— Research Letter —

Inhibitory Effect of Bovine Lactoferrin on Human Parainfluenza Virus Type 2 Infection

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Lactoferrin (Lf) is a multifunctional protein that has inhibitory activity against microorganisms. In this study, the effects of Lf on the growth of human parainfluenza virus type 2 (hPIV-2) in $LLCMK_2$ cells were investigated. Lf inhibited cell fusion and hemadsorption induced by hPIV-2. However, virus RNA synthesis was only slightly inhibited by Lf. In addition, indirect immunofluorescence study showed that virus protein syntheses were not completely inhibited by Lf. Using a recombinant, green fluorescence protein-expressing hPIV-2 (rghPIV-2), it was found that virus entry into cells were considerably inhibited by Lf, but cell-to-cell spread was not inhibited. The number of viruses produced by the cells were determined, and it was found that Lf reduced the number of released viruses to about 1/300 compared with that of positive control. Lf bound to cell surface within 10 min at early phase of infection, it assembled gradually, and many aggregates were observed at 120 min. These results indicated that Lf considerably inhibited virus adsorption to the surface of the cells by binding to the cell surface and prevented hPIV-2 infection.

Key words —— lactoferrin, inhibition, parainfluenza virus type 2

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INTRODUCTION

Human parainfluenza virus type 2 (hPIV-2) is one of the major human respiratory tract pathogens of infants and children. hPIV-2 is a member of the genus *Rubulavirus* in the family *Paramyxoviridae*, and it possess a single-stranded non-segmented and negative stranded RNA genome of approximately 15 k bases.[1] The gene order of hPIV-2 is 3′-(leader)-NP-P/V-M-F-HN-L-(trailer)-5′. All genes of hPIV-2 were sequenced by our group.[2–7] Monoclonal antibodies (mAbs) were made by Tsurudome.[8] The infectious hPIV-2 from cDNA clone was constructed by Kawano.[9]

Lactoferrin (Lf) is an 80 kDa iron-binding glycoprotein which presents mainly in milk, tears and saliva. Lf has many functions such as promotion of iron absorption, immunomodulation and host defense against bacterial and virus infections. Lf has been shown as an inhibitor of different enveloped viruses such as herpes simplex virus type 1 (HSV-1),[10] human immunodeficiency virus,[11] hepatitis B virus,[12] hepatitis C virus,[13] and respiratory syncytial virus (RSV),[14] and non-enveloped viruses, such as rotavirus,[15] poliovirus[16] and echovirus.[17] Lf affects early phase of viral infection[18,19] by a competitive binding interaction with cell surface heparan sulphate (HS) containing proteoglycans.[20] However, Lf can prevent not only entry but also cell-to-cell spread of HSV-1.[10,21] The antiviral effect of Lf on viruses which infect respiratory organs has been reported only on RSV,[14] but its effects on other respiratory viruses remain to be clarified.

In the present study, we investigated the effect of bovine Lf on the replication of hPIV-2 in $LLCMK_2$ cells. Virus RNA was prepared and amplified by polymerase chain reaction (PCR). Virus protein expression was observed by indirect immunofluorescence study using mAbs against NP, F and HN proteins of hPIV-2. The inhibitory effect of Lf on hPIV-2 infection to the cells was analyzed using rghPIV-2. The number of viruses released from infected cells cultured with Lf was determined.

MATERIALS AND METHODS

Bovine Lf —— Lf was a kind gift of Morinaga Milk Co. Ltd. (Tokyo, Japan). Lf was dissolved at 100 mg/ml in 10 mM phosphate buffered saline, pH 7.2 (PBS), and sterilized by filtration.

Virus and Cell Line —— hPIV-2 (Toshiba Strain) was used. Recombinant, green fluorescence protein-expressing hPIV-2 (rghPIV-2) was constructed by the method reported by Kawano *et al.*[9] There was no significant difference in the replication of the rghPIV-2 compared with that of hPIV-2 (data not shown). $LLCMK_2$ cells (rhesus monkey kidney cell line) were used.

Cultivation of Cells —— $LLCMK_2$ cells were cultured in a flat-bottomed 24-well plate in 1 ml culture medium. Minimum essential medium α (MEMα: Wako, Osaka, Japan) supplemented with 2% fetal calf serum (FCS) and 0.1 mg/ml kanamycin was used. The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. After 3 days, the cells became confluent (5×10^5 cells), and the medium was changed to MEMα with 0.5% FCS and 0.1 mg/ml kanamycin. Lf was added to the cells, and the cells were infected with hPIV-2 [3×10^2 tissue culture infections dose $(TCID)_{50}$].

Cytopathogenic Assay —— The cell fusion and hemadsorption (Had) were observed at 4 days post infection. Had test was carried out using sheep red blood cells (SRBC). The cells were incubated with 0.4% SRBC at room temperature for 30 min, washed 4 times with PBS, and Had was observed under a light microscope for cell culture.

RNA Preparation, cDNA Synthesis and PCR —— RNA was extracted from the cells (2×10^6 cells) cultured in a flat-bottomed 6-well plate using TRIZOL reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's method. cDNA was synthesized with 2 μg of RNA using superscript II reverse transcriptase (Invitrogen) with forward primers for NP, F and HN genes of hPIV-2.[22] PCR was done with cDNA using forward and reverse primers for NP, F and HN genes[22] and Ex Taq (Takara, Shiga, Japan).

Immunofluorescence Study —— To detect virus proteins in the infected cells, the cells were fixed with 10% formaldehyde-PBS at room temperature for 15 min, washed with PBS, and incubated with mouse mAbs against NP, F and HN proteins of hPIV-2 at room temperature for 30 min. After washing with PBS, the cells were incubated with Alexa 488 conjugated secondary antibody to mouse IgGs (Invitrogen) at room temperature for 30 min.

To determine the binding of Lf to the cells at early phase of infection, Lf was added and the cells were infected with hPIV-2 and cultured for 120 min. The cells were fixed at 10, 30, 60 and 120 min, and incubated with anti-bovine Lf polyclonal antibody produced in goat (Bethyl, Montgomery, TX, U.S.A.) at room temperature for 30 min, and then incubated with Alexa 594 conjugated rabbit anti-goat IgG antibodies (Molecular Probes, Eugen, OR, U.S.A.) at room temperature for 30 min.

The cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan).

RESULTS AND DISCUSSION

Lf (28 mg/ml) completely inhibited both cell fusion and Had induced by hPIV-2 (data not shown). Lf itself did not disturb normal cell morphology at the concentration used in the experiments.

Effect of Lf on Viral RNA Synthesis

RNA was prepared from the infected cells at 4 days post infection, and virus-synthesized RNA was analyzed with the use of hPIV-2 specific primers. As shown in Fig. 1, the syntheses of NP (Fig. 1, lane 7), F (Fig. 1, lane 8) and HN (Fig. 1, lane 9) genes of hPIV-2 were slightly inhibited in the hPIV-2 infected cells cultured with Lf, com-

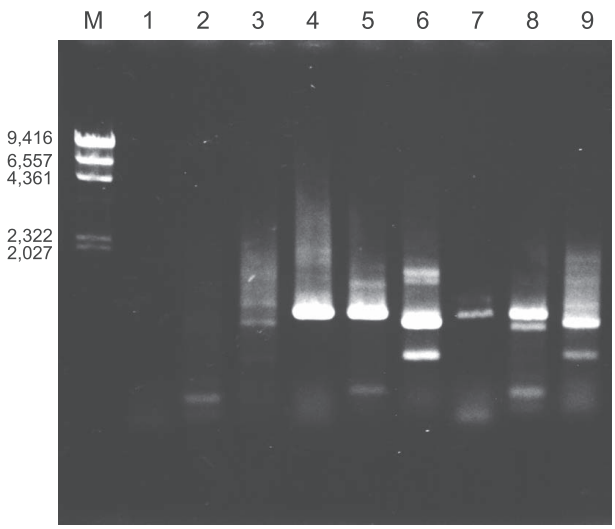

Fig. 1. Effect of Lf on Virus RNA Synthesis

Lane M: marker (base pair), lanes 1, 2, 3 :non-infected cells, lanes 4, 5, 6: hPIV-2 infected cells, lanes 7, 8, 9: hPIV-2 infected cells cultured with Lf. Lanes 1, 4, 7: NP gene, lanes 2, 5, 8: F gene, lanes 3, 6, 9: HN gene. Lf only partly inhibited RNA synthesis of hPIV-2.

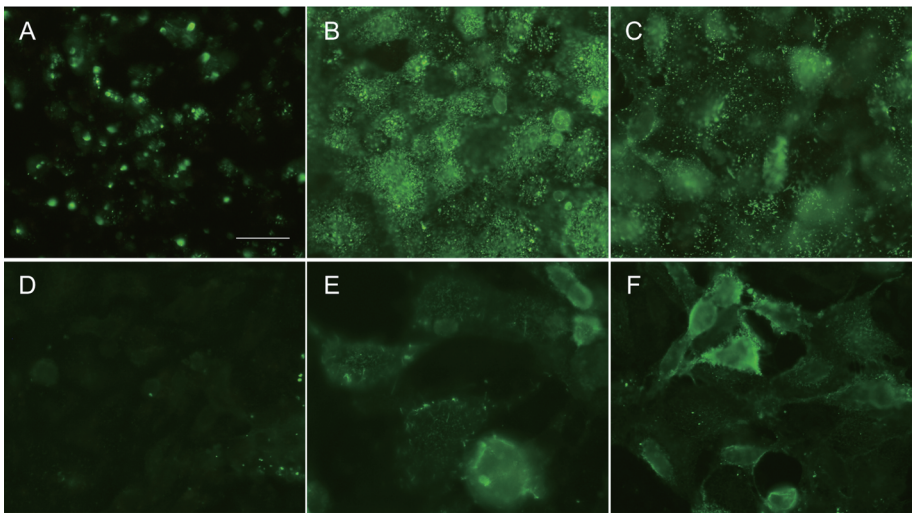

Fig. 2. Effect of Lf on the Expression of NP, F and HN Proteins of hPIV-2

The expression of NP (A), F (B) and HN (C) proteins of hPIV-2 infected cells. Lf partly inhibited the expression of NP (D), F (E) and HN (F) proteins. Bar: 50 μm.

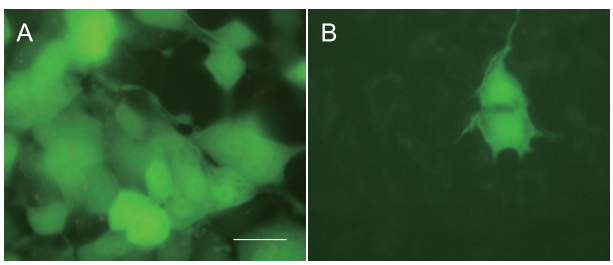

Fig. 3. Effect of Lf on the Entry of hPIV-2 into Cells

Cells infected with rghPIV-2 (A), cells infected with rghPIV-2 cultured with Lf (B). Lf largely prevented the entry of rghPIV-2, but did not prevent cell-to-cell spread of the virus. Bar: 50 μm.

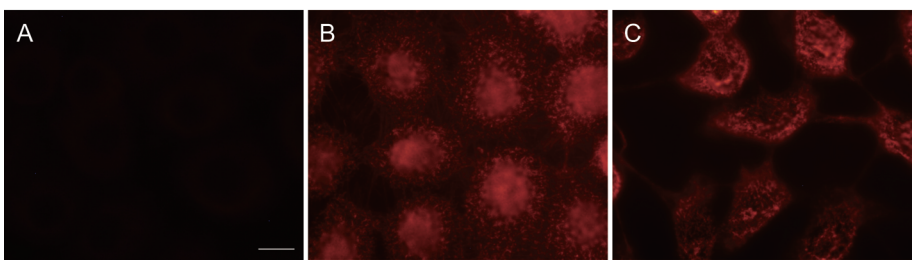

Fig. 4. Binding of Lf to the Cells at Early Stage of Infection

Control cells incubated with Alexa 594 conjugated secondary antibody (A): no fluorescence was seen. Lf bound to the surface of the cells within 10 min of addition (B) and some aggregates were seen at 120 min (C). Bar: 50 μm.

pared with those of NP (Fig. 1, lane 4), F (Fig. 1, lane 5) and HN (Fig. 1, lane 6) genes of positive controls.

Effect of Lf on Viral Protein Synthesis

Indirect immunofluorescence study was performed to investigate the effects of Lf on hPIV-2 protein expression. Lf was added to $LLCMK_2$ cells and they were infected with hPIV-2. At 4 days post infection, the cells were fixed and stained with the mAbs against NP, F and HN proteins of hPIV-2. Figs. 2A, B and C show the NP, F and HN protein expression in hPIV-2 infected cells, respectively. In hPIV-2 infected cells, NP, F and HN proteins were

observed in almost all the cells: NP protein was observed in many strong fluorescent dots mainly in the cytoplasm, F and HN proteins were in small dots in the cytoplasm and on the cell surface. Lf partly inhibited the expression of NP (Fig. 2D), F (Fig. 2E), and HN (Fig. 2F) proteins: NP, F and HN proteins were observed in some cells. However, intensity was weaker than that of hPIV-2 infected cells and the number of fluorescent dots decreased, indicating that Lf partly inhibited hPIV-2 infection in the cells.

Effect of Lf on the Entry of hPIV-2 into Cells

The above results suggested that Lf partly inhibited both hPIV-2 RNA and protein syntheses. In the following experiment, we determined the effect of Lf on the entry of hPIV-2 using rghPIV-2. Lf was added to the cell culture, the cells were infected with rghPIV-2 (3×10^5 $TCID_{50}$) and cultured for 24 hr. The cells were fixed with 3.3% formaldehyde-PBS and observed under a fluorescence microscope. Figure 3A is a positive control: almost all the cells had strong fluorescence. On the contrary, in several parts of the specimen of the infected cells cultured with Lf, development of 2 cellular foci was seen and around the 2 cells, many uninfected cells were observed. Figure 3B shows one of them. The result indicates that Lf considerably inhibited the virus entry into cells, and that it could not inhibit cell-to-cell spread of hPIV-2.

Titration of Virus Released from the Infected Cells

We determined the titer of virus released from cells cultured with and without Lf at 4 days post infection. Without Lf, the titer was 2×10^6 $TCID_{50}$/ml, and with Lf it reduced to 6.3×10^3 $TCID_{50}$/ml, indicating that Lf largely prevented the virus entry and, as a result, the release of virus from the cells decreased.

Binding of Lf to the Cells at Early Phase of Infection

The binding of Lf to the cells was analyzed by indirect immunofluorescence study. Lf bound to the cell surface within 10 min of addition (Fig. 4B), they assembled gradually (data not shown), and some aggregates were observed at 120 min (Fig. 4C). Lf bound to the cell surface and largely prevented the binding of hPIV-2 to the cells.

Lf is known to bind to the cell surface HS containing proteoglycans which are receptors for herpetic virus and for adenovirus type 2.[23] Also, human papilomavirus[24] requires HS for its infection. The inhibiting activity of Lf on these viruses has been ascribed to a competition for receptors which, as a consequence, prevents virus adsorption to cells. Moreover cell-to-cell spread of HSV-1 was inhibited by Lf.[10] The receptors for hPIVs are sialic acids,[1] so that the prevention of hPIV-2 adsorption to cells by Lf might be indirect. The Lf that bound to HS might prevent the binding of hPIV-2 by steric hindrance, not by competition.

In conclusion, the effect of Lf on hPIV-2 infection was analyzed, and we showed that Lf had a partial inhibitory effect on virus RNA and protein syntheses. In addition, it was shown that Lf inhibited the entry of hPIV-2 into cells considerably by binding to the cell surface, but cell-to-cell spread of hPIV-2 was not inhibited.

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