Vaccine (2008) **26**, 891–898

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/vaccine

Recombinant porcine lactoferrin expressed in the milk of transgenic mice protects neonatal mice from a lethal challenge with enterovirus type 71

Hsiao-Ling Chen[a,1], Li-Chung Wang[b,c,1], Chi-Hsuan Chang[b], Chih-Ching Yen[b,d], Winston T.K. Cheng[e,1], Shinn-Chih Wu[e], Che-Ming Hung[b], Meng-Fu Kuo[b], Chuan-Mu Chen[b,*]

[a] *Department of Molecular Biotechnology, Da-Yeh University, Changhwa 51501, Taiwan*
[b] *Department of Life Sciences, National Chung Hsing University, No. 250, Kuo Kuang Road, Taichung 40227, Taiwan*
[c] *Taichung Veterans General Hospital, Taichung 40705, Taiwan*
[d] *China Medical University Hospital, Taichung 404, Taiwan*
[e] *Department of Animal Science and Technology, National Taiwan University, Taipei 106, Taiwan*

Received 18 August 2007; received in revised form 28 November 2007; accepted 13 December 2007
Available online 3 January 2008

KEYWORDS
Porcine lactoferrin;
Antiviral activity;
Transgenic mice;
Enterovirus 71;
Neonatal viral infection

Summary The human *Enterovirus* genus of the piconavirus family causes most of the febrile illnesses that affect children during the summer season in Taiwan. Enterovirus type 71 (EV71) plays a key role in patients with hand-foot-and-mouth disease (HFMD) combined with severe paralysis or encephalitis. It is important to find a method for preventing infection with EV71 since there is no antiviral agent or vaccine for humans. In this study, we developed a transgenic mouse model for demonstrating the protective effects of recombinant lactoferrin (LF) against EV71 infection. Transgenic mice carrying alpha-lactalbumin-porcine lactoferrin (αLA-pLF) and BALB/c wild-type mice were subjected to EV71 inoculation. First, we analyzed the expression efficiencies of recombinant pLF (rpLF) in hemizygous and homozygous transgenic mice. Following EV71 inoculation on the 4th day of life, pups ingesting transgenic milk showed the significantly higher survival rate and heavier body weight compared to wild-type mice. RT-PCR analysis for EV71 viral RNA showed that the recombinant pLF had a blocking effect on EV71 infection. Our data suggest that oral intake of pLF-enriched milk exhibited the ability to prevent infection with EV71. The study also provides an animal model for validating the protective effects of pLF.

* Corresponding author. Tel.: +886 4 22856309; fax: +886 4 22851797.
E-mail address: chchen1@dragon.nchu.edu.tw (C.-M. Chen).
[1] These authors contributed equally.

0264-410X/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2007.12.013

Introduction

Enterovirus type 71 (EV71) is a non-enveloped single-stranded RNA virus which belongs to the *Picornaviridae* family. It was first described in 1974 in cases of central nervous disease in California [1] and over the last three decades has caused 13 small and large outbreaks throughout the world, causing an array of clinical complications [2]. The reported conspicuous manifestations caused by EV71 include hand-foot-and-mouth disease (HFMD) [3], polio-like paralysis [4,5], herpangina, aseptic meningitis, and fatal encephalitis [6,7]. Now, EV71 has become internationally infamous owing to the outbreaks in the Asia-Pacific area. In the Taiwanese epidemic of 1998, more than 129,000 cases of HFMD or herpangina were reported, and there were more than 400 patients, mostly less than 5 years old, hospitalized with severe complications. Among these patients, 78 children unfortunately died [7]. Due to the great panic caused by this catastrophe, a large number of investigations were performed to understand this virus and ways to prevent infection. However, almost 9 years after the pandemic, there is still no available antiviral agent or vaccine used for humans to prevent infection with this virus.

Lactoferrin (LF) is an 80-kDa multifunctional, monomeric glycoprotein present in external secretions of mammals, such as breast milk, tears, saliva and mucous secretions [8]. Because the protein is mostly found in external secretions and mucosa, it is believed that LF plays an important role in the non-specific immune response. It has been demonstrated that LF can display several protective functions, including antibacterial, antifungal [9], and immunomodulatory [10] activities. Furthermore, lactoferrin also displays potent antiviral activity, mostly in *in vitro* experiments. For example, a large variety of viruses, including herpes simplex virus (HSV) [11], cytomegalovirus (CMV), human immunodeficiency virus (HIV), hepatitis B and C viruses, poliovirus, respiratory syncytial virus (RSV) [12] and EV71 [13,14], were reported to be prevented from entering into host cells by LF in the early phase of infection. It has been demonstrated that bovine lactoferrin (bLF) has a higher efficiency for preventing EV71 infection *in vitro* than human lactoferrin (hLF), and bLF can protect mice from EV71-induced lethality in animal tests [13,14].

Porcine lactoferrin (pLF), which had been demonstrated to exist at a higher concentration in colostrum than other mammalian lactoferrins [15,16], is suspected to be an important factor for protecting piglets from several lethal infections [17—19]. Therefore, detecting the biological functions and the antimicrobial efficiency of pLF is a high priority, especially because we have knowledge to build on obtained from studies related to human and bovine LF from the past decade [20]. Furthermore, it has been demonstrated that synthetic porcine lactoferricin, a peptide derived from gastric pepsin cleavage of LF, exhibits a higher antimicrobial activity than human lactoferricin [21]. We therefore hypothesize that pLF exhibits effective antiviral activity. To test this idea, we produced a transgenic mouse model for detecting the antiviral activity of pLF [22].

In our previous study, the pLF gene was constructed under the control of the bovine α-lactalbumin (αLA) promoter to express the recombinant transgene, specifically in the mammary gland of lactating αLA-pLF transgenic mice [22]. It had been demonstrated that the transgene can be successfully transmitted to descendants and that transgenic mice can express a high level of pLF — up to 120 mg/l — in the milk. In this study, we detected the genotypes of pLF transgenic mice by slot-blot and compared the anti-EV71 activities of the pLF-enriched milk of hemizygote and homozygote transgenic mice in an animal model for the protection of neonatal mice from a lethal challenge with EV71 virus.

Materials and methods

Virus preparation and titer detection

The virus used in this study was isolated from the throat swab of a 26-month-old female infant who was infected with EV71 and unfortunately died in July 2000. The swab was decontaminated by 500 μg/ml penicillin, 500 μg/ml streptomycin sulfate and 10 μg/ml amphotericin B. The virus was then inoculated into Vero cells (ATCC CCL-81, USA) which were incubated in minimum essential medium (MEM; Gibco BRL Life Tech., Grand Island, NY) containing 2% fetal bovine serum (FBS). After the appearance of an 80% cytopathic effect (CPE), the cultured cells were frozen and defrosted twice; cell debris was removed by 3000 $\times$ *g* centrifugation for 5 min. The supernatants containing virus particles were then stored at −80 °C before use. The virus titer was determined by plaque assay as described previously [23]. Briefly, Vero cells at the concentration of 1×10^5 cell/ml were added into each well of a 24-well culture dish and incubated at 37 °C for about 2—3 days to produce a confluent monolayer. These cells were then infected by the serially diluted supernatants which contained EV71 via a 1-h incubation, and were then overlaid with Sea Plaque (BMA, Rockland, ME) in MEM plus 2% FBS before the plaque numbers were counted by staining with 0.5% crystal violet.

Genotype determination of pLF transgenic mice

To generate transgenic mice with the pLF transgene in their genomes capable of expressing the pLF protein in their mammary gland, a DNA construct which carried a 2.0-kb regulatory sequence of the bovine α-lactalbumin (αLA) gene and a 2.0-kb pLF cDNA sequence (Fig. 1A) was first engineered in the cloning vector pCR3. For pronuclear embryo microinjection, the mammary gland-specific expression cassette of the αLA-pLF-bGH-poly(A) transgene was excised from the vector by double digestion with AvrII and XhoI. The purified transgene was microinjected into the male pronuclei of fertilized eggs from superovulated female mice and transferred to recipient pseudo-pregnant females as previously described [22]. To detect the αLA-pLF transgene in the transgenic mice with hemizygote ($pLF^{+/-}$) or homozygote ($pLF^{+/+}$) genotype, slot blot was performed as previously described [24]. Ten micrograms of genomic DNA from pLF transgenic mice was first denatured by 3 M NaOH at 68 °C for 30 min; and then neutralized by 2 M NH_4OAc. The single-stranded denatured DNA was added into a slot-blot Minifold II (Schleichef & Schuell, Germany) containing a nitrocellulose membrane. The DNA was then attached to the membrane by a vacuum system. The membrane was put into a UV crosslinker (Strata-

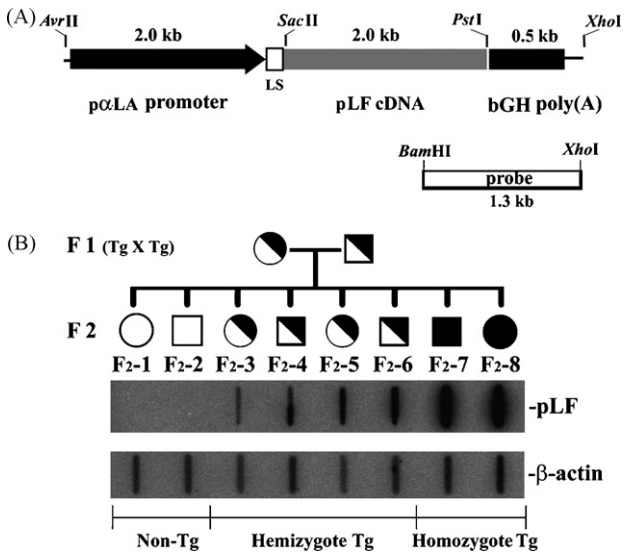

Figure 1 Schematic map of the constructed αLA-pLF transgene and detection of transgene copies in transgenic mice. (A) The structure of the αLA-pLF-bGH-poly(A) fusion gene is shown; the BamHI/XhoI double digested fragment was used as a probe for slot blot. (B) Slot-blot hybridization analysis of the transgene copies in the F2 generation of 8 different transgenic mice. The blots were re-hybridized with a β-actin probe that was used as an internal control.

gene, USA) to immobilize the DNA with 120 mJ of UV light. For the subsequent hybridization reaction, the full length αLA-pLF transgene was double digested by BamHI and XhoI, and the 1.3-kb fragment was used as a radioactive probe. Blots were subjected to autoradiography for 3 days. In order to confirm the results of the slot blot, the suspected homozygous mice were subjected to offspring test-cross.

Detection of pLF recombinant protein expression by immunohistochemical (IHC) staining and enzyme-linked immunosorbent assay (ELISA)

To detect the expression of recombinant pLF (rpLF) protein in the mammary glands of the transgenic mice, lactating female pLF transgenic mice were sacrificed on the 14th day of the lactation period and the mammary glands were gathered and cut into slices for IHC analysis. Rabbit anti-pLF polyclonal primary antibody (LTK BioLaboratories Inc., Taiwan) at a 1/500 dilution was dropped on the slides for identifying the pLF protein. A HRP-conjugated goat anti-rabbit IgG antibody was used as the secondary antibody. After staining with 3,3′ diaminobenzidine (DAB) dye and washing with PBS, the slides were then observed under a light microscope (Axiovert 135, Carl Zeiss, Germany). Quantitative ELISA was performed using a rabbit anti-pLF antibody and HRP-conjugated goat anti-rabbit IgG antibody. The pLF protein was detected using *o*-phenylenediamine (OPD; Sigma, St. Louis, MO) dissolved in citrate buffer (pH 5.0) in the presence of 0.01% H_2O_2. Results were read using an ELISA microplate reader (Bio-Rad Lab., USA) at 490 nm.

Animal model for antiviral test

BALB/c wild-type mice from the National Laboratory Animal Center, Taipei, Taiwan and αLA-pLF transgenic mice were raised in constant temperate (25 °C) and humidity (50%) with a 12-h light period and a 12-h dark period. Animals were housed and handled according to guidelines of the Animal Care Committee of the National Chung Hsing University. In order to develop an EV71-infection model for pre-weanling mouse pups, we randomly separated 2-, 4- and 7-day-old BALB/c wild-type pups into two groups. Six to ten pups of each group were intraperitoneally inoculated with either 100 μl EV71 solution containing 1.1×10^5 pfu/ml or 100 μl Vero cell lysate without EV71 virus as a negative control. These mice were then observed until 3 weeks of age; body weights were monitored daily. After establishment of the animal model, we chose a moderate condition for the experiment performed on αLA-pLF transgenic mice. The BALB/c pups were fed by three different kinds of female milk, including milk from either BALB/c wild-type mice, hemizygous αLA-pLF transgenic mice or homozygous αLA-pLF transgenic mice. The pups were then intraperitoneally injected with 100 μl EV71 solution (1.1×10^5 pfu/ml) on the 4th day after birth. Observation of the mice and detection of body weights continued for 3 weeks.

Virus re-culture and fluorescence in situ hybridization (FISH)

Three weeks after EV71 infection, animals were sacrificed and 8 different tissues, including liver, kidney, stomach, heart, lung, intestine, cerebrum and cerebellum, were taken to detect the existence of EV71. These tissues were homogenized and then decontaminated with a high concentration of antibiotics as described above. The samples were then centrifuged under $3000 \times g$ for 10 min and the supernatant was inoculated into the culture medium of Vero cells. These cultured cells were observed for the appearance of cytopathology. In the mean time, the existence of EV71 was detected by immuno-fluorescence staining with mouse anti-EV71 VP1 monoclonal antibody (Chemicon International Inc., Temecula, CA) and goat anti-mouse IgG conjugated FITC antibody (Chemicon). The image was observed under the laser scanning confocal microscope (LSM 510, Carl Zeiss Inc., Germany).

Reverse-transcription polymerase chain reaction (RT-PCR) for tissue viral detection

In order to further confirm the RNA genome amplification of infected EV71, we chose the same tissues as above for RT-PCR analysis with the primers for EV71 cDNA. First, tissues were homogenized and the total RNA was extracted using TriReagent (Molecular Research Center, Inc., USA). RNA pellets were resuspended in DEPC-water and then treated with 10 units of DNase I (Promega, USA) to remove DNA contamination, after which cDNA was synthesized with an oligo-dT primer and the SuperScript reverse transcriptase (Gibco BRL Life Tech., USA). The primers used for EV71 analysis were 5′-ACC ATG AAA CTG TGC AAG G-3′ and 5′-CCG GTA GGG GTG CAT GCA AC-3′.

Statistical analysis

Experimental values are expressed as the mean ± standard deviation (S.D.). All data were analyzed in the randomized complete block design using SAS General Linear Model procedures (SAS Institute Inc., Cary, NC). The difference between two means is presented as $P < 0.05$ (*) and $P < 0.01$ (**).

Results

Genotyping of hemizygote and homozygote pLF transgenic mice

A series of transgenic mice harboring a pLF gene driven by the mammary gland-specific promoter of the bovine αLA gene (Fig. 1A) had been generated in our previous report [22]. The αLA-pLF hybrid gene was confirmed to have been successfully integrated in transgenic mice genomes and transmitted stably through the germ-line to their offspring. For identifying hemizygote ($pLF^{+/-}$) and homozygote ($pLF^{+/+}$) transgenic lines, we first detected the genotypes of αLA-pLF transgenic mice by slot blots and offspring test-crosses. The probe used in slot blot analysis is shown in Fig. 1A as the product of BamHI and XhoI double digestion of the αLA-pLF transgene. Results showed that two transgenic mice (αLA-pLF-F2-7 and -F2-8) contained higher copies of the transgene than others (αLA-pLF-F2-3, -F2-4, -F2-5 and -F2-6) after the input DNA was normalized to the β-actin hybridization signal (Fig. 1B). After offspring test-crosses, we ascertained that these two transgenic mice were homozygous carriers of the transgene whereas the other four were hemizygous carriers.

Expression of recombinant pLF protein in mammary glands

After identification of hemizygote and homozygote transgenic lines, we further confirmed the expression levels of the recombinant pLF protein driven by the bovine αLA regulating sequence and leader peptide during the lactation period in these two transgenic lines. The mammary glands of the lactating hemizygous, homozygous, and wild-type mice were freshly dissected and the tissue sections were hybridized with a rabbit anti-pLF polyclonal antibody and a HRP-conjugated goat anti-rabbit IgG antibody for IHC analysis. Representative results are shown in Fig. 2; the recombinant pLF protein was expressed only within the lumen and around the epithelial cells of lactiferous tubules in the mammary gland of the transgenic mice. As shown in Fig. 2B, recom-

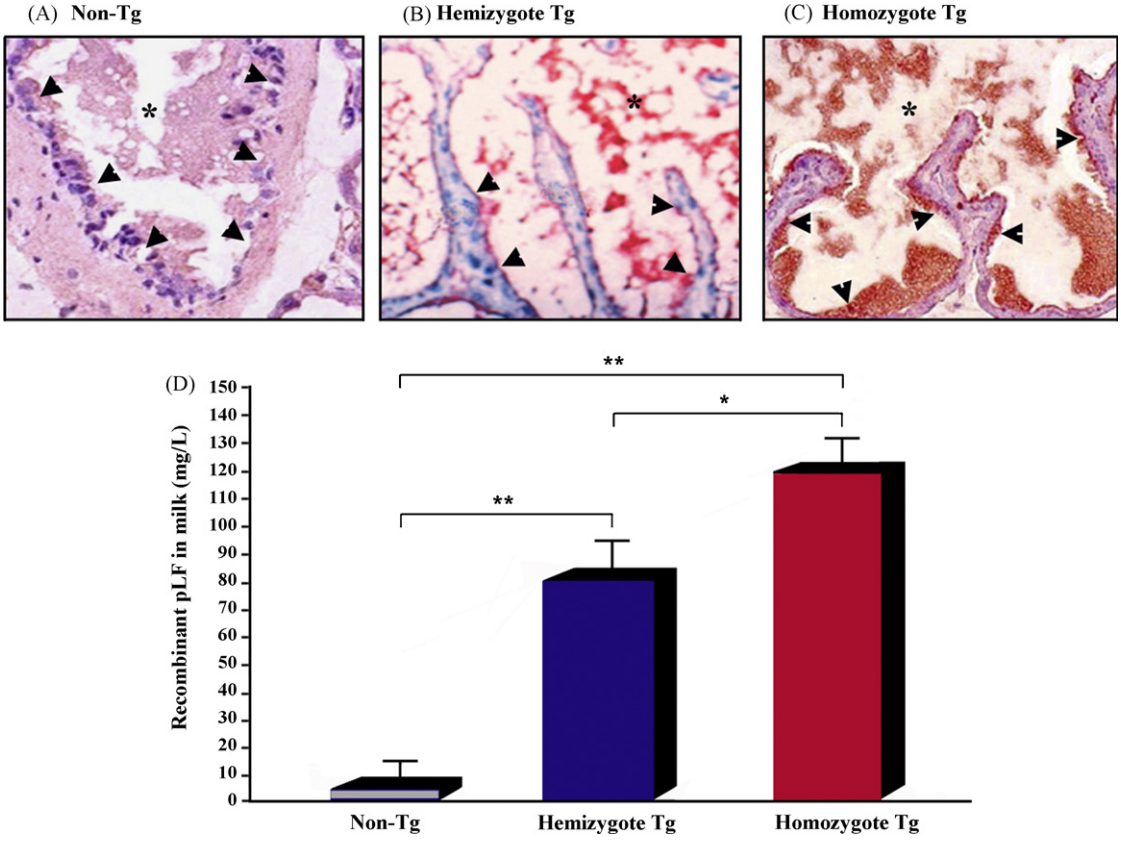

Figure 2 Immunohistochemical staining of mammary gland tissues from (A) lactating non-transgenic, (B) hemizygote transgenic and (C) homozygote transgenic mice for detecting the expression of recombinant pLF. The symbol ''*'' indicates the lumen of lactiferous tubules and the arrows represent the epithelial cells of lactiferous tubules. Samples were viewed using an Olympus IX71 microscope and DP71 image analyzer (original magnification, ×100). The stained pLF in the transgenic mammary gland is shown in red. (D) Quantitation of recombinant pLF secreted in the milk of transgenic mice was analyzed by ELISA assay. Results are representative of three experiments. The significant difference between two means is presented as (*) ($P < 0.05$) and (**) ($P < 0.01$).

binant pLF protein signals were detected in the lumen of lactiferous tubules, and also accumulated around the mammary epithelial cells in hemizygous transgenic females. The greater pLF accumulation observed in Fig. 2C illustrated that the expression and secretion efficiency of recombinant pLF was much higher in the homozygous transgenic mice than the hemizygous mice. There was no signal detected in the mammary gland sections of the wild-type mice (Fig. 2A). A sensitive ELISA protocol was applied to quantify the amounts of pLF secreted in the milk of transgenic mice. The concentration of recombinant pLF in the milk produced by homozygous transgenic lines (120 ± 13.6 mg/l) exhibited a significantly higher yield than that of hemizygous mice (80 ± 15.1 mg/l) ($P < 0.05$) as shown in Fig. 2D.

Establishment of an animal model for EV71 challenge

In order to investigate the direct effects of orally administrated recombinant pLF on the EV71-infected mice, we designed and developed an animal model, first through testing the wild-type mice for EV71 infection. Three different ages, including 2-day-old, 4-day-old and 7-day-old BALB/c wild-type pups were chosen. Each group was separated into two sub-groups for EV71 viral medium (1.1×10^4 pfu/pup) and normal Vero cell lysate (as a placebo control) intraperitoneal inoculation. During the post-inoculation observation period, 5 of 10 EV71-infected mice in the 2-day-old group (50% lethality) and 2 of 6 in the 4-day-old group died (33.3% lethality) and no mouse died in the 7-day-old group (0% lethality). There were significant differences ($P < 0.05$) between the body weights of surviving pups after EV71 infection and placebo-inoculated mice in all three groups (Fig. 3A), indicating that EV71 was indeed the injurious agent in all neonatal age groups. Among these three age groups, the 4-day-old group showed the most apparent difference in body weight immediately after inoculation and on the 21st day after challenge as shown in Fig. 3B. We therefore chose 4-day-old neonatal mice as the inoculation recipients in our animal model.

Anti-EV71 activity of pLF transgenic mice

The anti-EV71 activities of recombinant pLF produced in the milk of the αLA-pLF transgenic mice were validated under the conditions of an established viral infection animal model. First, the pregnancy of female wild-type, hemizygous and homozygous transgenic mice were timed in order to obtain pups of the same age. After birth, we introduced wild-type pups to the litters of transgenic mice. All of the BALB/c pups ($n = 10$ in each group) nursed by wild-type female ($pLF^{-/-}$), hemizygote ($pLF^{+/-}$) or homozygote ($pLF^{+/+}$) transgenic mice were intraperitoneally inoculated with EV71 viral medium on the 4th day after birth and observed for 3 weeks. Three, one and zero pups died during the 3 weeks after EV71-innoculated experimental periods in nursing different groups wild-type mice ($pLF^{-/-}$), hemizygote ($pLF^{+/-}$) and homozygote ($pLF^{+/+}$) transgenic mice, respectively. As shown in Fig. 4A, the growth rates of surviving pups were significantly different ($P < 0.01$) between the transgenic groups and the wild-type group. The body

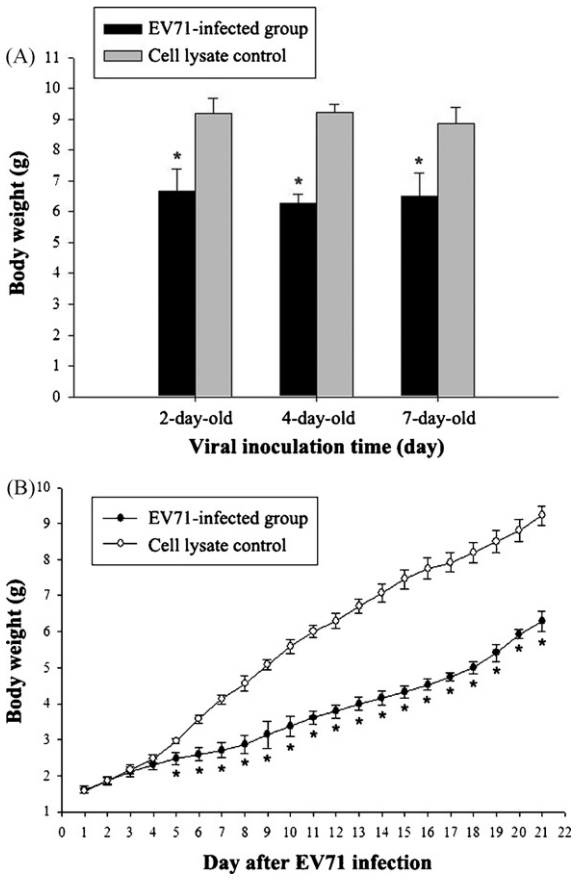

Figure 3 Body weight changes between EV71-infected and cell lysate placebo-inoculated mice in the animal model. (A) Significant differences ($P < 0.05$) between the mean body weight of EV71-infected mice and cell lysate placebo-inoculated mice at three different inoculation times, including 2-day-old, 4-day-old and 7-day-old neonatal pups. The body weights with mean ± S.D. of 21-day-old mice are shown. The statistic difference between two means is presented as (*) ($P < 0.05$). (B) The daily plot of body weight gains in the 4-day-old EV71- and placebo-inoculated neonatal groups. The mean body weight of EV71-inoculated mice was significantly different from the cell lysate placebo-inoculated mice ($P < 0.05$) after the experiment performed on the 5th day post-treatment.

weight gain of pups from the homozygous transgenic group was slightly heavier than the hemizygous group, although there was no statistically significant difference ($P > 0.05$). We observed an apparent difference in body size between these experimental groups 3 weeks after EV71 inoculation (Fig. 4B). This observation further illustrated the protective effects of orally administrated pLF *in vivo*.

The existing EV71 in infected animal tissues

Following 3 weeks of body weight measurements, all EV71-innoculated experimental pups were sacrificed for detection of EV71 viral particles in various tissues. For each mouse, 8 different tissues were dissected and homogenized, including liver, kidney, stomach, heart, lung, intestine, cerebrum and cerebellum. After centrifugation, the supernatant of the tissues was added into the culture medium of normal

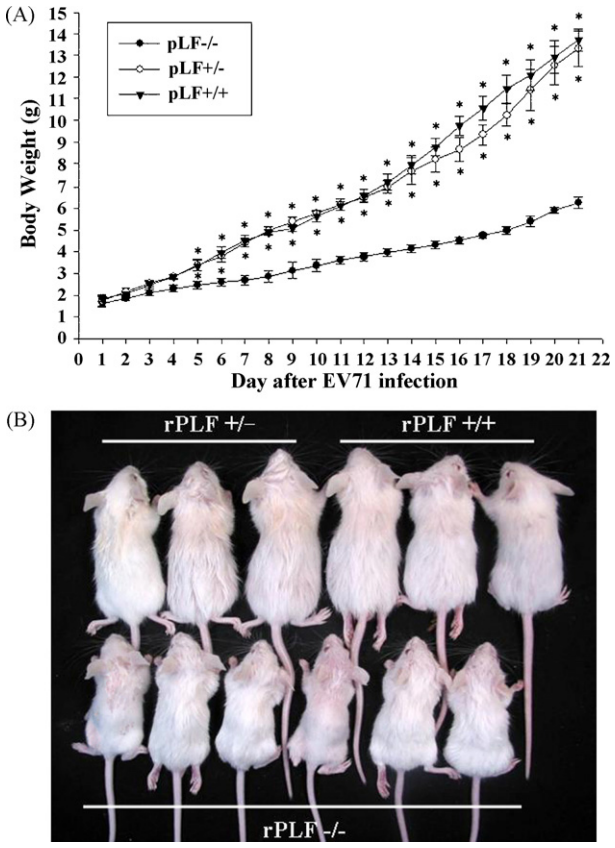

Figure 4 The protective effects of recombinant pLF against EV71 infection in αLA-pLF transgenic mice. (A) The daily body weights after EV71 infection were measured in mice pups nursing with the milk from wild-type BALB/c, hemizygote or homozygote transgenic females. (B) The body size of pups in the three different experimental groups was photographed at 3 weeks post-EV71 infection.

Vero cells. Vero cells that received cerebellum tissue lysates derived from EV71-inoculated pups which nursed from wild-type females ($pLF^{-/-}$) exhibited a severe cytopathic effect (Fig. 5B). In these cells, EV71 viral particles were detected by FISH assay using anti-EV71 VP1 specific antibody immunofluorescence staining (Fig. 5D), indicating that EV71 indeed existed in the cerebellum. However, the CPE (Fig. 5A) and viral particle signal (Fig. 5C) were not observed in Vero cell culture when the cerebellum tissue lysates were obtained from EV71-inoculated pups nursing by homozygous ($pLF^{+/+}$) transgenic females.

Discussion

Early in this study, we detected different expression rates of recombinant pLF in hemizygous and homozygous αLA-pLF transgenic mice and demonstrated that the protein was highly expressed in the lactiferous tubules of the homozygous transgenic mice (Fig. 1). These results not only indicated that the transgenic mice can successfully express recombinant pLF in mammary glands but also further demonstrated that we successfully produced a lactation-specific mammary expression model of pLF by using the αLA promoter and its 19-aa secretion signal peptide (Fig. 2). Confining the expression of recombinant protein to the mammary gland during the lactation period may be important for observing the specific biological function of the protein because it may be totally encompassed by the tight junctions of mammary epithelial cells, and may not have any function in the transgenic animal [25].

In the animal model we developed, about 30% of EV71-infected 4-day-old BALB/c wild-type mice died after EV71 inoculation (Fig. 3). The existence of the viral RNA genome in the cerebellum of infected mice was determined by RT-PCR (data not shown). Normally cultured Vero cells grown with cerebellum tissue lysates, which were derived from EV71-inoculated pups nursing from wild-type females, exhibited a severe cytopathic effect (Fig. 5). These results demonstrated that the mice indeed died due to the infection by EV71 and may be largely a result of the damage caused by EV71 in the cerebellum. The clinical records also showed that EV71 can invade the cerebellum of humans through the blood stream and can cause fatal encephalitis [7,26]. This relationship further confirmed the possibility that the mice in our model died because of EV71-induced encephalitis. In this point of view, we successfully produced an EV71 infection animal model which has the same infection pattern as seen in human infants [2,5].

Moreover, during the development of the animal model, we observed that five 2-day-old mice (5/10) died after EV71 infection, whereas only two mice (2/6) in the 4-day-old group and none in the 7-day-old group died after EV71 inoculation. These results indicate an age dependent character of EV71-induced death in BALB/c mice. Previous studies found that there was a 100% mortality rate when 1-day-old mice were inoculated with EV71 [27]. The mortality rate dropped apparently when the inoculated mice were 2 days old, and the mortality rate was zero when 6-day-old mice were infected. Our results correspond to the previous reports and we found the best conditions for an animal model to evaluate the properties of immuno-modulators of antiviral function *in vivo*.

In the determination of anti-EV71 activity of recombinant pLF, we demonstrated that pLF had a protective effect *in vivo*, not only from the differences in lethality rate, body weights, and body size (Fig. 4), but also from detecting the existence of EV71 in tissue through its induction of a severe cytopathic effect of co-cultured Vero cells (Fig. 5). The mechanism by which pLF may prevent EV71 infection may be the same as the mechanism used by both bovine and human LF. Other investigations have demonstrated that bovine or human LF can inhibit EV71 infection by blocking the adsorption or receptor-mediated binding of the virus to the target cell membrane *in vitro* [13,14]. The results of amplifying the EV71 RNA genome detected in the cerebellum of infected mice may correspond indirectly to the blocking effect of LF *in vitro*, and illustrated that LF can still block the entrance of EV71 into the cells *in vivo*. The other reason for the anti-EV71 activity of pLF in animals may be due to the immunomodulatory effects of recombinant pLF. It has been widely demonstrated that immunomodulatory properties were included in the protective effects of orally administered LF [28,29]. Since the αLA-pLF transgenic mouse model provided a high dose of recombinant pLF to the mouse pups immediately after birth, we attributed part of the anti-infection effects to the immunomodulatory

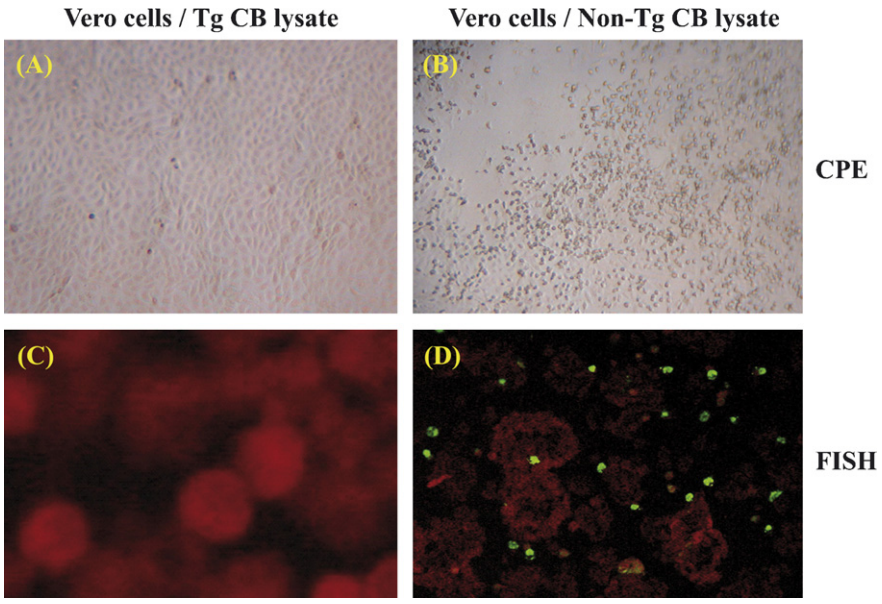

Figure 5 The existence of EV71 in infected mice tissues was assayed by Vero cell inoculation and fluorescence in situ hybridization (FISH). The cytopathic effects (CPE) of normal Vero cells inoculating cerebellum (CB) lysates of EV71-infected mice obtained from homozygote transgenic (A) and wild-type BALB/c (B) nursing groups were determined by an Olympus IX71 microscope and DP71 image analyzer (original magnification, $\times$100). Viral particles present in Vero cell culture medium were observed by fluorescence in situ hybridization against the anti-EV71 VP1 antibody in the transgenic group (C) and the non-transgenic group (D). The images were observed under a laser scanning confocal microscope (original magnification, $\times$400).

activity. In addition, it has been reported that the antiviral activity of LF has a dose-dependent character, which means that the higher the amount of LF, the higher the antiviral activity [30]. In our animal model, correspondingly, the milk of homozygous ($pLF^{+/+}$) transgenic mice contained a higher concentration of recombinant pLF, protecting the EV71-inoculated pups thereby increasing the survival rate and body weight gains compared to those of hemizygous ($pLF^{+/-}$) mice (Fig. 4).

To further compare the anti-EV71 effect of the mice pups fed with different sources of lactoferrin including porcine LF-enriched milk (pLF-Tg), bovine LF purified from milk, and recombinant pLF (rpLF) derived from yeast, the animal model of 4-day-old neonatal mice challenging with EV71 virus have been done, and body weight gain and survival rate have been quantitatively measured. In this result, pups suckling pLF-enriched milks from transgenic mice, oral administrating bLF purified from milk or recombinant pLF derived from yeast [15] had significantly greater body weight gain and survival rate than pups suckling normal milks from wild-type mice after EV71 infection. We demonstrated that the anti-EV71 activity of pLF is comparable to that of bLF in *in vivo* test, and the recombinant pLF produced by methylotrophic yeast also possess the similar antiviral activity.

In conclusion, this is the first animal study that demonstrates the anti-EV71 activity of porcine lactoferrin. The study not only elucidates the *in vivo* anti-EV71 activity of porcine lactoferrin, but also provides an extremely practical animal model for detecting the *in vivo* protective role of pLF, since we can substitute any infectious agent that is of interest. All these features indicate that porcine lactoferrin may be an effective and tolerable natural anti-EV71 agent. The production of recombinant pLF-enriched milks in transgenic animals is effective, and it delivers the same advantages of mammalian cells, such as a sophisticated refolding machinery and glycosylation. Other features of transgenic production are scale-up flexibility, since herd size can be increased rapidly and inexpensively, as well as the relatively low-cost and low-complexity of the raw product manufacturing facilities as compared to traditional cell culture facilities. This successful transgenic mouse model for protecting neonatal against EV71 infection shows that it is of great value to develop transgenic cow and goat for the mass production of recombinant pLF-enriched milks. In view of the high mortality and severe morbidity of cerebellar infections caused by EV71, a clinical trial to assess the efficacy of lactoferrin in the prevention of EV71 infections should be evaluated.

Acknowledgements

The authors would like to thank Dr. Jeffrey Conrad for critically reading the manuscript. We also thank Mr. Chieng-Yu Lin for his assistance in transgenic mice breeding. This research was supported in parts by grant NSC-95-2313-B-005-012 from the National Science Council, grant COA-96-1.2.1-U1(27) from the Council of Agriculture, and the Ministry of Education, Taiwan, Republic of China, under the ATU plan.

References

[1] Schmidt NJ, Lennette EH, Ho HH. An apparently new enterovirus isolated from patients with disease of the central nervous system. J Infect Dis 1974;129:304—9.

[2] Ho M. Enterovirus 71: the virus, its infections and outbreaks. J Microbiol Immunol Infect 2000;33:205—16.
[3] Hagiwara A, Tagaya I, Yoneyama T. Epidemic of hand, foot and mouth disease associated with enterovirus 71 infection. Intervirology 1978;9:60—3.
[4] Chumakov KM, Lavrova IK, Martianova LI, Korolev MB, Bashkirtsev VN, Voroshilova MK. Investigation of physicochemical properties of Bulgarian strain 258 of enterovirus type 71. Arch Virol 1979;60:359—62.
[5] Melnick JL. Enterovirus type 71 infections: a varied clinical pattern sometimes mimicking paralytic poliomyelitis. Rev Infect Dis 1984;2(Suppl.):387—90.
[6] Ishimaru Y, Nakano S, Yamaoka K, Takami S. Outbreaks of hand, foot, and mouth disease by enterovirus 71: high incidence of complication disorders of central nervous system. Arch Dis Child 1980;55:583—8.
[7] Ho M, Chen ER, Hsu KH, Twu SJ, Chen KT, Tsai SF, et al. An epidemic of enterovirus 71 infection in Taiwan: Taiwan Enterovirus Epidemic Working Group. N Engl J Med 1999;341:929—35.
[8] Brock JH. The physiology of lactoferrin. Biochem Cell Biol 2002;80:1—6.
[9] Farnaud S, Evans RW. Lactoferrin—a multifunctional protein with antimicrobial properties. Mol Immunol 2003;40:395—405.
[10] Baveye S, Elass E, Mazurier J, Spik G, Legrand D. Lactoferrin: a multifunctional glycoprotein involved in the modulation of the inflammatory process. Clin Chem Lab Med 1999;37:281—6.
[11] Jenssen H. Anti-herpes simplex virus activity of lactoferrin/lactoferricin—an example of antiviral activity of antimicrobial protein/peptide. Cell Mol Life Sci 2005;62:3002—13.
[12] van der Strate BW, Beljaars L, Molema G, Harmsen MC, Meijer DK. Antiviral activities of lactoferrin. Antiviral Res 2001; 52:225—39.
[13] Lin TY, Chu C, Chiu CH. Lactoferrin inhibits enterovirus 71 infection of human embryonal rhabdomyosarcoma cells *in vitro*. J Infect Dis 2002;186:1161—4.
[14] Weng TY, Chen LC, Shyu HW, Chen SH, Wang JR, Yu CK, et al. Lactoferrin inhibits enterovirus 71 infection by binding to VP1 protein and host cells. Antiviral Res 2005;67:31—7.
[15] Chen HL, Lai YW, Yen CC, Lin YY, Lu CY, Yang SH, et al. Production of recombinant porcine lactoferrin exhibiting antibacterial activity in methylo-trophic yeast, *Pichia pastoris*. J Mol Microbiol Biotechnol 2004;8:141—9.
[16] Steijns JM, van Hooijdonk AC. Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. Br J Nutr 2000;84(Suppl.):11—7.
[17] Chu RM, Wang SR, Weng CN, Pursel VG. Isolation and characterization of porcine milk lactoferrin. Am J Vet Res 1993; 54:1154—9.
[18] Hanson LA, Korotkova M, Haversen L, Mattsby-Baltzer I, Hahn-Zoric M, Silfverdal SA, et al. Breast-feeding, a complex support system for the offspring. Pediatr Int 2002;44:347—52.
[19] Lee WJ, Farmer JL, Hilty M, Kim YB. The protective effects of lactoferrin feeding against endotoxin lethal shock in germfree piglets. Infect Immun 1998;66:1421—6.
[20] Nuijens JH, van Berkel PH, Schanbacher FL. Structure and biological actions of lactoferrin. J Mamm Gland Biol Neoplasia 1996;1:285—95.
[21] Chen HL, Yen CC, Lu CY, Yu CH, Chen CM. Synthetic porcine lactoferricin with a 20-residue peptide exhibits antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. J Agric Food Chem 2006;54:3277—82.
[22] Wu SC, Chen HL, Yen CC, Kuo MF, Yang TS, Wang SR, et al. Recombinant porcine lactoferrin expressed in the milk of transgenic mice enhances offspring growth performance. J Agric Food Chem 2007;55:4670—7.
[23] Cheng WTK, Chen BC, Chiou ST, Chen CM. Use of nonautologous microencapulated fibroblasts in growth hormone gene therapy to improve growth of midget swine. Hum Gene Ther 1998;9:1995—2003.
[24] Chen HL, Yen CC, Tsai TC, Lai YW, Yu CH, Chen CM. Production and characterization of human extracellular superoxide dismutase (ECSOD) in methylotrophic yeast, *Pichia pastoris*. J Agric Food Chem 2006;54:8041—7.
[25] Chen CM, Wang CH, Wu SC, Lin CC, Lin SH, Cheng WTK. Temporal and spatial expression of biologically active human factor VIII in the milk of transgenic mice driven by mammary-specific bovine alpha-lactalbumin regulation sequences. Transgenic Res 2002;11:257—68.
[26] Lum LC, Wong KT, Lam SK, Chua KB, Goh AY, Lim WL, et al. Fatal enterovirus 71 encephalomyelitis. J Pediatr 1998;133:795—8.
[27] Yu CK, Chen CC, Chen CL, Wang JR, Liu CC, Yan JJ, et al. Neutralizing antibody provided protection against enterovirus type 71 lethal challenge in neonatal mice. J Biomed Sci 2000;7:523—8.
[28] Shin K, Wakabayashi H, Yamauchi K, Teraguchi S, Tamura Y, Kurokawa M, et al. Effects of orally administered bovine lactoferrin and lactoperoxidase on influenza virus infection in mice. J Med Microbiol 2005;54:717—23.
[29] Tomita M, Wakabayashi H, Yamauchi K, Teraguchi S, Hayasawa H. Bovine lactoferrin and lactoferricin derived from milk: production and applications. Biochem Cell Biol 2002;80: 109—12.
[30] Arnold D, Di Biase AM, Marchetti M, Pietrantoni A, Valenti P, Seganti L, et al. Antiadenovirus activity of milk proteins: lactoferrin prevents viral infection. Antiviral Res 2002;53:153—8.