Recombinant Listeria Vaccines Containing PEST Sequences Are Potent Immune Adjuvants for the Tumor-Associated Antigen Human Papillomavirus-16 E7

Duane A. Sewell, Vafa Shahabi, George R. Gunn III, Zhen-Kun Pan, Mary E. Domieniecki, and Yvonne Paterson

1Department of Microbiology and the University of Pennsylvania Cancer Center, and 2Department of Otorhinolaryngology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

Abstract

Previous work in our laboratory has established that the fusion of tumor-associated antigens to a truncated form of the Listeria monocytogenes virulence factor listeriolysin O (LLO) enhances the immunogenicity and antitumor efficacy of the tumor antigen when delivered by Listeria or by vaccinia. LLO contains a PEST sequence at the NH2 terminus. These sequences, which are found in eukaryotic proteins with a short cellular half-life, target proteins for degradation in the ubiquitin-proteasome pathway. To investigate whether the enhanced immunogenicity conferred by LLO is due to the PEST sequence, we constructed new Listeria recombinants that expressed the HPV-16 E7 antigen fused to LLO, which either contained or had been deleted of this sequence. We then compared the antitumor efficacy of this set of vectors and found that Listeria expressing the fusion protein LLO-E7 or PEST-E7 were effective at regressing established macroscopic HPV-16 immortalized tumors in syngeneic mice. In contrast, Listeria recombinants expressing E7 alone or E7 fused to LLO from which the PEST sequence had been genetically removed could only slow tumor growth. Because CD8+ T cell epitopes are generated in the ubiquitin-proteasome pathway, we also investigated the ability of the vaccines to induce E7-specific CD8+ T cells in the spleen and to generate E7-specific tumor-infiltrating lymphocytes. A strong correlation was observed between CD8+ T-cell induction and tumor homing and the antitumor efficacy of the Listeria-E7 vaccines. These findings suggest a strategy for the augmentation of tumor antigen-based immunotherapeutic strategies that may be broadly applicable.

Introduction

Listeria monocytogenes is an intracellular pathogen that induces potent cellular immune responses because of its unusual life cycle (1). Upon phagocytosis by an antigen-presenting cell, the bacteria are able to escape into the cytoplasm of the cell by perforating the phagolysosome. Once in the cytoplasm, bacterial proteins are effectively presented to cytotoxic T lymphocytes, thus initiating a cellular immune response against bacterial antigens. By engineering recombinant Listeria vaccines to secrete not only bacterial antigens but also tumor antigens, immune responses have been generated sufficient to cause the regression of established tumors in animal models (1-5).

In a previous study, two Listeria strains were engineered to secrete a tumor antigen associated with human papillomavirus (HPV) (4). The most effective of the two vaccines secreted the tumor antigen as a fusion with another protein, listeriolysin O (LLO). LLO is a hemolytic protein encoded by the hly gene that leads to perforation of the phagolysosome (6). However, the form of LLO included in the fusion was a truncated, nonhemolytic form, so the reason for enhanced efficacy was not due to increased virulence of the recombinant strain.

A possible reason for the enhanced efficacy may be the presence of a 19-amino acid sequence within LLO called a PEST sequence. PEST sequences are included so that the COOH terminus hemolytic domain is deleted. LLO-E7 has been described in detail previously (4). In short, it is a recombinant L. monocytogenes strain identical to Lm-LLO-E7 except that it contains or has been deleted of this sequence. We then compared the antitumor efficacy of this set of vectors and found that Listeria expressing the fusion protein LLO-E7 or PEST-E7 were effective at regressing macroscopic HPV-16 immortalized tumors in syngeneic mice.

Materials and Methods

Mice. Six- to 8-week-old C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Animals were cared for and used in accordance with protocols approved by the Animal Care and Use Committee of The University of Pennsylvania (Philadelphia, PA).

Cell Line. The TC-1 cell line, a generous gift from Dr. T. C. Wu (Johns Hopkins University School of Medicine, Baltimore, MD) is a lung epithelial cell immortalized with HPV-16 E6 and E7 and transformed with the c-Ha-ras oncogene (8). TC-1 was grown in RPMI 1640 supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 50 µmol/L 2-ME, 400 µg/mL G418, and 10% National Collection Type Culture 109 medium at 37°C with 10% CO2.

L. monocytogenes Strains and Propagation. The Listeria strain Lm-LLO-E7 has been described in detail previously (4). In short, it is a recombinant bacterial strain that secretes E7 as a fusion protein joined to a nonhemolytic LLO via an episomal expression system. The fusion gene contains the hly promoter, the hly gene encoding the first 441 amino acids of LLO (including the signal sequence and the PEST region), the E7 gene, and the Listeria plupotential transcription factor prfA. Only the first 441 amino acids of LLO are included so that the COOH terminus hemolytic domain is deleted.

Lm-PEST-E7 is a Listeria strain identical to Lm-LLO-E7 except that it contains only the promoter and PEST sequence of the hly gene. This includes the first 50 amino acids of LLO. It was constructed as follows: First, the hly promoter and PEST regions were fused to the full-length E7
gene. This was accomplished with SOEing PCR techniques (gene splicing by overlap extension). The E7 gene and the hly-PEST gene fragment were amplified from the plasmid pGG-55, which contains the first 441 amino acids of LLO, and spliced together by conventional PCR techniques. To create a final plasmid, pVS16.5, the hly-PEST-E7 fragment and the L. monocytogenes transcription factor prfA were cloned into the plasmid pAM401, which includes a chloramphenicol resistance gene for selection in vitro (9). The prfA gene fragment was generated as described previously (4). After ligation, the resultant plasmid was then used to transform XFL-7, a L. monocytogenes strain that is identical to Lm-LLO-E7 except that it lacks the PEST region, but contains the hly promoter, the signal sequence, E7, and the remainder of the truncated LLO. Lm-PEST-E7 contains only the hly promoter, the signal sequence, and E7.

Results

Construction of L. monocytogenes Strains with and without the PEST Region of Listeriolysin O. We have made three new L. monocytogenes strains to test the hypothesis that the PEST amino acid sequence is critical for the efficacy of our Listeria-based vaccines. The PEST-like sequence of LLO is 19 amino acids long and resides near the NH₂ terminus (7). The new constructs are based on the previously described recombinant Listeria strain Lm-LLO-E7 (4), which secretes the HPV-16 protein E7 as a fusion protein with the Listeria virulence factor LLO. Similar to Lm-LLO-E7, the new constructs are based on an episomal expression system, but the E7 gene is now linked to different, smaller portions of hly, the gene which encodes LLO. The episomal expression system relies on chloramphenicol resistance for in vitro selection and the expression of prfA for in vivo selection (4). The gene insert used to create the Lm-PEST-E7 vaccine contains E7 fused to only the PEST region and the signal sequence of LLO. Lm-ΔPEST-E7 contains E7 linked to most of the LLO sequence of LLO. Lm-PEST-E7 contains E7 linked to only the PEST region and the signal sequence, and Lm-PEST-E7 contains E7 linked to the C-terminus of the LLO sequence.

Vaccines Containing PEST Sequences Cause Effective Tumor Regression. Lm-LLO-E7, Lm-PEST-E7, Lm-ΔPEST-E7, and Lm-E7ss were compared for their ability to cause regression of tumors that express HPV-16 E7 protein. Subcutaneous tumors were established on the left flank of 40 C57BL/6 mice with the cell line TC-1. TC-1 is immortalized with HPV-16 E6 and E7 (8). After tumors had reached a palpable size of 4 to 5 mm, mice were divided into five groups of eight mice. Each of the groups was treated with one of four recombinant L. monocytogenes vaccines, and one group of mice was left untreated. The previously described PEST-containing vaccine, which expresses E7 linked to the C-terminus of the LLO sequence, was utilized as a positive control. Lm-LLO-E7 vaccine caused a significant decrease in the rate of tumor growth compared to the untreated group. This result suggests that the PEST domain of LLO is a critical component for tumor regression. The Lm-PEST-E7 vaccine also caused a significant decrease in tumor growth, but the results were not as dramatic as those observed with Lm-LLO-E7. The Lm-ΔPEST-E7 vaccine did not cause a significant decrease in tumor growth, indicating that the PEST domain of LLO is necessary for tumor regression. The Lm-E7ss vaccine also did not cause a significant decrease in tumor growth, suggesting that the lack of the PEST domain of LLO is not sufficient for tumor regression.
Lm-LLO-E7, successfully caused the regression of established tumors in five of eight cases. Administration of Lm-PEST-E7 also led to regression of established tumors in three of eight cases. There was no statistical difference between the average tumor size of mice treated with Lm-PEST-E7 or Lm-LLO-E7 at any time point. However, the vaccines that expressed E7 without the PEST sequences, Lm-ΔPEST-E7 and Lm-E7_epi, failed to cause tumor regression in all mice except one (Fig. 2A). This was representative of two experiments. For statistical analysis, the mean tumor sizes at day 28 were compared for the two experiments. A statistically significant difference in tumor sizes was seen between those tumors treated with PEST constructs (Lm-LLO-E7 or Lm-PEST-E7) and those treated without PEST regions (Lm-E7_epi or Lm-ΔPEST-E7; \( P = 0.001 \), Student’s \( t \) test; Fig. 2B).

**Vaccines Containing PEST Sequences Cause Increased Percentages of Antigen-Specific CD8⁺ Lymphocytes within the Spleen.** The vaccines were administered to tumor-bearing mice to compare the levels of E7-specific lymphocytes generated by the vaccine in the spleen. Mice were treated on days 7 and 14 with 0.1 \( \text{LD}_{50} \) of the four vaccines. Spleens were harvested on day 21 and stained with antibodies to CD62L, CD8, and the E7/Db tetramer. Lm-E7_epi and Lm-LLO-PEST-E7 induced similar low levels of E7 tetramer–positive activated CD8⁺ T cells in the spleen. Lm-PEST-E7 induced approximately five times more and Lm-LLO-E7 induced approximately 15 times more (Fig. 3A). Thus, increased percentages of tetramer-positive splenocytes were seen in mice vaccinated with PEST-containing vaccines. This result was reproducible over three experiments performed on different occasions.

The mean and SE for data obtained from the three experiments are depicted in Fig. 3B. The average values for the number of tetramer-positive CD8⁺ cells induced was statistically higher for Lm-LLO-E7 and Lm-PEST-E7 than for Lm-LLO-PEST-E7 and Lm-E7_epi (\( P < 0.05 \)) by Student’s \( t \) test.

**Vaccines Containing PEST Sequences Cause Increased Percentages of Antigen-Specific Tumor-Infiltrating Lymphocytes.** We have previously observed that there is a better correlation between antitumor efficacy and the number of tumor-infiltrating antigen-specific CD8⁺ T cells than with the numbers induced in peripheral lymphoid organs (10). Therefore, the vaccines were administered to tumor-bearing mice to compare the levels of E7-specific lymphocytes generated by the vaccine within the tumor. Mice were treated on days 7 and 14 with 0.1 \( \text{LD}_{50} \) of the four vaccines. Tumors were harvested on day 21 and stained with antibodies to CD62L, CD8, and the E7/Dβ tetramer. Lm-E7_epi and Lm-ΔPEST-E7 induced similar low levels of E7 tetramer–positive activated CD8⁺ T cells in the spleen. Lm-PEST-E7 induced approximately five times more and Lm-LLO-E7 induced approximately 15 times more (Fig. 3A). Thus, increased percentages of tetramer-positive splenocytes were seen in mice vaccinated with PEST-containing vaccines. This result was reproducible over three experiments performed on different occasions. The mean and SE for data obtained from the three experiments are depicted in Fig. 3B. The average values for the number of tetramer-positive CD8⁺ cells induced was statistically higher for Lm-LLO-E7 and Lm-PEST-E7 than for Lm-ΔPEST-E7 and Lm-E7_epi (\( P < 0.05 \)) by Student’s \( t \) test.

**Fig. 2.** A. *Listeria* constructs containing PEST regions lead to greater tumor regression. C57BL/6 mice (eight per group) received \( 1 \times 10^7 \) TC-1 cells by subcutaneous injection in the left flank. Mice were treated on days 7 and 14 after tumor challenge with 0.1 \( \text{LD}_{50} \) Lm-LLO-E7, Lm-PEST-E7, Lm-ΔPEST-E7, or Lm-E7_epi, or they were left untreated. The average tumor diameter is shown for each mouse. Tumor measurements for each time point are shown only for surviving mice; mice were sacrificed if their tumors reached 20 mm. On day 28, five of eight mice are tumor-free in the Lm-LLO-E7 group, and three of eight mice are tumor-free in the Lm-PEST-E7 group. These data are representative of two similar experiments. B, average tumor size in mice treated with *Listeria* vaccines. This graph depicts the average tumor sizes at day 28 post tumor challenge. There is a statistically significant difference between the Lm-PEST-E7 group and the Lm-ΔPEST-E7 group (\( P = 0.004 \), Student’s \( t \) test). Depicted is the average of two experiments.

**Fig. 3.** *Listeria* constructs containing PEST regions induce a higher percentage of E7-specific lymphocytes in the spleen. Spleens were harvested from two or three mice 14 days after the first vaccination and 7 days after a booster vaccination. Pooled splenocytes were stained with anti-CD8 and anti-CD62L antibody and with E7/Db tetrarmers. A, data from one experiment. These data are representative of three similar experiments. B, average and SE of data from all 3 experiments.
phocytes within the tumor were seen in mice vaccinated with PEST-containing vaccines Lm-LLO-E7 and Lm-PEST-E7 (Fig. 4A). This result was reproducible over three experiments. The mean and SE for the three experiments are depicted in Fig. 4B. The average values for the number of tetramer-positive CD8+ TILs induced was statistically higher for Lm-LLO-E7 than for Lm-ΔPEST-E7 and Lm-E7epi (P < 0.05) by Student’s t-test. The difference between Lm-PEST-E7 and either Lm-Δ-PEST-E7 or Lm-E7epi did not achieve statistical significance between the three experiments (P > 0.1), but the same trend was observed in each experiment. It is of interest that even though the number of E7-specific CD8+ T cells actually induced in the spleen was lower in mice immunized with Lm-PEST-E7 than in mice that received Lm-LLO-E7, very similar numbers of E7-specific CD8+ TILs were seen in mice that received either vaccine (about 22%).

Discussion

An important goal of tumor immunotherapeutic strategies is the optimal presentation of tumor antigens so that a rapid and potent immune response against the tumor is achieved. Recombinant L. monocytogenes vaccines that express tumor antigens have been shown to lead to the generation of tumor-specific T-lymphocytes and tumor regression in mouse models (1–5). In the present study, we have determined that the fusion of the antigen to a PEST sequence is critical for the efficacy of these vaccines. PEST regions are thought to target proteins for rapid degradation (7, 11–17). A study that compared the amino acid sequences of 10 proteins with short intracellular half-lives found regions rich in proline, glutamic acid, serine, and threonine in all 10 proteins (11). Subsequent studies have suggested that PEST regions are recognized and bound by components of the ubiquitin proteolytic pathway (18) and that the 26S proteasome is involved in the rapid degradation of PEST proteins (14).

Our results indicate that the fusion of a PEST sequence to the tumor antigen E7 is essential for the efficacy of the vaccine. The two constructs that express the PEST sequence within the fusion protein generated more E7-specific lymphocytes and cured more mice of their tumors than those constructs that do not contain the PEST region. It is unlikely that this difference is due to different levels of antigen expression by the four constructs, because expression of the fusion protein in vitro was highest for Lm-Δ-PEST-E7. Instead, we propose that the PEST region facilitates protein degradation and enhances antigen presentation to T-lymphocytes. These results have important implications for cancer vaccine design in general. Many tumor immunotherapeutic strategies currently under investigation use proteins to generate immune responses to tumor antigens. Often this strategy does not generate sufficient immune responses without an adjuvant such as CpG DNA or bacterial cell wall components (19, 20). Addition of a PEST region may improve not only these strategies, but also other live recombinant strategies such as vaccinia-based vaccination. Indeed, we have previously shown that the LLO-E7 fusion protein shows enhanced immunogenicity and antitumor efficacy compared with other forms of the E7 antigen when delivered by recombinant vaccinia virus (10).

Additional studies to examine this phenomenon should be considered, including in vitro degradation assays of translated fusion proteins and immunofluorescence studies to localize the proteins within an infected cell. It will also be important to determine whether the effect is due to more rapid degradation of the PEST fusion constructs, as opposed to an increase in the total amount of protein degraded by the proteasome.

Within our model, antigen presentation also occurs in the absence of PEST, because Lm-ΔPEST-E7 also caused some tumor regression and generation of E7-specific lymphocytes. Furthermore, there was a slight difference in efficacy between Lm-LLO-E7 and Lm-PEST-E7 and between Lm-ΔPEST-E7 and Lm-E7epi. In both cases, the vaccine that contained LLO sequences was superior. The question of whether the presence of other LLO sequences also promotes antigen presentation is unknown. One hypothesis is that the size of the fusion protein plays a role in the ability to process it. In this case, the larger protein was slightly more effective regardless of the presence or absence of PEST. There are also other peptide motifs known to target proteins for rapid degradation, including KFERQ motifs of Rnase A (21) and the cyclin destruction box from cyclin B (22), among others (23). Studies to determine whether there is a critical portion of LLO besides PEST are warranted.

In summary, our results indicate that the PEST sequence of amino acids enhances the efficacy of E7-specific L. monocytogenes vaccines. This result has broad implications for the augmentation of live and protein antigen-based immunotherapeutic strategies.

Acknowledgments

We thank Alex Rodriguez and Dennis Douven for excellent technical assistance and Drs. S. Farzana Hussain, Thorsten Verch, Christian Peters, and Amy Decatur for helpful advice and discussions.
References


Recombinant *Listeria* Vaccines Containing PEST Sequences Are Potent Immune Adjuvants for the Tumor-Associated Antigen Human Papillomavirus-16 E7


*Cancer Res* 2004;64:8821-8825.

**Updated version**
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/24/8821

**Cited articles**
This article cites 22 articles, 11 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/24/8821.full#ref-list-1

**Citing articles**
This article has been cited by 11 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/64/24/8821.full#related-urls

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/64/24/8821.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.