotherapy

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Introduction

Year number three was an exciting one on many fronts yet disappointing in others. Importantly, I have been able to show, and will describe in greater detail below, that fever-range whole body hyperthermia (WBH) treatment of mice bearing the 4T1 mammary adenocarcinomas results in a tumor growth delay. It also leads to an increase in the diameter of blood vessels in the tumor bed and an increased infiltration of eosinophils into the tumor bed. Interestingly, WBH led to a decrease in lung weight (used as a measure of mammary tumor metastasis), while slightly increasing the number of tumor clones established in a clonogenic assay. When combined with free IL-12, WBH treatments have been shown to increase survival of 4T1 bearing mice and increase the number of total tumor cures, decrease the number of lung tumor metastasis and somewhat surprisingly, decrease the number of IFN- γ spots produced by cells from the draining lymph node in an ELISPOT assay. These data are in accordance with work done in year number two using the mouse colon adenocarcinoma, CT26. Unfortunately, the examination of the effect of WBH and Doxil and free doxorubicin has not been addressed in the 4T1 model up to the present time. Disappointingly, the availability of microsphere encapsulated IL-12 has been restricted and therefore I have been unable to address whether or not WBH and microsphere encapsulated IL-12 shows the same efficacy in the 4T1 model as has been shown using the CT26 model.

Body

The effect of fever range whole body hyperthermia (WBH) on the growth and metastasis of the 4T1 mammary adenocarcinoma grown in BALB/c mice

Although not specifically written in the approved Statement of Work, it was necessary for me to determine whether or not the growth of the murine mammary carcinoma, 4T1, would be inhibited by WBH treatments prior to the use of this mouse model for examination of the effect of WBH with interleukin 12 (IL-12) or Doxil, doxorubicin treatment. Our lab has already shown, in many different mouse models (1, 2), that WBH inhibits the growth of tumors. I have been able to show that WBH treatment of mice bearing 4T1 tumors results in a tumor growth reduction (refer to Figure 1 (and all future Figures) in the Appendix). Other parameters by which we gauge success of WBH such as increased diameter of tumor blood vessels and immune cell infiltration of the tumor bed have also been observed in the 4T1 model (Figure 2, A and B). Interestingly, while the changes in blood vessel diameter are similar in all models thus far examined, I have observed a greater increase in infiltrating eosinophils in tumors of WBH treated, 4T1 bearing, animals (Figure 2, C and D) as compared to our other data where neutrophils and NK cells appear to be more prevalent. Interestingly, the eosinophil infiltration is restricted mostly to the periphery of primary 4T1 tumors from control mice, while the eosinophil infiltration can be found deep in the center of the WBH treated 4T1 tumors as well as the periphery. Suprisingly, the eosinophils in heated tumors are found quite some distance from the blood vessel from which we believe they extravasated.

The effect of WBH on lung metastasis of 4T1 from the primary tumor located in the mammary fat pad to the lung is less clear. While the weight of lungs, which is often used as a measure of tumor burden, is less in WBH treated animals compared to control animals (Figure 3, A) the number of tumor clones generated by WBH treated lungs in a clonogenic assay (also used to measure tumor burden) is slightly greater in the WBH treated mice. However, this difference is not statistically significant.

The effect of the combination of WBH and interleukin 12 (IL-12) on the growth and metastasis of 4T1 tumors in BALB/c mice

In year number two of this predoctoral grant, I was able to show that the combination of WBH and IL-12, whether given as free drug or encapsulated in a polylactic acid microspheres, resulted in a greater tumor growth delay, greater number of cures and a larger amount of IFN- γ measured in the serum of CT26 bearing BALB/c mice receiving the combination treatment when compared to controls. This past year, I have begun to characterize the combination of these two treatments on the growth and metastasis of 4T1 mammary adenocarcinomas, also syngeneic in BALB/c mice. When combined with free IL-12 given for three cycles (100ng/mouse/day for 5 days, rest for two days then repeated 2 more times), WBH has been shown to enhance survival of animals whose tumors were not completely resolved by the treatment combination as well as result in a greater number of tumor cures (Figure 4). In this experiment, 1×10^5 4T1 cells were implanted in the mammary fat pad and the first IL-12 dose and the single WBH treatment were given when the tumors were palpable (2x2mm, day 0). The survival of the mice was monitored over the next 70 days. Not only was survival enhanced (Figure 4), but, there were a greater number of tumor cures in the animals receiving the combination treatment compared to those receiving IL-12 alone (Figure 5). Finally, of tumors that were not completely cured by the combination treatment, their growth was significantly inhibited compared to those treated with IL-12 alone.

I used the ELISPOT assay in one experiment as a way to look for the number of activated cells in the tumor draining lymph nodes from mice from all experimental groups. By counting the number of IFN- γ + spots, an assessment of the number of activated cells in a total number of cells plated can be made. A greater number of IFN- γ spots suggests a larger number of tumor reactive cells. Although slightly enhanced, the number of IFN- γ positive spots between control and IL-12 treated mice was not significant. If one examines the number of IFN- γ spots in wells containing cells from IL-12 and WBH treated mice, there seems to be a decrease when compared to those treated with IL-12 alone, although this decrease is also not a significant one. It is interesting to conjecture, however, that maybe the IFN- γ producing cells have relocated to the site of the tumor in these particular animals. Work is still underway to look for the presence of these cells in the tumor bed.

Training accomplishments of year number three

In addition to systematically working through a series of revealing experiments, a significant training accomplishment for year number three included the implementation and characterization of a new and highly relevant mammary adenocarcinoma tumor model (4T1) in the laboratory of the PI's mentor which will be used in many future studies. It has been as rewarding as it has been educational to be responsible for establishing a model of such relevance in the lab.

Two new techniques were learned in year number three. The ELISPOT assay is widely used and accepted as a way to measure activated T cells by means of a cytokine, IFN- γ , they produce when they are activated. This technique, as well as intracellular cytokine staining for flow cytometric determination of cytokine producing cells which was also learned in year number three, will be valuable tools for the PI's future work.

Another very important training accomplishment due to the funding provided by this predoctoral grant actually involves the work completed in year number two. In that year, the PI gave a poster presentation and short seminar at the North American Hyperthermia Society and Radiation Research Society Annual Meeting which took place in San Juan, Puerto Rico in April 2001. Dennis Leeper, Ph.D. head of the Radiation Oncology Department at Thomas Jefferson University in Philadelphia, PA was in attendance at this meeting. During the summer of 2001, Dr. Leeper contacted the PI and invited her to give a talk in his Department as part of a recruitment mission. At the end of the visit to Philadelphia in November 2001, the PI was offered a postdoctoral position upon completion of her Ph.D. (set to occur by winter 2002-2003) (see announcement flyer in the Appendix).

Key Research Accomplishments

- Characterization of the 4T1 model, a new model for the mentor of the PI, has been a significant contribution to the lab and a good training experience for it has increased the number of mouse models for studying cancer with which the PI is familiar.
- It has been shown that WBH can induce a tumor growth delay, as well as induce the enlargement of blood vessels in and infiltration of immune effector cells into the tumor bed in the 4T1 model as previously seen in other models in the laboratory of the PI.
- WBH and IL-12 seem to at least function in an additive way on the growth of 4T1 tumors by enhancing survival and increasing the number of tumor cures. However a mechanism for this enhancement, i.e. a definitive increase in the number of tumor reactive cells has yet to be determined.

Reportable Outcomes

An invited book chapter for the Methods journal describing whole body hyperthermia treatment of mice is currently in its final stages. The PI is the first author on this chapter.

A single, one hour student seminar was given by the PI in November 2001 at Roswell Park Cancer Institute, Department of Immunology.

The PI gave a one hour seminar at Thomas Jefferson University, Department of Radiation Oncology in November 2001 upon invitation by Dennis Leeper, Ph.D.

No patents or licenses were applied for and/or issued

No degrees were conferred

No cell lines, tissue or serum repositories have been developed.

No additional funding has been applied for

Conclusions

A strong foundation has been made for the successful completion of the PI's Ph.D. in the next 6 to 8 months based solely on the support of this predoctoral grant. A great deal has been learned both scientifically and in other less tangible ways particularly with regards to how to cope with technical and other challenges. Endurance has paid off in many regards although progress was not as quick or as complete as the PI had anticipated. Herein lay another training accomplishment of this predoctoral grant.

Conclusions based on the data outlined in this report can be summarized as follows:

- Fever-range whole body hyperthermia inhibits the growth of 4T1 primary tumors.
- Fever-range whole body hyperthermia enlarges tumor blood vessels and induces an infiltrate of eosinophils into the center of the tumor.
- The combination of WBH and IL-12 treatments results in an increase in the survival of mice bearing 4T1 tumors.
- WBH +IL-12 treatment increases the number of tumor cures and significantly decreases tumor growth compared to IL-12 treatment alone.
- IL-12 treatment may slightly increase the number of IFN- γ producing cells from the draining lymph node of 4T1 bearing mice.

References

1. Burd, R, Dziedzic, T.S., Xu, Y., Caligiuri, M.A., Subjeck, J.R., Repasky, E.A. Tumor cell apoptosis, lymphocyte recruitment and tumor vascular changes are induced by low temperature, long duration whole body hyperthermia. *Journal of Cellular Physiology*, 177:137-147, 1998.
2. Tims, E., Burd, R., Pritchard, M., Repasky, E.A., *Infectious Diseases in Obstetrics and Gynecology*, 7:91-97, 1999.

Appendix

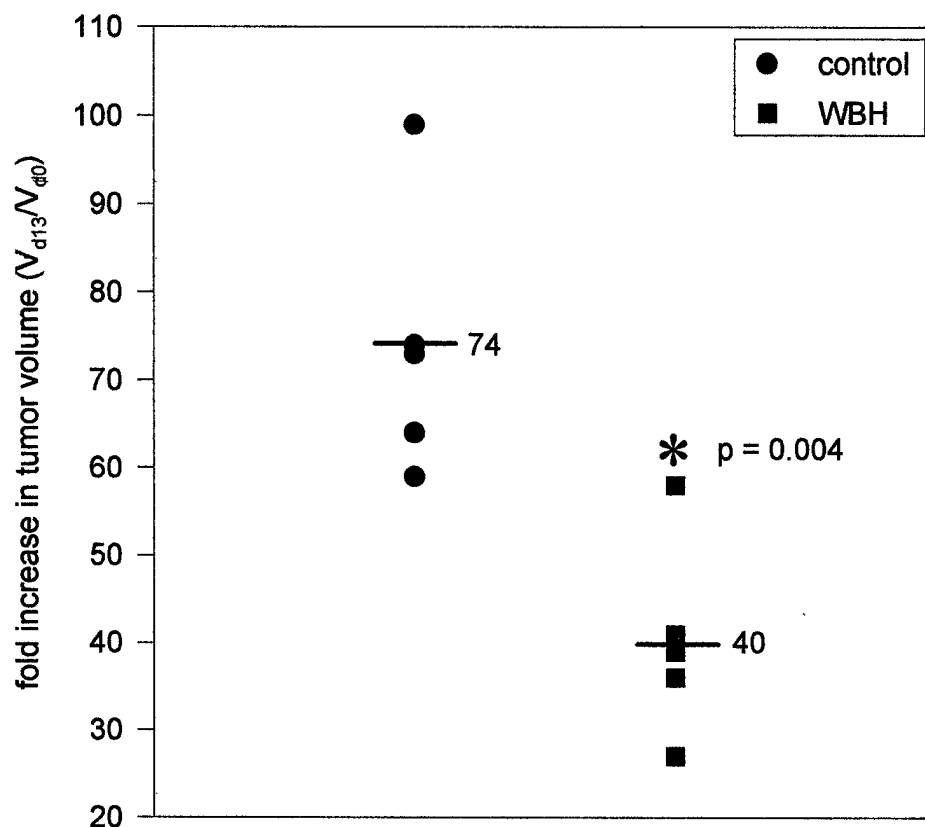


Figure 1. Fever-range whole body hyperthermia inhibits the growth of 4T1 primary tumors. On day 0, just prior to WBH, and day 13, the smallest (a) and largest (b) diameter of control and WBH treated tumors were measured from which tumor volumes were calculated using the formula: V (in mm^3) = $(a^2)(b)(0.4)$. Then fold increase in tumor volume was determined by dividing the volume of each tumor on day 13 by the volume of the same tumor on day 0. The black bars indicate the experimental mean of each group. * $p = 0.004$ by unpaired Student's t test and is representative of 2 separate experiments ($n = 5$ mice per group). These data are representative of 3 similar experiments.

Appendix (continued)

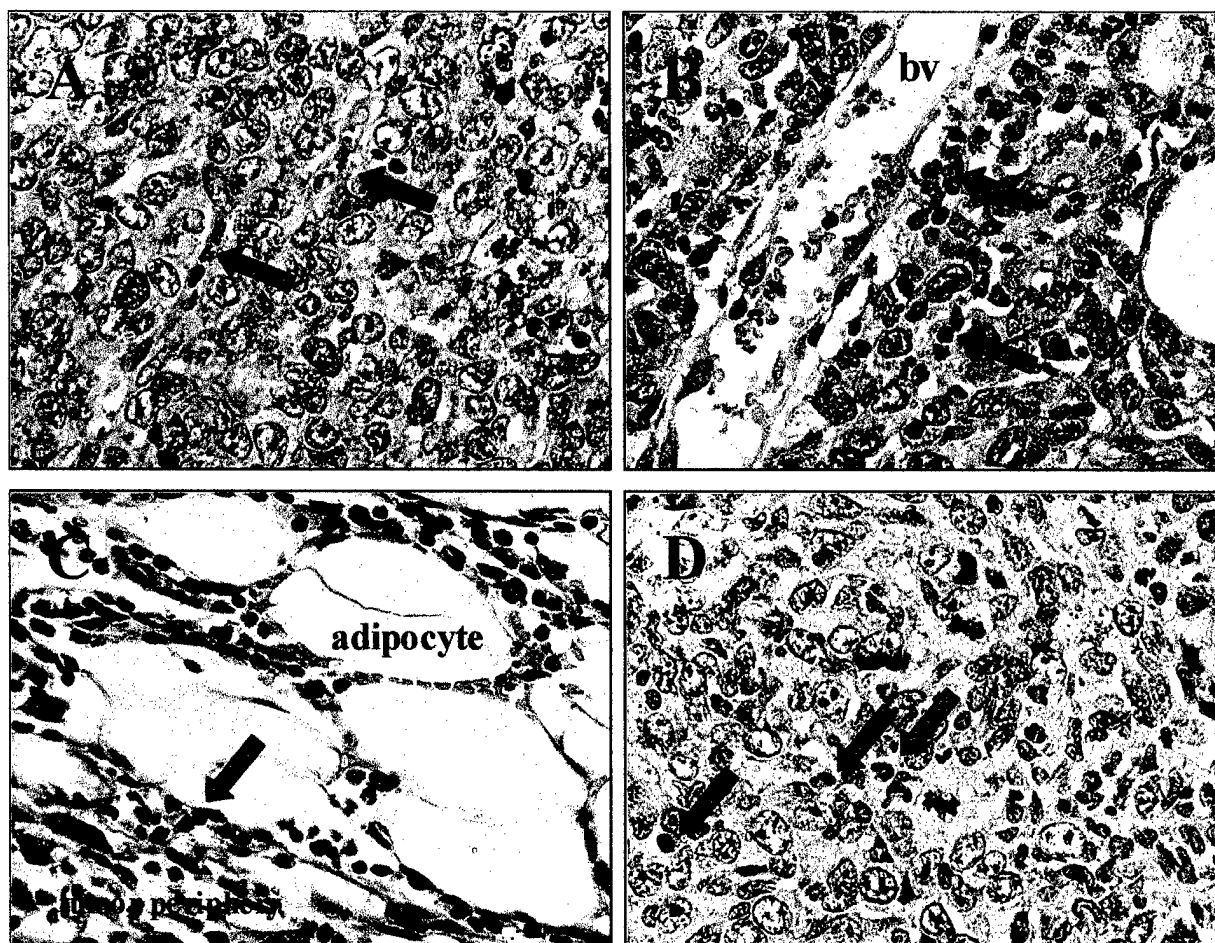


Figure 2. Fever-range whole body hyperthermia enlarges tumor blood vessels and induces an infiltrate of eosinophils into the center of the tumor. Eight hours after WBH, tumors from control and WBH treated mice were excised, fixed in formalin for histological analysis. A. Control tumors exhibit few noticeable blood vessels (red arrows). Tumors from WBH treated 4T1 bearing mice show enlarged blood vessels (B) containing granulocytes including eosinophils that have extravasated from the blood vessel and are seen in the tumor bed (red arrows). Control tumors show the greatest number of eosinophilic infiltrate around the periphery of the tumor as shown in C. The adipocytes present in the image are part of the mammary fat pad into which the 4T1 tumor cells were implanted. The red arrow indicates an area of the tumor periphery containing eosinophils. D. In a heated tumor, the infiltrating eosinophils (red arrows) are found not only at the periphery of the tumor but also in the tumor center at quite some distance from the closest blood vessel. These data are representative of three separate experiments.

Appendix (continued)

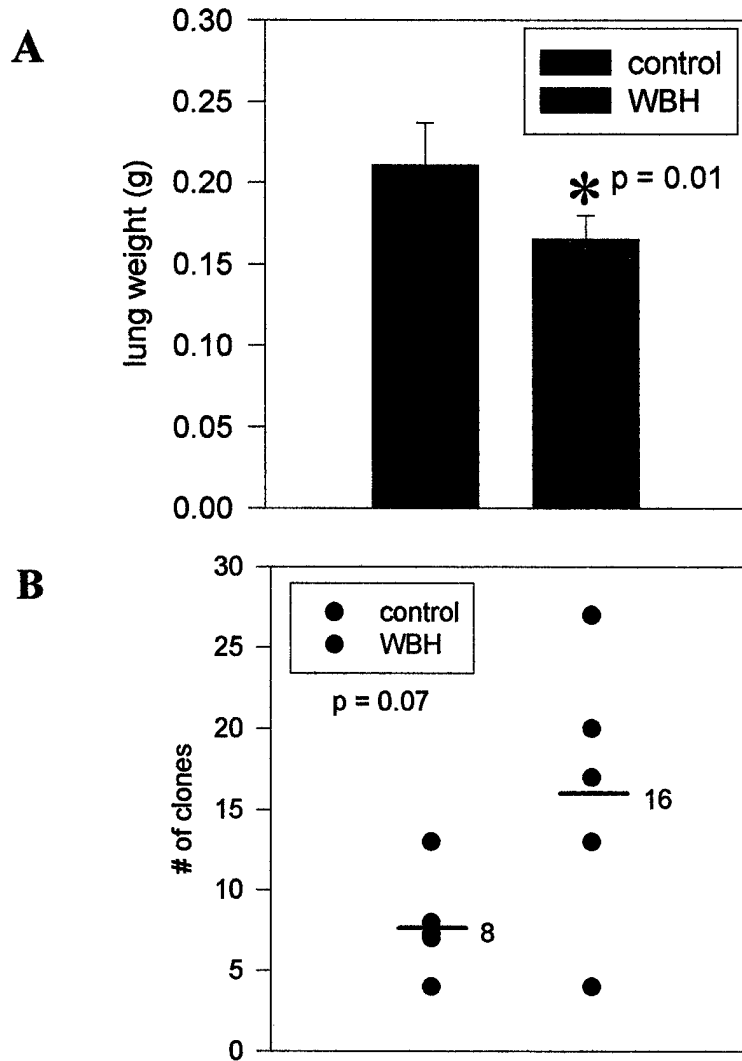


Figure 3. The effect of WBH on the amount of tumor metastasized to lung in 4T1 bearing mice is equivocal. A. Six days after a single WBH treatment, all mice were sacrificed, their lungs excised and weighed. Error bars indicated standard deviations of the data from the experimental mean. * $p = 0.01$ as calculated using the Student's t test. ($n = 5$ per group) B. The same lungs as in A were digested, filtered and the resultant lung digest was used in a clonogenic assay to determine the number of tumor cell clones in the lungs of 4T1 bearing mice. These results are in contrast to the results in A and imply that WBH may slightly enhance the metastatic burden of mouse lungs. $p = 0.07$ using an unpaired Student's t test. These data are representative of 3 separate experiments.

Appendix (continued)

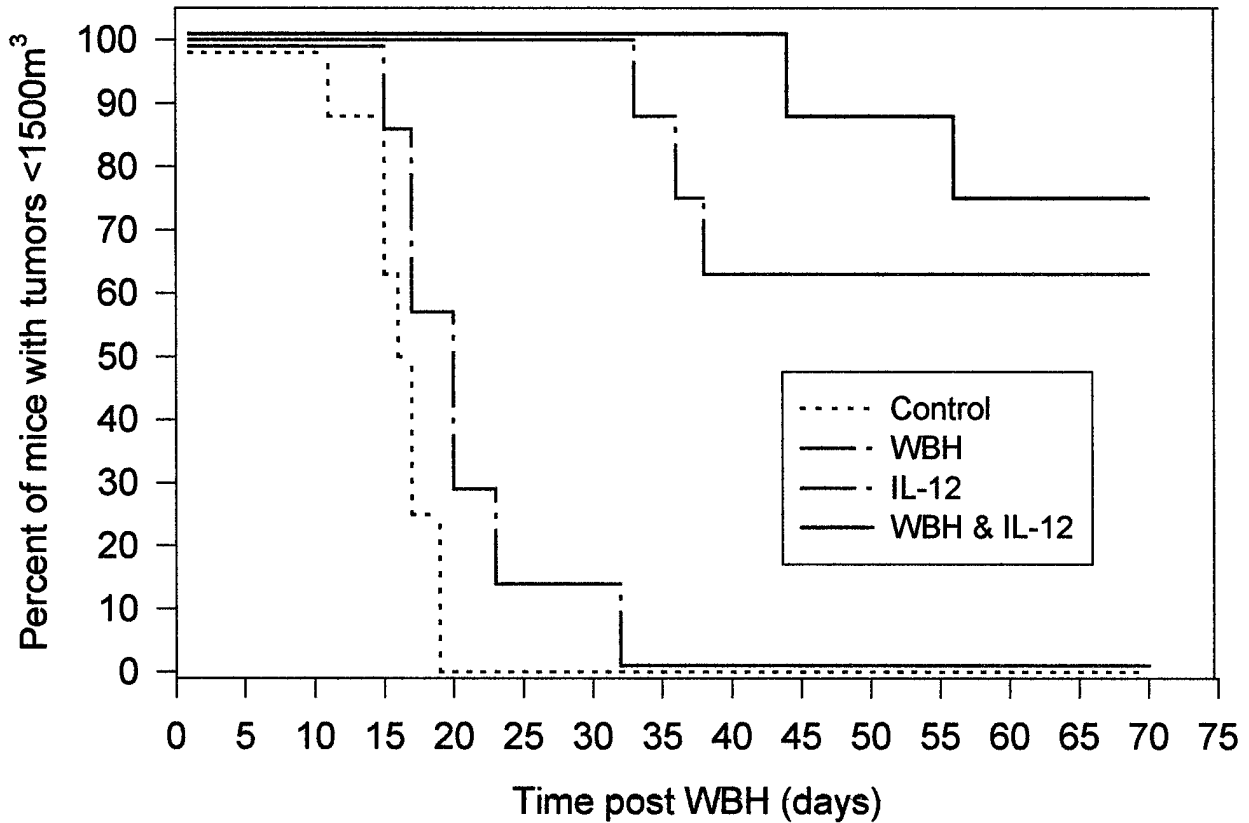


Figure 4. The combination of WBH and IL-12 treatments results in an increase in the survival of mice bearing 4T1 tumors. Mice were treated with IL-12 (100ng/mouse/day) for 3 cycles (5 days of treatment followed by 2 days of rest repeated 3 times). WBH treatment was given only once on day 0. The time for each mouse's tumor to reach 1500mm³ was determined taking tumor measurements every other day. The Kaplan-Meier graph illustrates an enhanced survival of mice treated with both IL-12 and WBH compared to those treated with IL-12 alone. These data are representative of two separate experiments.

Appendix (continued)

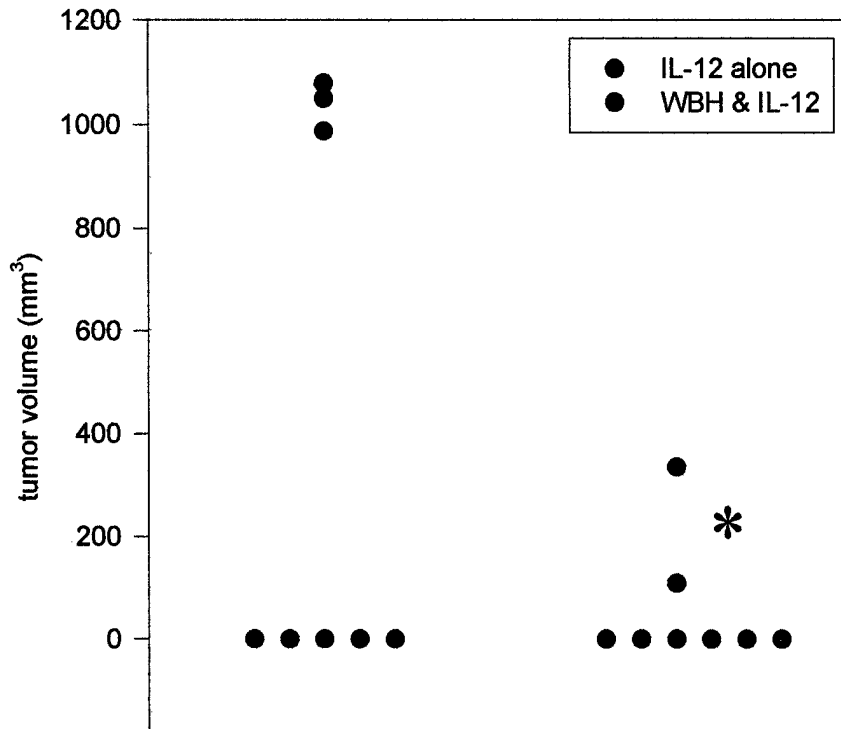


Figure 5. WBH +IL-12 treatment increases the number of tumor cures and significantly decreases tumor growth compared to IL-12 treatment alone. By day 31, all tumors in control animals had reached 1500mm³ while only one WBH treated animal bore a tumor of less than 1500mm³. Five of 8 IL-12 treated mice went on to be cured of their disease (63%) while 6 of 8 mice receiving both IL-12 and WBH were cured of their disease (75%). Of the remaining tumor bearing animals, all progressed. However, the tumor growth was delayed significantly in the mice receiving the combination treatment compared to those receiving only IL-12 alone. (n = 8 per group, Statistical significance was determined using the Student's t test. *p <0.01). These data are representative of three separate experiments.

Appendix (continued)

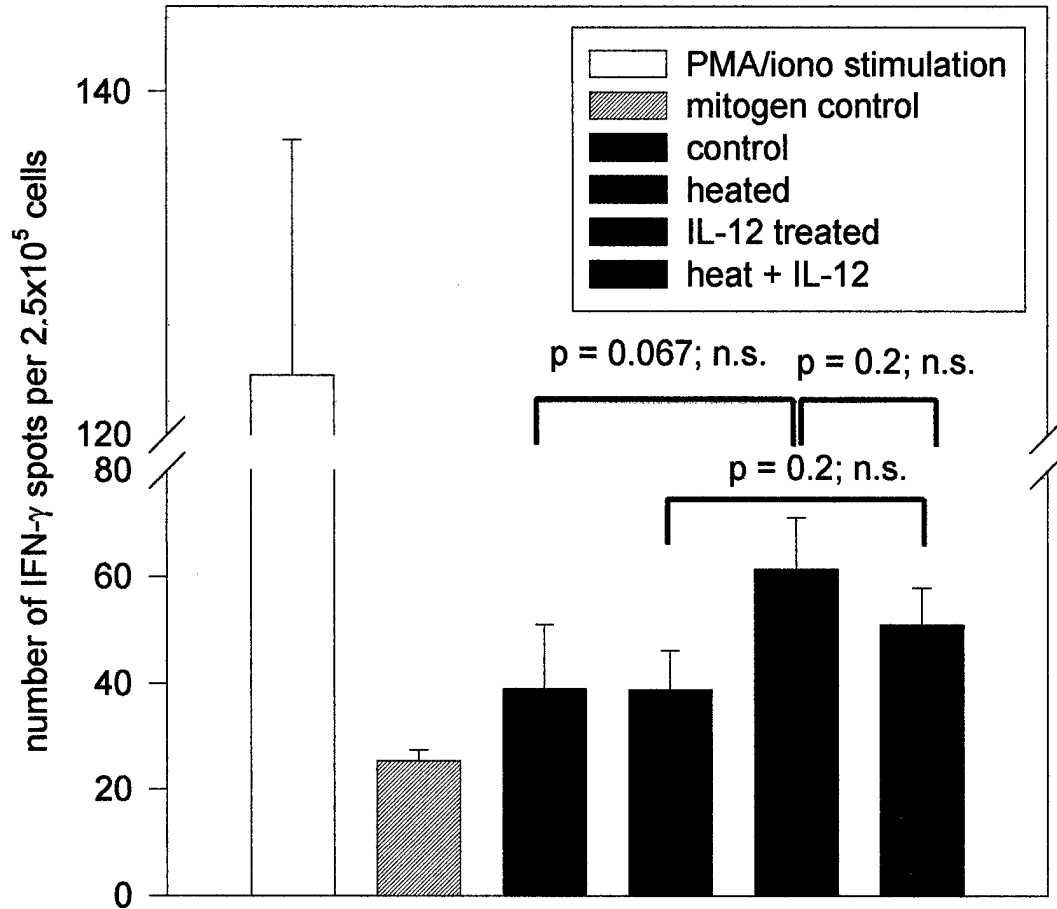
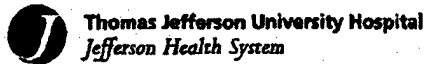


Figure 6. IL-12 treatment may slightly increase the number of IFN- γ producing cells from the draining lymph node of 4T1 bearing mice. Forty-eight hours after the WBH (after two IL-12 treatments), mice were sacrificed and the tumor draining lymph nodes were harvested and used in an ELISPOT assay to determine the number of IFN- γ producing cells in that organ. Lymph node cells from a lymph node contralateral to the tumor draining lymph node from a control animal were used as a negative control, while other cells from that same lymph node were treated with PMA (final concentration $1 \mu\text{g}/\text{mL}$) and ionomycin (final concentration $0.375 \mu\text{g}/\text{mL}$) to serve as a positive control. 2.5×10^5 cells were plated per well. Error bars represent standard deviations of the mean from 3 mice in each group. This experiment has only been done once.

Appendix (continued)

(TJU accidentally listed me as Ph.D., however, I have not yet completed the degree)



RADIOBIOLOGY SEMINAR

Division of Experimental Radiation Oncology

Department of Radiation Oncology

***"Enhancement of the Anti-Tumor
Immune Response in Mice Treated with
Fever Like Whole Body Hyperthermia"***

Michele Pritchard, Ph.D.

Department of Immunology

**Roswell Park Cancer Institute
Buffalo, New York**

**Monday, November 12, 2001
12:00 Noon**

**Bodine Center for Cancer Treatment
The Simon Kramer Conference Room (G312)
11th & Sansom Sts., Philadelphia, PA**

Contact Information: Nancy Mott 5-2046