Bovine Herpesvirus-1 (BHV-1) Recombinant Expressing Pseudorabies Virus (PrV) Glycoproteins B and C Induces Type 1 Immune Response in BALB/c mice

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ABSTRACT. Bovine herpesvirus 1 (BHV-1) attached poorly and penetrated into a mouse cell line, BALB 3T3/A31, but a recombinant BHV-1/TF7-6, which expresses pseudorabies virus (PrV) gB and gC genes, did attach and penetrated into cells more efficiently. In this study the gene green fluorescent protein (GFP) has been integrated into genome of BHV-1/TF7-6 and its parental line of BHV-1. When the mouse mesenteries were incubated in vitro and infected with BHV-1/TF7-6/GFP, strong fluorescence was observed while BHV-1/GFP infection hardly demonstrated fluorescence, suggesting that BHV-1 recombinant expressing PrV gB and gC can infect mouse tissue cells more efficiently than the parental BHV-1 does. When BALB/c mice were inoculated with purified BHV-1/TF7-6 or its parental BHV-1, the former induced lower level of anti-BHV-1 immunoglobulin G (IgG) than the latter did. When sub-clones of anti-BHV-1 IgG were analyzed, it was found that mice immunized with BHV-1/TF7-6 or the parental BHV-1 demonstrated the same level of IgG2a. Since anti-BHV-1 IgG1 level was lower in mice inoculated with BHV-1/TF7-6, the IgG2a:IgG1 ratio was higher in BHV-1/TF7-6 inoculated mice than in the parental BHV-1 inoculated ones. These results indicate that BHV-1/TF7-6 induces type 1 predominant immune to BALB/c mice.

KEY WORDS: BHV-1, IgG1/IgG2a, PrV.

Pseudorabies virus (PrV), a member of the Alphaherpesvirinae, is the etiological agent of the Aujeszky’s disease (AD) which is often fatal in newborn pigs, causes respiratory disorders in older pigs and reproductive failures such as abortions and stillbirths in sows [1, 5, 6]. In other animals such as cattle, sheep, dogs and cats, PrV causes a severe neurological, and often fatal disease. PrV can infect experimentally small laboratory animals. Bovine herpesvirus-1, another member of Alpha-herpesvirinae, causes diseases in cattle such as infectious bovine rhinotracheitis, vulvovaginitis, abortion, encephalitis in calves and multisystemic infection in newborns. BHV-1 infects not only cattle but also pigs [3, 20], goats [17], minks, ferrets [25], and rabbits [11] but infection in animals other than cattle is rare. BHV-1 does not grow in mice at all [4]. Therefore it would appear that BHV-1 has narrower host range than PrV. The wide host range of PrV correlates with wide host range in vitro. In tissue culture PrV can grow in many cell lines derived from pigs, cattle, hamster, rats, rabbits and mice while BHV-1 can grow well in bovine derived cells but poorly in hamster cells and not at all in mouse cells.

We have reported earlier that a BHV-1 recombinant which expresses PrV gB and gC exhibited remarkable competence in virus attachment and penetration into non-bovine cells [18]. If this recombinant, BHV-1/TF7-6, can enter into the cells and express genes on its genome in cells of animals other than cattle, BHV-1/TF7-6 may be used as a vector for recombinant vaccines not only for cattle but also for other animals. In this study we investigated the infectivity and immunogenic properties of BHV-1/TF7-6 in mice, which are refractory to BHV-1 infection.

MATERIALS AND METHODS

Viruses and cells: IBRV(NG)dltk is a thymidine kinase (tk) deletion mutant of BHV-1 (LA) [21]. BHV-1/TF7-6 was a recombinant derived from IBRV(NG)dltk containing the PrV gB and gC genes [21]. Viruses were propagated in bovine kidney derived MDBK cells. MDBK cells and hamster lung derived HmLu-1 cells were cultured in Eagle’s minimum essential medium (EMEM) supplemented with 7.5% fetal calf serum (FCS) and 60 µg/ml of kanamycin. BALB/c 3T3, A31–1–1 cells (A31 cells) were cultured in Dulbecco’s modified Eagle’s minimum essential medium (DMEM) supplemented with 10.0% FCS and 60 µg/ml of kanamycin.

Purification of virus: MDBK cells were infected with IBRV(NG)dltk or BHV-1/TF7-6, at a multiplicity of infection (MOI) of 0.1 and incubated at 37°C for 72 hr. The medium was collected from the infected cultures and centrifuged twice at 2,000 rpm for 5 min at room temperature to remove cell debris. The supernatant was then centrifuged at 100,000 × g for 120 min at 4°C to pellet the virus. The virus was re-suspended in phosphate-buffered saline (PBS), loaded onto a 12 ml of linear 10% to 40% potassium tartrate gradient in TNE (50 mM Tris HCl, pH 7.5; 130 mM NaCl, 1 mM EDTA) and centrifuged at 100,000 × g for 120 min at 4°C. The gradient was fractionated into 12 fractions by puncturing the bottom of the tube and collecting fractions drop-wise. The purified virus fraction was centrifuged at
100,000 x g for 120 min at 4°C. The precipitates were resuspended in EMEM containing 1% FCS and stored at −80°C as purified virus.

Immunization: Five mice were immunized intraperitoneally with 10⁶ plaque formation unit (PFU) of purified BHV-1/TF7-6 or IBRV(NG)dtlk. After immunization serum samples were collected once a week.

ELISA for detection of the antibody against BHV-1: Purified IBRV(NG)dtlk was diluted in carbonate buffer (74 mM NaHCO₃, 26 mM Na₂HCO₃, pH 9.6) to 1 x 10⁶ PFU/ml and 100 µl aliquots were added to each well of a 96-well ELISA plate (Corning, U.S.A.). The plate was incubated at 4°C overnight, washed 3 times with PBS containing 0.1% Tween 20 (PBS-T) and 100 µl of PBS was inoculated right into each well. The reaction was stopped by adding 1:3000 diluted 1/3000 diluted rabbit anti mouse IgG antibody (ZYMED, U.S.A.) and incubated at 37°C for 1 hr. After washing with PBS-TritionX100, 100 µl of 0.04% o-phenylanediamine and 0.003% H₂SO₄ and the absorbance at 490 nm was determined.

Detection of virus entry to tissue of adult mouse mesenteries: Mesenteries of 8 weeks old female BALB/c mouse was harvested and cut into approximate 5 mm x 5 mm pieces. The piece of mesenteries was incubated for 9 hr at 37°C with 2 x 10⁷ PFU of IBRV/TK-GFP or BHV-1/TF7–6/TK-GFP in RPMI1640 medium (GIBCO BRL, U.S.A.) containing 10% FCS. After the incubation, the pieces of mesenteries were picked up and incubated again in fresh RPMI1640 medium (GIBCO BRL, U.S.A.) containing 10% FCS for 6 hr. And then the pieces were observed under a fluorescent microscope to detect specific fluorescence of GFP.

Statistical analysis: The data of different groups were analyzed for statistical significance by the student t test.

RESULTS

Construction of BHV-1 recombinant expressing GFP as a marker of virus entry: GFP expressing recombinants of BHV-1, which formed fluorescent plaques in permissive MDBK cells, were constructed to investigate the entry of BHV-1 recombinants into semi- or non-permissive cells. BHV-1/TK-GFP was constructed by integrating the GFP gene under the control of the PrV IE promoter at the site of the PrV IE gene of PrV at the XbaI site of the plasmid vector, pUC19 and resulting plasmid was designated as pLATK/pie. The resulting plasmid was designated as pLATK/pie. The Hind III-XbaI fragment of BHV-1 containing the promoter of the PrV immediate early (IE) gene was obtained by digesting this plasmid with KpnI and EcoRI sites of pA3/MCSdelH to construct pLAH-K/pTK. The Hind III-XbaI fragment containing the promoter of the PrV immediate early (IE) gene was obtained from the plasmid pie [30], treated with Klenow fragment and inserted at the XbaI site of pLAH/K-pTK. The resulting plasmid was designated with pLAH-K-pTK/pIE. The Hind III-XbaI fragment of pCX-EGFP, containing eGFP cDNA and poly A site of the rabbit beta-globin gene, was inserted into XbaI site of the plasmid. pLAH/K-pTk/pIE. The resulting plasmid was designated as p25/GFP. The recombinant BHV-1/GFP was constructed by homologous recombination between pLATK/GFP and BHV-1 LA strain. BHV-1/TF7–6/TK-GFP was constructed by homologous recombination between p25/GFP and BHV-1/TF7–6. The details of the procedures for homologous recombination and cloning of recombinant viruses were described previously [27, 28].
Fig. 1. Schematic diagrams of GFP expressing recombinants, BHV-1/GFP and BHV-1/TF7-6/TK-GFP. (A) BHV-1. Open boxes indicate repeat region. Solid bars indicate unique long (UL) and unique short (US) regions. (B) BHV-1/GFP. GFP gene is inserted into the tk gene of BHV-1. (C) BHV-1/TF7-6/TK-GFP. PrV gB gene is inserted into the tk gene of BHV-1. PrV gC gene is replaced with BHV-1 gC gene. PrV tk gene and GFP gene are inserted into the BHV-1 gG gene.

Fig. 2. Expression of GFP by BHV-1/GFP and BHV-1/TF7-6/TK-GFP in semi-permissive HmLu cells and non-permissive A31 cells. The cells were infected with BHV-1/GFP or BHV-1/TF7-6/TK-GFP and observed at 24 hr post infection. (A) HmLu cells infected with BHV-1/TF7-6/TK-GFP. (B) HmLu cells infected with BHV-1/GFP. (C) A31 cells infected with BHV-1/TF7-6/TK-GFP. (D) A31 cells infected with BHV-1/GFP.
expressed. BHV-1/GFP was thymidine kinase negative while BHV-1/TF7-6/TK-GFP was thymidine kinase positive and gG negative. Since the thymidine kinase and the gG are not contained in the BHV-1 virion [9], the deletion of these genes are not involved in the early events of BHV-1 infection such as attachment, penetration and expression of the IE genes. Therefore we used these recombinants to investigate the entry of BHV-1 and BHV-1/TF7-6 into cells or tissues.

Entry of BHV-1 recombinants into semi-permissive and non-permissive cells: BHV-1 grows poorly in HmLu-1 cells and not at all in A31 cells although PrV can grow in both cell lines very well. Therefore HmLu-1 cells are semi-permissive and A31 cells are non-permissive for BHV-1 and both cells are permissive for PrV [18]. As shown in the Fig. 2, BHV-1/TF7-6/TK-GFP showed much greater level of specific fluorescence than BHV-1/GFP both in A31 cells and in HmLu cells. However, plaque formation of BHV-1/TF7-6/TK-GFP was not observed in A31 cells. These results confirmed the earlier data obtained by measuring BHV-1 DNA by quantitative competitive PCR (QCPCR) that PrV gB and gC on the BHV-1 recombinant increased the level of viral DNA entry into non-permissive A31 cells but they did not make BHV-1 to multiply [18].

Entry of BHV-1 recombinants into cells of mouse mesenteries: To determine whether the BHV-1 recombinants expressing PrV gB and gC, can infect mouse tissue cells, following experiments were carried out. The mesenteries were harvested from mouse and cut into small pieces. The pieces were incubated with BHV-1/GFP or BHV-1/TF7-6/
TK-GFP and then the expression of GFP was followed as a marker of virus entry into the mesentery cells. As shown in Fig. 3, GFP is hardly expressed in the mesentery cells infected with BHV-1/GFP but strong fluorescence of GFP was observed in mesenteries cells infected with BHV-1/TF7-6/TK-GFP. Therefore it can be concluded that PrV gB and gC on the BHV-1 recombinant increase the ability of entry into not only tissue culture cell line but also mouse mesentery cells.

**Induction of IgG in mice immunized with IBRV(NG)dltk and BHV-1/TF7-6:** When BHV-1 is injected into intraperitoneal cavity of a mouse, virus contacts with mesenteries immediately. BHV-1/TF7-6 virions may attach and be taken up by cells of the mesenteries but BHV-1 virions may stay in the peritoneal cavity without being taken up by the mesentery. It is of interest to know whether the immunogenic properties of BHV-1/TF7-6 and BHV-1 are different when inoculated intraperitoneally into mice. BALB/c mice were immunized with 10^6 PFU of purified virus, BHV-1/TF7-6 or IBRV(NG)dltk interperitoneally. Both were thymidine kinase negative and gG positive. BHV-1/TF7-6 derived from IBRV(NG)dltk expressed PrV gB and gC. Sera were collected from each mice every week and anti-BHV-1 IgG was measured. As shown in Fig.4, immunization with BHV-1/TF7-6 induced lower level of IgG against BHV-1 virion than with IBRV(NG)dltk. Especially, at one week and three week post immunization significant difference was observed (p<0.01).

**Induction of DTH response in immunized mice:** For the evaluation of cell mediated immunity, the DTH responses were measured. Six weeks after the immunization, the UV inactivated IBRV(NG)dltk was injected as a test antigen in the left footpad of a mouse. As a control, the same volume of PBS was injected in the right footpad of a mouse. After 2 days from the injection, positive DTH responses were observed as the swelling of left footpad, in the both groups immunized with BHV-1/TF7-6 and IBRV(NG)dltk (Fig. 5). The average of swelling value of BHV-1/TF7-6 immunized group was slightly higher than that of IBRV(NG)dltk group. (0.29 ± 0.19 mm and 0.2 ± 0.15 mm, respectively). But statistical difference was not observed (p>0.05).

**IgG1 and IgG2a production in immunized mice:** Antigens on BHV-1 virion specific serum antibody isotype was measured as an *in vivo* indicator of type 1 and type 2 immune response. At four weeks post immunization, BHV-1/TF7-6 immunized mice produced lower level of IgG1 comparing that induced in IBRV(NG)dltk immunized mice. However, the levels of induced IgG2a were not different statistically between the two groups of mice immunized with BHV-1/TF7-6 or IBRV(NG)dltk (p>0.05) (Fig. 6, A). And BHV-1/TF7-6 immunized mice have a significantly higher
IgG2a:IgG1 ratio than that of IBRV(NG)dltk immunized mice (Fig. 6, B). These results indicate that immunization with BHV-1/TF7-6 induce type1 predominant immune response than IBRV(NG)dltk control.

DISCUSSION

Several kind of attenuated live PrV strain was reported and used as live vaccine to control Aujeszky’s disease in pigs [2, 8, 12–16, 23, 24, 26, 29]. Piglets born from sows that have been latently infected with PrV have maternal antibodies against PrV. Although, this passively acquired immunity usually reduces the severity of clinical signs of AD after infection of PrV [10, 16], it does not last very long and pigs are not protected after the immunity wanes. Furthermore the passively acquired immunity interferes with the induction of the active immune response by attenuated live PrV vaccines. In our laboratory BHV-1 recombinants, which express PrV glycoproteins in combinations, had been constructed to be used as the vaccine against AD in piglets with maternal antibodies because BHV-1 vector based vaccine is less likely to be interfered by the maternal antibodies against PrV [7, 21, 22]. Immunization with the recombinant BHV-1, which expresses PrV glycoproteins, protected mice from death by lethal PrV infection and induced cell mediated immune responses against PrV [7, 22]. We also reported that when the BHV-1 recombinant was treated by formaldehyde, the protective ability was reduced [22] suggesting that the BHV-1 recombinant expressing PrV glycoproteins might be growing in mice.

During the course of the study it was found that BHV-1 recombinants which express PrV gB and gC had marked increase in attachment and penetration abilities to semi-permissive HmLu-1 and non-permissive A31 cells. The increase of virus titer was observed in HmLu-1 cells but not in A31 cells [18, 19].

In this study the marker GFP gene was integrated into the genomes of BHV-1/TF7-6 and BHV-1 to construct BHV-1/TF7-6/TK-GFP and BHV-1/GFP, respectively. As expected from the previous studies, stronger fluorescence of GFP was observed in HmLu-1 or A31 cells infected by BHV-1/TF7-6/TK-GFP than by BHV-1/GFP. The stronger
fluorescence by BHV-1/TF7-6/TK-GFP infection was observed not only in the established cell lines but also in the mesentery cells freshly obtained from mouse peritoneal cavity. The results suggest that PrV gB, gC on BHV-1 virion increase the competence of attachment and penetration into the mouse mesentery cells.

When the immunogenic properties of BHV-1 and BHV-1/TF7-6 was investigated in mice, it was found that BHV-1/TF7-6 and BHV-1 induced about the same level of IgG2 but the former induced much lower level of IgG1. This suggests that BHV-1/TF7-6 induced more of the type 1 immune response than parental BHV-1 did. This may be due to cell types where the virus antigens might be expressed.

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REFERENCES


