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A recombinant rabies virus expressing luciferase

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Summary. – A recombinant Rabies virus (RV) expressing firefly luciferase (rRV-luc) was generated by an improved reverse genetics system. Its biological properties were compared with those of the parental RV. The rRV-luc grew in BHK-21 cells similarly to RV, but its virulence for mice was weaker as shown by the lower infectious titers in brain. Rising infectious titers of rRV-luc during its passaging in BHK-21 cells indicated a virus adaptation, while the luciferase (luc) expression was stable. These results suggest that the recombinant RV carrying luc gene might prove a useful tool for further analysis of pathogenesis of RV in small animal models.

Keywords: Rabies virus; luciferase; reverse genetic

Introduction

Rabies is one of the oldest diseases and viral infections of the CNS that causes fatal encephalitis in many warm-blooded mammals (Murphy, 2008). So far, rabies is still an incurable disease with a mortality rate of almost 100% (Rim *et al*., 2009). In countries of Asia and Africa, where rabies has become an important public health problem is estimated that this disease is responsible for over 50,000 human deaths annually (WHO, 2005). RV, the causative agent of rabies, is a negative-sense single-stranded RNA virus (the genus *Lyssavirus,* the family *Rhabdovirida*) and has a relatively simple, modular genome organization containing five genes designated N, P, M, G, and L. The sequences in 3′- and 5′-ends are the promoter and terminator of the genome. The intergenic region between two genes is a no-coding region (NCR). The longest NCR is between G and L genes that is known as a pseudogene and designated ψ (Wunner, 2007). Until recently, our knowledge of RV pathogenesis has been limited and many of the mechanisms involved are still unclear (Dietschold *et al*., 2005). The speed of virus uptake, the ability of the virus to spread efficiently from cell-to-cell and the rate of virus replication are the major factors that determine the pathogenicity of RV. Although substantial progress has been made in the identification of RV elements that play a role in the pathogenesis of rabies, it is still unclear which host cell factors are involved in the disease process. The same is true for the mechanisms by which these factors determine the outcome of the disease. Hence, basic research related to the infection and RV pathogenesis should be enhanced in order to provide a basis for the development of treatment.

Bioluminescence imaging (BLI) has emerged as a powerful alternative to the conventional studies of viral infection and pathogenesis in small animal models. This technology captures the light emitted from different luc enzymes to detect the sites of viral infection and to quantify a viral replication, dissemination, host immune response, and signaling pathways in the context of a living animal (Luker and Luker, 2008). Importantly, these processes can be interrogated repetitively in the same animal over the course of hours to weeks. Firefly (*Photinus pyralis*) luciferase, whose substrate is D-luciferin, has been used in several imaging studies (Contag and Bachmann, 2002; Contag and Ross, 2002). This enzyme has a minimal background activity and can even cross the blood-brain barrier after intraperitoneal or intravenous (i.v.) injection. Consequently, the imaging *in vivo* can be taken in any organ (Luker and Leib, 2005).

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Abbreviations: BLI = bioluminescence imaging; luc = luciferase; CAT = chloramphenicol acetyltransferase; i.c. = intracerebral; NCR = no-coding region; pH-G,- L, -N, -P = plasmid containing the full-length cDNA of G, L, N, P gene; p.i. = post infection; pRV = plasmid containing the full-length cDNA of RV; pRV-luc = plasmid containing the full-length cDNA of rRV-luc; RLU = relative light units; RV = Rabies virus; rRV-luc = recombinant RV expressing luciferase

Recombinant viruses expressing firefly luc have been used to study the virus-host interaction with BLI and demonstrate several significant advantages (Cook and Griffin, 2003; Luker *et al.*, 2002, 2003, 2005, 2006). However, this technology has not yet been used to investigate RV-host pathogenesis.

In order to develop an effective tool to analyze RV infection and dissemination using BLI in a small animal model, we constructed a recombinant RV expressing luc gene. We estimated its biological properties. In addition we found that the rRV-luc can efficiently express luc and may be a promising tool for further analysis of RV pathogenesis.

Materials and Methods

Virus and cells. RV HEP-Flury strain was derived from the HEP-Flury cDNA clone. Baby hamster kidney (BHK-21) cells and African green monkey kidney (Vero) cells were purchased from the Chinese Institute of Veterinary Drug Control, Beijing, and grown at 37°C in DMEM supplemented with 10% FCS (both Invitrogen).

Plasmid construction. Full-length cDNA plasmid used to generate a recombinant RV expressing luc was constructed (Fig. 1). The luc gene from the pGL3 Basic vector (Promega) was amplified by PCR (luc-FP: 5'-AGTCGTACGATGGA GACGCCAA-3', BsiWI, underlined; luc-RP: 5'-GTTCT GCAGTTACACGGCGATCT-3', PstI, underlined, and inserted into the G-L NCR of the full-length plasmid pRV, which contains the full-length cDNA of RV HEP-Flury strain forming pRV-luc plasmid.

Recovery of recombinant virus from pRV-luc. Transfection experiments and recovery of the recombinant virus were carried out as described (Inoue *et al.*, 2003). Briefly, BHK-21 cells were grown overnight to approximately 80% confluence (cca 5×10^5 cells/well) in 12-well plates. Cells were transfected with 1 μg of pRV-luc and 0.25, 0.125, 0.05, and 0.075 μg/well of pH-P, -L, -N, and -G using transfection reagent (Qiagen) according to the manufacturer's protocol. After 3 hrs, the cells were washed once and maintained at 37°C in DMEM supplemented with 10% FCS for 2 days and incubated for a further 4 days at 34°C. Cells were examined for the recovered virus by immunofluorescence staining with FITC-labeled antibody against N protein of RV (Fujirabio). The culture fluid was collected as a virus stock and stored at -80°C until use. The recovered viruses were then passaged in BHK-21cells for preparation of a virus stock for further experiments. The recovered viruses generated from the full-length plasmids pRV-luc were designated rRV-luc.

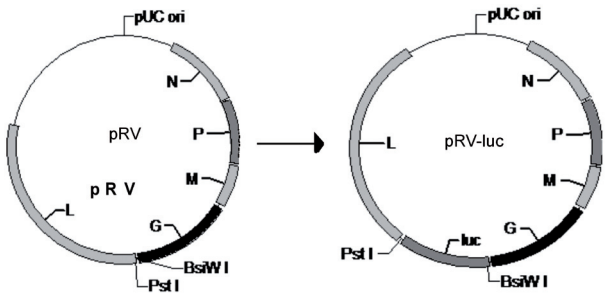

Fig. 1

Construction of the recombinant plasmid containing the full-length RV genome and the luc gene

Virus titration in BHK-21 cells. The titers of rRV-luc and HEP-Flury were determined using the fluorescent antibody test (FAT) on BHK-21 cells. Monolayers of BHK-21 cells in 96-well plates were infected with serial 10-fold dilutions of virus suspension and incubated at 37°C for 4 days. Cells were fixed with 80% acetone for 1 hr and stained with FITC-labeled antibody against N protein. Antigen-positive foci were counted under a fluorescent microscope (Olympus) and calculated as focus forming unit per ml (FFU/ml). All titrations were carried out in triplicates and the mean ± SD was used as a titer.

RT-PCR for luc gene. To confirm that the recovered RV was derived from pRV-luc, the luc gene was amplified by RT-PCR with the primers luc-FP and luc-RP described above and sequenced. Viral RNA was extracted from the recovered RV-infected BHK-21 cells using Trizol reagent (Invitrogen) according to the manufacturer's protocol (de Felipe *et al.*, 2003).

Passaging of rRV-luc strain in BHK-21 cells. To examine the genetic stability of the recombinant virus, serial passages of the rRV-luc strain in BHK-21 cells were performed. Monolayers of BHK-21 cells were infected with the virus at a MOI = 0.01 and the culture fluid was harvested at day 4 post infection (p.i.). This procedure was repeated 10 times. Then the titer of each virus were examined by the direct FAT as described above and the sequences of luc gene from rRV-luc virus of the passage 10 were confirmed by sequencing of RT-PCR fragments.

Assay of luc activity. Monolayers of BHK-21 cells in 96-well plates were infected with 3×10^5 FFU in 100 μl of virus suspension of passage 5 to 10 and incubated at 37°C for 4 days. The cultivation media were removed and cells were lysed using the appropriate buffer provided with ONE-Glo™ Luciferase Assay System (Promega). Intracellular luc expression was measured according to the manufacturer's instructions and the relative light units (RLU) were determined in a GloMax 20/20 Luminometer.

Virus growth in BHK-21 and Vero cells. Monolayer cultures of 3×10^6 BHK-21 cells or Vero cells were respectively infected with rRV-luc or HEP-Flury at a MOI = 0.01. After incubation at 37°C for 1 hr, the cells were washed 3 times with PBS to remove unabsorbed virus. The cells were incubated

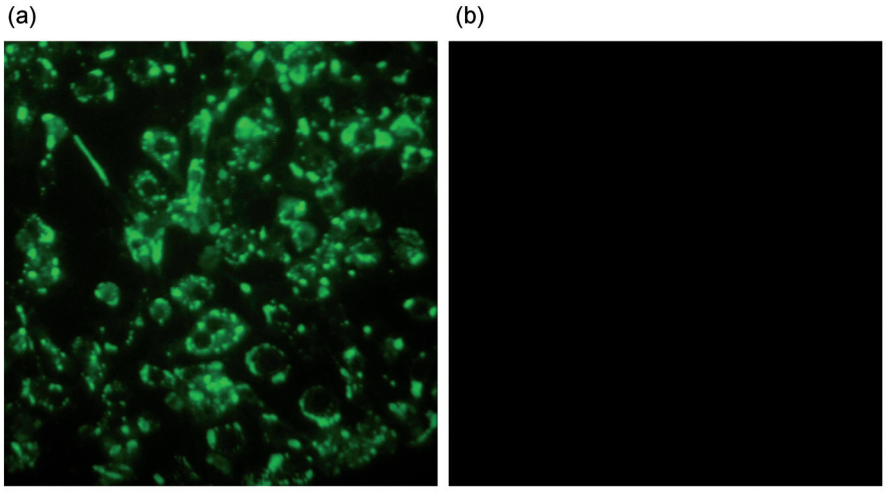

Fig. 2

FAT detection of RV antigen in BHK-21 cells transfected with the full-length RV genome plasmid and helper plasmids

Transfected cells (a), mock-transfected cells (b).

with the fresh medium at 37°C for 5 days. 100 µl of culture supernatants was removed at each time point (24, 48, 72, 96, and 120 hrs p.i.). The titers of each harvested virus were examined by the focus assay as described above.

Virus titration in suckling mice. The mice, strain ddY, were purchased from the Center of Laboratorial Animals in Southern Medical University, China and used in animal experiments. Groups of 10 suckling mice (3-day-old) were inoculated by intracerebral (i.c.) route with 20 µl of serial 10-fold dilutions of each virus and the 50% lethal dose (LD_{50}) of each virus was calculated by the method of Reed and Müench (1938).

Determination of body weight changes in the virus-infected mice. Groups of 5 adult mice (6-week-old female) were inoculated i.c. with rRV-luc or HEP-Flury including 3.9×10^4 FFU in 30 µl and monitored for a body weight and survival over a period of 14 days.

Results

Recovery of the recombinant virus carrying luc gene from the respective plasmid

At day 6 after transfection of the full-length genome plasmid pRV-luc and three helper plasmids into BHK-21 cells, the recombinant N protein was detected by FAT using a FITC-labeled N-specific antibody. Abundant expression of the N protein was seen in the BHK-21 cells indicating that the rRV-luc virus propagated and expressed viral proteins efficiently (Fig. 2). To confirm that the recovered RV was carrying the luc gene, stock virus of the rRV-luc strain was used to amplify the fragment of luc by RT-PCR with a pair of specific primers luc-FP and luc-RP. DNA band of 1,665 bp was observed from rRV-luc strain (Fig. 3). This result indicated that the recombinant RV was carrying the luc gene.

Passage of the recombinant virus in BHK-21 cells

In order to examine the genetic stability of the rRV-luc strain in BHK-21 cells, serial passages from 1 to 10 were carried out. The titer of the rRV-luc strain in culture medium gradually rose with the number of passage in BHK-21 cells and reached the value 1.3×10^6 FFU/ml (Fig. 4). Examina-

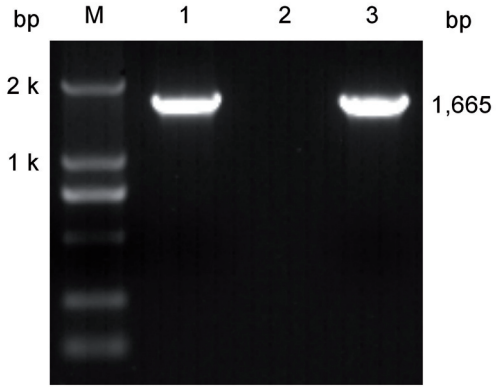

Fig. 3

Confirmation of rRV-luc recovery by RT-PCR

Agarose gel electrophoresis of RT-PCR products. rRV-luc after the first (lane 1) and tenth passage (lane 3), RV (lane 2), DNA size markers (lane M).

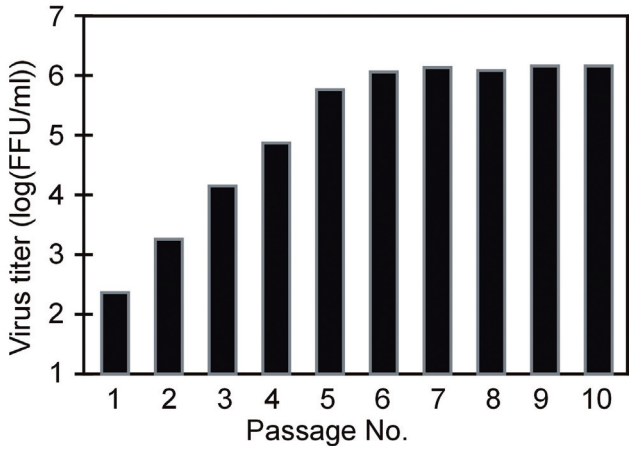

Fig. 4

Effect of rRV-luc passaging in BHK-21 cells on its titer

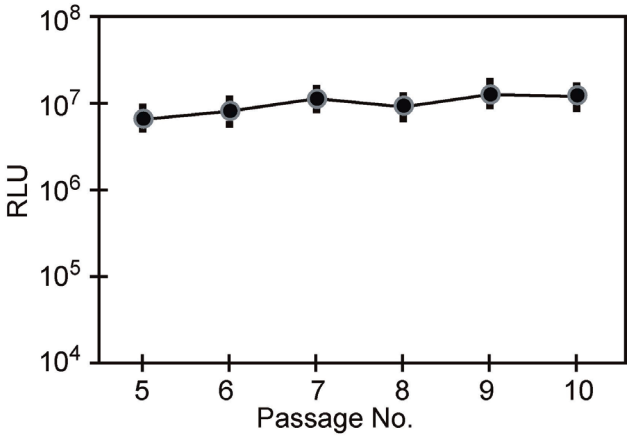

Fig. 5

Effect of rRV-luc passaging in BHK-21 cells on the luc gene expression

tion of rRV-luc strain after 10 passages showed that the virus contained luc gene as was confirmed by RT-PCR and direct sequencing of PCR product (Fig. 3).

The luc expression in rRV-luc-transfected BHK-21 cells was determined with ONE-Glo™ Luciferase Assay System (Promega) and RLU were determined in a GloMax 20/20 Luminometer. The expression of luc gene remained stable during serial passages of the recombinant virus (Fig. 5).

Growth of the recombinant virus in BHK-21 and Vero cells

Replication of the rRV-luc strain was examined by the multiple-step growth curve in BHK-21 and Vero cells. Growth curve of the rRV-luc strain of passage 10 was similar to one of the HEP-Flury strain in BHK-21 and Vero cells (Fig. 6). Maximum virus titers of the rRV-luc strain were 1.3×10^6 FFU/ml and 1.0×10^5 FFU/ml in BHK-21 and Vero cells, respectively.

Pathogenicity of the recombinant virus for adult and suckling mice

To assess the virulence of rRV-luc strain *in vivo*, i.c. inoculation of adult mice with rRV-luc and HEP-Flury strains was performed and the mice were observed for 14 days. Neither rRV-luc strain nor HEP-Flury strain killed the infected animals. The body weight ratios of the mice infected with rRV-luc and HEP-Flury strains had dropped till day 8 p.i. and then gradually increased (Fig. 7). However, rRV-luc strain caused less change of body weight than the HEP-Flury strain.

After i.c. inoculation of suckling mice with rRV-luc and HEP-Flury strains, the mice developed hind limb paralysis and succumbed to the infection. The virus titers of rRV-luc strain and HEP-Flury strain were 1.4×10^5 LD_{50}/ml and 2.0×10^6 LD_{50}/ml, respectively.

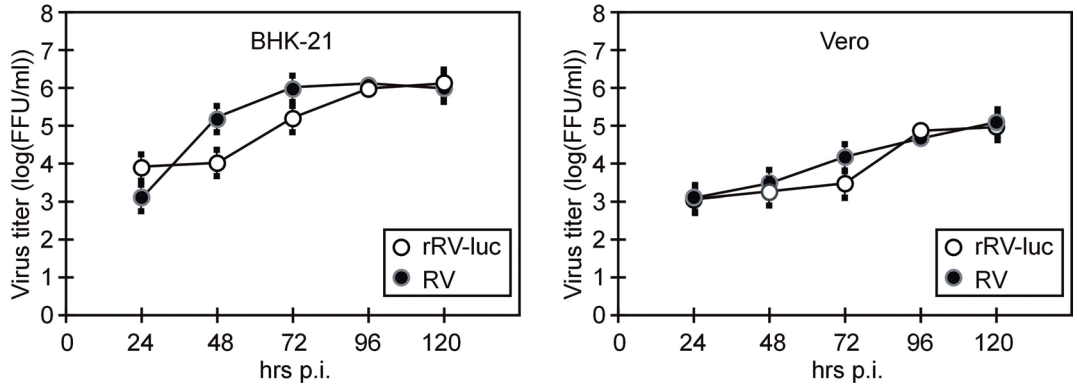

Fig. 6

Growth of rRV-luc and RV in BHK-21 and Vero cells

Discussion

RV causes a fatal CNS disease and its pathogenesis has puzzled investigators for more than a century (Matthias *et al.*, 2005). Using the reverse genetic system to generate recombinant RV has provided tools for a more detailed analysis of the RV pathogenesis. However, the complex interactions between virus and host immunity that determine pathogenesis of RV infection remain incompletely defined. Because of the threat of RV in Asia and Africa, it is essential to develop new techniques and reagents to investigate viral and host determinants of disease in the relevant animal models. It is expected that identifying and characterizing specific viral and host mediators of pathogenesis will lead to the new strategies for vaccination against RV infection and novel targets for therapy. A method for recovering a virus of the order *Mononegavirales* from the full-length cDNA was first developed for RV (Schnell *et al.* 1994). Since then, variations of the RV recovering systems pertaining to the manner of expression of the plasmid-encoded viral components have been reported. As a source of T7 RNA polymerase, recombinant poxviruses have been used (Schnell *et al.*, 1994; Ito *et al.*, 2001) or non-viral systems including the widely used BSR-T7/5 (Buchholz *et al.*, 1999) and BHK T7-9 cells (Ito *et al.*, 2003). More recently, RNA polymerase II has also been found to be suitable for recovery of RV (Inoue *et al.*, 2003). The rRV-luc strain was easily recovered in BHK-21 cells using the improved reverse genetics system that utilizes cellular RNA polymerase II. The titers of recovered virus in culture medium gradually rose with the serial passage in BHK-21 cells and reached the value of 1.3×10^6 FFU/ml (Fig. 4).

The rRV-luc strain was able to infect a variety of cells as similar as the parental HEP-Flury strain (Inoue *et al.*, 2003). The growth curves of two viruses were similar and the titers reached a peak by 96–120 hrs p.i. in the BHK-21 and Vero cells indicating that the insertion of luc gene does not affect a virus replication (Fig. 6). The highest amounts of virus were 1.3×10^6 FFU/ml in the BHK-21 cells, but 1.0×10^5 FFU/ml in the Vero cells. It may relate the fact that the viruses were recovered from BHK-21 cells, so that they are better adapted to the multiplication in BHK-21 cells than that in Vero cells.

Analysis of the rRV-luc strain pathogenicity indicated that this strain is less virulent than the parental HEP-Flury strain. It was reported that the neurotropism is largely determined by the G protein (Dietzschold *et al.*, 2008). These findings suggested that the G protein is not the sole determinant of attenuated phenotype of HEP-Flury strain. A genomic region such as the ψ sequences other than the G gene may also be related to the RV pathogenicity (Faber *et al.*, 2004). Furthermore, these results have other significance: (i) inserting foreign gene into the G-L NCR of RV was effective and safe, what indicated that the RV is suitable as a viral vector, (ii) the size of RV's genome increased by the insertion of foreign gene and may affect the pathogenicity of RV (Faber *et al.*, 2005).

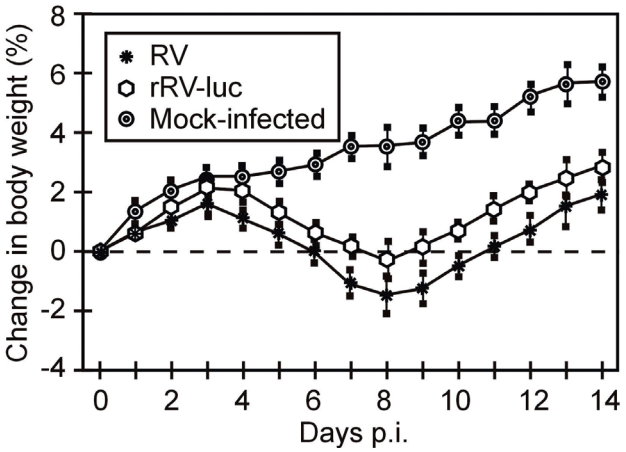

Fig. 7

Body weight changes in adult mice infected with rRV-luc and RV

The rRV-luc retained the extra luc gene and maintained the expression level of luc during multiple virus passages in the cultured cells. Similar findings have been reported: recombinant RV with chloramphenicol acetyltransferase (CAT) gene inserted into the G-L NCR also maintained CAT activity during multiple passages in cultured cells (Mebatsion *et al.*, 1996). Since it is thought that recombination seldom occurs in the un-segmented negative-sense RNA viruses including RV, the observed genetic stability of CAT and extra luc genes might be due to the lack of recombination.

In conclusion, a recombinant RV carrying firefly luc gene was constructed from the HEP-Flury cDNA clone using an improved reverse genetics system. The biological properties of the recombinant virus were determined and compared with the parental HEP-Flury strain. It was found that the recombinant rRV-luc strain was extremely stable after serial passages in the cultured cells and the intracellular luc expression was efficient. The present findings suggest that the rRV-luc strain might be a valuable tool for the investigation of RV-host interaction using BLI technology.

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