Fusion to Listeriolysin O and Delivery by Listeria monocytogenes Enhances the Immunogenicity of HER-2/neu and Reveals Subdominant Epitopes in the FVB/N Mouse

Reshma Singh, Mary E. Dominiecki, Elizabeth M. Jaffee and Yvonne Paterson

J Immunol 2005; 175:3663-3673; ;
doi: 10.4049/jimmunol.175.6.3663
http://www.jimmunol.org/content/175/6/3663

References
This article cites 51 articles, 27 of which you can access for free at: http://www.jimmunol.org/content/175/6/3663.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Fusion to Listerialysin O and Delivery by *Listeria monocytogenes* Enhances the Immunogenicity of HER-2/neu and Reveals Subdominant Epitopes in the FVB/N Mouse

Reshma Singh,* Mary E. Dominiecki,2* Elizabeth M. Jaffee,† and Yvonne Paterson3*‡

Five overlapping fragments of rat HER-2/neu have been expressed in recombinant *Listeria monocytogenes*. Each fragment of HER-2/neu is secreted as a fusion protein with a truncated, nonhemolytic form of listerialysin O (LLO). Lm-LLO-EC1, Lm-LLO-EC2, and Lm-LLO-EC3 overlap the extracellular domain of HER-2/neu, whereas Lm-LLO-IC1 and Lm-LLO-IC2 span the intracellular domain. All five strains controlled the growth of established NT-2 tumors, a rat HER-2/neu-expressing tumor line derived from a spontaneously arising mammary tumor in a FVB/N HER-2/neu-transgenic mouse. The antitumor effect of each of these vaccine constructs was abrogated by the in vivo depletion of CD8+ T cells, although only one known epitope has been defined previously and is present in Lm-LLO-EC2. Anti-HER-2/neu CTL responses were generated by each of the rLm vaccine constructs. With the use of a panel of 3T3 cell lines expressing overlapping fragments of HER-2/neu, regions of HER-2/neu with potential CD8+ T cell epitopes have been defined. DNA vaccines expressing either a fragment or full-length HER-2/neu were constructed in LLO-fused and non-LLO-fused forms. CTL analysis of the DNA vaccines revealed a broadening in the regions of HER-2/neu recognizable as targets when the target Ag is fused to LLO. These studies show the efficacy of *L. monocytogenes*-based HER-2/neu vaccines in a murine model of breast cancer and also that the immunogenicity of self-Ags can be increased by fusion to LLO and delivery by *L. monocytogenes* revealing subdominant epitopes.

A member of the epidermal growth factor receptor family of tyrosine kinases, HER-2/neu is a 185-kDa glycoprotein. It consists of an extracellular domain, a transmembrane domain, and an intracellular domain, which is known to be involved in cellular signaling (1–4). It is overexpressed in 25–40% of breast cancers and is also overexpressed in many cancers of the ovaries, lung, pancreas, and gastrointestinal tract (5–7). The overexpression of HER-2/neu is associated with uncontrolled cell growth and signaling, both of which contribute to the development of tumors (2, 8). Patients with cancers that overexpress HER-2/neu exhibit tolerance even with detectable humoral (9), CD8+ T cell (10), and CD4+ T cell (11) responses directed against HER-2/neu.

*Listeria monocytogenes* is an intracellular pathogen that primarily infects APCs and has adapted for life in the cytoplasm of these cells (12, 13). Host cells, such as macrophages, actively phagocytose *L. monocytogenes*, and the majority of the bacteria are degraded in the phagolysosome (14). Some of the bacteria escape into the host cytosol by perforating the phagosomal membrane through the action of a hemolysin, listerialysin O (LLO) (13, 15). Once in the cytosol, *L. monocytogenes* can polymerize the host actin and pass directly from cell to cell further evading the host immune system and resulting in a negligible Ab response to *L. monocytogenes* (13).

*L. monocytogenes* is an attractive vaccine vector because proteins produced by this bacterium can be presented as short peptides via both the MHC class I and class II pathways generating both CD8+ and CD4+ T cell responses to these Ags (16). Direct killing of tumor cells occurs through the function of CD8+ T cells, but this killing may be enhanced through the activation of CD4+ T cells. MHC class II molecules present peptides derived from listerial proteins in the phagolysosome to activate CD4+ T cells (16). Conversely, proteins that are produced by *L. monocytogenes* in the host cytosol are presented to CD8+ T cells by MHC class I molecules (17, 18). Taking advantage of the ability of *L. monocytogenes* to target Ags to both of these pathways should lead to a strong cellular immune response against the Ags it produces. Our lab has previously shown this in response to the human papillomavirus (HPV) E7 protein and the influenza nucleoprotein (NP) (17, 19–21). Both of these systems used recombinant *L. monocytogenes* containing a plasmid with a truncated, nonhemolytic, LLO fused to the N terminus of either E7 (Lm-LLO-E7) or NP (Lm-LLO-NP) (17, 19). In both these cases, complete tumor regression can be seen upon vaccination of mice-bearing tumors with the target Ag (19, 20). In particular, Lm-LLO-E7 induces the complete regression of >75% of established E7-expressing murine tumors. This vaccine is also capable of generating an E7-specific CD8+ T cell response that can be used to effectively kill target tumor cells in a CTL assay, and the antitumor response is abrogated in vivo upon depletion of CD8+ T cells (19).

---

*Abbreviations used in this paper: LLO, listerialysin O; HPV, human papillomavirus; NP, nucleoprotein; PEST, proline, glutamic acid, serine, and threonine.*

---

1 Address correspondence and reprint requests to Dr. Yvonne Paterson, Department of Microbiology, 323 Johnson Pavilion, 36th Street and Hamilton Walk, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6076. E-mail address: yvonne@mail.med.upenn.edu

2 Current address: Department of Biology, Slippery Rock University, Slippery Rock, PA 16057.

3 Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104; and Department of Oncology, Johns Hopkins School of Medicine, Baltimore, MD 21237

Received for publication April 21, 2005. Accepted for publication July 5, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 R.S. was partly supported by a Cancer Research Institute training grant, “Predoctoral Emphasis Pathway in Tumor Immunology,” and partly by Department of Defense Predoctoral Training Grant W81XWH-04-1-0338. M.E.D. was supported by Department of Defense Postdoctoral Training Grant DAMD 17-BC02-1-0545.

2 Current address: Department of Biology, Slippery Rock University, Slippery Rock, PA 16057.

3 Abbreviations used in this paper: LLO, listerialysin O; HPV, human papillomavirus; NP, nucleoprotein; PEST, proline, glutamic acid, serine, and threonine.
Based on these results, we have adapted the Lm-LLO-Ag system for the self-Ag HER-2/ neu. Several other vaccines using the HER-2/neu tumor Ag in the mouse have been studied previously. In wild-type FVB/N mice, whole cell vaccination has been effective in preventing tumor growth upon tumor challenge but is not effective as a treatment for established tumors (22). Recombinant vaccinia virus transduced with HER-2/neu has also been shown to be fairly effective in a tumor prevention model (23). HSP-110-HER-2/neu chaperone complex vaccines containing the intracellular domain of HER-2/neu have also been described previously (24). It has been shown previously that depletion of either CD4+ or CD8+ T cells in tumor-bearing mice results in decreased tumor regression vs undepleted mice (25). Because of the ability of L. monocytogenes to generate both an Ag-specific CD4+ or CD8+ T cell response, it appears to be a promising vaccine vector for HER-2/neu. In addition, the use of a bacterium such as L. monocytogenes to deliver fragments of HER-2/neu fused to LLO may make self-Ags, such as HER-2/neu, immunogenic enough to induce an anti-HER-2/neu tumor response in mice with HER-2/neu-positive tumors.

In this study, we describe a series of L. monocytogenes-based vaccines for HER-2/neu. Each of these vaccines is capable of significantly impacting on tumor growth and is able to induce an anti-HER-2/neu CTL response, despite the fact that there is only one known CD8+ T cell epitope, which falls into a region covered by only one vaccine (Lm-LLO-EC2) (26). These results suggest that there are subdominant HER-2/neu epitopes that are revealed through this vaccination strategy that are immunogenic enough to induce CD8+ T cells that can control tumor growth. This study further attempts to determine whether these subdominant epitopes are revealed through 1) the use of L. monocytogenes as a vaccine vector, 2) fusion of the Ag to LLO, 3) splitting HER-2/neu into fragments, or 4) a combination of these possibilities.

Materials and Methods

L. monocytogenes and vaccine construction

The L. monocytogenes strains used for these studies are Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, and Lm-LLO-IC2, all of which contain a fragment of rat HER-2/neu fused to the listerial hly gene in an episomal expression system that has been described previously by our lab (19, 21). Fragments of HER-2/neu have been cloned into the plasmid pGG-55, and the amino acids in each fragment are 20–326 in Lm-LLO-EC1, and 1020–1260 in Lm-LLO-IC2 (see Fig. 1). Each of these fragments, or a combination of these possibilities.

Western blotting

The HER-2/neu L. monocytogenes vaccines were grown overnight in Luria-Bertani medium with 50 μg/ml chloramphenicol at 37°C. Supernatants were TCA precipitated and resuspended in 1 x LDS sample buffer (Invitrogen Life Technologies). Fifteen microliters of each sample were loaded on a 4–12% Bis-Tris SDS-PAGE gel (Invitrogen Life Technologies). The gel was then transferred to a Immobilon-P polyvinylidene fluoride membrane (Millipore) and blotted with a polyclonal rabbit serum (No. 3628, Santa Cruz Biotechnology, Santa Cruz, CA) raised to the first 30 residues of the LLO protein (anti-proline, glutamic acid, serine, and threonine (PEST)). The secondary Ab was an HRP-conjugated anti-rabbit Ab (Amersham Biosciences).

Mice

Six- to 8-wk-old female FVB/N mice were purchased from Charles River Laboratories.

Cell lines

The FVB/N syngeneic NT-2 tumor cell line was developed from a spontaneously occurring mammary tumor in a FVB/N HER-2/neu-transgenic mouse (27). NT-2 tumor cells constitutively express low levels of rat HER-2/neu and are tumorigenic in wild-type syngeneic mice. NT-2 cells were grown in RPMI 1640 medium supplemented with 10% FCS, 10.2 mM HEPES, 2 mM l-glutamine, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 50 U/ml penicillin G, 50 μg/ml streptomycin, 20 μg/ml insulin, and 2 μg/ml gentamicin at 37°C with 5% CO2. NIH 3T3 cells are a mouse fibroblast line and the 3T3-neu cell lines were made from these wild-type cells as described previously (26). Briefly, wild-type 3T3 cells were transduced with overlapping fragments of the rat HER-2/neu gene, creating nine 3T3 HER-2/neu fragment lines, and one 3T3 line expressing the full-length rat HER-2/neu. The 3T3 HER-2/neu fragment lines encompass the following overlapping alanine substitution regions: 3T3-neu-1 spans 1–165; 3T3-neu-2 spans 148–291; 3T3-neu-3 spans 274–426; 3T3-neu-4 spans 410–553; 3T3-neu-5 spans 531–687; 3T3-neu-6 spans 665–820; 3T3-neu-7 spans 979–952; 3T3-neu-8 spans 929–1085; and 3T3-neu-9 spans 1063–1260. The NIH 3T3 and all of the derived cells were cultured in DMEM supplemented with 10% FCS, 2 mM l-glutamine, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 50 U/ml penicillin G, and 50 μg/ml streptomycin. Culture medium for the 3T3-neu cell lines was supplemented with 1 mg/ml G418. Cells were grown at 37°C with 5% CO2.

Tumor regression

Six- to 8-wk-old female FVB/N mice were injected s.c. on the right flank with 2 x 106 NT-2 tumor cells in 200 μl of PBS. One week postinoculation, the tumors reached a palpable size at 4–5 mm. Each treatment group consisted of eight mice, which were vaccinated with 0.1 LD50 i.p. Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, Lm-LLO-IC2, Lm- LLO-E7, or PBS on days 7, 14, and 21 after tumor inoculation.
Measurement of tumor growth

Tumors were measured every 2 days with calipers spanning the shortest and longest surface diameters. Plots show the mean of these two measurements as the tumor size in millimeters vs time. Mice were sacrificed when mean tumor diameter reached 20 mm, and tumor measurements for each time point are shown only for the surviving mice.

CD8\(^+\) cell depletion

CD8\(^+\) T cells were depleted in NT-2 tumor-bearing mice with 0.5 mg of 2.43 (21) on days 6, 7, 8, 11, 14, 17, 20, and 23 posttumor injection. The 2.43 (anti-CD8\(^+\) T cell) Ab was affinity purified from ascites on a protein G-Sepharose column (Amersham Biosciences). This Ab has been shown by many investigators to specifically deplete only CD8\(^+\) T cells. In contrast, a similarly purified, isotype-matched rat Ab, GL117.41, which proliferates an anti-E. coli \(\beta\)-galactosidase, had no effect on tumor growth in similar transplantable tumor models (19, 28). CD8\(^+\) T cell populations were reduced by \(>95\%\) as measured by flow cytometric analysis on day 24 (data not shown). Mice were vaccinated and tumors measured as described above.

Flow cytometric analysis

FVB/N mice, 6–8 wk old, were immunized with either PBS, 0.1 LD\(_{50}\) Lm-LLO-EC2, or 0.1 LD\(_{50}\) Lm-LLO-E7 and then boosted 21 days later. Three-color flow cytometry for CD8 (53-6.7, FITC conjugated), CD62 ligand (Mel-14, APC conjugated) (BD Biosciences), and HER-2/neu H-2\(^i\) tetramer (PE conjugated) was performed using a FACS Calibur flow cytometer with CellQuest software (BD Biosciences). The tetramer was loaded with a H-2\(^i\)-specific PDSLRDLSVF peptide. Tetramers were provided by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility of Emory University (Atlanta, GA), under the direction of Dr. J. Altman, and the National Institutes of Health AIDS Research and Reference Reagent Program. Splenocytes were harvested 5 days after the boost and were stained at room temperature with the tetramer for 1 h at 1/200 dilution. Cells were then stained on ice with anti-CD8 and anti-CD62L Abs for 30 min. The splenocytes were then analyzed as described above comparing CD8\(^+\),CD62L\(^low\),tetramer\(^+\) cells generated by either PBS, Lm-LLO-EC2, or Lm-LLO-E7 vaccination. Analysis was done using FlowJo software (Tree Star).

\(^{51}\)Cr release assay

Six- to 8-wk-old FVB/N mice were immunized i.p. with 0.1 LD\(_{50}\) Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, Lm-LLO-IC2, Lm-LLO-E7, or PBS or with 50 \(\mu\)l i.m. of pcDNA neu, pcDNA LLO-neu, pcDNA LLO-EC1, or pcDNA LLO-EC1. Mice were then sacrificed 9 days after immunization, and spleens were harvested. Splenocytes were then cultured with irradiated NT-2 (20,000 rad) tumor cells at a 100:1 ratio of splenocytes to tumor cells with 20 U/ml IL-2 (Roche). Following 4 days of culture, the splenocytes were used as effector cells in a standard \(^{51}\)Cr release assay. Briefly, target cells (3T3-wt, 3T3-neu-1, 3T3-neu-2, 3T3-neu-3, 3T3-neu-4, 3T3-neu-5, 3T3-neu-6, 3T3-neu-7, 3T3-neu-8, and 3T3-neu-9 lines) were labeled with chromium and were then cultured with splenocytes at the E/T ratios of 200:1, 100:1, 50:1, and 25:1 in triplicate for 4 h. In addition, 3T3-wt cells were pulsed for a minimum of 1 h before labeling with \(^{51}\)Cr with 1 \(\mu\)M of the H-2\(^i\)-specific PDSLRDLSVF peptide. Following the incubation, 100 \(\mu\)l of supernatant were assayed for \(^{51}\)Cr release. The percent-specific lysis was determined as (experimental counts per minute − spontaneous counts per minute)/(total counts per minute − spontaneous counts per minute)) \times 100.

DNA vaccination

Female FVB/N mice that were 6–8 wk old were given 7 \times 10^5 NT-2 tumor cells s.c. on the right flank. Mice were then vaccinated i.m. with either 50 \(\mu\)g of GM-CSF, 50 \(\mu\)g of pcDNA neu + GM-CSF, 50 \(\mu\)g of pcDNA LLO-neu + GM-CSF, 50 \(\mu\)g of pcDNA LLO-neu + GM-CSF, 50 \(\mu\)g of pcDNA LLO-EC1 + GM-CSF, or i.p. with PBS or 0.1 LD\(_{50}\) Lm-LLO-EC1. The vaccines were given on days 3, 10, and 18 and tumors were measured every 3 days. Mice were sacrificed when their tumors reached 20 mm in size.

Statistics

The Student \(t\) test was used for statistical analyses, and significant differences (\(p < 0.05\)) are noted.

Results

Generation of L. monocytogenes strains that secrete fragments of HER-2/neu

Five recombinant L. monocytogenes strains that express and secrete overlapping fragments of the rat HER-2/neu gene have been designed and constructed (Fig. 1A). In the design of these vaccine constructs, the signal sequence and transmembrane domains of HER-2/neu have been deleted due to the hydrophobicity of these regions and the inability of L. monocytogenes to secrete extremely hydrophobic domains. All the fragments have been cloned into the pGG-55 expression system described previously (19). The Lm-LLO-HER-2/neu constructs have been designed based on the effectiveness of an artificial tumor Ag, NP, and the HPV tumor Ag, E7, in this system (19–21). The secretion of each of the HER-2/neu fragment vaccines has been confirmed by Western blot of secreted Listerial proteins (Fig. 1B). Secretion of the fusion protein LLO-E7 by Lm-LLO-E7 as a control is shown at 67 kDa. An 83-kDa recombinant protein is secreted by Lm-LLO-EC1, 70 kDa by Lm-LLO-EC2, 68 kDa by Lm-LLO-EC3, 92.5 kDa by Lm-LLO-IC1, and 74 kDa by Lm-LLO-IC2. Each of these strains is highly attenuated as compared with the wild-type 10403S, but the virulence of each strain is comparable to Lm-LLO-E7, which is \(\sim 1 \times 10^9\) CFU.

Vaccination with each Lm-based HER-2/neu construct leads to stasis in tumor growth

Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, and Lm-LLO-IC2 were each compared with PBS controls and Lm-LLO-E7-vaccinated mice for their ability to impact on the growth of HER-2/neu-expressing tumor cells. The tumor burden of HER-2/neu-expressing tumor cells in mice vaccinated with Lm-LLO-EC1, Lm-LLO-EC2, or Lm-LLO-EC3 was significantly reduced compared with mice vaccinated with Lm-LLO-E7 or PBS (Fig. 2A). Mice vaccinated with Lm-LLO-IC1 or Lm-LLO-IC2 showed a similar trend, with a decrease in tumor growth compared with control mice. These results are consistent with previous studies showing that vaccines targeted against HER-2/neu can induce antitumor immune responses and lead to tumor stasis in vivo.

FIGURE 1. HER-2/neu has been broken into fragments, and recombinant L. monocytogenes is capable of secreting each of these vaccines as an LLO-fusion protein. A, Rat HER-2/neu was broken into fragments for the construction of a series of Lm-LLO-HER-2/neu vaccines as described under Materials and Methods. B, Following the construction of each of these vaccines, the secretion of the fusion peptides was confirmed by Western blot analysis. Marker (lane 1), Lm-LLO-E7 (lane 2), Lm-LLO-IC1 (lane 3), Lm-LLO-EC2 (lane 4), Lm-LLO-EC3 (lane 5), Lm-LLO-IC1 (lane 6), and Lm-LLO-IC2 (lane 7). * denotes the band for endogenous LLO secreted by all L. monocytogenes. The Listeria vaccines were grown overnight at 37°C in Luria-Bertani broth. Supernatants were TCA precipitated, and 15 \(\mu\)l of each sample were analyzed by Western blot analysis. The blot was probed with an anti-PEST rabbit serum, followed by HRP-conjugated anti-rabbit secondary Ab. The blot was developed using ECL detection reagents.
of the rat HER-2/neu-expressing tumor line, NT-2. FVB/N mice were given s.c. NT-2 tumor injections on the right flank that produced palpable tumors of 4–5 mm 7 days later. Mice were given weekly i.p. injections of 0.1 LD50 Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, Lm-LLO-IC2, or the controls Lm-LLO-E7 or PBS. Neither the PBS nor Lm-LLO-E7 injections had any impact on tumor growth, but within a week of the first vaccination with either Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, or Lm-LLO-IC2, NT-2 tumor growth became static and remained so until the experiment was terminated at 90 days (Fig. 2, A and B). This cessation in tumor growth remained well after the last HER-2/neu vaccination on day 21, and a subsequent regression of tumors in some of the mice was observed. Three of eight of the mice with Lm-LLO-EC2 and Lm-LLO-EC3 vaccinations were completely cured of their tumors, while one of eight mice vaccinated with Lm-LLO-EC1 or Lm-LLO-IC1 showed complete tumor regression. None of the tumors in the Lm-LLO-IC2 vaccinated group underwent a complete regression, but the tumor growth of the mice in this group remained static and in some cases the tumor size decreased.

**CD8+ T cells are necessary for Lm-LLO-HER-2/neu vaccine effectiveness**

For both Lm-LLO-E7 and Lm-LLO-NP, CD8+ T cells are absolutely necessary for vaccine effectiveness (19). FVB/N mice were depleted of CD8+ T cells after establishment of NT-2 tumors. Upon depletion of CD8+ T cells with the anti-CD8 Ab 2.43, each of the Lm-LLO-HER-2/neu vaccines lost all effectiveness (Fig. 3, A and B). Tumor growth in mice that were not depleted of CD8+ T cells could be controlled with vaccination of the mice with Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, and Lm-LLO-IC2 on days 7, 14, and 21. Growth of tumor in PBS mock-vaccinated mice from a similar experiment is shown in Fig. 6 C for comparative purposes. The growth of tumor in PBS vaccinated mice is similar but slightly slower than the growth of tumors in the Lm-LLO-HER-2/neu-vaccinated mice that were depleted of CD8+ T cells (Fig. 3C). This suggests that endogenous CD8+ T cells induced by the tumor may be slowing tumor growth in unvaccinated mice as we have previously observed in other tumor models (21).

**Lm-LLO-EC2 vaccination induces an increase in HER-2/neu-specific CD8+ T cells**

The CD8+ T cell depletion tumor regression experiment shown in Fig. 3 clearly shows that CD8+ T cells have an essential role in the control of NT-2 tumor growth. Measurement of the HER-2/neu-specific CD8+ T cells activated through vaccination with the rLm constructs can be performed by tetramer analysis. This analysis is only possible for the Lm-LLO-EC2 construct because the only CD8+ T cell epitope known for the FVB/N mouse is from aa 420–429, which is contained within the EC2 fragment (26). Mice were vaccinated with 0.1 LD50 Lm-LLO-EC2 or Lm-LLO-E7 or injected with PBS and then boosted 21 days later. Splenocytes were then stained with the H-2q tetramer loaded with the PD-SLRDLVF peptide, and the activated CD8+ T cells were analyzed to determine the percentages that were tetramer positive (Fig. 4). A 5-fold increase in tetramer-positive cells in the spleens was observed in non-tumor-bearing FVB/N mice after vaccination with Lm-LLO-EC2 vs injection with either Lm-LLO-E7 or PBS.

**CTL analysis shows the induction of CD8+ T cells against subdominant epitopes**

Because each Lm-LLO-HER-2/neu vaccine is capable of impacting on tumor growth, yet only one FVB/N CD8+ T cell epitope has been described, CTL assays were performed using 3T3 cell lines expressing HER-2/neu fragments to determine which regions of HER-2/neu contain a potential epitope. Mice were immunized with one of the Lm-LLO-HER-2/neu vaccines, and the splenocytes were used as effector cells in a standard 51Cr release assay. Initially each vaccine was tested with wild-type 3T3 cells as targets for a negative control and 3T3 cells transduced with full-length rat HER-2/neu (26) as a positive control. Each vaccine is capable of inducing an anti-HER-2/neu CTL response as evidenced by the specific lysis of 3T3-neu cells vs wild-type 3T3 cells (Fig. 5, A and B). Splenocytes from PBS-vaccinated mice produced little lysis regardless of the E:T ratio, while >20% lysis of 3T3-neu cells was produced by each of the Lm-LLO-HER-2/neu vaccine constructs at E:T ratios of 200:1 or 100:1. Splenocytes from PBS-vaccinated mice were used as a control for all subsequent CTL assays because no difference in lysis of 3T3-wt and 3T3-neu cells was seen using either PBS or Lm-LLO-E7-primed CTLs (Fig. 5, C and D). Both PBS and Lm-LLO-E7 vaccinations induced a very low level of
nonspecific lysis that was virtually equal. The levels of lysis seen from the Lm-LLO-HER-2/neu vaccinations with any of the HER-2/neu-expressing cell lines is similar to the lysis observed by pulsing 3T3-wt target cells with the known FVB/N CD8/H11001 T cell epitope peptide at high E:T ratios (Fig. 5E). However, the lysis observed when not targeting a specific peptide titrates out by 50:1, whereas with a known peptide, a significant level of lysis can still be seen at an E:T ratio of 25:1 (Fig. 5E). This illustrates the fact that although these assays can be used as a guide to where in the HER-2/neu sequence potential CD8/H11001 T cell epitopes are located, once a specific peptide sequence is identified, more sensitive CTL assays can be done.

Following the determination that each of the vaccine constructs was capable of eliciting an anti-HER-2/neu immune response in a CTL assay, a panel of 3T3 cells expressing fragments of HER-2/neu (26) were used as target cells for vaccinations with each of the corresponding rLm vaccines (Table I). Each vaccine is capable of eliciting a CTL response to at least one fragment of HER-2/neu. Several fragments of HER-2/neu likely contain a subdominant epitope as evidenced by lysis that was significantly different from controls (p < 0.05). Based on the CTL analysis of this panel of target cells, regions of HER-2/neu with potential subdominant epitopes for the FVB/N mouse have been identified.

**Fusion to LLO and delivery by L. monocytogenes enhances the immunogenicity of HER-2/neu**

There are several possibilities for why these recombinant L. monocytogenes vaccines are capable of revealing subdominant epitopes when other vaccination strategies have not. The Lm-LLO-HER-2/neu vaccines may be revealing these subdominant epitopes through 1) delivery by L. monocytogenes, 2) fusion of the target tumor Ag to LLO, 3) breaking HER-2/neu into fragments, or 4) a combination of these factors. To determine which of these factors contributes to the enhanced immunogenicity of HER-2/neu in the Lm-LLO-HER-2/neu system, a series of DNA vaccines were constructed. Only the EC1 fragment was made to study the effect of breaking HER-2/neu into pieces because each Lm-LLO-HER-2/neu vaccine worked equally well in tumor regression studies. These vaccines consist of a pcDNA 3.1 backbone containing one of the following inserts: full-length HER-2/neu (pcDNA neu), full-length HER-2/neu fused to LLO (pcDNA LLO-neu), the EC1 fragment (pcDNA EC1), or the EC1 fragment fused to LLO (pcDNA LLO-EC1).

Following the construction of the pcDNA-HER-2/neu vaccines, a tumor regression experiment was done comparing these constructs with Lm-LLO-EC1. Mice were given 7 \times 10^6 NT-2 cells s.c. and were vaccinated i.m. with the DNA vaccines plus GM-CSF on a separate pcDNA 3.1 plasmid and i.p. with Lm-LLO-EC1 or PBS on days 3, 10, and 18. GM-CSF was included as an adjuvant for the DNA vaccines because of its ability to enhance the efficacy of DNA vaccines (29). The best vaccine based on this experiment was Lm-LLO-EC1 (Fig. 6A). Two Lm-LLO-EC1-vaccinated mice never developed tumors and two more later regressed their tumors compared with the pcDNA LLO-EC1 group in which one mouse never developed a tumor. By day 62, the differences in tumor sizes of the Lm-LLO-EC1 and pcDNA LLO-EC1 groups are statistically different (p < 0.05) and remain so on day 76 by which time there are four mice in the Lm-LLO-EC1 group without
tumors, compared with only one mouse in the pcDNA LLO-EC1 group. Fusion to LLO was also compared through pcDNA EC1 vs pcDNA LLO-EC1 and pcDNA neu vs pcDNA LLO-neu (Fig. 6, B and C). The constructs where either the full-length HER-2/neu or the EC1 fragment are fused to LLO are clearly better in terms of tumor regression as compared with the unfused versions. Mice vaccinated with either of the unfused vaccines developed tumors that grew at the same rate as those in control mice vaccinated with just GM-CSF, which grew slightly slower than the tumors in mice vaccinated with only PBS. These differences in tumor sizes for pcDNA EC1 vs pcDNA LLO-EC1 and pcDNA neu vs pcDNA LLO-neu vaccinated mice are statistically significant (p < 0.01) on day 62 posttumor inoculation. The effect of breaking HER-2/neu up into smaller pieces was tested by comparing pcDNA LLO-neu vs pcDNA LLO-EC1 and pcDNA neu vs pcDNA EC1 (Fig. 6, D and E). Although there does seem to be minor differences in the tumor sizes between pcDNA LLO-EC1 and pcDNA LLO-neu between days 62 and 90 (Fig. 6D), this difference is not statistically significant. No difference could be seen between the pcDNA neu and pcDNA EC1-vaccinated mice, and tumors in both of these groups grew out as rapidly as the GM-CSF controls (Fig. 6E).

**Broad CTL response to LLO-fused DNA vaccines vs unfused DNA vaccines**

Each of the DNA vaccines was further analyzed using the standard ⁵¹Cr release assay as described above. The LLO-fused versions of either full-length HER-2/neu or the EC1 fragment were capable of inducing T cells that lysed more of the 3T3-neu target cell lines than did the non-LLO-fused DNA vaccines (Table II). pcDNA neu induced a strong CTL response against only the 3T3-neu-4 target cell line, the line that contains the H-2q epitope previously described by Ercolini et al. (26). The fusion of the full-length HER-2/neu to LLO resulted in a broadening of the regions that could be recognized by CTLs, and the same result was seen with the EC1 fragment fused to LLO vs the non-LLO-fused EC1. Thus, whereby only 3T3-neu-1 and 3T3-neu-4 were targeted for lysis by pcDNA neu splenocytes, 3T3-neu-1, -4, -5, and -9 were targeted by splenocytes from pcDNA-LLO-neu.
the Lm-LLO-EC2 vaccine was expected to generate an anti-HER-2/neu tumor response because this vaccine contains the only previously described rat HER-2/neu epitope for the FVB/N mouse, although there is evidence that this may not be the immunodominant epitope in the HER-2/neu-transgenic mouse (26, 30). Surprisingly, each of the five vaccine constructs generated an antitumor immune response that resulted in stasis of tumor growth and eventually a late onset of regression in a subset of the vaccinated mice. The antitumor immunity observed absolutely required CD8+ T cells. The fact that each of these vaccine constructs could generate HER-2/neu-specific CD8+ T cells and an antitumor response suggests that subdominant MHC class I-restricted HER-2/neu epitopes are being revealed through the use of these vaccines. This study shows that these epitopes emerge not only through the use of *L. monocytogenes* as a vaccine vector but also through the fusion of the HER-2/neu fragments to LLO.

Fusion to LLO was originally performed to ensure that the target Ag would be secreted by recombinant *L. monocytogenes*, resulting in presentation of the secreted target Ag by the MHC class I pathway and the generation of a CD8+ T cell response (17). However, subsequent to these earlier experiments, we found that fusion of target Ags to either LLO or a PEST-like sequence contained in LLO resulted in an increased antitumor response (31). The exact reason for this enhancement in immunogenicity of fused Ags is not yet clear, but we hypothesize that these fused Ags are more readily degraded by the proteasome and subsequently targeted to the class I pathway. Decatur and Portnoy (32) have shown that deletion of a PEST-like sequence toward the N terminus of LLO results in an accumulation of LLO in the cytosol of the host cells, which suggests that this sequence may target LLO for degradation. However, we believe that LLO is doing more to increase the immunogenicity of target Ags than simply increasing the degradation and subsequent presentation of these Ags. We compared vaccines where the tumor Ag is fused to LLO vs fusion to PEST and showed that both elicited similar antitumor immune responses (31). However, even fusing the Ag to a LLO molecule from which the PEST sequence has been deleted resulted in better antitumor efficacy than using the Ag alone, suggesting that LLO may have an adjuvant effect quite apart from enhancing Ag processing. We have shown previously using vaccines that deliver the HPV-16 E7 Ag that Lm-LLO-E7 can mature bone marrow-derived dendritic cells and up-regulate costimulatory molecules, whereas Lm-E7 does not, which also points to an adjuvant effect for LLO (33).

Potential regions of HER-2/neu with subdominant epitopes have been identified through CTL analysis following vaccination of mice with DNA vaccines consisting of either full-length or the EC1 fragment of HER-2/neu both fused to LLO and unfused. Lysis of regions of HER-2/neu not containing the identified dominant epitope (26) was seen with each of the four DNA vaccines. A broadening of the regions of HER-2/neu with potential epitopes based on this CTL analysis can be observed with fusion to LLO, further solidifying the theory that LLO enhances the immunogenicity of target Ags leading to the revelation of subdominant epitopes. Comparing the DNA vaccines, it is clear that fusion of the full-length neu to LLO increases the regions of neu that are recognized from two, with no LLO fusion, to four with LLO fusion (Table II). However, this expanded response was not equal to the responses seen with the *Listeria*-based HER-2/neu vaccines, as a total of nine regions with potential epitopes have been identified with these vaccines (Table I). Furthermore, the comparison of tumor regression induced by pcDNA LLO-EC1 with that of Lm-LLO-EC1 shows that Lm-LLO-EC1 is superior to pcDNA LLO-EC1 (Fig. 6A). Therefore, the increased epitope recognition of the *Listeria*-based vaccines vs the DNA vaccines is likely due to enhancement of the magnitude of the immune response through delivery by *Listeria*, which enhances the detection level of weak epitopes. These DNA vaccine results suggest that some epitopes, but not all, can be revealed through fusion to LLO, but some need the additional boost of delivery by *L. monocytogenes* as well, perhaps because of the added danger signals provided by a live vaccine vector (34) in addition to direct delivery to the MHC class I pathway of Ag processing by professional APCs.

DNA-based vaccines targeting HER-2/neu have been evaluated by other groups in transplantable tumor models (35–38). A major difference between the Lm-LLO- or DNA-LLO-HER-2/neu vaccine studies described here and those performed by other groups is that all of our vaccines were tested in a therapeutic, rather than a prophylactic, mode. In our studies, the first DNA vaccination was given 3 days after injection of the tumor but before the appearance of palpable tumor. In the pcDNA LLO-EC1 group, one mouse never developed a tumor and one mouse later rejected its tumor, whereas there was a slow growth of tumor in the pcDNA LLO-neu

---

**Table 1. Regions of HER-2/neu with potential H-2d epitopes based on percent-specific lysis from CTL analysis and the corresponding vaccines**

<table>
<thead>
<tr>
<th>Listeria Strain</th>
<th>Neu Region Spanned</th>
<th>Percent-Specific Lysis of Target Cellsa</th>
<th>Neur Regions Containing an Epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lm-LLO-EC1</td>
<td>20–326</td>
<td>3T3-neu-1: 14.3* 0.7</td>
<td>20–148</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3T3-neu-2: 0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3T3-neu-3: 6.5* 3.5</td>
<td>291–326</td>
</tr>
<tr>
<td>Lm-LLO-EC2</td>
<td>303–501</td>
<td>3T3-neu-3: 10.9* 7.4</td>
<td>303–426</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3T3-neu-4: 23.8* 8.4*</td>
<td>410–501</td>
</tr>
<tr>
<td>Lm-LLO-EC3</td>
<td>479–655</td>
<td>3T3-neu-5: 34.4* 25.3*</td>
<td>531–655</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3T3-neu-6: 6.9* 9*</td>
<td>690–797</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3T3-neu-7: 0 2.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3T3-neu-8: 18.2* 6.4</td>
<td>952–1081</td>
</tr>
<tr>
<td>Lm-LLO-IC1</td>
<td>690–1081</td>
<td>3T3-neu-9: 16.5 0</td>
<td>1063–1260</td>
</tr>
<tr>
<td>Lm-LLO-IC2</td>
<td>1020–1260</td>
<td>3T3-neu-8: 10.3* 8.2*</td>
<td>1020–1085</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3T3-neu-9: 16.5 0</td>
<td></td>
</tr>
</tbody>
</table>

*Percent specific lysis minus background lysis by PBS control vaccinated mice for the E:T ratios of 200:1 and 100:1. Each well was set up in triplicate, and the results of a representative experiment are shown. The results were calculated as percent lysis minus background lysis/(total lysis−spontaneous lysis).*
The Lm-LLO-HER-2/neu vaccines are given 7 days posttumor injection, at which point there is a 4- to 5-mm macroscopic tumor present. A subset of mice vaccinated with these vaccines do completely regress their tumors, which is a very important result because it is extremely difficult to cause the regression or induce a halt in the growth of established tumors. The
We intended to explore this hypothesis by testing the reactivity of the majority of HER-2/neu DNA vaccines produced by other groups were used in the less stringent prophylactic mode to determine whether vaccination will lead to protection against a subsequent tumor challenge (35–38). In this study, the DNA vaccines studied were comprised of the extracellular domain of HER-2/neu, the transmembrane domain of HER-2/neu, or the intracellular domain of HER-2/neu. The extracellular domain DNA vaccine protected 25–35% of mice and that percentage was increased to 60–75% with the addition of IL-12. Approximately 50% of the mice were protected from tumor challenge after treatment with the intracellular DNA vaccine, and the administration of IL-12 increased this percentage of protected animals to between 80 and 100%. Sixty to 75% of mice were protected from tumor challenge with the administration of the transmembrane DNA vaccine, and this percentage remained the same with the addition of IL-12 (35).

In our study, each of the vaccines resulted in the stasis of tumor growth immediately following the first vaccination (Fig. 2). However, none of the tumors decreased in size until >1 mo following the last vaccination. Ag loss or mutation and the down-regulation of MHC class I molecules frequently result in the lack of tumor regression (39). In the case of HER-2/neu-overexpressing tumors, where the overexpression of the tumor Ag contributes to the transformed phenotype of the tumors, the loss of the tumor Ag could also result in a lack of tumor growth. Neu-negative variants have been observed after anti-HER-2/neu therapies (40). We are currently attempting to determine whether the tumors that do not decrease in size or undergo regression in response to the Listeria-based vaccines are down-regulating HER-2/neu.

The induction of tumor regression at >1 mo after the final boost is in contrast with our observations using other tumor Ags where tumor regression will often begin after the first immunization and is completed within 28 days (19, 21, 31). The delay in tumor regression observed with the Lm-LLO-HER-2/neu vaccines is not likely due to the reactivation of a memory response. Although, it is likely that memory cells are generated following the recombinant L. monocytogenes vaccines, no additional vaccination or stimulus is given to reactivate these memory cells at the time that the tumor regression begins. We think it is more plausible that the increase in antitumor reactivity at a delayed time point is due to epitope spreading by cross-presentation of tumor-derived HER-2/neu or other Ags.

Epitope spreading has been seen previously in many different tumor models and can result in the generation of a specific antitumor response to cryptic and subdominant epitopes that were not initially part of the treatment administered (41–43). The Listeria-based vaccines containing fragments of HER-2/neu may be generating sufficient initial antitumor CD8+ T cells to limit the growth of the tumor early after vaccination. Although the tumor growth is stabilized with new tumor cells being generated at the same rate that tumor cells are dying, APCs such as dendritic cells may be acquiring pieces of the dying cells, traveling to the draining lymph nodes, and cross-presenting these Ags to activate a much broader T cell response than could be generated by vaccination targeting one region of the HER-2/neu molecule. This phenomenon has been observed previously, and cross-presentation by CD11c+ dendritic cells is seen as a key factor in the control of tumor growth because the CD8+ T cell response elicited by a specific immunotherapy can be significantly enhanced by this mechanism (44, 45). We intend to explore this hypothesis by testing the reactivity of the
lymphocytes in the tumor-draining lymph nodes to see whether T cells specific to a region of HER-2/neu that was not included in that particular vaccine construct emerges at the time that regression begins. However, this task will be easier once we have completed our epitope mapping of the entire HER-2/neu molecule, which is currently underway. We have already determined that there are a large number of CD11c⁺ dendritic cells infiltrating the tumors following the vaccinations before the onset of regression (data not shown) that could cross-present Ags.

The most effective antitumor response will likely be generated by enhancing low-affinity anti-HER-2/neu-specific CD8⁺ T cells that were not deleted during thymic selection (46). This will be particularly important in transgenic mouse models of cancer in which the mice are tolerant to the tumor Ag and the high-affinity T cells that can be generated to target the tumor in wild-type (non-tolerant) mice will not be present (47, 48). Makki et al. (49) and Kedl et al. (50) have shown that targeting an immunodominant tumor epitope can fail to result in a strong enough antitumor response that will result in the eradication of a tumor. Anti-HER-2/neu therapies that can lead to the amplification of subdominant epitopes are likely be more effective than vaccination against dominant epitopes.

Potentially confounding the efficacy of these vaccines is the fact that the vaccines are based on the rat HER-2/neu gene but that these vaccines are being tested in mice. In this study, the wild-type FVB/N mice are not specifically tolerant to the rat HER-2/neu gene, although the mouse and rat HER-2/neu genes are >90% homologous (51), so some degree of tolerance is expected. Although these sequences are extremely homologous, there are differences in the degree of homology for the different vaccine constructs. The IC1 construct containing the highly conserved kinase domain is 98% homologous to the mouse protein sequence, and this sequence shows the highest level of homology among the five vaccine constructs. Both the EC1 and EC2 regions are 94% homologous with the corresponding mouse regions, and the EC3 and IC2 regions are 93% homologous. In all of the vaccines, excluding Lm-LLO-IC1, the amino acid differences are spread throughout the entire fragment, whereas in IC1 the differences flank the kinase domain with only one nonhomologous amino acid in the kinase domain making the kinase domain 99.6% homologous. Interestingly, the CTL analysis of the Listeria-based vaccines (Table I) shows that of the three 3T3 lines that can be targeted by this fragment only two, 3T3-neu-6 and 3T3-neu-8, were capable of being lysed by effector cells induced by Lm-LLO-IC1 vaccination. Although we do not yet know the exact sequences of the MHC class I epitopes that are recognized in these fragments, it is possible that there are no epitopes present in the kinase domain, as this region is almost entirely homologous between the mouse and the rat. In addition, the portion of HER-2/neu contained in 3T3-neu-7 falls entirely in the kinase domain, and there is no CTL response against this region.

We do not observe a strong correlation between CTL activity and tetramer analysis of HER-2/neu-specific T cells from mice immunized with Lm-LLO-EC2, for which we have a tetramer available. This is because for most of the vaccines, the only source of Ag available to stimulate bulk populations of T cells in splenocytes is irradiated tumor cells. The NT-2 cells do not express a high level of Ag and thus are not very potent stimulators of effector cells. Furthermore, the effector cells used are a polyclonal T cell population that is derived from whole splenocytes, so CD8⁺ T cells are not the only cells being counted as effector cells. In contrast, tetramer-positive T cells can be analyzed in the activated CD8⁺ T cell population. Despite these limits to the CTL assay, we do see statistically significant levels of killing of the target cells from which we are able to narrow down potential areas that contain CTL epitopes.

We have shown in this study that vaccines generated from fragments of HER-2/neu that do not contain the dominant epitope are as effective as the vaccine that does result in CD8⁺ T cells specific to the dominant epitope when fused to LLO and delivered by L. monocytogenes. Work is now underway to identify these subdominant epitopes and to test these vaccines in a HER-2/neu-transgenic mouse model for breast cancer (30).

Acknowledgments

We greatly appreciate the advice of Dr. Zhen-Kun Pan in the tumor regression experiments. We also thank Dr. Paul Neeson for helpful discussions and for assistance with FACS analysis.

Disclosures

Y. Paterson has a financial interest in Advaxis, a vaccine and immunotherapeutic company that is developing L. monocytogenes as a cancer vaccine vector.

References


