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13. ABSTRACT (Maximum 200 Words)

Current breast cancer therapy consisting of surgery, radiation and chemotherapy, fails in 44,000 women per year. Radiation and chemotherapy can also cause significant side effects. New and improved therapies need to be developed to cure patients who fail other therapies. Recently, Her2/neu has been described as a breast cancer antigen because it is overexpressed on 25-40% of all breast cancers. Her2/neu is only expressed at very low levels on normal cells; therefore, it is an appropriate target for the design of immunotherapeutics specific for breast cancer. Our lab has used *Listeria monocytogenes* as a vaccine vector for cancer because it induces strong $CD4^+$ and $CD8^+$ T cell responses. We have shown that recombinant listerial strains expressing tumor-associated antigens such as Her2/neu can induce regression of established tumors expressing those antigens. In this study, we have designed and constructed vaccines against portions of Her2/neu using *Listeria monocytogenes*. In the next phase of our study, we will test these vaccines and evaluate the immune responses induced in mice. If these studies are successful, the vaccines could be tested in clinical trials in human patients and could serve as an effective, targeted treatment for breast cancer with minimal side effects.

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Table of Contents

Cover..1

SF 298..2

Introduction...4

Body..4

Key Research Accomplishments...8

Reportable Outcomes...8

Conclusions...8

References...8

Appendices..None

I. Introduction

Current breast cancer therapy consists of surgery, radiation and chemotherapy. Although these therapies are successful, 44,000 women per year fail current therapies. Immunotherapy is an attractive alternative because it is specific and targeted to cancer cells, thereby causing fewer side effects and increased effectiveness. Her2/neu is a 185-kDa protein that is overexpressed on 25-40% of all breast cancers and many ovarian cancers. Although Her2/neu is present on normal noncancerous cells, it is not immunologically silent. Her2/neu-specific humoral, $CD4^+$ T cell and $CD8^+$ T cell responses have been observed in patients with breast cancer. Because Her2neu is expressed on the surface of cells and specifically overexpressed on a high percentage of breast cancer cells, it is a good target for the design of anti-cancer therapeutics. Our lab and others have shown that intracellular bacteria can be successfully used as vaccine vectors. *Listeria monocytogenes* are particularly attractive as vectors because they preferentially infect antigen-presenting cells, specifically macrophages and dendritic cells. Since Listeria spend part of their life cycle in the phagolysosome and part of their life cycle in the host cytosol, antigens expressed by the bacteria are presented via both the MHC Class I and Class II pathways. Previous studies have shown that expression of endogenous and exogenous antigens by *Listeria monocytogenes* results in induction of $CD4^+$ T cells with Th1 type properties and strong $CD8^+$ T cell responses. Recombinant listerial strains expressing tumor-associated antigens have been shown to induce regression of established tumors expressing those antigens (Gunn *et al.*, 2001).

II. Body

The goal of our present study is to use *Listeria monocytogenes* as a vector for vaccines against Her2/neu, which is expressed in a high percentage of breast cancers. Prior results from our laboratory have shown that recombinant listerial strains expressing fusions of the listerial protein, listeriolysin O (LLO), to the HPV protein E7 (E7) are effective in inducing regression of established tumors expressing E7 in mice (Gunn *et al.*, 2001). Listerial strains expressing the fusion protein were considerably better at inducing regression than strains expressing only E7. At that time, we thought that the PEST domain of LLO mediated this effect, because this domain is thought to target proteins to be ubiquitinated and degraded by the proteasome. Therefore, the presence of the PEST domain may increase antigen presentation. Subsequent studies have shown that constructs containing the PEST domain fused to E7 are significantly better than constructs only containing E7 but are not as good as constructs containing more of the LLO protein (unpublished data). Consequently, we have decided to make all of our initial fusions to LLO.

We set out to make four constructs each containing overlapping fragments of Her2/neu. Since Her2/neu is very large and contains a transmembrane domain, we knew we would be unable to express the entire protein in Listeria. Figure 1 contains a diagram showing each of the Her2/neu fragments.

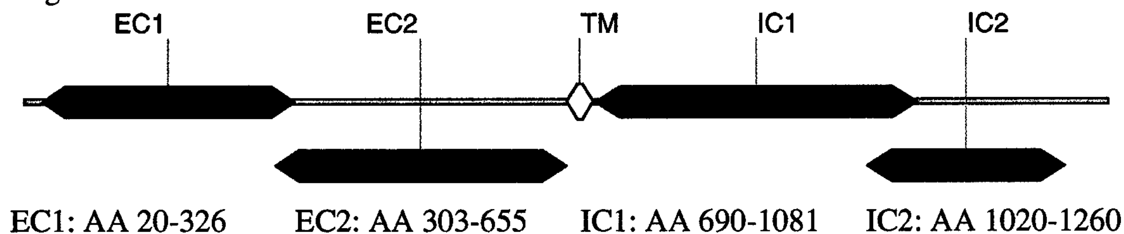

Figure 1. Overlapping fragments of Her2/neu inserted into listerial vector.

Our general scheme was to amplify each fragment of Her2/neu from the pNINA plasmid obtained from Dr. Elizabeth Jaffee (John Hopkins University) with specific primers, amplify it in a cloning vector, digest it and ligate it to a vector that already contains the promoter, and the region encoding the first 441 amino acids of LLO (Figure 2). Once the constructs were made in *E. coli,* we were able to purify the plasmid and electroporate the *prfA*- strain of *Listeria monocytogenes* (gift from Dr. Hao Shen, University of Pennsylvania). Putting the constructs into *prfA*- listerial strain allows us to select *in vivo* because the *prfA* gene contained on the plasmid is required for growth *in vivo.* We confirmed expression of the construct using PCR and Western blotting. We have been successful in creating LLO-EC1, LLO-IC1, and LLO-IC2 (Figures 3 and 4). These strains have been passaged twice in mice. Passaging has been shown to solidify the expression of proteins expressed in recombinant listerial strains (Peters *et al.*, 2003). The LD_{50} has also been determined for LLO-EC1 and LLO-IC2 ($1x10^8$ for both strains) and they are identical but significantly less virulent than the wild type strain, 10403S ($LD_{50} = 1x10^5$). The LD_{50} of LmLLO-IC1 is still being determined.

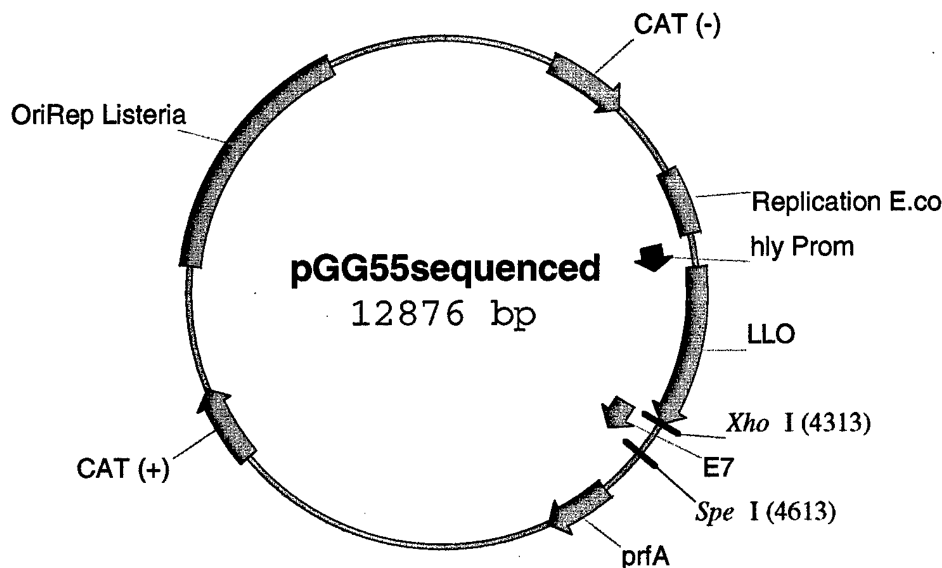

Figure 2. Listerial vector containing promoter and 5' portion of LLO gene.

Figure 3. PCR of LLO-EC1, LLO-IC1, and LLO-IC2 constructs in Listerial strain XFL7.

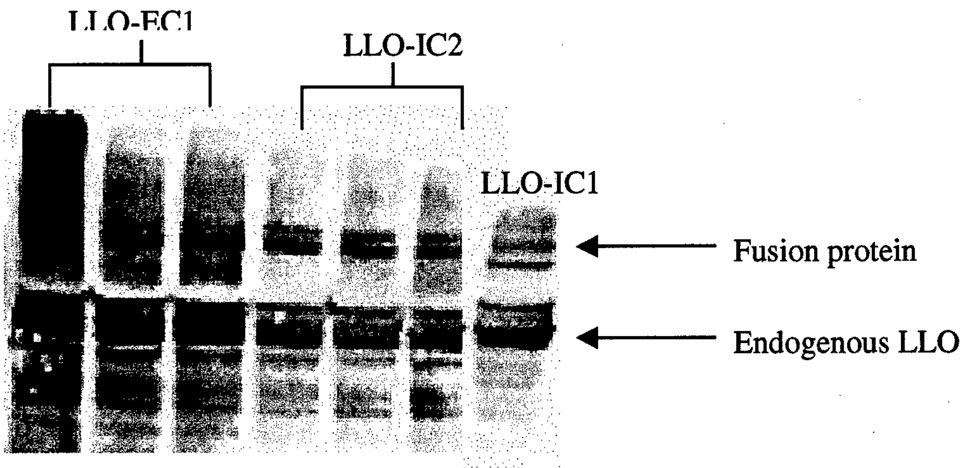

Figure 4. Western blot of LLO-EC1, LLO-IC1, and LLO-IC2.

We have encountered some problems with the LLO-EC2 construct. We have successfully made this construct several times and the protein is not secreted from Listeria. Previous studies have shown that both CD4^{+} and CD8^{+} T cells are required for regression of tumors in our system (Reilly *et al.* 2001). In order to induce both a CD4^{+} and CD8^{+} T cell response, the recombinant proteins need to be secreted from Listeria. We know that LLO-EC2 is being made because we can detect it on Westerns of cell lysates (Figure 5). We have decided to break the original EC2 into two smaller overlapping pieces (Figure 6) to try to circumvent the secretion problems. We have made both LLO-EC2' and LLO-EC3 and electroporated them into *Listeria monocytogenes*. We have confirmed the presence of each construct by PCR (Figure 7). When we analyzed the supernatants of each strain, we found that LLO-EC2' and LLO-EC3 were both secreted but that LLO-EC2' is degraded after secretion (Figure 8). We don't anticipate this being a problem because degradation may enhance antigen processing. We are in the process of passaging both strains..

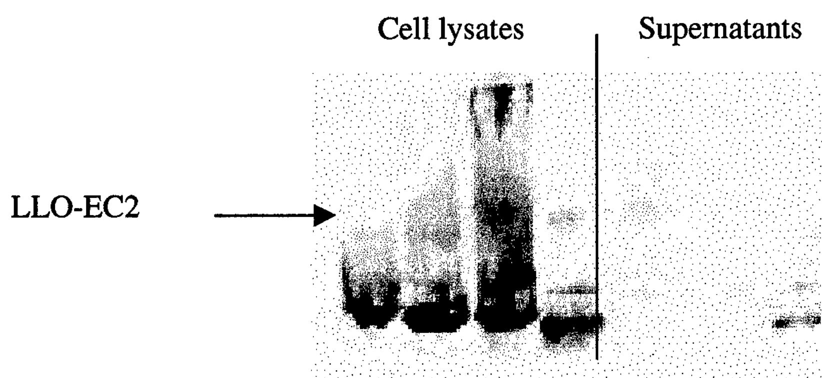

Figure 5. Western blot of clones of LmLLO-EC2 cell lysates vs. supernatants

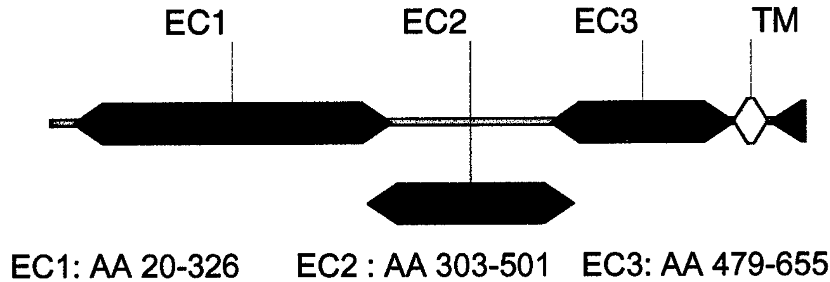

Figure 6. Further fragmentation of EC2 into EC2' and EC3.

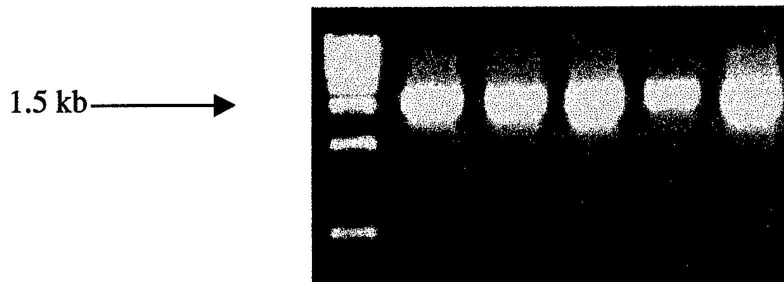

Figure 7. PCR of clones of LLO-EC2 in *Listeria monocytogenes.*

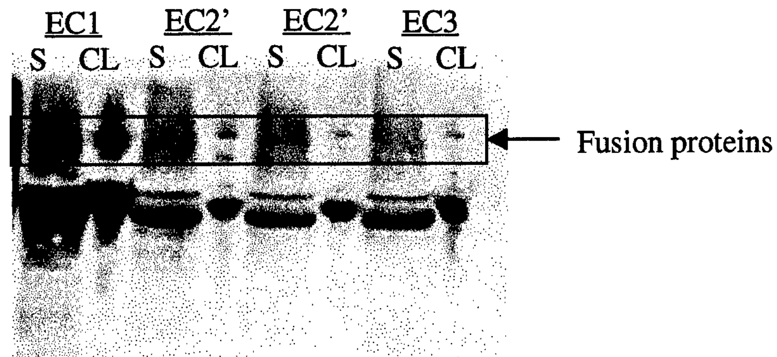

Figure 8. Western blot of LLO-EC2' and LLO-EC3 supernatants (S) and cell lysates (CL).

In summary, we have 4 constructs made and we are still working on the fifth one. We plan to test each of these vaccines for their ability to regress established tumors expressing rat *neu* in FvB/n mice and for their ability to protect Her2/neu transgenic mice from the formation of spontaneous tumors, which occurs in 50% of these mice by 6 months of age. We would like to make all of the vaccine constructs before we start testing the efficacy of each vaccine so we can compare the vaccines directly.

We initially outlined a design for a DNA vaccine vector that would encompass the entire Her2/neu gene. The design was to use the pcDNA3.1-NT-GFP vector from Invitrogen and add elements that we need to work in a Gram-positive strain, e.g. Gram-positive origin of replication, Gram-positive selection marker, etc. We attempted to make a DNA vaccine based on this system with HPV E7. The system is not ideal because the vector contains many elements not required for our system and does not contain enough unique restriction enzyme sites to use for cloning. We are in the process of

designing a DNA vaccine from scratch which will only contain necessary elements but will also contain enough unique sites throughout the vector that we will be able to add elements where necessary and to use the vector for other antigens.

III. Key Research Accomplishments

- Construction of LmLLO-EC1, LmLLO-EC2, LmLLO-EC3, LmLLO-IC1, and LmLLO-IC2
- Confirmation of expression and secretion of each fusion protein by Western blotting
- Passaging in mice of LmLLO-EC1, LmLLO-IC1, and LmLLO-IC2
- Determination of the LD_{50} for LmLLO-EC1 and LmLLO-IC2

IV. Reportable Outcomes

- Mary E. Dominiecki has accepted an Assistant Professor of Biology position at Slippery Rock University that will start in the fall of 2003. This grant has provided her with valuable training that she will use in her future position.

V. Conclusions

Since this work is in the initial phases, no conclusions can be drawn about the efficacies of these vaccines at this time.

VI. References

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