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ORIGINAL ARTICLE

Construction and immunogenicity of a recombinant pseudotype baculovirus expressing the glycoprotein of rabies virus in mice

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Abstract A pseudotype baculovirus with the glycoprotein of vesicular stomatitis virus (VSV-G) on the envelope was used as a vector for the construction of recombinant baculovirus expressing the G protein of rabies virus (RABV) under the cytomegalovirus (CMV) promoter. The generated recombinant baculovirus (BV-G) efficiently expressed the RABV G proteins in mammalian cells. Intramuscular vaccination with BV-G (10^9 PFU/mouse) induced the production of RABV G-specific neutralizing antibodies and strong T cell responses in mice. Our data clearly indicate that pseudotype baculovirus-mediated gene delivery can be utilized as an alternative strategy to develop a new generation of vaccine against RABV infection.

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Introduction

Rabies virus (RABV), a negative-stranded RNA virus, is a member of the genus *Lyssavirus* within the family *Rhabdoviridae*. The 12-kb RABV viral genome contains five regions, encoding nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase protein (L), in the order 3'-N-P-M-G-L-5' [1]. The glycoprotein is the major immunogenic protein and is associated with the production of RABV-specific neutralizing antibodies [2–8]. G-protein-based experimental vaccines, such as DNA vaccines, recombinant protein vaccines, and live-virus-vectored vaccines (recombinant pseudorabies virus), have been developed and tested against rabies infection [9–20].

Baculoviruses are useful vectors for gene delivery and vaccine development because of their highly efficient gene delivery [21–25]. Baculoviruses can enter into mammalian cells but usually do not replicate in the recipient cells. For example, a modified Autographa californica nuclear polyhedrosis virus (AcMNPV) containing a eukaryotic promoter such as the cytomegalovirus immediate early (CMV-IE) promoter can express foreign genes in mammalian cells. Baculoviruses have been used as vehicles for the development of vaccines, and vaccination with a recombinant baculovirus expressing glycoprotein B (gB) of pseudorabies virus can induce vigorous gB-specific antibody responses in mice [26]. Recently, baculoviruses have been utilized as vehicles to generate recombinant pseudotype baculoviruses, and vaccination with these recombinant pseudotype baculoviruses has been demonstrated to induce high levels of humoral and cellular responses to various antigens, such as influenza virus HA [27], *Plasmodium berghei/falciparum* circumsporozoite protein [28], and hepatitis C virus E2 glycoprotein [29].

In the present study, a recombinant pseudotype baculovirus expressing the RABV G protein was constructed. Vaccination with the recombinant pseudotype baculovirus induced the expression of RABV G proteins in mammalian cells and stimulated vigorous humoral and T cell immunity against RABV in mice.

Materials and methods

Cells and virus

BHK-21 and Vero cells were cultured in Dulbecco's modified essential medium (DMEM, Invitrogen) containing 10 % (v/v) heat-inactivated fetal bovine serum (FBS; Gibco), 100 μg/ml streptomycin, and 100 IU/ml penicillin at 37°C and 5% CO_2. *Spodoptera frugipera* (Sf-9) cells were cultured in 10% FBS Grace's insect medium (Invitrogen) at 27°C and used to propagate recombinant baculovirus.

The RABV ERA strain was propagated in Vero cells. The standard strain of challenge virus (CVS-11) was used as a challenge virus for fluorescent antibody virus neutralization (FAVN). BV-VSVG-EGFP is a pseudotype baculovirus containing an CMV-IE promoter that controls the expression of EGFP [30], and this was used as a control strain of the virus.

Construction of recombinant baculoviruses

The modified baculovirus vector, pFastBac-VSV/G, contained the polyhedron promoter of pFastbac1, which was used to control the expression of the VSV G gene (Invitrogen). The recombinant baculovirus, BV-VSV/G-CMV-RABV/G (BV-G), was generated by the following procedures: A DNA fragment containing the full-length G gene from the ERA strain of RABV was amplified by PCR. The sequences of the forward and reverse primers were AGAGAATTCATG GTTCCTCAGGCTCTC AAGTCTAGATCACAGTCT GGTCTCACC, respectively (*EcoRI* and *XbaI* sequences are underlined; the fragment is 1575 bp long, corresponding to positions 3317-4891 of the ERA genome). The PCR products were cloned into the vector pCMVPA to yield pCMVPA-ERAG. The pCMVPA was a gift from Dr. Wenzhou Hong (State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, China) and is a modified pBluescript SK (+) vector containing the complete hCMV immediate-early promoter, multiple cloning sites, and an SV40 late polyA signal from the eukaryotic expression vector pCI-neo (Promega). The CMV-ERAG DNA fragment was excised from pCMVPA-ERAG and inserted into pFastBac-VSV/G, resulting in the recombinant plasmid pFastBac- VSV/G-CMV- RABV/G.

The recombinant baculovirus BV-G was generated using the Bac-to-Bac® system, according to the manufacturer's instructions (Invitrogen). The resultant viruses were further amplified by propagation in Sf-9 cells and purified as described [31]. The titer of the virus was determined using a BacPAK rapid titer assay kit in Sf-9 cells according to the manufacturers' instructions (Clontech).

Baculovirus transduction and western blot

BHK cells were transduced with BV-G or control for 48 h as described previously [32]. The cells were harvested and lysed in SDS-loading buffer containing 0.1 M beta-mercaptoethanol and subjected to western blot analysis using a RABV-G-specific monoclonal antibody (kindly provided by Professor Zhenfang Fu, Department of Pathology, College of Veterinary Medicine, University of Georgia, Athens, GA, USA).

Immunization of mice

Female ICR mice at 4-5 weeks of age were purchased from Beijing Vital River Experimental Animal Co., China. The mice were divided randomly into four groups (10 mice per group) and injected intramuscularly in the hind limb with 100 μl of inoculum containing 10^8 or 10^9 PFU (plaque forming units) of BV-G, 10^9 PFU of control BV-VSVG-EGFP, or Grace's medium. The mice were boosted with the same dosage of virus three weeks later. Serum samples were prepared from blood collected from the tail vein of individual mice before the second injection and from the retro-orbital plexus two weeks after the second injection and stored at −20°C for serological analysis.

Fluorescent antibody virus neutralization (FAVN)

Viral antigens were detected with an FITC-labeled anti-rabies monoclonal antibody (Laboratory of Epidemiology, Veterinary Institute, Changchun, China). The titers of neutralization antibodies in individual sera were determined using the Kärber method and expressed as international units (IU/ml). Seroconversion is defined here as a response of 0.5 IU/ml or greater.

Lymphocyte proliferation assay

To examine the specific T cell response to the RABV G protein, individual mice that had been immunized with recombinant BV-G virus or controls were sacrificed two weeks after boosting. Their splenic mononuclear cells were prepared as described previously [31]. The cells (10^6 cells/well) were challenged in triplicate with inactive RABV (CVS11, inactivated by UV) in 96-well plates for 48 h, and

antigen-specific T cell proliferation was determined by MTT assays using a CellTiter 96 ® Aqueous One Solution Cell Proliferation Assay kit, according to the manufacturer's instructions (Promega). The cells cultured in medium alone were used as negative controls. Lymphocyte proliferation was quantified using the stimulation index (SI), which was calculated as the ratio of the OD_{490nm} of stimulated cells to the OD_{490nm} of negative controls.

Statistical analysis

Data are expressed as mean ± SD. The difference among groups was analyzed by Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

Results

Expression of the G protein in BV-G -transduced mammalian cells

To generate recombinant BV-G, a DNA fragment was amplified by PCR from RABV and then cloned into pFastBac-VSV/G, forming the recombinant plasmid pFastBac-VSV/G-CMV-RABV/G. After DNA sequencing, the recombinant plasmid was transduced into sf9 cells for the generation of BV-G virus with a titer of 10^{10} PFU/ml, and G protein was detected by western blot assay (Fig. 1). As expected, recombinant GP could be detected by PAGE analysis (data not shown), and a notable band corresponding to a molecular weight of about 64 kDa was detected in lysates of BV-G-transduced BHK cells (Fig. 1, lane 1) but not in BV-VSVG-EGFP- transduced cells (Fig. 1, lane 2). Apparently, the pseudotype baculovirus BV-G efficiently mediated RABV G gene delivery and expression of the G protein in transduced mammalian cells.

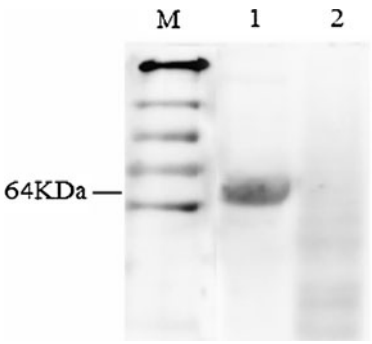

Fig. 1 Expression of pseudotype baculovirus BV-G and western blot analysis. BV-G-transduced BHK cell lysates (lane 1) or BV-VSVG-EGFP- tranduced cell lysates (lane 2) were prepared at 48 h post-transduction and subjected to western blot. M: prestained protein ladder SM0671 (Fermentas); lane 1, BV-G-transduced BHK cells; lane 2, BV-VSVG-EGFP-transduced BHK cells

Induction of RABV-specific antibody responses in mice by vaccination with BV-G

To determine the effect of the BV-G vaccine *in vivo*, groups of mice were immunized intramuscularly with different doses of BV-G or control, and their sera were tested for neutralization antibodies against RABV (Fig. 2). There was no detectable antibody against RABV in the control-virus-immunized mice or in the mice that had been vaccinated with 10^8 PFU of BV-G. In contrast, vaccination with 10^9 PFU of BV-G induced variable levels of humoral responses in mice two weeks after boosting, and the neutralizing antibody titers ranged from 0.5 to 10.26 IU/ml. However, the levels of anti-RABV antibodies were very low two weeks after primary immunization (data not shown).

Induction of RABV-specific cellular immune responses in mice by vaccination with BV-G

We tested T cell immunity induced by vaccination with BV-G. After immunization of mice with different doses of BV-G vaccine or controls, splenic mononuclear cells were isolated from individual mice, and the ability of their T cell responses to inactive RABV was determined by MTT assays (Table 1). While there was no RABV-specific T cell proliferation in the mice vaccinated with control BV-VSVG-CMV-EGFP or those injected with PBS, strong T cell responses to inactive RABV were detected in the mice vaccinated with BV-G. Interestingly, T cell responses to

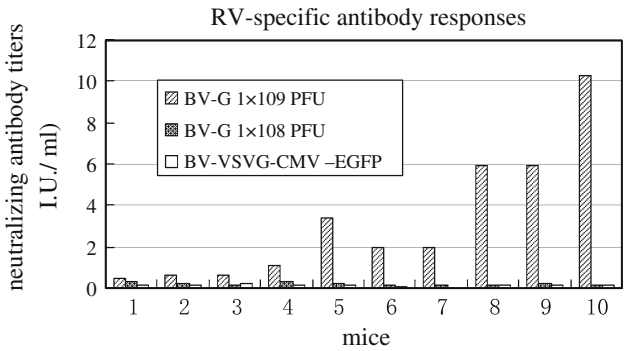

Fig. 2 Antibody responses to RABV. Groups of female ICR mice were injected intramuscularly in the hindlimb with 100 µl of inoculum containing 10^8 or 10^9 PFU of BV-G, 10^9 PFU of control BV-VSVG-EGFP, or Grace's medium and boosted with the same dosage of virus three weeks later. Blood samples were collected from individual mice two weeks after the primary and secondary injections. The levels of RABV-specific antibodies were analyzed by FAVN assays. The data shown are mean titers of neutralization antibodies in sera collected two weeks after boosting from individual mice from three repeated experiments. There was no detectable neutralization antibody in sera collected two weeks after primary immunization (data not shown)

Table 1 Splenic T cell proliferative responses to inactive RABV

Group	Stimulation index
10^9 PFU of BV-G	15.17367 ± 0.592880*#
10^8 PFU of BV-G	10.597 ± 0.293429#
10^9 PFU of BV-VSVG-CMV-EGFP	1.544333 ± 0.195664
PBS	1.186 ± 0.070682

Data are expressed as mean ± SD of each group (n = 10 per group) from three independent experiments. * $p < 0.05$ vs. the group of mice received 10^8 PFU of BV-G; # $p < 0.05$ vs. the group of mice that received 10^9 PFU of BV-VSVG-CMV-EGFP

RABV in the mice vaccinated with 10^9 PFU of BV-G were significantly higher than that in the mice vaccinated with 10^8 PFU of BV-G ($P < 0.05$). Apparently, vaccination with BV-G induced antigen-specific T cell responses in a dose-dependent manner in our experimental system.

Discussion

The baculovirus expression system (BES) provides an efficient and rapid approach for generating recombinant baculoviruses [33]. As a vaccine vector, baculoviruses possess several striking features: (1) they are able to accommodate a large exogenous DNA genome (>30 kb) [34], which makes them suitable for co-expression of multiple heterologous antigens under independent promoters; (2) construction, manipulation, production and scale-up of recombinant baculovirus are relatively simple and fast, and high yields of viruses (>10^{10} PFU/ml) can be achieved easily [35]; (3) they are non-cytotoxic and non-replicative in mammalian cells, even at a high multiplicity of infection (MOI); (4) animals do not contain pre-existing antibodies against baculovirus, which could interfere with the action of recombinant genes in the host; (5) because insect cells can modify recombinant proteins posttranslationally, the BES can express eukaryotic proteins in a form that more closely resembles their natural state. Most importantly, baculoviruses are derived from insect hosts and have no capacity to replicate in human cells. Indeed, previous studies have demonstrated that baculoviruses failed to replicate in HeLa cells [24], and the baculovirus polyhedrin promoter is unable to control the expression of the downstream genes in Huh7 cells, even when highly sensitive RT-PCR is used for detection [22]. Furthermore, inoculation with baculoviruses by different routes, including intravenous, oral, intracerebral and intramuscular appears to be safe in experimental animals, and oral feeding with baculoviruses in volunteer subjects did not cause any adverse effect [36, 37]. Therefore, the high levels of glycoprotein expression and relative safety of baculoviruses suggest the pseudotype baculovirus expressing the RABV glycoprotein-based vaccine may be suitable for use in humans, especially since vaccination with 10^9 PFU of BV-G induced a moderate neutralizing antibody response. Whether BV-G-based vaccines, like the commercially available vaccines, can induce a strong immunity remains to be determined. Given that this vaccine would not replicate in humans, multiple vaccinations with high dose of the vaccine may be required to achieve immunity against RABV infection.

Baculoviruses are usually sensitive to inactivation *in vivo* by the complement system, although the glycoprotein of vesicular stomatitis virus (VSV G) displayed on the envelope of a pseudotype baculovirus can exhibit greater resistance to inactivation. Furthermore, the VSV G proteins can increase gene transfer efficiency, particularly into mouse skeletal muscle, which extends the host range [38].

We employed a pseudotype baculovirus with the glycoprotein of vesicular stomatitis virus (VSV G) on the envelope as the vector to construct a recombinant pseudotype baculovirus, BV-G, that expressed the G protein of rabies virus (RABV) controlled by the cytomegalovirus (CMV) promoter. We found that vaccination with the recombinant pseudotype baculovirus induced the expression of the RABV G protein in mammalian cells. More importantly, vaccination with RABV-G induced detectable neutralization antibody responses to RABV in mice and strong T cell proliferative responses to inactive RABV ex vivo. Therefore, the BV-G recombinant pseudotype baculovirus can effectively deliver the target gene to mouse tissues and mediate the expression of target proteins with high immunogenicity in mice.

There are two possible explanations for the immunogenicity of recombinant baculovirus: high transduction efficiency *in vivo* and efficient antigen presentation. Previous studies have demonstrated that pseudotype baculovirus can enter mouse skeletal muscle cells with higher efficiency [38–40], although the intramuscularly injected plasmid DNA can only be captured by low frequency of muscle cells [41]. The higher transduction efficiency of pseudotype baculovirus may result in higher levels of antigen expression, which should promote efficient presentation by professional antigen-presenting cells (APCs) and subsequently induce vigorous immune responses *in vivo*. More importantly, baculovirus vectors have been demonstrated to efficiently transduce dendritic cells (DCs), the most important APCs [28]. Theoretically, the more efficient the antigen presentation is, the stronger the immune response that is induced. In addition, baculovirus has an adjuvant effect, which could also enhance the induction of an antigen-specific immune response *in vivo*.

It is widely accepted that vaccination-induced neutralization antibodies are important for protection against rabies virus [43], but experimental infections in mice

suggest that cell-mediated immune responses are required for efficient viral clearance [44]. Innate effector cells can amplify and direct the subsequent adaptive response [45–47], whereas effector T cells can secret the proinflammatory cytokines that activate NK and APCs and enhance their cytotoxicity against pathogens and pathogen-infected cells [42, 48]. Indeed, NK cells are important immune effectors that rapidly contain an infection. NK cells become activated when the balance of activating and inhibitory signals that they receive is disturbed [49]. Apparently, NK cells are major players in response to rabies virus infection and vaccination.

We are interested in further investigating the immunogenicity of the RABV-G in dogs and humans, the hosts of RABV.

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