Immunogenic Properties of a Bovine Herpesvirus-1 (BHV-1) Recombinant Expressing Major Pseudorabies Virus (PrV) Glycoproteins in Combination

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ABSTRACT. A recombinant bovine herpesvirus type 1 (BHV-1), designated BHV-1/TF17-1, which expresses pseudorabies virus (PrV) glycoproteins gB, gC, gD, gE and gI in combination was constructed. To test the protective immunity, 10 mice were inoculated with BHV-1/TF17-1 and three weeks later 10 mice were intraperitoneally (i.p.) challenged with 20 LD₅₀ virulent PrV (YS-81). BHV-1/TF17-1 protected all the mice from the PrV lethal challenge while all the control mice died in around 3 days. Mice vaccinated with BHV-1/TF17-1 acquired high PrV-neutralizing antibody titers and demonstrated strong delayed type hypersensitivity responses and moderate *in vitro* lymphocyte proliferative responses to PrV antigen. Since the major PrV glycoproteins were integrated into virions (probably into viral envelope), BHV-1/TF17-1 was neutralized with anti-PrV antiserum. However, the susceptibility of BHV-1/TF17-1 to anti-PrV antiserum is 2- to 4-fold lower than that of PrV vaccine lines. Our results demonstrated the possibility of BHV-1/TF17-1 as a vaccine to protect piglets from Aujeszky's disease where maternal antibodies against PrV interfere attenuated live PrV vaccines.

KEY WORDS: Aujeszky's disease, BHV-1, glycoprotein, PrV, recombinant vaccine.

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Aujeszky's disease (AD), caused by pseudorabies virus (PrV), a member of the Alphaherpesvirinae, is a serious economic problem in swine industry. Attempts have been made to control PrV infection by vaccination with several kinds of attenuated live PrV strains [4, 9, 16, 17, 19, 20, 27, 28, 30, 34]. Currently, vaccination programs are based on the use of marker vaccines in which one of the non-essential glycoproteins, either gC, gE or gG, is deleted. But existing attenuated live vaccines have two major problems to control AD. First, vaccination is effective to prevent the clinical disease but not necessarily able to prevent PrV infection, replication and establishment of latency. Vaccinated pigs can be infected with field virus and subsequently shed the virus [6, 13, 21, 31]. Second, piglets born from sows latently infected with PrV have maternal antibodies against PrV. This passively acquired immunity usually reduces the severity of clinical signs of AD after infection of PrV [1, 10, 22]. High level of maternally derived antibody (MDA) can prevent virus excretion in contact-infected pigs and can reduce transmission of PrV among maternally immune pigs [3]. Thus, maternal immunity is apparently of benefit for young piglets but it wanes with the age of pigs. Sooner or later the pigs become susceptible to PrV infection and need vaccination. The presence of maternal antibodies at the time of vaccination, however, is often a disadvantage, as it interferes with the induction of the active immune response against live attenuated PrV vaccines. After vaccination, maternally immune pigs often do not develop an increase in serum antibody titer [2, 32, 33]. Moreover, a single

vaccination could not prevent transmission of PrV among maternally immune pigs, whereas it could prevent transmission among non-maternally immune pigs [3]. Therefore, it is likely that vaccination with existing attenuated live vaccine strains is inadequate to control AD.

Bovine herpesvirus type-1 (BHV-1), another member of the Alphaherpesvirinae, is an etiological agent for cattle diseases such as infectious bovine rhinotracheitis (IBR) and BHV-1 infection constitutes one of the most important infectious diseases of cattle and causes significant economic losses to live stock around the world. BHV-1 has been proposed to be a vector for recombinant vaccines to protect cattle against various diseases. BHV-1 fulfills the important requirement for a vaccine vector, i.e. biological safety because of its narrow host range, while others have broader host ranges capable of productive infection of diverse vertebrate species (e.g., PrV). BHV-1 vectors which express foot-and-mouth disease virus capsid protein (VP1) epitopes as parts of fusion proteins with gC have been constructed [11, 12].

In our laboratory, BHV-1 recombinants, which express PrV glycoproteins gB, gC, gD, gE or gI, either individually or in some combinations, had been constructed [25] and found that these BHV-1 recombinants were able to induce moderate protective immune responses against PrV [26]. However, none of these recombinant expressed PrV gB and gD together, both of which are known to be important antigens to induce protective immunity against PrV. In this study we constructed a new BHV-1 recombinant, which express five PrV glycoproteins, gB, gC, gD, gE and gI together, in a hope to obtain efficacious recombinant vaccine against PrV. We characterized this BHV-1 recombinant and found that this induced not only humoral immunity but also cell mediated immunity against PrV and protected mice from the lethal challenge with PrV.

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This BHV-1 recombinant may be useful as an alternative vaccine to the attenuated live PrV vaccine in piglets which have high maternal antibodies.

MATERIALS AND METHODS

Viruses and cells: IBRV(NG)dltk is a thymidine kinase (tk) deletion mutant of BHV-1 (LA) [25]. BHV-1/TF17-1 was a recombinant derived from IBRV(NG)dltk containing the PrV gB, gC, gD, gE, and gI genes constructed by the method described by Otsuka and Xuan [25]. PrV Indiana S strain [PrV (IND)] and PrV Yamagata S-81 strain (YS-81) were the wild types of PrV. The PrV vaccine strains used for this study were gC(-) vaccine, OMNIMARK-PrV, tk(-) vaccines, OMNIVAC-PrV and BUCHAREST-PrV, gE(-) vaccine, DUPHER-PrV, tk(-)-gE(-) vaccine, INTERVET-PrV. BHV-1 recombinants were propagated in a bovine kidney cell line, MDBK. PrV strains were propagated in the porcine kidney cell line, CPK. Viruses were titrated in MDBK cells by plaque titration. Cells were cultured in Eagle's minimum essential medium (EMEM, Nissui, Japan) supplemented with 7.5% fetal calf serum (FCS) and 60 µg/ml of kanamycin.

Viral growth curves: MDBK cells were cultivated in 35 mm dishes. The confluent monolayers were inoculated with viruses at a multiplicity of infection (MOI) of 10 (for analysis of one step growth) or at an MOI of 0.1 (for multi step growth) at 37°C for 1 hr. Then prewarmed medium was added after remaining extracellular virus was removed by washing with PBS and the cultures were incubated at 37°C for the appropriate times. Cells and supernatant were harvested at 0, 3, 7.5, 19, 38, 49, 57, 96 hr post infection. Virus progeny was titrated on MDBK cells.

Purification of virions: MDBK cells were infected with IBRV(NG)dltk or TF17-1, at an MOI of 0.1 and incubated at 37°C for 72 hr. CPK cells were infected with PrV (IND) at an MOI of 0.1 and incubated at 37°C for 48 hr. The medium was collected from the infected cultures and centrifuged twice at 2,000 rpm for 5 min at room temperature in a KUBOTA 2010 centrifuge to remove cell debris. The supernatant was then centrifuged at $100,000 \times g$ for 120 min at 4°C in a Beckman SW 41 rotor (Beckman Spinco, Palo alto) to pellet the virions. The virions were resuspended in PBS buffer, 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 $100,000 \times g$ for 120 min at 4°C in a Beckman SW 41 rotor. Thereafter, the gradient was fractionated into 12 fractions by puncturing the bottom of the tube and collecting fractions dropwise. The virus antigen (PrV gB) was located in the fractions by the dot blot assay. The fractions containing the virus antigen were diluted 1:4 in PBS and then subjected to the same density gradient centrifugation procedure. The reason for performing the second density gradient centrifugation procedure is to make sure that the virion fraction is not contaminated with light density defective virus particles or virus glycoproteins associated with cell membrane. The fractions from the second density gradient centrifugation were monitored by plaque titration and dot blot analysis to confirm that

the peak of the infectious titer corresponds with the peak of the virus antigen. The purified virus fraction was dialyzed with PBS by ultrafiltration with microconcentrator (MICROCON; AMICON, Inc) and used for Western blot analysis, delayed type hypersensitivity (DTH) and lymphocyte proliferation assays. This purified virion preparation is referred to 'gradient purified virus'.

Polyacrylamide gel electrophoresis and Western blot analysis: Gel electrophoresis was carried out by the procedure of Laemmli [14]. Virus infected cells were harvested and suspended in PBS. Equal volume of $2 \times$ SDS-sample buffer (0.02% bromophenol, 20% glycerol, 4% SDS, 125 mM Tris-HCl pH 6.8) was added. For reducing conditions, 1.4 mM 2-mercaptoethanol was added and incubated at 100°C for 5 min. Samples of virus infected cell lysate were electrophoresed in a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride sheet (Immobilon Transfer Membrane; Millipore) at a 30 mA per gel for 90 min by the electro-blotting procedure using power supplier (Savant PS 250). The membranes were soaked for 30 min with blocking solution, PBS containing 3% (W/V) skim milk (Yukijirushi Nyugyu, Co.), and then washed with PBS. Each membrane was treated with polyclonal antibodies; R33 (anti-PrV gB), gI 5-3 (anti-PrV gE) or PgI 3 fusion (anti-PrV gI), or monoclonal antibodies; 1E8 (anti-PrV gC) or 7B5 (anti-PrV gD) [25, 26]. These antibodies were described elsewhere [25]. Membranes were incubated at 37°C for 1 hr in 100 to 1,000 fold diluted antibodies in blocking solution, and then washed with PBS. They were further incubated for 1 hr at 37°C with horseradish peroxidase conjugated goat anti-mouse (or rabbit in the case of PrV gB) Ig G, which was diluted 1,000 fold with blocking solution, washed with PBS, and then finally developed for peroxidase activity with ECL system (Amersham Pharmacia Biotech. UK) for 1 min at room temperature. The fluorescence was detected by exposing to the X-ray film (Kodak X-Omat).

Dot blot analysis: Gradient-purified virion fractions were directly transferred onto an Immobilon membrane with a dot blotter (MINIFORD SRC96D; Schleicher & Schuell, Co. Germany). The membrane was washed once with PBS, soaked for 30 min with blocking solution (PBS containing 3% skim milk) and then washed with PBS. And then it was incubated for 1 hr at 37°C with specific anti-PrV glycoprotein antibodies described above, which were diluted 100 to 1,000 fold in blocking solution, and washed with PBS. The membrane was further incubated for 1 hr at 37°C with the secondary antibodies diluted 1,000 fold in blocking solution, washed with PBS, and then finally developed for peroxidase activity with ECL system for 1 min or with 3,3'-Diaminobenzidine tetrahydrochloride (DAB) solution (PBS: DAB: 35% hydrogen peroxide (H_2O_2) = 25 ml: 12 mg : 40 µl) at room temperature until the desired color intensity was achieved (4 to 5 min). The membrane stained with DAB solution was washed two or three times with de-ionized water for total period of 10 to 20 min.

Mice: four- to eight-week-old female ddY mice were purchased from Saitama experimental animals supply, Co. (Saitama, Japan). Four-week-old mice were used for chal-

challenge experiments, seven- to eight-week-old mice were used for DTH assay and five- to six-week-old mice were used for lymphoblast proliferation assay.

Vaccination and challenge experiments: Female ddY mice were vaccinated i.p. once with 0.5 ml of virus suspensions in tissue culture media. In each group (15 mice per group) mice were immunized with $1 \times 10^{3.45}$ TCID₅₀, $1 \times 10^{4.45}$ TCID₅₀ or $1 \times 10^{5.45}$ TCID₅₀ of BHV-1/TF17-1 per mouse. As controls, one group was immunized with $1 \times 10^{5.45}$ TCID₅₀ of the parental line IBRV(NG)dltk and another control group was not vaccinated at all. Three weeks after the vaccination, 5 mice from each group were bled and virus neutralization (VN) titer was assayed. Ten mice from each group were challenged i.p. with 20 LD₅₀ PrV (YS-81), virulent strain of PrV, and examine daily for clinical signs. Mice surviving 2 weeks after the challenge were regarded as protected.

Delayed type hypersensitivity (DTH) assay: Female ddY mice were vaccinated i.p. once with 0.6 ml of BHV-1/TF17-1 or parental IBRV(NG)dltk suspended in tissue culture media. Groups of mice (5 mice per group) were immunized with 1×10^6 PFU or 1×10^7 PFU of BHV-1/TF17-1 and one group was immunized with 1×10^7 PFU of the parental IBRV(NG)dltk. Twenty-five days after the vaccination, the test antigen suspended in 20 μ l PBS was injected intradermally in the right footpad of a mouse. Gradient purified PrV (IND) was UV inactivated and used as the test antigen. Each mouse was inoculated with 1×10^6 PFU equivalent of UV inactivated PrV. At 2 days after injection of the test antigen, the mouse was anesthetized with 1.5 mg of sodium pentobarbital (Nembutal; DAINABOT) and the DTH reaction was measured with vernier calipers (Kanon, Japan) as net swelling (post-challenge minus pre-challenge footpad thickness). Differences of DTH response in different groups were analyzed for statistical significance by the student *t* test.

Lymphocyte proliferation assay: Mice were vaccinated once as described in DTH assay. Four weeks after the vaccination, spleens from immunized mice were removed, single cell suspensions were prepared and 2×10^5 lymphocytes were inoculated into a well of a 96 well plate. To stimulate the proliferative response, UV-inactivated PrV at 2×10^5 or 4×10^5 PFU equivalent per well was added and no antigen was added to control wells. On the day 2, cells were labeled for 6 hr with 1 μ Ci of [Methyl-³H]-thymidine (NENTM Life Science Products, Inc.) per well, harvested with a semiautomatic cell harvester and incorporated radioactivity was measured by liquid scintillation counter (LSC-5100, ALOKA, Co., Ltd). The index of lymphoblast proliferation was calculated as counts per minute after stimulation with antigen divided by counts per minute after stimulation without antigen.

Virus neutralization (VN) assay: Virus neutralizing activity of antiserum from vaccinated mice was tested in a 50% plaque reduction assay. Appropriate dilutions (ca. 100 PFU per well) of PrV (IND) were incubated for 1 hr at 37°C with the heat inactivated mouse antisera and 5% normal rabbit serum as a source of complement and plated onto monolayers of MDBK cells. After 1 hr incubation at 37°C to permit absorption, the monolayers were overlaid with EMEM containing 0.5%

methylcellulose and 7.5% FCS. Plaques were allowed to develop for 2 to 3 days and the cells were then stained with crystal violet. The end point was determined by the serum dilution required for 50% plaque reduction.

To compare the susceptibility of BHV-1/TF17-1 and commercial attenuated live PrV vaccine strains, VN assays were carried out using anti-PrV antiserum, PS3, which was derived from AD positive sow and a kind gift from the Nippon Institute for Biological Science. Serially diluted heat inactivated PS3 was incubated with approximately 100 PFU of virus and 10% rabbit serum as a source of complement. The resulting VN titers of PS3 were used as an index of determining differences of susceptibility to PrV-neutralizing antibody among PrV commercial vaccines and BHV-1/17-1.

RESULTS

Growth characteristics of BHV-1/TF17-1: BHV-1/TF17-1 was constructed by the method described by Otsuka and Xuan [25] by inserting the PrV *Bam* HI-7 fragment containing the PrV gD, gI and gE gene at the tk locus of the genome of IBRV(NG)dltk, replacing the coding sequence of the BHV-1 gC gene with the coding sequence of the PrV gC gene, and inserting the PrV gB gene at the BHV-1 gG locus. The genome map of BHV-1/TF17-1 is shown in Fig. 1. The genome of BHV-1/TF17-1 contains 11 Kbp PrV DNA. To see if the integrated foreign DNA has any effect on the growth of BHV-1 recombinant, the growth characteristics of BHV-1/TF17-1 were analyzed. BHV-1/TF17-1 formed distinctly smaller plaques on MDBK cells than IBRV(NG)dltk and PrV (IND) (data not shown). One step and/or multi step growth curves of BHV-1/17-1 in MDBK cells were compared with those of the parental IBRV(NG)dltk and PrV (IND). After infection of MDBK cells with each virus at an MOI of 10 or 0.1, the cells and media were collected at various times, freeze-thawed and plaque-titrated on MDBK cells. The titers of plaque forming units included cell-associated and cell-free virus are plotted (data not shown). At the stationary phase, BHV-1/TF17-1 reached a titer at least 10-fold less than that of IBRV(NG)dltk but at the similar level with that of PrV (IND).

Detection of recombinant PrV glycoproteins in BHV-1/TF17-1-infected MDBK cells by Western blot analysis: To detect the recombinant PrV glycoproteins in BHV-1/TF17-1-infected cell lysate, Western blot assay was performed. MDBK cells were infected at an MOI of 0.1 with IBRV(NG)dltk, BHV-1/TF17-1 or PrV (IND). Virus-infected cells were harvested when extensive CPE was observed, and proteins were fractionated by gel electrophoresis, blotted onto a membrane and reacted with monoclonal antibodies against PrV gC (Fig. 2a) and PrV gD (Fig. 2b) or polyclonal antibodies against PrV gE (Fig. 2c), PrV gI (Fig. 2d) and PrV gB (Fig. 2e). Proteins were analyzed in reducing conditions (PrV gE and gI) or non-reducing conditions (PrV gB, gC and gD). As expected, in PrV (IND) infected cell extracts 98 kDa-mature form of PrV gC, 50 kDa-mature form of PrV gD, 123 kDa-mature form of PrV gB, 130 kDa-mature form of PrV gE, 63 kDa-mature form and the 52 kDa-precur-

Fig. 1. The genomic construction of BHV-1/TF17-1. The BHV-1 genome consists of a unique long region (UL) and a unique short region (US). The latter is flanked by inverted repeats (IR, internal repeat; TR, terminal repeat) shown as boxes. BHV-1/TF17-1 was constructed based on the IBRV(NG)dltk by inserting the PrV *Bam* HI-7 fragment containing the PrV gD, gI and gE gene at the tk locus, by replacing the coding region of the BHV-1 gC gene with the coding region of the PrV gC gene, and by inserting the PrV gB gene at the BHV-1 gG locus (abbreviations: Hn, *Hind* III; B1, *Bgl* II; Xh, *Xho* I; Bm, *Bam* HI; P, *Pst* I).

sor form of PrV gI were detected. In BHV-1/17-1 infected cell extracts, the presence of the mature form of PrV glycoproteins, gC, gD, gE, gI and gB and the precursor form of gI were detected. In contrast, neither IBRV(NG)dltk nor mock control did not show PrV glycoproteins at all.

Detection of recombinant PrV glycoproteins in purified virions by dot blot analysis: Extra-cellular virions of BHV-1/17-1 were purified as described in Materials and Methods. After the first density gradient centrifugation, the gradient was fractionated into 12 fractions and each fraction was subjected to plaque titration and dot blot assay. Anti-PrV gB polyclonal antibody (R33) was used for dot blot assay in order to locate the cell debris and immature virus particles. The most of plaque forming units were located in the fraction 5 while the dot blot assay demonstrated the presence of PrV gB in the fractions 5-9 (Fig. 3b). Therefore, it was possible that the fraction 5 still contained cell debris and immature virus particles. To ensure that the virion preparation was free of contam-

ination of cellular component, the fraction 5 in the first gradient purification step was further purified by another gradient centrifugation. The result of the second purification is shown in Fig. 3c and 3d. The fraction 5 in the second gradient purification step contained the peak of infectious units and PrV gB antigen suggesting that the virion fraction was free from less dense contaminants. Virions were located at a density of about 1.3 g per ml and well separated from the lower density (< 1.05 g/ml) fractions (the fraction 11-12), where endoplasmic reticulum (ER), plasma membrane and Golgi predominate [8]. These results confirm the effectiveness of the purification protocol.

Dot blot analysis was performed to detect the presence of PrV gC, gD and gI in the purified virion. The 2 × purified fraction 5 of BHV-1/TF17-1 and IBRV(NG)dltk were transferred onto the membrane at a dose of 1×10^5 PFU per dot and were reacted with antibodies. As shown in Fig. 3e, expressions of all five recombinant PrV glycoproteins were confirmed in

Fig. 2. Expression of PrV gB (gII), gC (gIII), gD (gp50), gE (gI) and gI (gp63) in BHV-1/TF17-1 infected cells. MDBK cells were infected with IBRV(NG)dltk, BHV-1/TF17-1 and PrV (IND) at an MOI of 0.1. Whole cell extracts were collected and analyzed by Western blots. The blots were incubated with monoclonal antibodies against PrV gC (a) and PrV gD (b) or with polyclonal antibodies against PrV gE (c), PrV gI (d) and PrV gB (e). Samples used are mock (lane 1), PrV (IND) (lane 2), IBRV(NG)dltk (lane 3) and BHV-1/TF17-1 (lane 4). The arrowheads indicate the expected migration positions of processed mature species of each glycoprotein. The dots indicate the positions of the non-glycosylated precursor proteins. Molecular weight markers in kDa were shown on the left.

BHV-1/TF17-1 virions.

Protection of mice against PrV challenge: Mice were vaccinated as described in the Materials and Methods and three weeks later vaccinated and control mice were challenged with 20 LD₅₀ of virulent PrV (YS-81). All the non-vaccinated mice died and the average survival time was 3.2 days. Among the 10 mice immunized with IBRV(NG)dltk, one survived and the average survival time of the 9 mice was 3.0 days (Table 1). All the 10 mice immunized with $1 \times 10^{5.45}$ TCID₅₀ BHV-1/TF17-1 were protected (the group 1). However, 4 mice immunized with $1 \times 10^{4.45}$ TCID₅₀ (the group 2) and 3 mice immunized with $1 \times 10^{3.45}$ TCID₅₀ (the group 3) did not survive.

The average survival time of the dead mice in both group 2 and 3 was 4.3 days, which was clearly longer than those of IBRV(NG)dltk- or mock-vaccinated mice.

Induction of virus neutralization antibodies in mice: Sera collected from 5 mice in each group three weeks after the immunization (just prior to challenge infection) were subjected to virus neutralization (VN) tests against PrV virulent strain. Sera were also collected from the mice which survived for two weeks after the challenge with 20 LD₅₀ of PrV (YS-81). As shown in Table 2, high VN titers against PrV were detected in BHV-1/TF17-1-vaccinated mice. However, no dose dependent relationship was observed between neutraliz-

ing activity and the amount of virus used for vaccination. After the challenge infection of virulent PrV, the VN titer of the survivors further increased.

Induction of DTH response in vaccinated mice: For the evaluation of cell mediated immunity in mouse system induced with BHV-1/TF17-1, the DTH responses were measured by footpad swelling after the injection of UV-inacti-

vated purified PrV virions. At day 1 after injection no swelling of the foot-pad was observed but 2 to 3 days later, positive DTH responses were observed in the groups vaccinated with TF17-1 (Fig. 4). In the group vaccinated with 107 PFU of TF17-1 (the group I), the average of swelling value ($37 \pm 10, \times 10^{-2}$) was significantly higher than that of IBRV(NG)dltk vaccinated mice ($p < 0.01$ by the student *t* test).

Fig. 3. Density gradient purification of virion. MDBK cells were infected with IBRV(NG)dltk and BHV-1/TF17-1, at an MOI of 0.1. Virus particles were purified from the culture media as described in Materials and Methods. The fractions from first (a) and second (c) purification were monitored by plaque titration. Anti-PrV gB polyclonal antibody (R33) was used for dot blot analysis in order to locate the cell debris and immature virus particles in first (b) and second (d) purification steps. Expression of PrV glycoproteins on the surface of the BHV-1/TF17-1 virion was examined with the anti-PrV gB, gC, gD, gE and gI antibodies as described in Materials and Methods (e).

On the other hand, the average of swelling value ($16 \pm 17, \times 10^{-2}$) in the mice vaccinated with 10^6 PFU of TF17-1 (the group II) was slightly higher than that of negative control (the group III). However, this difference was not statistically significant ($p > 0.05$).

Lymphocyte proliferation assay: Groups of mice (5 mice per group) were vaccinated with 10^6 PFU or 10^7 PFU of TF17-1 or 10^7 PFU of IBRV(NG)dltk, respectively. Spleen cells were harvested 4 weeks later and stimulated *in vitro* with

UV-inactivated PrV (IND). As shown in Fig. 5, in the absence of *in vitro* priming with UV-inactivated PrV (IND) at an MOI of 1.0, none of the groups showed any incorporation above the IBRV(NG)dltk vaccination control (the group III) levels. Following the stimulation of the spleen cells with UV-inactivated virus, significant lymphocyte proliferation was observed in 2 mice from the group I (the mouse no. 3 and 5) and 4 mice from the group II (the mouse no. 7, 8, 9 and 10) compared with results for the mouse no. 11–15 each in the IBRV(NG)dltk vaccinated control group (the group III) ($p < 0.05$ by student *t* test). In the mouse no. 1 of the group I, mild lymphocyte proliferation was found ($p = 0.08$). No statistical difference was detectable in the mouse no. 2, 4 (the group I) and no. 6 (the group II), although the morphological blastation of splenocytes from all 10 mice inoculated with BHV-1/TF17-1 were observed after *in vitro* stimulation with PrV antigen. The variable response observed in the BHV-1/TF17-1-vaccinated groups may be due to the fact that the ddY mouse is a closed line.

In vitro susceptibility to PrV-neutralizing antibody: Susceptibility of BHV-1/TF17-1 to the neutralizing antibody against PrV was compared with that of attenuated live PrV vaccine strains. BHV-1/TF17-1 or various PrV lines were incubated with serially diluted anti-PrV swine serum, PS3, and the reciprocal of the dilution, at which the plaque numbers of the virus reduced by 50%, was used as an index for susceptibility to anti-PrV antibodies. Results are shown in Table 3. The indices of PrV vaccine strains were similar to each other (1,600–3,200) except for that of the Omnimark strain which was very high (25,600–51,200). The index of BHV-1/TF17-1 (800–1,600) was lower than that against PrV vaccine strains, although it was significantly higher than that of IBRV (Table 3, exp. 4 and 5). The results suggest that BHV-1/TF17-1 is neutralized by anti-PrV antibodies but it is much less susceptible to anti-PrV antibodies than PrV vaccine strains.

DISCUSSION

For the purpose of providing an alternative vaccine against Aujeszky's disease to supplement or replace the currently

Table 1. Protection of mice against challenge infection of virulent PRV after immunization with BHV-1 recombinants^{a)}

Group	Virus	Titer (TCID ₅₀ /0.5 ml)	Mice protected/total	Survival time ^{b)} of unprotected mice (day)
1	BHV-1/TF17-1	$10^{5.45}$	10/10 ^{c)}	
2		$10^{4.45}$	6/10	4.3
3		$10^{3.45}$	7/10	4.3
4	IBRV(NG)dltk	$10^{5.45}$	1/10	3.0
5	mock infected	—	0/10	3.2

a) Mice were immunized with 0.5 ml of virus suspension containing indicating dose of BHV-1 recombinants and challenged i.p. with 20 LD₅₀ PRV (YS 81) 3 weeks later. Ten mice per group.

b) Average survival time of the mice died after the challenge infection.

c) Expressed as the number of mice surviving challenged per total number of mice in group.

Table 2. Virus neutralization titer of immunized mice sera before and after the challenge infection

Group	Virus (titer)	Virus neutralization titer ^{a)}	
		Before challenge ^{b)}	After challenge ^{c)}
1	BHV-1/TF17-1 ($1 \times 10^{5.45}$ TCID ₅₀)	50	100
		50	100
		50	400
		800	>1600
		>1600	>1600
			>1600
			400
			800
			800
			400
2	BHV-1/TF17-1 ($1 \times 10^{4.45}$ TCID ₅₀)	200	100
		200	200
		200	800
		200	400
		50	
3	BHV-1/TF17-1 ($1 \times 10^{3.45}$ TCID ₅₀)	200	>1600
		100	
		100	
		<50	
		50	

a) VN titers are expressed as the reciprocal geometric means of the dilution that produces a 50% reduction in plaque numbers.

b) A group of 15 mice (4-week-old ddY) were intraperitoneally (i.p.) immunized with 0.6 ml of BHV-1/TF17-1 virus. Three weeks later sera were collected from 5 out of 15 mice per group.

c) Three weeks after the immunization, 10 mice per group were i.p. challenged with PrV (YS-81) as described in Table 2. Two weeks later sera were collected from survivors. All 10 survivors in group 1, 4 out of 6 survivors in group 2 and 1 out of 7 survivors in group 3 were tested.

Fig. 4. DTH responses in mice vaccinated with BHV-1/TF17-1. The footpad thickness at 48 hr after injection with antigen (UV-inactivated PrV) was measured in mice vaccinated with BHV-1/TF17-1. Each bar represents the mean $\pm$ S.D. of 5 mice in the group. Asterisk (**) denote statistically significant differences from the value of IBRV(NG)dltk control (group III) ($p < 0.01$ by the student *t* test).

a BHV-1 based recombinant, BHV-1/TF17-1, in which the PrV genes of gB, gC, gD, gE and gI were integrated. We here demonstrated that vaccination of mice with BHV-1/TF17-1 could provide perfect protection (10 survivors per 10 mice)

against lethal i.p. challenge of virulent PrV. We observed BHV-1/TF17-1 induced high virus neutralizing antibody titers against PrV and strong cell mediated immune responses against PrV antigens in both DTH assay (Fig. 4) and lympho-

Fig. 5. Lymphocyte proliferation assay in mice vaccinated with BHV-1/TF17-1. Spleen cells were prepared from vaccinated mice, cultured with UV-inactivated PrV purified virions, and labeled with [3 H]-thymidine as described in Materials and Methods. Each bar represents the mean $\pm$ S.D. for three replicates. Solid bar, group I; hatched bar, group II; open bar, group III. Asterisk (*) denote statistically significant differences from the control (group III) ($p < 0.05$ by the student t test).

cyte proliferation assay (Fig. 5). The perfect protective immunity shown in challenge experiment of BHV-1/TF17-1 may be correlated with the strong induction of cellular immunity.

The susceptibility of BHV-1/TF17-1 to anti-PrV antiserum is lower than that of PrV vaccine strains (Table 3). BHV-1/TF17-1 expresses authentic gB, gD, gE and gI and PrV counterparts on the virion envelope. Since PrV glycoproteins are foreign to BHV-1, it is speculated that although PrV glycoproteins are incorporated in the virion envelope, the amounts might be much less than authentic glycoproteins or that the structure of incorporated PrV glycoproteins is such that antibodies have reduced accessibility. Which ever the reason, it would appear that the recombinant BHV-1 based vaccine is able to circumvent the interference by maternally derived antibodies (MDA) better than the attenuated PrV based vaccines do.

BHV-1/TF17-1 contains more than 11 kbp of PrV DNA which might interfere with the growth of this recombinant virus. If the titer of BHV-1/TF7-1 is too low, it is unsuitable

as a practical vaccine. The one step and multi step growth curves indicate that BHV-1/TF17-1 was able to grow in MDBK and CPK cells to reach relatively high titers and (data not shown). BHV-1 infects not only cattle but also pigs [5, 24], goats [23], minks, ferrets [28] and rabbits [14]. Mice are thought to be refractory to BHV-1 infection [7] but had been used extensively as a model system for PrV vaccine [18, 30] and for testing the efficacy of PrV vaccines. Mice are not the best animals to test the efficacy of BHV-1 vector vaccines, generally. This is apparently supported by the data that BHV-1/TF17-1 and IBRV(NG)dltk were not able to grow in A31 cells, derived from BALB/c mouse. However, our preliminary observations suggested that BHV-1/17-1 attached to and penetrated into A31 cells more efficiently than IBRV(NG)dltk did and this enhanced attachment and penetration were the results of the expression of PrV gC and gB in the recombinant BHV-1 (manuscript in preparation). The fact that live BHV-1/TF17-1 induced perfect protection, significant high VN titers, strong cell mediated immune responses (DTH and Lym-

Table 3. VN titers and *in vitro* susceptibility of BHV-1 recombinants and PrV commercial vaccines to PrV-neutralizing antibody^{a)}

Virus	Strains ^{b)}	VN titer ^{c)}				
		exp. 1	exp. 2	exp. 3	exp. 4	exp. 5
PrV	Indiana S	>1600	2560	3200	3200	ND
	Omnimark	ND	ND	<25600	>25600	51200
	Omnivac	ND	ND	1600	ND	1600
	Dupher	ND	ND	3200	ND	ND
	Intervet	ND	ND	3200	ND	ND
	Bucharest	ND	ND	3200	3200	3200
BHV-1	IBRV(NG)dtk	ND	ND	ND	<160	<50
	TF17-1	800	1280	1600	ND	1600

a) PrV-neutralizing antibody was derived from an Aujeszky's disease positive sow.

b) PrV strains used in this study are commercial vaccines except strain Indiana S.

c) Experiment 1 to 5 were performed under same conditions. VN titers are expressed as the reciprocal geometric means of the dilution that produces a 50% reduction in plaque numbers.

d) ND, not done.

phocyte proliferation) suggests that BHV-1/TF17-1 may grow in mice and that mouse model can be used to evaluate the efficacy of BHV-1 based-live vaccines. This also indicates that BHV-1/TF17-1 may confer stronger immunity against PrV in animals such as pigs, sheep and cattle that are more susceptible to BHV-1 infection since excellent efficacy of live a vaccine is generally dependent on its growth *in vivo*.

In swine herds heavily contaminated with PrV, newly born piglets contain high titer of MDA against PrV. Attenuated live PrV vaccines currently in use in pigs are not effective to induce protective immunity in the presence of a high level of MDA. To eradicate PrV from infected herds, it is very important to protect newborn piglets with an effective vaccine, which induces active immunity in these environments. Data in this study supports that the BHV-1 recombinant expressing multiple PrV glycoproteins may be practical as an alternative swine vaccine for AD or as a secondary vaccine to boost the protective effect of the PrV based vaccines. In order to further evaluation of this BHV-1 based vector as a practical vaccine against PrV, tests in swine will be needed.

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