Suppression of influenza virus infection by the orf virus isolated in Taiwan

Fong-Yuan LIN, Yeu-Yang TSENG, Kun-Wei CHAN, Shu-Ting KUO, Cheng-Hsiung YANG, Chi-Young WANG, Masaki TAKASU, Wei-Li HSI and Min-Liang WONG

1) Department of Veterinary Medicine, College of Veterinary Medicine, National Chung Hsing University, Taichung 402, Taiwan
2) Graduate Institute of Microbiology and Public Health, College of Veterinary Medicine, National Chung Hsing University, Taichung 402, Taiwan
3) Department of Beauty Science, MeiHo University, Neipu, Pingtung County, Taiwan
4) Department of Veterinary Medicine, National Chiayi University, Chiayi, Taiwan
5) Animal Health Research Institute, Council of Agriculture, Tamsui, Taiwan
6) Formerly at the Livestock Disease Control Center of Taichung County, Taichung, Taiwan
7) Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University, Gifu 501–1193, Japan

ABSTRACT. Orf virus (ORFV), a member of parapoxvirus, is an enveloped virus with genome of double-stranded DNA. ORFV causes contagious pustular dermatitis or contagious ecthyma in sheep and goats worldwide. In general, detection of viral DNA and observing ORFV virion in tissues of afflicted animals are two methods commonly used for diagnosis of orf infection; however, isolation of the ORFV in cell culture using virus-containing tissue as inoculum is known to be difficult. In this work, the ORFV (Hoping strain) isolated in central Taiwan was successfully grown in cell culture. We further examined the biochemical characteristic of our isolate, including viral genotyping, viral mRNA and protein expression. By electron microscopy, one unique form of viral particle from ORFV infected cellular lysate was demonstrated in the negative-stained field. Moreover, immunomodulating and anti-influenza virus properties of this ORFV were investigated. ORFV stimulated human monocytes (THP-1) secreting proinflammatory cytokines IL-8 and TNF-α. And, pre-treatment of ORFV-infected cell medium prevents A549 cells from subsequent type A influenza virus (IAV) infection. Similarly, mice infected with ORFV via both intramuscular and subcutaneous routes at two days prior to IAV infection significantly decreased the replication of IAV. In summary, the results of a current study indicated our Hoping strain harbors the immune modulator property; with such a bio-adjuvanticity, we further proved that pre-exposure of ORFV protects animals from subsequent IAV infection.

KEY WORDS: cell culture, immunomodulating, influenza virus, orf virus, plaque assay

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Orf virus (ORFV), a member of genus parapoxvirus, has a double-stranded DNA genome of approximate 138 kilo-base pairs [10]. The ovoid-shaped viral particles with a crisscross textures have an average size of 260 nm × 160 nm [6]. It can cause contagious pustular dermatitis or contagious ecthyma in sheep, goats, cattle and other ruminants, manifesting extensive vesicles, papules and scabs and distributing at lips, gums, muzzles and udders [4, 29, 33]. Moreover, lesions around mouths and lips of kids and lambs make them reluctant to suck, and their mortality can be as high as 93% under secondary complications [12]. In severe outbreaks, nearly 100% morbidity can be reached, but the mortality for adult animals was often less than 20%. Veterinarians, farmers and inspectors may get this disease through small wounds in the gums, muzzles or udders [32]. The strains of viruses and types of cells determined the outcome of an adaption. For example, Nara strain and OKA strain were adapted in testes and kidney cells as well as lung tissue of fetal lambs and cell lines, CSL503 and ovine testis (OT), respectively [33]. Results from capripoxvirus showed that despite tedious procedures for preparations, primary cells were more susceptible to the infection of field isolates than cell lines and the titer of progeny virus from primary cells was consistently higher than that from the ovine testis cell line [2]. However, this was not likely to be the case for those laboratory strains, which had been adapted to cell lines and exhibited stable titers in serial passages. Therefore, the choice of primary cells or cell lines for the culture of ORFV mostly depended on the research purposes.
Since 2006, several outbreaks of orf were found in the central Taiwan, and studies focused on viral DNA sequencing and phylogenetic analysis were reported. [5, 6]. Although the morbidity of these cases were only approximately 2–6% and mild lesions with the mortality of less than 0.8% were found in lambs, more and more cases indicated Taiwan had become an endemic area of orf. Differential diagnostic techniques based on the heterogenic variations in the C-terminals of ATPase genes have been developed for those strains in Taiwan [5]. The initial goal of the present study is to grow ORFV found in Taiwan in cell cultures. One interesting aspect of ORFV biology is the ability to modulate immune response [11]. Hence, the cytokine stimulating activity of our isolate and its effect on following influenza virus replication in cultured cells and animal model were also studied.

MATERIALS AND METHODS

Preparation of primary goat testis cells: One two-week old lamb (Nubian breed) was sacrificed, and tissue including his testis and ears was removed. The use and experimental protocols were approved by the Committee on the Ethics of Animal Experiments of National Chung Hsing University (approval numbers: 101-40). The testis cells derived from removed testis and fibroblasts from ears were cultured in 1× RPMI 1640 medium with 10% fetal bovine serum (FBS). When cells grew to full confluency, cells were trypsinized and subcultured in 1:1 or 1:2 ratios.

Virus inoculation and adaptation to cell culture: Samples were collected from ORFV affected animals in central Taiwan (Hoping) in 2009. Half gram of collected scab from mouth of infected goats was soaked and homogenized in 5 ml TE buffer (50 mM Tris-HCl and 10 mM EDTA, pH 8.0) with additional antibiotic. After centrifugation at 3,500 rpm for 10 min, the supernatant was transferred, filtrated through a 0.45 µm filter and stored as a crude viral stock. The homogenate was subsequently inoculated into the primary goat testis cells. During viral absorption, the cells were gently shaken forward and backward every 15 min. After 1 hr, the unattached virus was removed by washing the cells twice with pre-warmed 1 x PBS, and the cells were maintained in complete medium 1× RPMI 1640 medium with 10% fetal bovine serum (FBS).

Restriction enzymes digestion: The primary goat testis cells were seeded on the 10-cm cell culture dish (approximate 3.5 × 10⁶ cells) and were infected with 0.1 MOI (multiplicity of infection) of ORFV. The infected cells were trypsinized and collected when 80% infected cells showing cytopathy (~24 hr). Cells were harvested by brief centrifugation and washed with 1 x PBS twice. The total DNA was obtained (−24 hr). Cells were harvested by brief centrifugation and washed with 1 x PBS twice. The total DNA was obtained by following a method previously described [20]. For each reaction, 5 µg of extracted DNA was digested with 20 units of a restriction enzyme at 37°C for 2 hr. The mixture was analyzed by electrophoresis at 70 volts in a 0.7% agarose gel.

Examination of viral gene expression by RT-PCR: The primary goat testis cells (3.5 × 10⁵) were infected with 10 MOI of ORFV and incubated at 37°C with 5% CO₂. Total

PCR using 2 sets of B2L gene specific primers: outer primer set OVB2LF1 and OVB2LR1 [17], and inner primer set OVB2LF2 and OVB2LR2 (sequences are listed in Table 1) for detecting existing viral DNA. The expected size of PCR products obtained from the first and second round amplification is 1.2 kbp (OVB2LF1 and OVB2LR1; full length B2L gene) and 889 bp (OVB2LF2 and OVB2LR2), respectively. In the first round of nested PCR, the PCR condition started with a denaturation step at 94°C for 4 min, followed by 29 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (annealing) and 72°C for 1 min (extension), and the reaction was ended by a final extension at 72°C for 7 min. One microliter of the first round PCR product was used for the next round of PCR which was followed the same conditions, except with 25 amplification cycles. PCR products were analyzed by 1% agarose gel electrophoresis with Health safe nucleic acid stain. For detection of viral gene during the plaque purification, the PCR was conducted using the inner set of B2L-specific primers (OVB2LF2 and OVB2LR2) following the same PCR condition with a 35 amplification cycles.

Table 1. List of the primers

| Primer | Sequence |
|--------|----------|
| OVB2LF1 | 5′−TCCCTGAAGCCGATTATTATTTGTG−3′ |
| OVB2LR1 | 5′−GCTTGGCGGCGGCTCGGACCTTC−3′ |
| OVB2LF2 | 5′−GCAGCCTTCGCTGCAAATCTGAG−3′ |
| OVB2LR2 | 5′−AAGGCGGGCGGCTCGGATGT−3′ |
| FP     | 5′−GTGGTGATCACTGGAGAAGTCCGGTG−3′ |
| RP1    | 5′−GTGCCCTTGTGCCCTTTATGCTC−3′ |
| RP2    | 5′−CGCGCGTCAGAGTCGAGTCGCCCGC−3′ |
cellular RNA was extracted from the infected cells following the instruction of the RNeasy Mini Kit (Qiagen, Limburg, Netherlands). After RNA quantification, one microgram of RNA was treated with RNase-Free DNase (Promega, Madison, WI, U.S.A.) to eliminate DNA contamination. Reverse transcription was conducted with 0.5 μg of RNA, and the cDNA was synthesized by SuperScript III reverse transcriptase (Invitrogen, Waltham, MA, U.S.A.). Subsequent PCR was performed with OVB2LF2 and OVB2LR2 primers. The condition of PCR was a first denaturation at 94°C for 4 min, following with 35 cycles of brief denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec and extension at 72°C for 45 sec, and a final extension at 72°C for 7 min.

**Western blot analysis:** The primary goat testis cells were infected with 1 plaque forming unit (PFU) of ORFV and maintained with 1 × RPMI 1640 medium containing 2% FBS at 37°C with 5% CO2. The cellular lysate was prepared by rupturing cells with sample buffer after twice washing with PBS. Following boiling at 100°C for 6 min, proteins were separated by the SDS-15% PAGE and transferred onto a PVDF membrane (Amersham, GE Healthcare, Buckinghamshire, U.K.). After blocking with TBST (20 mM Tris, 150 mM NaCl and 0.1% Tween 20, pH 7.6) containing 5% skim milk, the membrane was incubated with 1:2,000 diluted mouse polyclonal anti-OV20.0 antibody generated from mice immunized with purified OV20.0 recombinant protein at 4°C overnight. Then, the membrane was washed 3 times with TBST. The secondary antibody, the horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, Suffolk, U.K.), was added and incubated for 1 hr at room temperature. The signal was then developed using an enzyme-linked chemiluminescence system (ECL, Amersham, GE Healthcare).

**Electron microscopy:** Electron microscopy was used to examine the morphology of isolated virus particles. The plaque purified virus was inoculated into the goat primary testis cells. The cells were maintained at 37°C with 5% CO2 and observed the CPE formation. When 80% of the infected cells show severe CPE, the cells and medium were collected by scraping off the attached cells with tip. After brief sonication on ice, the cell debris was removed by centrifugation. The cell supernatant was transferred to a new tube and processed for negative-strain electron microscopy by staining with 2% phosphotungstic acid (PTA).

**Cytokines expression in ORFV-infected THP-1 cells:** Total 1 × 10⁶ THP-1 cells kindly provided by Professor S.S. Chiou in Graduate Institute of Microbiology and Public Health, National Chung Hsing University were seed in 6 well multiple plates within 1 ml of RPMI 1640 medium containing 10% FBS. Subsequently, 10 MOI of ORFV was inoculated into the cells and incubated with the human monocyte THP-1 cells. Forty-eight hr later, the supernatant was collected by brief centrifugation and transferred to a new tube which was then kept at -80°C. The cytokine expression in the culture medium was examined by using the Human IL-8 and tumor necrosis factor alpha (TNF-α) ELISA kit (KOMA BIOTECH INC., Seoul, Korea) performed following the manufacturer’s procedure. A set of standard cytokines, provided within the kit, with known concentrations were analyzed in parallel for establishment of standard curve. Finally, the OD value of each sample was obtained from reading the plate at 450 nm wavelength. Concentrations of the cytokines were estimated by comparing with the standard curve. The mean value of the results was obtained from three independent experiments and was then assessed by T-test. *P* value (P<0.05) indicated the significant difference between two groups of samples.

**Effect of ORFV on subsequent influenza virus infection in cells:** Goat fibroblast primary cells were infected with ORFV at MOI of 1, and the cell media were collected at 0, 6, 12 and 24 hr post-infection (hpi). A549 cells were pre-treated with the ORFV infected cell medium for 24 hr. After removal of the cell medium and PBS washing, the A549 cells were infected with 1 MOI of influenza virus (PR8 strain) [7]. Cellular lysate of infected human lung carcinoma A549 cells obtained from ATCC (CCL-185) was harvested at 12 hpi and resolved by SDS-PAGE electrophoresis followed by Western blot analysis using IgY antibody against influenza virus NP generated from chickens immunized with purified NP recombinant protein. The overall procedures of Western blot analysis followed the description in the previous section. Densitometric quantifications of each band indicating NP expression level were carried out using National Institutes of Health IMAGEJ software version 1.43 (http://rsb.info.nih.gov/ij).

**Effect of ORFV on subsequent influenza virus infection in mice:** The inhibitory activity on influenza virus infection by ORFV was further tested in mice. Six weeks old, female BALB/C mice received UV-inactivated 2 × 10⁵ plaque formation unit (PFU) of isolated ORFV, Hoping strain (n=4) or PBS (n=4, as negative control) by intramuscular (IM) or subcutaneous (SC) routes. Two days after infection, the mice were intranasally challenged with 1 × 10⁶ influenza virus (PR8 strain). Seven days post infection, blood was taken from each mouse for determining the concentrations of IL-6 and TNF-α in serum by ELISA kits (BioLegend; San Diego, CA, U.S.A.) performed following the manufacturer’s procedure. Subsequently, the mice were scarified, and the lungs of the mice were collected. The whole lung tissue was homogenized in 1 ml of DMEM without FBS. After brief centrifugation, the supernatant was used for measurement of the influenza virus titer on MDCK cells. After two to three days post-infection, the infected MDCK cells were fixed in methanol and stained with crystal violet. The number of influenza viral plaque was calculated. The biostatic analysis was performed by using the *t*-test model.

**RESULTS**

**Viral infection and adaptation to the primary testis cells:** The crude virus stock was serially diluted and inoculated into the primary goat testis cell. Cells were observed daily for appearance of CPE, and the presence of viral DNA was determined by PCR in each run (1 week interval). After 3–5 rounds of blind passages, stronger bands were amplified in the second round of nested PCR, although no apparent CPE was observed. In subsequent cycles of blind passages, ORFV was adapted and enriched in primary goat testis cells; viral
DNA can be detected in the first round of the nested PCR.

**Plaque purification of the ORFV:** With continued viral passages, the infected primary goat testis cells began to show the cytopathic effect (CPE) and form a viral plaque. The CPE in primary goat testis cells was local and limited on the area of affected cells after four to five days after infection (Fig. 1A) that is consistent with one previous study that parapoxviruses form plaques in primary bovine testis cells [22]. After three times of plaque purification, the purity of isolated ORFV was examined by PCR using primers targeting viral B2L gene. The size of PCR product is proximate 900 bp (Fig. 1B), and subsequent DNA automated-sequencing confirmed the nucleotide identity of ORFV B2L gene (data not shown).

**Identification of isolated ORFV:** A single-step PCR developed in our laboratory that shows distinct amplification patterns of three ORFV strains in Taiwan was used for identifying the isolated viruses [5]. The Nantou and Taiping strain could amplify 180 and 217 bp product, respectively, and 2 different length fragments (180 and 254 bp) can be produced at the same time in the primary cells from goats. Results showed our purified ORFV was the Hoping strain (Fig. 1C). The nucleotide sequences of the Hoping strain were further confirmed by automated DNA sequencing (data not shown). Furthermore, the restriction enzyme digestion pattern of viral DNA of the Hoping strain was also confirmed (Fig. 1D). In comparison with the smearing DNA of uninfected sample (lane 5 in Fig. 1D), all the DNA of virus-infected samples treated with restriction enzymes showed characteristic cutting patterns.

**Viral gene expression examined by RT-PCR and immunoblotting:** To examine viral gene expression in the primary goat testis cell, the viral RNA was detected by RT-PCR. Despite the weaker expression, the transcripts of B2L can be detected at the early stage of infection (2-3 hpi), and it was largely synthesized after 12 hpi (Fig. 2A). Moreover, the ORFV gene expression was verified by Western blotting by using the mouse polyclonal anti-OV20.0 antibody. Results of Fig. 2B demonstrated the viral OV20.0 protein (25 kDa) was produced after 6, 12 and 24 hpi. These data indicated that genes of isolated ORFV can be actively expressed in the primary goat testis cells.
Electron micrograph observation: Morphological confirmation of orf viruses in the infected goat testis cells was achieved with electron microscopy. The electron micrograph results demonstrated the presence of ovoid-shape virions with a spiral crisscross pattern (Fig. 2C).

Detection of cytokines produced in THP-1 cells with ORFV: The ORFV infection elicits expression of proinflammatory cytokines, such as several interleukins (ILs) and TNF-α, which contributes to the immune regulation of ORFV and has been demonstrated in many studies [8, 9, 15, 26, 37]. It is important to explore whether ORFV (Hoping strain) harbors the immunostimulating activity. The human monocyte cell line, THP-1 cell, was infected with our local isolate at MOI of 10 for 48 hr. The cell medium was collected for examination of the cytokine IL-8 and TNF-α expressions by using the ELISA kit (KOMABIOTECH INC.) The results demonstrated that IL-8 and TNF-α expression level significantly increased in cells infected with ORFV, compared with that of PBS control. The column of each group was the mean (+/–SD) of three independent experiments. Statistical analysis was performed using unpaired T-test, and P value <0.05 (shown with a star symbol) indicates the statistical significance.

ORFV inhibited influenza virus replication in A549 cells: As an immune modulator, ORFV has been shown to act as an inhibitor to prevent other virus infection and also to work as a tumor killer [3, 16, 19, 31]. The primary goat fibroblast cells were infected with 1 MOI of ORFV. The ORFV infected cell medium, of which the infectivity of ORFV was under detection, was collected at 6, 12 and 24 hpi, and overlaid onto A549 cells. After 24 hr treatment, the A549 cells were then infected with influenza virus PR8 strain for 12 hr. A significant decrease of viral NP protein expression was observed in cells pre-treated with ORFV infected cell medium (Fig. 4). Noticeably, increase of inhibitory strength coincided with the length of ORFV infection; the longer ORFV infection, the stronger inhibitory effect was observed. It indicates ORFV (Hoping strain) can prevent influenza virus infection in A549 cells.

Inhibition of subsequent influenza virus replication in mice inoculated with UV-inactivated ORFV: To further investigate the inhibitory activity on influenza virus infection by UV-inactivated ORFV, 6 weeks old female BALB/C mice were inoculated with either 2 × 10^5 PFU of UV-inactivated ORFV (n=4) or PBS (n=4, as a negative control) by IM and SC routes for 2 days and subsequently infected with influenza virus. As shown in Fig. 5A, in mice groups received the inactivated ORFV via both IM and SC routes, the titer of influenza viruses significantly decreased (Fig. 5A). The cytokine profile was also determined in mice inoculated with...
DISCUSSION

ORFV, an epitheliotropic parapoxvirus, causes proliferative dermatitis in goats and sheep, and persistence infection usually occurs in an outbreak farm. Although molecular identification by PCR and phylogenetic analysis of viral DNA purified from the animal tissues are common, the isolation of ORFV from the field and production of virions in cell culture are still formidable tasks [1]. The isolation of ORFV from the field and production of virions in cell culture are still formidable tasks [1].

Recently, Rohde et al. generated 2 ORFV recombinants expressing the hemagglutinin (HA) or nucleoprotein (NP) of the highly pathogenic avian influenza virus (HPAI, strain H5N1), and via IM route immunization of two doses (10^7

The primary tests of cell medium were infected with ORFV (Hoping strain). At two hr post infection (hpi), the unattached virus was removed by washing with PBS. The cells were maintained in complete RPMI 1640 medium with 10% FBS. At 6, 12 and 24 hpi, the medium was collected and used for treatment of human A549 cells. After 24 hr treatment, the A549 cells were infected with 1 MOI of IAV (PR8 strain). At 12 hpi, expression of viral nucleoprotein (NP) of the IAV was analyzed by immunoblotting (panel A), and the quantitative analysis of NP production was shown in panel B. The column of each group was the mean (+/-SD) of three independent experiments. The results were analyzed by the T-test. The P value <0.05 (shown with a star symbol) indicates the difference between 2 groups is statistically significant.
PFU of each) of HA-expressing ORFV protects mice from lethal HPAIV infection [35]. The present study showed that without expressing any influenza specific antigen, treatment of ORFV significantly inhibited expression of NP protein in cells, and also, one dose (10^5 PFU) of ORFV immunization is able to reduce the influenza viral load in mouse lung. Based on the cytokine expression profile, it appears ORFV infection elevates levels of proinflammatory cytokines (IL-6 or IL-8). However, further studies are required to elucidate the mechanisms of suppressing influenza virus infection. In summary, our findings indicate the immunostimulating effect of ORFV.

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