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Protection of Mice by a λ-Based Therapeutic Vaccine against Cancer Associated with Human Papillomavirus Type 16

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Key Words
Phage-based nanoparticles · Cervical cancer · Gene therapy · Human papillomavirus E7 · C57BL/6 mice

Abstract
Objective: Human papillomavirus (HPV) oncoproteins (i.e. E6 and E7) are constitutively expressed in cervical cancer cells. The proteins are ideal targets to be used for developing therapeutic vaccines against existing HPV-associated carcinomas. To date, whole bacteriophage ('phage')-λ particles, rather than purified 'naked' DNA, have been described as highly efficient delivery vehicles for a DNA vaccine. ***Methods:*** In this study, a safe and efficient λ-based therapeutic cancer vaccine, recombinant λ-ZAP E7 phage, was developed by inserting a HPV16 E7 gene into the Lambda ZAP® cytomegalovirus vector. λ-ZAP E7 phages were employed to immunize mice against the E7-expressing murine tumor cell line (TC-1), which is used as a tumor model in an H-2b murine system. ***Results:*** The tumor-bearing mice indicated a significant inhibition of tumor growth after 3 injections of 2×10^{12} particles of recombinant phages. Released lactate dehydrogenase, interferon-γ and granzyme B from spleen cells and lymphocyte proliferation of spleen cells, which all demonstrate the enhancement of cell-mediated immunity, suggested the phages could be a potent gene delivery system in animal models. ***Conclusion:*** Our results suggest the recombinant phages can be used as effective biological tools for inducing E7-specific protective immune responses. Hence, the study introduces a possible therapeutic strategy against cervical cancer and other HPV-related neoplasia.

Introduction

Human papillomaviruses (HPV) have been identified as the etiological agent of cervical carcinomas, the second most common malignancy in women worldwide [1]. HPV are suspected to be also involved in the induction of other tumors. Hence, the development of prophylactic and therapeutic vaccines against HPV is of the utmost priority [2]. Since the regression of lesions in HPV16-infected humans appears to be associated with T helper cell (Th)1 and CD8+ T cell responses to the early proteins (especially E7 protein), an efficient therapeutic vaccination

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against HPV-associated disease will most likely depend on the induction of specific cellular immune responses [3]. Therefore, HPV E7 is an ideal target antigen for developing therapeutic vaccines against HPV-associated neoplasms [4].

Nucleic acid vaccines potentially have a number of advantages over traditional, attenuated, killed or subunit vaccines. They are cheaper and easier to produce than recombinant protein vaccines. The vaccines have fewer adverse side effects, and they induce both cellular (Th1) and humoral (Th2) arms of the immune system. Studies in mice have demonstrated good antibody and cellular responses with nucleic acid vaccines [5]. However, in some examples of protection against some diseases, trials in larger animals, humans and nonhuman primates have been plagued by a lack of efficacy when using naked DNA [6]. Various methods have been tested to improve responses to DNA vaccines, and they have been reviewed [7]. However, many of these methods increase the cost or reduce the ease of production of DNA vaccines significantly, while others can present regulatory hurdles, and alternative ways to improve responses are still being sought.

Whole phage nanoparticles are novel systems for gene delivery to animal cells [8]. In this strategy, the gene encoding the vaccine antigen, under control of a suitable eukaryotic promoter, is incorporated into the phage genome. Subsequently, the phage particle is used to immunize the host.

Bacteriophage vectors offer many advantages as vaccine delivery and transfer vectors due to their genetic tractability, inexpensive production, suitability for large-scale production as well as their physical stability and compatibility with simple storage and formulation methods such as desiccation [9, 10]. Bacteriophage-λ offers many additional advantages as a DNA vaccine delivery system. Apart from being highly stable, the DNA is contained within a protective protein matrix and is therefore not susceptible to nuclease degradation. Phage particles are targeted directly to antigen-presenting (AP) cells [11, 12], which appears to result in much better immune responses than equivalent plasmid constructs at much lower doses [8].

To date, a few researches have focused on the use of the well-characterized cloning vector phage-λ as a gene delivery vehicle [13]. The previous studies have demonstrated the expression cassettes containing the gene of interest under the control of cytomegalovirus (CMV) promoter using λ-gt11 as a standard λ-DNA cloning vector. Significant immune responses have been presented using a variety of different vaccine expression cassettes [8, 12].

In the present study, a Lambda ZAP® CMV DNA cloning vector has been employed to provide a bacteriophage particle formulation as a pharmaceutically acceptable carrier. It has been shown that recombinant λ-ZAP E7 phage induces therapeutic immunity against TC-1 tumors in mice.

Materials and Methods

Construction of λ-Vaccines

A lambda ZAP CMV vector (Stratagene, USA) was used for the construction of recombinant λ-bacteriophages. The vector has potential characteristics for expression in eukaryotic cells. Eukaryotic expression of inserts is driven by the CMV immediate-early promoter with the SV40 transcription terminator and polyadenylation signal. The λ-ZAP E7 vector was prepared by digesting the HPV16 E7 fragment from plasmid pcDNA3-E7 digested with *Eco*RI and *Xho*I, and cloning the whole plasmid into the *Eco*RI and *Xho*I sites of the Lambda ZAP CMV vector. The packaging extracts (Gigapack® III Gold Packaging Extract; Stratagene) were used to package the recombinant λ-phage vector with high efficiency [14]. A wild λ-ZAP phage was used as a negative control for further experiments.

Purification of λ-Phage Nanoparticles

The bacteriophages were amplified on *Escherichia coli* strain XL1-Blue MRF'. The phages were purified and concentrated using standard microbiological techniques, as described before. Briefly, lysed cultures were cooled to room temperature. DNase I and RNase A (both Sigma Ltd., UK) were added to a final concentration of 1 μg/ml and incubated for 30 min at room temperature. NaCl (1 M) was added, and the flasks were left to stand on ice for 1 h. Cell debris was removed by centrifugation at 11,000 *g* for 10 min at 4° and the collected supernatants were pooled. Solid polyethylene glycol (Sigma Ltd.) was added to a final concentration of 10% (w/v), dissolved slowly at room temperature, and incubated at 4° for at least 1 h. The precipitated bacteriophage particles were recovered by centrifugation at 11,000 *g* for 10 min at 4° and the pellet was resuspended in phage buffer (SM; diluent and storage buffer used for routine manipulation of phage suspension). An equal volume of chloroform was added, and the culture was centrifuged at 300 *g* for 15 min at 4°. The upper aqueous phase was then removed and the bacteriophages were pelleted by centrifugation at 26,000 rpm for 2 h at 4°. The bacteriophage pellet was resuspended in 1–2 ml of SM buffer at 4° overnight, pelleted once more and then resuspended in SM solution prior to storage or further manipulation [9].

Mice and Cells

C57BL/6 mice (6–8 weeks old) were purchased from the Pasteur Institute (Karaj, Iran). The mice were housed for 1 week before the experiment, given free access to food and water, and maintained in a light/dark cycle with lights on from 6.00 to 18.00 h. All experiments were done according to the guidelines for the care and use of laboratory animals by the ethical commission of the Tarbiat Modares University.

TC-1 (part of the Johns Hopkins Special Collection) was derived from primary epithelial cells of C57BL/6 mice cotransformed with HPV16 E6 and E7 and activated c-Ha-*ras* oncogene. The TC-1 cell line, which is HPV16 E7+, was used as a tumor model in an H-2b murine system. TC-1 and Chinese hamster ovary (CHO) cell lines were grown in RPMI medium (RPMI 1640; Gibco-BRL, UK) supplemented with 10% (v/v) fetal calf serum (FCS), penicillin/streptomycin 50 U/ml, 2 mM glutamine, 1 mM sodium pyruvate, 2 mM nonessential amino acids and G418 0.4 mg/ml at 37° with 5% CO_2.

In vitro Gene Delivery and Expression

Recombinant λ-ZAP HPV16 E7 phages at an MOI of 10^6 were added to the CHO cell line. Transduction of target cells was enhanced using spinoculation or centrifugal enhancement after addition of phage particles [15]. The cells were incubated in complete media for 48 h. Transfected CHO cells by wild-type λ-phages were used as a negative control. To confirm the expression of recombinant HPV E7 in the cell lines, Western blot analysis was performed on the extracted total protein. Then, the cells were lysed in SDS loading buffer containing 1 mM dithiothreitol. Cellular proteins were separated on 15% polyacrylamide gels by SDS-PAGE, blotted onto polyvinylidene difluoride membranes (Roche, Germany) and treated with the monoclonal HPV16 E7 mouse antibody (Abcam, UK), followed by detection with goat anti-mouse secondary antibody conjugated to alkaline phosphatase (Sigma, St. Louis, Mo., USA) in secondary antibody solution. Color was developed by incubating the membrane in alkaline phosphate buffer containing tetramethylbenzidine substrate solution.

Tumor Therapy Assay

For the in vivo therapeutic experiments, C57BL/6 mice (7 per group) were challenged by subcutaneous injection in the right flank with 2×10^5 of TC-1 cells suspended in 100 μl PBS. After 1 week, the mice were immunized with 2×10^{12} particles (2×10^{12} phages = 100 μg DNA per mouse) of recombinant λ-ZAP E7 phage, wild λ-ZAP phage (phage control) and PBS (negative control) via subcutaneous injection. The mice received 2 boosts with the same regimen 1 and 2 weeks later.

The subcutaneous tumor volume was estimated according to Carlsson's formula [16]. Hence, the largest (a) and the smallest (b) superficial diameters of the tumor were measured twice a week and then the volume (V) of the tumor was calculated ($V = a \times b \times b/2$). Statistical analysis was performed using Student's t test. All values were expressed as means ± SD.

Cytotoxic T Cell Assay

One week after the third immunization, the mice were sacrificed and their splenocytes isolated. For each sample obtained from individual mice, single-cell suspensions of mononuclear cells (used as the effector cells) were cocultured in RPMI 1640 medium with washed target cells (EL4) at various effector-to-target cell (E/T) ratios (25:1, 50:1 and 100:1) and in 96-well flat-bottom plates for 4 h in phenol red-free RPMI 1640 containing 3% FCS. For preparation of the target cells, EL4 cells were stimulated with 1 μg/ml E7-specific H-2Db cytotoxic T cell (CTL) epitope (E7, amino acids 49–57) or 4×10^5 TC-1 cells previously treated with mitomycin C (30 μg/ml for 3 h) and then incubated for 4 h.

After centrifugation, the supernatants (50 μl/well) were transferred to the 96-well flat-bottom plates, and lyses of target cells were determined by measuring lactate dehydrogenase (LDH) release using a LDH cytotoxicity detection kit according to the procedures stated by the manufacturer (Takara Company, Shiga, Japan). Several controls were used for the cytotoxicity assay.

The 'high control' was the total LDH released from the target cells, and all EL4 cells were lysed by medium containing 1% Triton X-100. The 'low control' was the natural release of LDH from the target cells, which was obtained by adding EL4 cells only to the assay medium. The 'T cell control' was used to measure the natural release of LDH from T cells and was obtained by adding the different ratios of T cells only to the assay medium. For all samples, including the controls, the assay was performed in triplicate.

The LDH-mediated conversion of tetrazolium salt into a red formazan product was measured at 490 nm after incubation at room temperature for 30 min. The percentage of specific cytolysis was determined by the following formula [17]:

$$\%\ \text{Cytotoxicity} = \left[\left(\text{experimental value} - \text{effector cell control}\right) - \frac{\text{low control}}{\text{high control} - \text{low control}}\right] \times 100$$

Lymphocyte Proliferation Assay

One week after the third immunization, a single-cell suspension of mononuclear cells obtained from immunized mice was used for the lymphocyte proliferation assay (LPA). Briefly, the suspension of isolated spleen cells was treated with lysis buffer (0.15 M NH_4Cl; 1 mM $KHCO_3$; 0.1 mM Na_2EDTA; pH 7.2) in order to clear red blood cells. In 96-well flat-bottom culture plates (Nalge Nunc International, Denmark), 2×10^5 cells per well were cultured. The preparations were cultured in RPMI 1640 supplemented with 10% FCS, 1% L-glutamine, 1% HEPES, 0.1% 2-mercaptoethanol and 0.1% penicillin/streptomycin, and incubated in the presence of 1 μg/ml E7-specific H-2Db CTL epitope (E7, amino acids 49–57) or 4×10^5 TC-1 cells previously treated with mitomycin C (30 μg/ml for 3 h) per well at 37° in 5% CO_2. 5 μg/ml T cell mitogen phytohemagglutinin (Sigma Chemicals) was used as the positive control. After 3 days, 5 μg/ml of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma chemicals] was added to each well and incubated for 5 h at 37° in 5% CO_2. DMSO (100 μl) was also added to dissolve formed formazan crystals. Then, the plates were read at 540 nm, and the results expressed as a stimulation index (SI). The SI was determined as follows: optical density (OD) values of stimulated cells (Cs) minus relative cell numbers of unstimulated cells (Cu) divided by relative OD values of unstimulated cells:

$$SI = (Cs - Cu)/Cu.$$

All tests were performed in triplicate for each mouse.

Granzyme B Release Assay

Seven days after the last immunization, the spleens were removed (3 mice/day) and a single-cell suspension of mononuclear cells (effector cells) was prepared in RPMI 1640 (Gibco).

The effector cells (100 μl/well) were added to triplicate wells at specified concentrations followed by 5×10^4 target EL4 cells/E7-specific H-2Db CTL epitope (E7, amino acids 49–57) at differ-

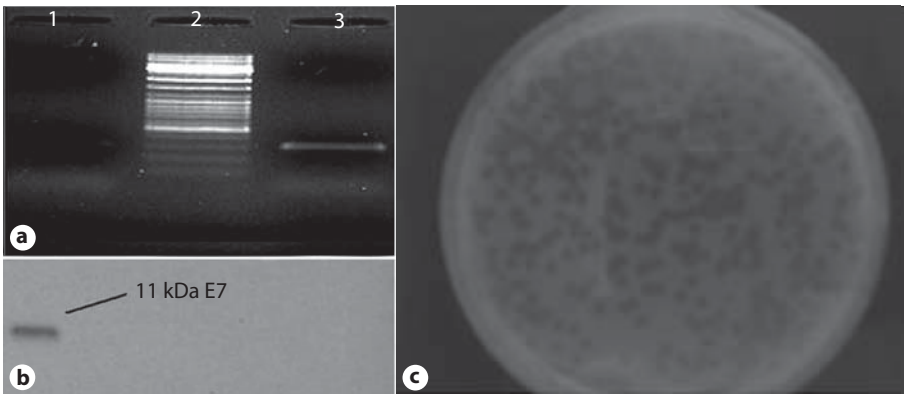

Fig. 1. a Polymerase chain reaction using E7 primers. The presence of E7 gene in λ-ZAP HPV16 E7 phages was confirmed by E7-specific primers. **b** Western blot analysis of CHO cells infected with λ-ZAP HPV16 E7 phages. After overnight incubation, the cellular proteins were extracted and analyzed by SDS-PAGE and Western blotting. **c** Plaque formation by recombinant λ-phages (agar on top).

ent E/T ratios of 25:1, 50:1 and 100:1 per well (100 μl). After effector-target cell incubation for 6 h, the plate was centrifuged at 1,000 *g,* the culture supernatant harvested and the granzyme B content measured by ELISA using a quantitative sandwich enzyme immunoassay technique (eBioscience Mouse Granzyme B ELISA Ready-SET) according to the manufacturer's instruction. Briefly, Corning Costar 9018 96-well ELISA plates (Nalge Nunc International) were coated overnight at 4° with purified mouse anti-human GrB mAb (clone eB288/16G6; eBioscience Inc., San Diego, Calif., USA) in 0.1 M bicarbonate buffer (pH 9.6). After washing and blocking, the cell culture supernatants from wells for each group after 6 h of stimulation or GrB standards were added to the wells and then incubated at room temperature for 2 h. Biotinylated mouse anti-human GrB (clone eBioLUEE) was added to the wells for 1 h, then washed extensively with washing buffer. Avidin-horseradish peroxidase was added for 30 min, then the ELISA plates were washed and GrB was detected after the addition of tetramethylbenzidine substrate solution. Absorbance was measured at 450 nm at various time points. The detection limit of the granzyme B ELISA system was 40 pg/ml.

Cytokine Secretion Assay

One week after the third immunization, mononuclear cells from spleens of immunized mice at a concentration of 2×10^6 cells per well were incubated in 24-well plates (Nalge Nunc International) for 2 days in a total volume of 1.5 ml of RPMI 1640 supplemented with 10% FCS, 1% L-glutamine, 1% HEPES, 0.1% 2-mercaptoethanol and 0.1% penicillin/streptomycin, and pulsed with 1 μg/ml E7-specific H-2Db CTL epitope at 37° in 5% CO_2. The cell supernatants were collected and assayed for the presence of interferon (IFN)-γ and interleukin (IL)-4, using commercially available sandwich-based ELISA kits (R&D Systems, Minneapolis, Minn., USA) following the manufacturer's instruction. All tests were performed in triplicate for each mouse.

Statistical Analysis

To compare results between the different groups, a one-way ANOVA test was used. The statistical software SPSS version 11.0 was utilized for statistical analyses. Differences were considered statistically significant when $p < 0.05$.

Results

Construction of λ-ZAP HPV16 E7 Phages

The presence of the E7 gene in packaged phages was confirmed by polymerase chain reaction using E7 primers (fig. 1a). Also, the plaque formation of the phages (agar on top) approved the subcloning and ligation steps [14] (fig. 1c). For protein analysis of λ-ZAP E7 phages, Western blot was performed on a CHO cell line which was transduced with recombinant λ-ZAP HPV16 E7 phages at an MOI of 10^6. 48 h after transduction, a signal corresponding to the E7 protein (11 kDa) was detected by Western blotting, as shown in figure 1b.

LDH Cytolytic Activity

The CTL response in immunized mice was examined in this study by the LDH release assay in 96-well plates. The mice were immunized 3 times, and the CTL activity was measured as described before (subsection Cytotoxic T Cell Assay). As shown in figure 2, lymphocytes in vaccinated mice with λ-ZAP HPV16 E7 phages (39.4 ± 1.3%) had a significantly increased specific cytolytic activity at an E/T ratio of 50:1 when compared to that of wild λ-ZAP phages (phage control; 22 ± 2.07%) and PBS (negative control; 5.7 ± 2.9%; $p < 0.05$). However, no statistically significant difference in cytolytic activity was found between the phage control and negative control groups at a 50:1 E/T ratio. The differences between all these groups at E/T ratios of 25:1 or 100:1 did not reach overall statistical significance. Based on these results, the CTL activities in mice immunized with λ-ZAP HPV16 E7 phages were stronger than those in control ones.

Lymphocyte Proliferation Response

Since lymphocyte proliferative responses are generally considered as a measure of cell-mediated immunity, HPV

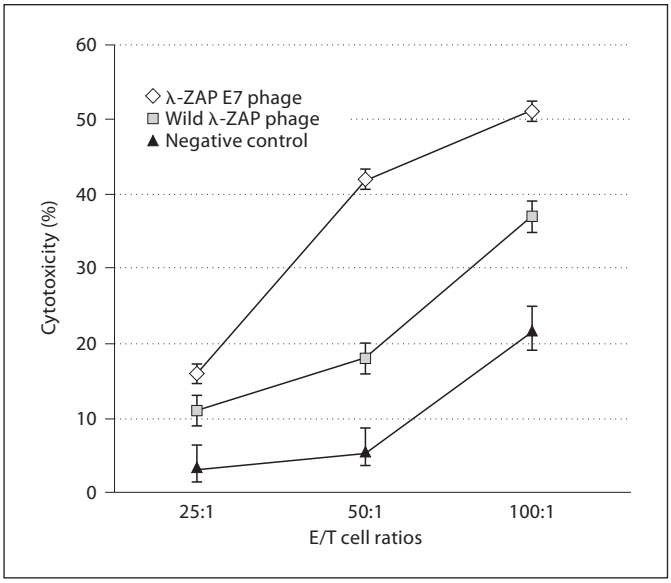

Fig. 2. CTL assays to demonstrate lymphocytes in mice vaccinated with different phage vaccines using quantitative measurement of LDH release. After mice were immunized with λ-ZAP phages 3 times, spleens were harvested as described in the Materials and Methods section. Data were collected from LDH results at various E/T ratios (25:1, 50:1, 100:1) and expressed as percent cytotoxicity ± SD. The data shown here are from 3 independent experiments in triplicate.

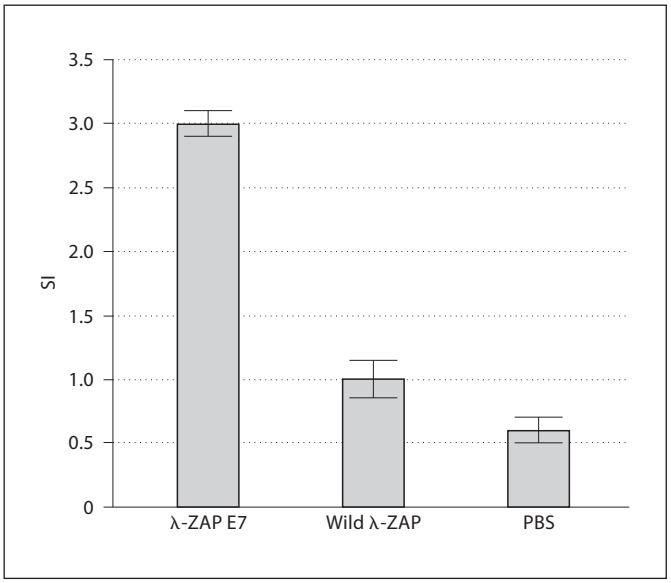

Fig. 3. Splenocyte proliferation levels after in vitro stimulation with HPV16 E7 epitope. C57BL/6 mice were challenged by subcutaneous injection of TC-1 cells. After 1 week, the mice were subcutaneously immunized 3 times with λ-ZAP HPV16 E7 phages, wild λ-ZAP phages (phage control) and PBS (negative control). One week after final immunization, spleens of individual mice (3/group) were removed and lymphocyte proliferation was evaluated by the MTT method. Formazan crystal formation after incubation with MTT was determined by solving the crystals in DMSO, and the OD was read at 540 nm. Bars: means. Whiskers: SEM. Lymphocyte proliferation in the λ-ZAP HPV16 E7 phage group was significantly higher than in the control groups ($p < 0.05$).

E7 antigen-specific lymphocyte proliferation was evaluated by MTT assay. As shown in figure 3, the mice immunized with λ-ZAP HPV16 E7 phages induced a much better E7-specific proliferation response than the control groups, which received wild λ-ZAP phages (phage control) and PBS (negative control; $p < 0.05$). These data indicate the capability of λ-ZAP phages as gene delivery vehicles to induce spleen cell proliferation as a marker of cellular immune response.

Induced Granzyme B Release Responses in Tumor C57BL/6 Mice

To determine whether bacteriophage-λ particles could elicit antigen-specific cytolytic responses to a vectored insert, HPV16 E7 was inserted into the Lambda ZAP CMV vector, under transcriptional control of the CMV immediate-early promoter. One week after final immunization, a single-cell suspension of splenocytes was incubated with EL4 target cells/E7-specific H-2Db CTL epitope. The cell supernatants were stored 6 h after incubation to assay the granzyme B level by ELISA.

Mice vaccinated with λ-ZAP HPV16 E7 phages (205 ± 20.4%) exhibited a significant increase in specific cytolytic activity at an E/T ratio of 50:1, as compared to that of wild λ-ZAP phages (phage control; 42 ± 12.8%) and PBS (negative control) (fig. 4). The PBS value was lower than the limit of detection of the granzyme B ELISA system (nondetectable). The λ-ZAP HPV16 E7 phage group results have been statistically significant at $p < 0.05$ using the paired Student t test compared to the control groups.

Secretion Levels of IFN-γ

To determine whether vaccination with the recombinant λ-ZAP phage vaccine could upregulate cytokine secretion and increase specific immune responses, we measured IL-4 and IFN-γ levels in the supernatants of mononuclear cells from vaccinated mice restimulated in vitro with HPV E7 epitope. As shown in figure 5, splenocytes taken from immunized mice which received λ-ZAP

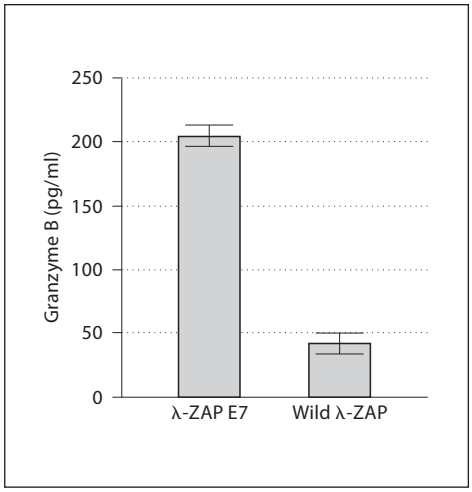

Fig. 4. Measured granzyme B in culture supernatants (stimulated spleen cells) by ELISA (quantitative sandwich enzyme immunoassay technique). One week after the last injection, mice were sacrificed and spleen cells pooled. Splenocytes were stimulated with EL4 target cells/E7-specific H-2Db CTL epitope. Samples were assayed in triplicate. Bars: means of released granzyme B concentrations. Whiskers: SD. The experiments were repeated twice more with similar results. The λ-ZAP HPV16 E7 phage group results were statistically significant at $p < 0.05$ using the paired Student t test in comparison to the control groups. The PBS value was lower than the limit of detection of the granzyme B ELISA system.

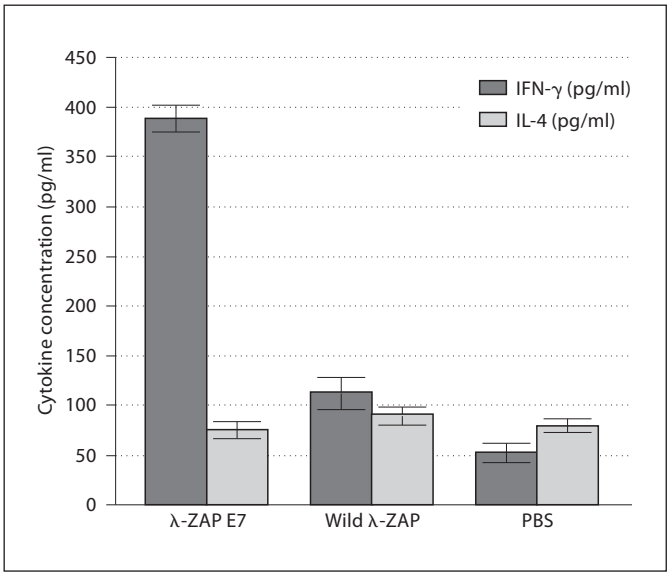

Fig. 5. Determination of the production of Th1 and Th2 cytokines. Collected supernatants were screened for the presence of IFN-γ and IL-4 to determine the phenotype (Th1 vs. Th2) of the immune responses. The concentration of the cytokines was determined by comparison to a standard curve of serially diluted positive control samples. Bars: means. Whiskers: SD. Each sample was examined in triplicate, and the results are representative of 2 experiments ($p < 0.05$).

HPV16 E7 phage vaccine produced higher levels of IFN-γ in comparison to the control groups, without any changes in IL-4 levels. This result indicates that the λ-ZAP HPV16 E7 phage vaccine regimen predominantly displays a Th1 cytokine profile.

λ-ZAP E7 Phage Vaccine Protection against HPV16 E7-Expressing Cells

To determine whether the observed increase in the number of E7-specific CD8+ T cells after vaccination with the recombinant λ-ZAP E7 phage could be translated into a better E7-specific antitumor effect, we performed an in vivo tumor treatment experiment via a previously characterized E7-expressing tumor model, TC-1 [18]. Mice were subcutaneously injected with 2×10^5 TC-1 cells per mouse into the right flank , and then immunized 3 times with recombinant λ-ZAP E7 phage, wild λ-ZAP phage and PBS in a 1-week interval. The tumors were measured twice a week once they became palpable. The controls consisted of unimmunized mice challenged with tumors. The tumor volume was monitored up to 30 days after the tumor challenge. As shown in figure 6, mice treated with the recombinant λ-ZAP E7 phage had significantly reduced tumor volumes when compared to mice treated with the wild λ-ZAP phage and PBS ($p < 0.05$). The results indicate that vaccination with the recombinant λ-ZAP E7 phage induces a more efficiently therapeutic antitumor effect than vaccination in the control groups.

Discussion

Bacteriophage-based vectors have many desirable properties of both animal viral and nonviral systems without any significant drawbacks. Much like nonviral vectors, phage particles are simple genetic packages lacking an intrinsic tropism for animal cells [10]. However, a phage can be produced at high titers in bacterial cultures, potentially making the production simpler and more economical than either nonviral or viral systems. Phage particles are also extremely stable under a variety of harsh conditions [10, 19].

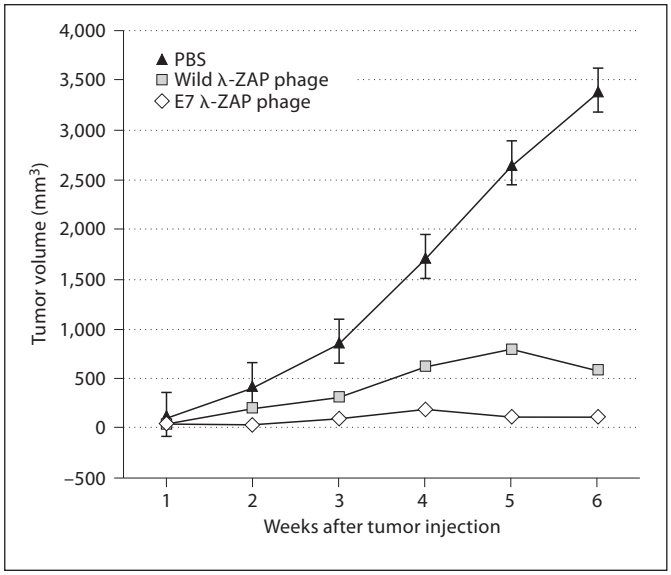

Fig. 6. Therapeutic vaccination against TC-1-induced tumors. Mice were inoculated with 2×10^5 TC-1 tumor cells into the right flank and then treated with recombinant λ-ZAP E7 phage, wild λ-ZAP phage (phage control) and PBS (negative control) 7 days after inoculation. The mice were monitored for evidence of tumor growth by palpation and inspection twice a week. To determine the tumor volume, each individual tumor size was measured. Line and scatter plot graphs depicting the tumor volume (in mm^3) are presented. The data presented are a representation of 2 independent experiments.

In addition, phages have been experimentally administrated to animals and used in a safe manner for human applications which include the treatment of bacterial infections and, more recently, immunization [18, 20]. March et al. [12] have shown the immunization by multiple injections of unmodified λ-phage particles containing hepatitis B surface antigen resulting in a strong antigen-specific humoral immune response in mice and rabbits. The authors speculated the response reflected antibody-mediated opsonization of the phages during the subsequent immunizations. The results indicate that even unmodified nontargeted λ-phage particles can mediate in vivo gene delivery, possibly due to their uptake by AP cells. Addressing the mechanism of phage-mediated gene transfer, and to test whether phagocytosis was required for this process, the findings by Lankes et al. [21] showed phagocytosis to be responsible for a portion but not the majority of phage-mediated gene transfer. It is more likely that nonphagocytic mechanisms contribute to the uptake of phage particles and expression of phage-encoded proteins. These include macropinocytosis, a process that is induced by many microbial pathogens [22]. The major cell types capable of performing macropinocytosis include dendritic cells and macrophages. Following uptake by AP cells, the DNA is released and the eukaryotic promoter directs a long-lasting expression of the vaccine antigen within the AP cells, maintaining a strong secondary response, which then induces the activated T and B cells to elicit an immune response to the antigen. This mechanism may explain enhanced immune responses to vector-encoded mammalian antigens.

In order to detect the elimination of tumors by our λ-ZAP E7 phages, 4 immunogenicity tests – the LDH cytotoxicity test and IFN-γ, LPA and granzyme B assays – were performed. The cytotoxicity test was carried out to test the immune response generated by the killing of the HPV16 oncogenic antigen-expressing cells (TC-1) by the λ-ZAP E7 phage. The IFN-γ assay was carried out to examine whether the immune response to TC-1 cells lay in a cell-mediated pathway or not. IFN-γ is a cytokine involved in cell-mediated immune response, especially for CTL and Th1 cells [23]. Primed T cells usually respond quickly and strongly when they come into contact with antigens they have previously encountered. Generally, a large amount of IFN-γ is produced when the primed T cells are in cell-mediated response.

To further evaluate cell-mediated immunity, the assays employing granzyme B were developed. Granzyme B is a lysosomal enzyme which mediates specific cytotoxic responses and has recently been implicated in antiviral defense [24, 25].

The LDH cytotoxicity test and LPA showed abilities approximately 2.3- and 3-fold higher in the λ-ZAP E7 phage group than in the phage control group. Granzyme B and IFN-γ demonstrated the best correlations with antitumor effect. Also, the tests showed approximately 4.8- and 3.5-fold higher values than the phage control group. The results seem to be logical as granzyme B is one of the major compounds of cytotoxic granules. To our knowledge, this is the first time the secretion of granzyme B was studied in cervical cancer models [26].

Our findings indicate that secreted IFN-γ and granzyme B by activated CTL play a critical role in cellular immune responses, which is important for antitumor and antiviral effects. Moreover, granzyme B seems to be an obvious candidate for the development of high-throughput screening assays, and subsequently for the evaluation of cell-mediated immune responses to vaccines and immunotherapy [26]. Relative antitumor effects induced by wild λ-phages can be related to the natural immunostimulation of λ-phages [27].

Here, we have attempted to characterize phage-mediated gene transfer in vivo. This study provides first proof-of-concept support for the notion that λ-phage vectors may result in efficient cell-mediated immune responses and antitumor effects. It seems that phage nanoparticles may be taken up, at least in part, via nonphagocytic mechanisms, possibly including macropinocytosis. Our findings offer important insight into the mechanism of phage-mediated gene transfer and suggest possible modifications to improve phage-based vaccine delivery systems.

References

1 Bosch FX, Lorincz A, Munoz N, Meijer CJ, Shah KV: The causal relation between human papillomavirus and cervical cancer. J Clin Pathol 2002;55:244–265.
2 zur Hausen H: Papillomaviruses in human cancers. Proc Assoc Am Physicians 1999;111:581–587.
3 Nakagawa M, Viscidi R, Deshmukh I, Costa MD, Palefsky JM, Farhat S, Moscicki AB: Time course of humoral and cell-mediated immune responses to human papillomavirus type 16 in infected women. Clin Diagn Lab Immunol 2002;9:877–882.
4 Eiben GL, da Silva DM, Fausch SC, le Poole IC, Nishimura MI, Kast WM: Cervical cancer vaccines: recent advances in HPV research. Viral Immunol 2003;16:111–121.
5 Tang DC, DeVit M, Johnston SA: Genetic immunization is a simple method for eliciting an immune response. Nature 1992;356:152–154.
6 Dietrich G, Gentschev I, Hess J, Ulmer JB, Kaufmann SH, Goebel W: Delivery of DNA vaccines by attenuated intracellular bacteria. Immunol Today 1999;20:251–253.
7 Leitner WW, Ying H, Restifo NP: DNA- and RNA-based vaccines: principles, progress and prospects. Vaccine 1999;18:765–777.
8 Clark JR, March JB: Bacteriophage-mediated nucleic acid immunization. FEMS Immunol Med Microbiol 2004;40:21–26.
9 Jepson CD, March JB: Bacteriophage lambda is a highly stable DNA vaccine delivery vehicle. Vaccine 2004;22:2413–2419.
10 Petty NK, Evans TJ, Fineran PC, Salmond GP: Biotechnological exploitation of bacteriophage research. Trends Biotechnol 2007;25:7–15.
11 Aronow R, Danon D, Shahar A, Aronson M: Electron microscopy of in vitro endocytosis of T_2 phage by cells from rabbit peritoneal exudate. J Exp Med 1964;120:943–954.
12 March JB, Clark JR, Jepson CD: Genetic immunisation against hepatitis B using whole bacteriophage lambda particles. Vaccine 2004;22:1666–1671.
13 Chauthaiwale V, Therwath A, Deshpande VV: Bacteriophage lambda as a cloning vector. Microbiol Rev 1992;56:577–591.
14 Ghaemi A, Soleimanjahi H, Gill P, Mohamad Hassan Z, Roohvand F: Design and construction of recombinant phage nanobioparticles as a candidate vehicle for oral-gene vaccine delivery. Daneshvar 2009;81:1–6.
15 O'Doherty U, Swiggard WJ, Malim MH: Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding. J Virol 2000;74:10074–10080.
16 Carlsson G, Gullberg B, Hafström L: Estimation of liver tumor volume using different formulas: an experimental study in rats. J Cancer Res Clin Oncol 1983;105:20–23.
17 Cheng WF, Hung CF, Chai CY, et al: Tumor-specific immunity and antiangiogenesis generated by a DNA vaccine encoding calreticulin linked to a tumor antigen. J Clin Invest 2001;108:669–678.
18 Dabrowska K, Switała-Jelen K, Opolski A, Weber-Dabrowska B, Gorski A: Bacteriophage penetration in vertebrates. J Appl Microbiol 2005;98:7–13.
19 Larocca D, Kassner PD, Witte A, Ladner RC, Pierce GF, Baird A: Gene transfer to mammalian cells using genetically targeted filamentous bacteriophage. FASEB J 1999;13:727–734.
20 Sulakvelidze A, Alavidze Z, Morris JG Jr: Bacteriophage therapy. Antimicrob Agents Chemother 2001;45:649–659.
21 Lankes H, Zanghi CN, Santos K, Capella C, Duke CM, Dewhurst S: In vivo gene delivery and expression by bacteriophage lambda vectors. J Appl Microbiol 2007;102:1337–1349.
22 Amyere M, Mettlen M, van der Smissen P, Platek A, Payrastre B, Veithen A, Courtoy PJ: Origin, originality, functions, subversions and molecular signalling of macropinocytosis. Int J Med Microbiol 2002;291:487–494.
23 Arai KI, Lee F, Miyajima A, Miyatake S, Arai N, Yokota T: Cytokines: coordinators of immune and inflammatory responses. Ann Rev Biochem 1990;59:783–836.
24 Shafer-Weaver K, Sayers T, Strobl S, Derby E, Ulderich T, Baseler M, Malyguine A: The Granzyme B ELISPOT assay: an alternative to the 51Cr-release assay for monitoring cell-mediated cytotoxicity. J Transl Med 2003;1:14.
25 Trapani JA, Smyth MJ: Functional significance of the perforin/granzyme cell death pathway. Nat Rev Immunol 2002;2:735–747.
26 Ammar M, Mokni M, Boubaker S, El Gaied A, Ben Osman A, Louzir H: Involvement of granzyme B and granulysin in the cytotoxic response in lichen planus. J Cutan Pathol 2008;35:630–634.
27 Clark JR, March JB: Bacterial viruses as human vaccines? Expert Rev Vaccines 2004;3:463–476.