

LETTER TO THE EDITOR

Construction and packaging of Semliki forest virus replicon particles efficiently expressing Influenza A virus (H5N1) hemagglutinin

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There are currently used more than 25 vaccines around the world, which include live attenuated, inactivated or subunit vaccines. These vaccines have only limited effect on the prevention of infections caused by some viral and bacterial pathogens (1). Moreover, there are still many infectious agents that cause serious diseases in humans, but no effective vaccines are available to prevent them. Advances in the fields of molecular biology, virology, and recombinant DNA technology have led to the new approaches in conception of the vaccines that can not only target pathogens, but also potentially extend the scope of vaccination to non-infectious diseases and therapeutic vaccination. Despite the fact that these vectored vaccines have showed a real promise, many researchers encountered such problems as a limited safety, anti-vector immunity, and low immunogenicity (2).

Semliki Forest virus (SFV) replicon vectors that are capable of self-replication and expression of the heterologous protein are being developed as a technology platform

for studying a vaccine against many infectious diseases and tumors (3). In SFV replicon vectors the structural protein genes are replaced by heterologous genes and the non-structural genes are retained to permit self-replication. This molecular design shows high expression levels of the heterologous proteins in cultured cells (4). SFV replicon particle vaccines assembled by SFV replicon vectors with structural proteins expressed by SFV helper vector in trans, have the potential to overcome many shortcomings of the DNA vaccines. Firstly, they present high expression levels of the heterologous proteins that could efficiently induce both humoral and cellular response (5). Secondly, SFV replicon particle vaccines, a type of RNA-based vaccines, are theoretically safer than DNA-based vaccines, since they cannot be incorporated into the cellular genome (6). Thirdly, since SFV structural proteins are not expressed in the vaccine recipients, anti-vector immune responses are generally minimal allowing multiple immunizations of the same individual (7). In addition, SFV replicon particle vaccines are single-cycle vectors that are incapable of spreading from the infected to non-infected cells. Their replication eventually causes cell death, thereby eliminating long-term antigen expression (8).

In order to assess whether a model antigen, the hemagglutinin (HA) of the human-avian influenza A virus (H5N1) could be efficiently expressed in SFV replicon particles, we constructed and assembled SFV-HA virus-like parti-

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Abbreviations: EGFP = enhanced green fluorescent protein; HA = hemagglutinin; IF = immunofluorescence assay; SFV = Semliki Forest virus; TU = transducing unit; VLP(s) = virus-like particle(s)

cle (SFV-HA VLP). The HA gene of influenza A virus (A/H5N1/human/Anhui/1/2005) was first amplified from plasmid pDC315-HA by PCR (kindly provided by Dr. Yue-long Shu, Virology Institute, Chinese Center for Disease Control and Prevention). SFV-HA vector was constructed by subcloning HA gene into the unique *Xho*I and *Not*I cloning sites of an SFV vector (GeneChem), downstream of the subgenomic promoter. An SFV helper vector was also used for assembling the SFV-HA VLPs by providing the structural proteins *in trans*. To examine the expression efficiency of SFV-HA VLP, we used the SFV-EGFP VLP expressing the enhanced green fluorescent protein (EGFP) as a control.

To assemble SFV-HA VLP and SFV-EGFP VLP, the SFV-HA, SFV-EGFP vectors and SFV helper vector were transcribed and capped *in vitro* by using a transcription kit that contained SP6 RNA polymerase and a capping analog. The integrity and quantity of RNA transcripts were further checked by an electrophoresis in denaturing gels. The transcripts were purified and divided into aliquots for transfection of BHK-21 cells. SFV-HA VLP and SFV-EGFP VLP expressing the target genes were generated by using a BHK-21 cell-based production system. Before packaging SFV-HA VLP and SFV-EGFP VLP, two groups of 4×10^6 BHK-21 cells were cultured in 15 ml of growth medium without antibiotics. Each group of cells was transfected with RNA of SFV-HA and SFV-EGFP vectors and co-transfected with SFV helper vector using Lipofectamine™ 2000 (Invitrogen). Transfected cells were incubated at 30°C in a CO₂ incubator for 48 hrs. Supernatants containing SFV-HA VLP or SFV-EGFP VLP were harvested 48 hrs post transfection and sterile filtered through a filter with a pore size of 0.2 µm. Afterward, the SFV-HA VLP and SFV-EGFP VLP were sedimented from the filtered medium by centrifugation at 20,000 x *g* for 4 hrs and resuspended in Hanks' balanced salt solution by gentle pipetting. Finally, the particle suspensions were divided into aliquots for infection of BHK-21 cells.

SFV-EGFP VLP titer was quantified by measuring the cell infection efficiency by a flow cytometry system (Coulter Epics XL, Beckman). The titer was calculated according to the following formula (9):

$$\text{Titer [TU/ml]} = (2 \times 10^5 \text{ target cells}) \times (\text{EGFP}^+ \text{ cells [\%]}) / \text{volume of supernatant [ml]}$$

Transducing unit (TU) refers to the number of vector genomes that can infect, enter, and integrate into a population of cells. TU/ml refers to the number of cells that are infected by the vector/ml of vector solution. SFV-HA VLPs were titrated by using SFV-EGFP VLPs as described previously.

In this study, EGFP expression was detected by observation under a fluorescence microscope (DP30BW, Olympus) and HA expression was detected by RT-PCR and immunofluorescence (IF). To examine the apoptosis caused by both types of VLPs, BHK-21 cells (10^7 cells) were infected with

different VLPs and non-infected BHK-21 cells were used as a negative control. Cells were collected 24 hrs after infection. The quantity of apoptotic or necrotic BHK-21 cells was analyzed by annexin V apoptosis detection kits (Pharmingen) according to the manufacturer's instructions. In addition, the flow cytometric analysis was also performed.

The sequences of HA from SFV-HA vector and EGFP from SFV-EGFP vector were confirmed by sequencing. Electrophoresis showed that the lengths of RNA transcripts of SFV-HA, SFV-EGFP vectors and SFV helper vector were approximately 9,500, 8,500, and 7,000 bp, respectively. SFV-EGFP VLP titer was quantified to 1.1×10^7 TU/ml by the flow cytometry based on the analysis of infection efficiency. The titer of SFV-HA VLP was 1.4×10^7 TU/ml titrated by using the SFV-EGFP VLP.

For the expression confirmation of HA and EGFP, two groups of 2×10^5 BHK-21 cells supplemented with protamine sulfate (5 µg/ml) were infected with SFV-HA VLPs and SFV-EGFP VLPs, respectively, and cultivated for 24 hrs. RT-PCR and IF of the BHK-21 cells infected with SFV-HA VLP demonstrated the presence of the HA expression. In addition, the apoptosis assay showed that the BHK-21 cells infected with both types of the VLPs showed significantly higher level of apoptosis than the control cells non-infected with the VLPs.

In conclusion, our results showed that SFV-HA VLPs could be successfully assembled. The HA of the human isolate of avian influenza A virus (H5N1) can be expressed effectively in the SFV-HA VLPs. In addition, these particles induced the apoptosis, what may be especially important for SFV-based vaccines. It was hypothesized that the apoptosis actually resulted in an enhanced efficacy of SFV-based vaccines as compared with DNA vaccines due to the enhanced uptake of the antigen from the dead cells by antigen-presenting cells (6). Our study also demonstrated that SFV-HA VLPs were able to deliver and express HA of influenza A virus in the cell culture and the SFV-HA replicon system could be used as a new approach for studying the effective vaccine against human-avian influenza. However, further experiments concerning the immune response of animals to the expressed antigens need to be performed.

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