Lactoferrin Inhibits Enterovirus 71 Infection of Human Embryonal Rhabdomyosarcoma Cells In Vitro

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Enterovirus 71 (EV71), the newest member of Enteroviridae, is notable for its etiological role in epidemics of severe neurological diseases in children. It appears to be emerging as an important virulent neurotropic enterovirus in the upcoming era of poliomyelitis eradication, whereas no effective vaccine or antiviral agents are available at this moment. Human and bovine lactoferrins, iron-binding proteins belonging to the nonimmune defense system, were assayed in vitro to assess their inhibiting capacity on the cytopathic effect of EV71 on human embryonal rhabdomyosarcoma (RD) cells. Both bovine and human lactoferrins were found to be potent inhibitors of EV71 infection (mean IC50, 10.5–24.5 μg/mL and 103.3–185.0 μg/mL, respectively). Lactoferrin probably exerts its effect at the level of viral adsorption, since the ongoing infection could not be further inhibited after the EV71 penetrated RD cells.

Materials and Methods

Cell lines, chemicals, and virus strains. Human embryonal RD cells and Vero cells were used in this study. Cells usually were cultured in Dulbecco’s modified Eagle medium (DMEM) with 4.5 g of glucose/L and 10% fetal calf serum. Bovine lactoferrin was kindly provided by the Nutritional Science Laboratory (Morinaga Milk Industry, Japan). Human lactoferrin, as well as dextran (10,000 molecular weight) and human serum albumin that were used as controls, were purchased from Sigma. The following EV71 strains that caused encephalomyelitis during outbreaks in Taiwan and Malaysia were used in this study: 2272, 1743, and 1470 (Taiwan) and 13091 (Malaysia). Other enteroviruses examined included coxsackievirus A16 and echovirus 9 (1 clinical isolate for each).
Anti-EV71 activity of bovine lactoferrin was assessed first by measuring the inhibitory effect on virus-induced cytopathogenicity in RD cells. Bovine lactoferrin readily inhibited EV71 infection of RD cells. A concentration-dependent antiviral effect of bovine lactoferrin was seen microscopically as a concentration-dependent decrease of cytopathic effect in the cells (data not shown). Human lactoferrin also was capable of inhibiting EV71 infections. By using plaque reduction assay, we found that bovine lactoferrin (mean IC$_{50}$, 10.5–24.5 µg/mL against 4 different strains of EV71) had a 10-fold stronger antiviral effect, compared with that of human lactoferrin (mean IC$_{50}$, 103.3–185.0 µg/mL against 2 different strains of EV71; table 1). The IC$_{50}$ of human and bovine lactoferrins against strain 2272 was estimated to be 400 and 40 µg/mL, respectively. By use of the same assay system, bovine lactoferrin showed effectiveness in the inhibition of coxsackievirus A16, but not echovirus 9, infection of Vero cells (table 1). Both dextran (10,000 molecular weight) and human serum albumin exerted no effect on enteroviral infection of RD or Vero cells.

The influence of lactoferrin on EV71 infection of RD cells was investigated further in a time-of-addition assay. Cells were preincubated with virus (strain 2272) for 15–180 min, after which optimally inhibiting concentration of bovine lactoferrin (final concentration, 250 µg/mL) was added. It was found that, when lactoferrin was added to the cells that were preincubated with virus for 2 h, there was virtually no inhibitory effect on viral infection (figure 1). In another experiment, lactoferrin that had been added to RD cells was removed by washing with medium before the addition of the virus (strain 2272). If the preincubation continued $\leq$30 min before virus was added, viral inhibition was markedly decreased (figure 2). The continuous presence of lactoferrin appeared to be sufficient to completely inhibit virus infection of the cells (figure 2).

Discussion

This is the first report that lactoferrin has a marked antiviral effect against EV71 and coxsackievirus A16. Lactoferrins from both bovine and human origins prevented EV71 infection of RD cells in vitro. A relatively stronger inhibitory effect was observed using bovine lactoferrin. The finding is important because there has been no effective antiviral agents available thus far for the prevention and control of EV71 infections. Our

![Figure 1. Time-of-addition assay. Bovine lactoferrin at 250 µg/mL (i.e., $>$IC$_{50}$) was added after the infection of human embryonal rhabdomyosarcoma (RD) cells with EV71 strain 2272 commenced; within 2 h, antiviral activity gradually decreased to zero.](Image 320x111 to 548x260)
results indicate that lactoferrin only can exert its effect at the levels of virus adsorption or receptor-mediated binding to the target cell membrane. If EV71 had been preincubated with RD cells for increasing periods before lactoferrin was added, the protecting effect gradually disappeared. Lactoferrin has been known to bind to heparan-like molecules at the cell surface [14]. Thus, it may interfere with the binding of EV71 to cell surface heparan receptors. This hypothesis could explain our results in the time-of-addition assays. On the other hand, when cells were preincubated with lactoferrin for ≥1 h and subsequently were washed, maximal protection against EV71 infection still was observed. This suggests that the cells can be loaded with or irreversibly bind lactoferrin. Therefore, administration of exogenous lactoferrin to hosts may result in some prophylactic effects on enteroviral infections.

It can be put forward that the lower activity of human lactoferrin against EV71, compared with that of bovine lactoferrin, is linked to the differences in the molecular structure. Bovine lactoferrin is 69% identical to human lactoferrin, but, despite this high degree of similarity, their comparison showed that the glycan chains of the molecules and the number of the disulphide bridges vary [15, 16]. These variations probably will contribute to differences in the functional domains responsible for the binding properties of the lactoferrins to host cells or viral particles. Another possible explanation for the observed difference between the effects of human and bovine lactoferrins is that commercially purchased human lactoferrin could have lost N-terminal arginine residues, which might interfere with some of the antimicrobial activities of the protein [12, 13].

Lactoferrin is produced by lactating women and secreted in their milk. An antibacterial domain has been identified in the lactoferrin polypeptide backbone [17]. In milk, some lactoferrin is associated with IgA. This complex serves as a first line of defense to protect the newborns from bacterial infections. Virtually no fatal EV71 infections took place in infants <2–3 months old in the 1998 outbreak [6], which contrasts with our observation that lactoferrin inhibits EV71 infection in vitro. A hypothesis is made that lactoferrin, transferred to infants through breast-feeding, plays at least a partial role in the protection of newborns and young infants from severe EV71 infections. In addition, lactoferrin is produced by neutrophilic leukocytes and is stored in their granules. Activated leukocytes can deposit the contents of these granules into the blood stream. During active bacterial infections, a significant increase in plasma lactoferrin level is often observed. It would be of interest to check serum lactoferrin level in patients with severe EV71 infections to determine whether lactoferrin also plays a physiologic role in the host response against EV71 infections.

Bovine lactoferrin inhibited EV71 infection of RD cells with an IC₅₀ of ~10–25 µg/mL, which is similar to that of lactoferrin against HSV-1 and lower than that against CMV and HIV-1 [10–13]. The RD cells appeared intact, even in the presence of lactoferrin at 500 µg/mL (data not shown). All these features indicate that lactoferrin may be an effective and tolerable natural anti-EV71 agent. In view of the high mortality and severe morbidity of CNS infections caused by EV71, a clinical trial to assess the efficacy of lactoferrin in the prevention of EV71 infections should be performed.

References

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