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TITLE: Evaluation of Listeria monocytogenes Based Vaccines for HER-2/Neu in Mouse Transgenic Models of Breast Cancer

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**Abstract:**
HER-2/neu is a member of the epidermal growth factor receptor family and is overexpressed in several cancers including breast, ovarian, and pancreatic cancers and is associated with poor prognosis. Patients with HER-2/neu overexpressing tumors are capable of mounting an immune response against their tumors, but this response is not enough to stop the growth and spread of the tumors. Our lab has developed *Listeria monocytogenes* as a vector to deliver cancer antigens to the host cell to elicit a tumor specific immune response. Overlapping fragments of rat HER-2/neu have been expressed in recombinant *Listeria monocytogenes*. Using a transplantable tumor model developed for the FVB mouse, the five Lm-based HER-2/neu vaccines are being tested. Upon immunization with any of the vaccines, growth of established tumors is stopped and the size remains stable following 2 boosts. Tumors begin to decrease in size 3-4 weeks after the last boost and 40-50% of the mice regress their tumors. Each vaccine is capable of eliciting an anti-tumor response of the same magnitude as the vaccine containing the known T cell epitope, and this leads to the conclusion that there are multiple epitopes that have not been identified and work is beginning to map these epitopes.

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I. Introduction

Immunotherapies that target tumor associated antigens (TAAs) are promising alternatives to the current breast cancer therapies of surgery, radiation, chemotherapy, and hormone replacement therapies, due to the fact that they can specifically target tumor cells. In addition to this is the fact that over 44,000 women fail one or more of these therapies each year. According to the American Cancer Society, breast cancer is projected to remain the second leading cause of cancer deaths among women in the United States through 2005. In addition, over 1500 men are diagnosed with breast cancer each year in the United States alone. HER-2/neu is of interest as a target tumor antigen because it is overexpressed in 20 to 40% of all breast cancers and is also overexpressed in cancers of the ovaries, lung, pancreas, and gastrointestinal tract (Disis et al., 1997; Knutson et al., 1999; Li et al., 1994). Progression of focal tumors to metastatic cancer and the subsequent poor prognosis of patients have been associated with the overexpression of HER-2/neu in breast adenocarcinomas (Knutson et al., 1999; Treurniet et al., 1992). Despite this, patients with cancers that overexpress HER-2/neu have humoral (Coronella, J.A., 2001; Disis et al., 1997b, Disisi et al., 2000), CD8+ T cell (Peoples et al., 1995), and CD4+ T cell (Tuttle et al., 1998) immune responses directed towards HER-2/neu, but these responses are not effective. It has been shown that intracellular bacteria, such as *Listeria monocytogenes* can be used as vaccines vectors due to their ability to generate strong and specific T cells responses (Pan et al., 1995; Pan et al., 1995b; Pan et al., 1999; Pardoll, 1996; Weiskirch et al., 1997). *Listeria* preferentially infects macrophages and other antigen presenting cells, and while most of the bacteria is killed in the phagolysosome, a small number escape into the cytosol resulting in the presentation of Listerial antigens by both the MHC class I and class II pathways (Hsieh et al., 1993; Weiskirch et al., 2001). Our lab has previously shown that *Listeria monocytogenes* expressing tumor-associated antigens can induce the regression of established tumors that express those antigens (Gunn et al., 2001). This system is being adapted for the breast cancer associated antigen HER-2/neu.

II. Body

This study is aimed at determining the effectiveness of *Listeria monocytogenes* based immunotherapies for HER-2/neu overexpressing breast cancers. *Listeria monocytogenes* that express fragments of HER-2/neu will prime CD4+ and CD8+ T cell responses that are robust enough to overcome tolerance in transgenic mouse models of HER-2/neu. Previous studies have shown that *Listeria monocytogenes* that express the viral protein E7 are capable for eliciting a strong enough anti-tumor response to E7 in tumor bearing mice to cause tumor regression (Gunn et al., 2001). The E7 system has been adapted for HER-2/neu and this study looks at the effectiveness of these vaccines and also at the immune responses generated with these treatments.
We had previously completed the cloning of HER-2/neu into the expression vector that was then transduced into *Listeria monocytogenes* (Figure 1) and had also confirmed the secretion of each construct by *Listeria*.

![Diagram of HER-2/neu domains and amino acid ranges](image)

**Figure 1.** Schematic of the overlapping extracellular and intracellular fragments of HER-2/neu cloned into an expression vector. The numbers represent the amino acids.

Upon confirmation that each of the above constructs fused to a truncated form of the Listerial protein Listeriolysin-O, the constructs were tested in a tumor regression study in accordance with task 1. In these experiments, wild type FVB mice were used. The background FVB strain was chosen because this is the background of the HER-2/neu transgenic mice that are used in future studies. Mice were implanted with NT-2 tumor cells, which is a transplantable tumor line for the FVB mice that was derived from a spontaneous mammary tumor in an FVB HER-2/neu transgenic mouse (Reilly et al., 2000). Mice were subcutaneously injected with 2.5 x 10^6 NT-2 cells each on Day 0. They were then vaccinated with 0.1 LD_{50} of the individual vaccines on days 7, 14, and 21, and tumor measurements were taken every three days (Figure 2). The mice were sacrificed when their tumor reached the size of 20 mm.

![Graph showing tumor regression](image)
Figure 2. Regression of NT-2 tumors in wild type FVB mice. Interestingly, both these experiments show that each vaccine construct can have a significant impact on tumor growth. The tumors in the experimental groups stop growing almost immediately following the first vaccination, while the control group of PBS and LLO-E7 (an irrelevant *Listeria* vaccine) continue to steadily grow out. Following the third vaccination on day 21, the mice are not vaccinated again, but the tumors remain at around 5 mm. Between days 50 and 60 after tumor inoculation, a subset of mice from a mix of the vaccination groups regress their tumors. In the end between 20-30% of the mice regress their tumors completely.

These experiments demonstrated that each of the vaccines is capable of eliciting a specific anti-tumor response in wild type mice with implanted tumors that express HER-2/neu. The next part of task 1 was to test the ability of each of the vaccines to regress implanted NT-2 tumors in the FVB HER-2/neu transgenic mice. This experiment was set up almost identically to the wild type experiment. The difference with this was that instead of 2.5 x 10^6 cells, mice were implanted with 1 x 10^6 cells each. This is due to the fact that the transgenic mice are tolerized to HER-2/neu and it takes fewer tumor cells to get a palpable tumor in seven days compared to the wild type mice. Mice were again vaccinated intraperitoneally with 0.1 LD_{50} of a vaccine on days 7, 14, and 21, and their tumors were measured every three days (Figure 3).

Figure 3. Regression of NT-2 tumors in FVB HER-2/neu transgenic mice. Representative graph of multiple experiments.

Following the vaccinations, the experimental groups all showed a significant difference in growth from the two control groups. While the controls steadily grow out, the experimental groups all show a much slower rate of growth. Based on these results, tolerance has been broken in these mice. These *Listeria*-based constructs are capable of
generating an immune response that is able to control the growth of HER-2/neu tumors enough so that the tumors grow very slowly compared to control vaccines. This experiment was not carried out as long as the previous wild type experiments due to the fact that within two days following the last measurement, all of the mice in the HER-2/neu vaccinated groups had scratched away their tumors. Every time this experiment has been done, that same result has occurred at the same time point. We believe that the vaccinations have generated a very large delayed type hypersensitivity (DTH) reaction at the site of the tumor in the transgenic mice that results in the mice scratching at the area until the tumor is gone and as a result the inflammation is also gone. This will be further studied in experiments that have been planned for the future.

The third point outlined in task one was to test the ability of each vaccine to prevent the development of spontaneous mammary tumors in the FVB HER-2/neu transgenic mice. This experiment is currently ongoing and there are no results to report at this time.

Following the observations from the tumor regression studies, the next step was to begin looking at the immune response generated by the non-transgenic and the transgenic mice upon injection with each of the vaccines. Preliminary experiments have been done addressing part d of task 2, which is to characterize the tumor infiltrating lymphocytes by tetramer analysis, FACs, and functional assays. Initial experiments have been done to look at the tetramer positive cells in the tumor and to FACs analyze all of the cells in the tumors. These studies are currently limited by the fact that there is only one known epitope in the FVB mouse for HER-2/neu, so the tetramer analysis can only be done from the Lm-LLO-EC2 construct. Currently, work is being focused on analyzing the CD8+ T cell response through CTL assays, which is part of task 2, part b. By analyzing the specific lysis of HER-2/neu expressing cells, regions containing potential CD8+ T cell epitopes can be identified. In order to do these experiments we are using target 3T3 cells that have been transduced with fragments of HER-2/neu to help narrow down the regions of interest (Ercolini et al., 2003). Mice are vaccinated on day 0 with 0.1 LD50 of a particular vaccine, and then are sacrificed on day 9 when spleens are harvested. The splenocytes are then cultured for four days at a 100:1 ratio of splenocytes to irradiated NT-2 tumor cells as feeder cells, with the addition of 20U/ml IL-2. On day 13, the target cells are labeled with 51 Chromium for one hour and then cultured with the effector cells at different effector to target ratios. Based on these experiments portions of HER-2/neu with potential unknown epitopes have been identified (Figure 4 and Table 1).
Figure 4: CTL analysis of each HER-2/neu vaccine construct with wild type 3T3 and 3T3 cells containing full length HER-2/neu as target cells.

<table>
<thead>
<tr>
<th>Listeria Construct</th>
<th>Neu Region Spanned</th>
<th>Killing of 3T3-neu Target Cells</th>
<th>Neu Regions Containing an Epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lm-LLO-EC1</td>
<td>30-326</td>
<td>Neu-1/Neu-2/Neu-3</td>
<td>20-148/291-326</td>
</tr>
<tr>
<td>Lm-LLO-EC2</td>
<td>303-501</td>
<td>Neu-3/Neu-4</td>
<td>303-426/401-501b</td>
</tr>
<tr>
<td>Lm-LLO-EC3</td>
<td>479-655</td>
<td>Neu-4/Neu-5</td>
<td>479-553/531-655</td>
</tr>
<tr>
<td>Lm-LLO-IC1</td>
<td>690-1081</td>
<td>Neu-6/Neu-7/Neu-8</td>
<td>690-797/952-1081</td>
</tr>
<tr>
<td>Lm-LLO-IC2</td>
<td>1020-1260</td>
<td>Neu-8/Neu-9</td>
<td>1020-1085/1063-1260</td>
</tr>
</tbody>
</table>

Table 1. Regions of HER-2/neu with potential FVB epitopes based on specific lysis from CTL analysis and the corresponding vaccine constructs. a Strong killing: greater than 20% above background lysis; weak killing: 10-20% above background lysis; no killing above background lysis. Background lysis varies between 0-12% for each experiment. b The epitope identified from Ercolini et al is from 420-429 and is partially contained in Neu-3 and fully in Neu-4.

Once summarized, these results show that there are clear regions where there do not seem to be any epitopes, while there are several regions, where there appear to be strong CTL epitopes present. It appears as though these sub-dominant epitopes are revealed by (1) breaking HER-2/neu into fragments; (2) fusing these fragments to listeriolysin O (LLO); (3) delivering the antigen through *Listeria monocytogenes*; or (4) a combination of these. To determine which of these factors is the key factor, we are testing DNA vaccines that compare the full length HER-2/neu with a fragment, and also compare LLO-fused with un-fused antigens.

To address this question, DNA vaccines for HER-2/neu were made. The vaccines were all made by inserting a portion or all of HER-2/neu into the pcDNA 3.1 backbone either unfused or fused to LLO. To test the fragments, to this point only one fragment has been made into a DNA vaccine. The vaccines developed are pcDNA with the full length HER-2/neu inserted (pcDNA neu), pcDNA with the full length HER-2/neu inserted fused to LLO (pcDNA LLO-neu), pcDNA with the EC1 fragment inserted (pcDNA EC1), and pcDNA with the EC1 fragment fused to LLO (pcDNA LLO-EC1). These vaccines are currently being tested in a tumor regression experiment with Lm-LLO-EC1 as the vaccine that uses *Listeria* as a delivery mechanism. Mice were implanted subcutaneously with 7.5 x 10^5 NT-2 tumor cells on day 0 and then were vaccinated on days 3, 10, and 17. Lm-LLO-EC1 and PBS were administered intraperitoneally, and the 50µg per mouse of each DNA vaccine was given intra-muscularly along with GM-CSF as an adjuvant.

Although the experiment has not yet been completed, the vaccine that works the best is Lm-LLO-EC1. Several of these mice did not develop tumors and some of the mice that did develop tumors have regressed their tumors completely. pcDNA neu and pcDNA EC1 did not work at slowing down tumor growth at all, whereas both pcDNA LLO-neu
and pcDNA LLO-EC1 resulted in the tumors growing more slowly that in the control. A small number of pcDNA LLO-EC1 mice have also undergone a complete regression of their tumors. Based on these results it appears as though both fusing the antigen to LLO along with delivery through *Listeria* seems to increase the immunogenicity of the target tumor antigen, and allows for the exposure of sub-dominant HER-2/neu epitopes.

The antibody response generated by vaccinating mice with each of the extracellular region vaccines has also been done to address part a of task 2 (Figure 5).

![Figure 5. FACs analysis of HER-2/neu serum antibody production upon vaccination with PBS, Lm-LLO-EC1, Lm-LLO-EC2, or Lm-LLO-EC3.](image)

For this experiment only the extracellular fragments of HER-2/neu were used because the assay used to determine serum antibody production is flow cytometry. Mice were vaccinated on day 0 and blood was collected on day 14. The serum was immediately separated from the blood cells and was then used to stain either wild type 3T3 cells or the 3T3 cells that express HER-2/neu (3T3-neu) to look to see if the antibody binds to the extracellular portion of the HER-2/neu expressed on the surface of the cell. An anti-mouse IgG conjugated to a fluorochrome was used as a secondary antibody and both wild type and 3T3-neu cells were stained with a commercial HER-2/neu antibody as a positive control. The commercial antibody was used at a dilution of 1:200 and a very clear positive peak can be seen with the 3T3-neu cells. The serum was tested at several dilutions and at the very low dilution of 1:50, only a very small shift in the peak can be seen. These vaccines result in a negligible amount of antibody production, which was to be expected since *Listeria monocytogenes* is an intracellular bacterium which passes from cell to cell with very little to no contact with the extracellular environment. Due to this fact a hybridoma that produces an anti-HER-2/neu antibody has been obtained from Dr. Mark Greene and is currently being used to harvest antibody for co-injection with vaccines as outlined in task 3 (Drebin et al., 1988). At this point, the experiments for task 3 have not been started.
In the coming year, several experiments have been planned and questions of interest identified. The spontaneous tumor protection experiment will be completed and repeated. Further CTL analysis to identify the exact sequences of the sub-dominant epitopes will be done using a peptide library to screen the regions with a projected epitope. Experiments will also be started to address the points for task 3, in which mice will be vaccinated with the *Listeria* vaccines and will also be given HER-2/neu antibody injections at the same time to see if the addition of the antibody will help to slow down and possibly regress the tumors in the transgenic mice. Along with this, experiments are being planned to study the suspected DTH immune response that the transgenic mice are undergoing around day 40-45 that causes them to scratch away their tumors. Of particular interest would be to identify tumor infiltrating lymphocytes in the days immediately prior to this occurring to determine if there is a particular cell type that is causing this reaction in each of the mice.

### III. Key Research Accomplishments

- Regression studies in wild type FVB mice with implanted NT-2 tumors showing stasis in tumor growth and the eventual regression of a subset of tumors.
- Regression studies in HER-2/neu transgenic FVB mice with implanted NT-2 tumors showing a much slower rate of tumor growth than control groups.
- CTL analysis of each vaccine with the corresponding target cells lines leading to narrowed down regions with potential HER-2/neu epitopes for the FVB mouse.
- Construction of DNA vaccines for full length HER-2/neu, full length HER-2/neu fused to LLO, the EC1 fragment, and the EC1 fragment fused to LLO.
- Regression study with DNA vaccines showing that the best system involves the fusion of the tumor antigen to LLO and also involves *Listeria monocytogenes* as a vehicle for delivery.
- Analysis of serum for HER-2/neu antibody production showing that negligible amounts of HER-2/neu antibody is made through vaccination with any of these constructs.

### IV. Reportable Outcomes

- Presentation of poster based on this data at the Cancer Research Institute meeting in October 2004.
- Applied for patent.
V. Conclusions

Listeria monocytogenes based vaccines for HER-2/neu are capable of revealing sub-dominant HER-2/neu epitopes through fusion of the target antigen with LLO and also through delivery by Listeria. Each of the five HER-2/neu vaccines made based on breaking HER-2/neu up into fragments results in the generation of an immune response that is capable of stopping implanted tumor growth and also regression of a subset of tumors in wild type FVB mice. These vaccines are also capable of significantly slowing down the growth of implanted tumors in HER-2/neu transgenic mice, which are highly tolerant to HER-2/neu. Cytotoxic T lymphocyte analysis has shown that there are several regions with a very strong possibility of identifying a potential CD8+ T cell epitope. The idea that the fusion to LLO and also delivery of the tumor antigen by Listeria monocytogenes enhances the immunogenicity of the antigen is further supported by the DNA vaccine regression experiment that shows that the most effective anti-HER-2/neu therapy is Lm-LLO-EC1 as compared to any of the DNA vaccines, but the DNA vaccines that seem to be partially effective are the vaccines where the tumor antigen is fused to LLO. It is currently unclear how LLO may be enhancing the immunogenicity of fused antigens, but this is being studied in the lab.

Although this work is being done with the rat HER-2/neu and all of the experiments are being done in a mouse model, this work is related to patients with HER-2/neu overexpressing breast cancers. The HER-2/neu transgenic mouse develops breast cancer similar to humans. In these mice, the cancer generally begins as a focal mammary tumor and then, once it metastasizes spreads rapidly to the lungs and liver before spreading throughout the body. This is very similar to what happens to people with breast cancer when the cancer metastasizes. In addition, the mice are very tolerant to HER-2/neu as evidenced by the fact that a smaller dose of tumor cells results in tumor growth versus wild type mice. Patients with HER-2/neu breast cancers have also been shown to be tolerant to HER-2/neu due to the fact that many patients are capable of developing only a weak and ineffective immune response to HER-2/neu. These mice have demonstrated that Listeria monocytogenes based vaccines to HER-2/neu are capable of eliciting an immune response in transgenic mice that is capable of significantly impacting on tumor growth and breaking tolerance. This means that this immunotherapy may be adapted for humans, and that it may be capable of generating a strong enough immune response in patients that leads to an impact on tumor growth and the breaking of tolerance.
VI. References


