T7 phage displaying latent membrane protein 1 of Epstein-Barr virus elicits humoral and cellular immune responses in rats

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Summary. – The latent membrane protein 1 (LMP1) encoded by Epstein-Barr virus (EBV) has become a potential target in EBV-associated tumor prevention and treatment due to its multiple biological effects. In this study, the recombinant T7 phage displaying full-length LMP1 protein was cloned and used as an immunogen to immunize rats. Results of flow cytometry, Western blot analysis, and ELISA confirmed that both humoral and cellular immune responses were elicited in the immunized rats. Our data suggested that T7 phage was an efficient antigen carrier. The recombinant T7-LMP1 phage reconstitutes the antigenic and immunogenic properties of LMP1 and can serve as a vaccine against EBV.

Keywords: Epstein-Barr virus; latent membrane protein 1; T7 phage; phage display; recombinant phage

Introduction

EBV-encoded LMP1 is an intriguingly modulated potential oncoprotein with multiple functions. Expression of LMP1 is observed in most malignancies associated with EBV infection. Genetic and biochemical studies together with the recombinant virus experiments uncovered that LMP1 hijacks the cell signaling adapters of tumor necrosis factor receptor superfamily to alter a cell gene expression via activation of NF-κB (nuclear factor κ-gene binding), mitogen activated protein kinases, c-Jun N-terminal kinase, and interferon regulatory factors (Huen et al., 1995; Eliopoulos et al., 1999a, b; Zhang and Pagano, 2000). LMP1 possesses diverse biological effects on the cell growth, development, differentiation, and survival. On the other hand, people hold considerable interest in the possibility of targeting virus-specific immune response to the viral antigens. Hence, LMP1 has been a potential target in the EBV-associated tumor prevention and treatment (Pan et al., 2009; Delbende et al., 2009).

Phage display technology was first published by Smith in 1985. In phage display, a foreign peptide or protein is expressed on the phage surface through transcriptional fusion with a protein coat gene. Recently, phage particles have been described as the highly efficient DNA vaccine delivery vehicles. The immune efficacy of phage-based vaccines containing certain epitopes was extensively investigated to learn new approaches in combating the bacterial, viral, and cancer diseases (Gao et al., 2010). Four kinds of display system have been developed so far, namely filamentous phage, phage λ, T4 phage, and T7 phage. Among those, T7 phage has the ability to display small peptides in high-copy number and large peptides or proteins in low- or mid-copy number. This system is widely used (Tan et al., 2005; Herrmann et al., 2007; Shadidi et al., 2008).

In this study, we attempted to construct a recombinant T7-LMP1 phage and examine its immunogenicity in rats. Results showed that vaccination with T7-LMP1 phage could efficiently elicit both humoral and cellular immune responses.

Materials and Methods

Cell lines and animals. MCF (human breast cancer cell line) and 5-8F (human nasopharyngeal carcinoma cell line expressing...
LMP1 were grown in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml of penicillin, and 100 μg/ml streptomycin at 37°C in the presence of 5% CO₂. Female 3-month-old Wistar rats were purchased from the Center of experimental animals, Hubei province, China.

**Construction of recombinant T7 phage.** Plasmid pSG5-LMP1 was a kind gift from Dr. Caoya's lab, Central South University Cancer Research Institute. Full-length LMP1 gene was obtained by PCR amplification using pSG 5-LMP1 plasmid as a template. EcoRI and HindIII sites were included in the upstream primer 5’-ATACGACTCTATAGGGCGAATTCC-3’ and the downstream primer 5’-GCAAGCTTGTTTCTGTTTTAATAAGA-3’, respectively. The PCR amplification was performed in 50 μl of reaction mixture under the following condition: 30 cycles of 94°C for 30 secs, 55°C for 1 min, and 72°C for 5 mins. LA Taq DNA polymerase and DNA markers were purchased from TaKaRa. Primer sequences were synthesized by Invitrogen Company. China.

The double enzyme digested PCR product was purified and ligated into T7 select 10-3b vector (Novagen) EcoRI/HindIII arms. Ligation reaction was carried out at 16°C overnight and the mixture included 0.5 μg insert, 1 μl T7 arms, 0.5 μl 10 × buffer, and 1 μl T4 DNA ligase (TakaRa). The ligation mixture (5 μl) was in vitro packaged using 25 μl T7 packaging extract (Novagen). Plaque assay of the packaging reaction was performed according to the manufacturer’s protocol. The recombinant T7 phage (T7-LMP1) was confirmed by picking out a single clone for PCR using T7-up and T7-down primers and sequencing.

**Production and purification of T7-LMP1 phage.** 500 ml of *Escherichia coli* strain BLT5403 grown in Luria-Bertani/carbenicillin medium in log phase was infected with T7-LMP1 (1 × 10⁶ PFU) and incubated with shaking at 37°C until lysis was observed. The lysate was clarified by spinning down at 8,000 x g for 10 mins. Then, 0.2 volume of 20% PEG made in 2.5 mol/l NaCl solution was added to the supernatant, placed at 4°C overnight, and spun down at 12,000 rpm for 30 mins. Finally, the T7-LMP1 pellet was resuspended in saline.

**Immunization of rats.** Thirty female 3-month-old Wistar rats were randomly divided into three groups and injected subcutaneously with 500 μl of T7-LMP1 particles (10¹⁰ PFU/ml), wild T7 phage, and saline, respectively, once a week for a month. In the whole process of immunization, the rats were closely observed as to local or systemic reactions would be recorded. Rats were sacrificed 7 days after the final immunization and the serum samples were collected by heart puncture under a general anesthesia. Pathologic changes of liver, lung, and kidney of the rats immunized with T7-LMP1 particles were examined.

**Flow cytometry.** Heparinized 500 μl blood samples of three groups of rats were stained with anti-CD3⁺ FITC, anti-CD4⁺ PE, and anti-CD8⁺ PE monoclonal antibodies (Lianke) and analyzed on a Beckman Coulter Epics XL flow cytometer.

**Western blot analysis.** Fifty μg of 5-8F and MCF-7 cell lysates were fractionated by SDS-PAGE and transferred onto a nitrocellulose membrane. The blots were incubated with T7-LMP1 antiserum (diluted 1:100), commercial LMP1 antiserum (DAKO), or normal serum. Then, the blots were incubated with a goat anti-rat IgG-HRP (Santa). Finally, the bound antibodies were visualized using the ECL PLUS Western blotting substrate kit (GE Healthcare) according to the manufacturer’s instructions.

**ELISA.** Wells of microtiter plates were coated with 100 μl of diluted 5-8F cell lysates (10 μg/ml) by incubation overnight at 4°C and washed 5 times with PBS containing 0.05% Tween-20. Next, 100 μl diluted antibody was added to the appropriate wells and incubated 1 hr at 37°C. After 5 washings, 100 μl of the diluted goat anti-rat IgG-HRP was added to each well. The plates were incubated 30 mins at 37°C, washed 5 times, and 150 μl of ECL substrate was added to each well and incubated for 15 mins at 37°C. Finally, 0.05 ml 2 N sulfuric acid was added to stop the reaction and the A₄₅₀ of the plate was read immediately. Each serum dilution was tested in triplicates.

**Statistical analysis.** The Student’s paired two-tailed t-test was used for a comparison between groups.

**Results**

**Production of T7-LMP1 phage**

The sequence coding for the full-length LMP1 of EBV was amplified from pSG5-LMP1 plasmid by PCR for cloning into T7 select 10-3b vector. The LMP1 protein was displayed on the T7 capsid protein in mid-copy number. The icosahedral shape of phage particle may facilitate antibody access to the fusion protein due to its 360° display. Plaque assay showed the titer of the packaging reaction reached 1.35 × 10¹⁰ PFU/ml.

**Immune responses of rats to immunization with T7-LMP1 phage**

Levels of blood CD3⁺, CD4⁺, and CD8⁺ T lymphocytes were analyzed using a flow cytometry (Table 1). The results of flow cytometry analysis indicated that the number of CD3⁺, CD4⁺, and CD8⁺ T lymphocytes in rats immunized with T7-LMP1 phage increased more than that of the other two groups (p<0.05), although a significant statistical differ-

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**Table 1. T lymphocyte subsets in rats immunized with T7-LMP1 phage**

<table>
<thead>
<tr>
<th>T lymphocytes subsets</th>
<th>T7-LMP1 phage</th>
<th>T7 phage</th>
<th>Saline</th>
</tr>
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<tbody>
<tr>
<td>CD3</td>
<td>72.3±5.25</td>
<td>57.0±6.51</td>
<td>46.5±8.94</td>
</tr>
<tr>
<td>CD4</td>
<td>60.7±9.98</td>
<td>44.1±5.87</td>
<td>36.3±7.55</td>
</tr>
<tr>
<td>CD8</td>
<td>17.7±1.97</td>
<td>14.8±1.47</td>
<td>12.6±2.72</td>
</tr>
</tbody>
</table>

Levels of T lymphocyte subsets in blood were assayed by flow cytometry. Results are means from triplicates ± SD. Asterisks indicate statistically significant differences compared with T7 phage and saline group.
ence was also found between the wild T7-immunized group and saline-immunized group.

Antisera of the immunized rats were investigated for their specificity against LMP1 protein by Western blot analysis. The data demonstrated that sera from T7-LMP1-immunized rats recognized a 63 K band of LMP1 as well as commercial LMP1 antiserum (Fig. 1), whereas sera from the wild T7-immunized rats and normal sera did not recognize this band (data not shown).

Cell lysate of 5-8F cells expressing LMP1 was first immobilized on the wells of microtiter plate. Antisera from rats immunized with the recombinant T7-LMP1 phage diluted by 1:500, 1:1,000, 1:2,000, and normal sera diluted by 1:500 were added to react with the immobilized LMP1 protein. Titer of serum antibodies in rats immunized with T7-LMP1 phage was higher than 1:2,000. This value was significantly higher than titer of normal serum used as a control (Fig. 2).

Safety of T7-LMP1 vaccine

During the four-week immunization, the weight gain, color of the fur, diet, excretion, and the activity of rats in all groups were similar and without abnormalities. No adverse local or systemic reactions were observed. Furthermore, no pathologic changes in liver, lungs, and kidneys of the rats immunized with T7-LMP1 particles were found by the microscopic examination.

Discussion

In this study, it was clearly shown that immunization of Wistar rats with T7 phage particles expressing full-length LMP1 protein elicited both antiviral antibody and cellular responses demonstrating the possible protective and therapeutic effect of this phage vaccine on the antiviral immunity.

The high prevalence of EBV in immune competent hosts indicated that EBV has evolved immune evasion mechanisms in such a way that it resides within the host immune system and establishes a persistent infection. Multiple immune evasion strategies are incorporated within LMP1. It’s a difficult task to precisely decipher the pleiotropic roles of LMP1, because it utilizes many cellular signaling pathways. Compared with its expression in healthy tissues containing EBV-infected cells, LMP1 protein is relatively highly expressed in most of the EBV-associated tumors, such as immunoblastic lymphoma or post-transplant proliferative disease, Hodgkin’s disease, and poorly or undifferentiated nasopharyngeal carcinomas (Liebowitz, 1998; Dukers et al., 2000; Khabir et al., 2005). The growth stimulus and oncogenic role of LMP1 are
Phage particles are also naturally immunostimulatory car-
the nuclease degradation under protective protein matrix.
protein is fused with the capsid protein, which is resistant to
and can be produced easily on a large scale. The displayed
the cell membrane (Russel, 1991). Phage vaccines are cheap
of phage particles replicate fast and assemble in the cytoplasm
stable to the harsh conditions that inactivate other phage. T7
it ideal for the protein display. It is extremely robust and is
benefit in patients with the EBV-associated tumors.
Hence, the reconstitution of LMP1 immunity responses may
be necessary for the sake of getting a long-term therapeutic
benefit in patients with the EBV-associated tumors.

T7 phage possesses some intrinsic properties that make
it ideal for the protein display. It is extremely robust and is
stable to the harsh conditions that inactivate other phage. T7
phage particles replicate fast and assemble in the cytoplasm
of E. coli cells. The phage progeny is released by the cell lysis
and displayed peptides do not need to be secreted through
the cell membrane (Russel, 1991). Phage vaccines are cheap
and can be produced easily on a large scale. The displayed
protein is fused with the capsid protein, which is resistant to
the nuclease degradation under protective protein matrix.
Phage particles are also naturally immunostimulatory car-
ying sufficient CD4+ T cell epitopes to elicit the immune
responses (Kleinschmidt et al., 1970; Meola et al., 1995).
Furthermore, high antibody titers against the phage do
not interfere with the immune response against displayed
protein and are more likely to efficiently target the phage to
antigen presenting cells (March et al., 2004). To some extent,
our data supports the idea that T7 phage may act as an ad-
juvant to facilitate LMP1-induced cytotoxic T lymphocyte
response and humoral immune response. No adverse reac-
tions occurred during the whole process of fourfold phage
immunization. Phages were also shown to be safe by other
studies (Bruttin and Brussow, 2005; Gamage et al., 2009).
However, like other phage display systems, T7 also has its
limitations in theory. The most important limitation is that
the displayed peptide is not post-translationally modified as
in eukaryotic systems. Another one is that only peptides less
than 50 amino acids can be expressed in high-copy number
(Rosenberg et al., 1996).

So far, three groups of vaccine design strategies have
been investigated in both animal and human clinical tri-
als. The first group is represented by the peptide-based
vaccines. The immunodominant peptide was used for
the immunization either alone or in combination with
adjuvant. The second group is represented by the dend-
ritic cell-based vaccines. Dendritic cells pulsed with
MHC class I-restricted peptides or with natural peptides
could induce a potent antitumor immunity (Banchereau
and Steinman, 1998). The last group is represented by
the recombinant viruses or nucleotide acid-based vac-
cines. Peptide-based vaccines and recombinant viruses or
nucleotide acid-based vaccines are limited by the lack of
appropriate delivery systems for the effective activation
of immune response. Production of dendritic cell-based
vaccines is laborious and expensive and moreover, it may
not be optimal for migrating to the tumor sites (Kalos,
2003). Using phage as the vaccine delivery vehicle would
be able to decrease the cost and time of the production
and purification of antigen epitopes.

In conclusion, our results suggested that T7 phage is an
efficient antigen carrier. The recombinant T7-LMP1 phage
can serve as a vaccine for eliciting of the EBV immunity.

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