Biol. Chem., Vol. 385, pp. 1029–1034, November 2004 • Copyright © by Walter de Gruyter • Berlin • New York. DOI 10.1515/BC.2004.133

Minireview

Secretory leukoprotease inhibitor and pulmonary surfactant serve as principal defenses against influenza A virus infection in the airway and chemical agents up-regulating their levels may have therapeutic potential

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Abstract

Influenza A virus (IAV) is one of the most common infectious pathogens in humans. Entry of this virus into cells is primarily determined by host cellular trypsin-type processing proteases, which proteolytically activate viral membrane fusion glycoprotein precursors. Human IAV and murine parainfluenza virus type 1 Sendai virus are exclusively pneumotropic, and the infectious organ tropism of these viruses is determined by the susceptibility of the viral envelope glycoprotein to cleavage by proteases in the airway. Proteases in the upper respiratory tract are suppressed by secretory leukoprotease inhibitor, and those in the lower respiratory tract are suppressed by pulmonary surfactant, which by adsorption inhibits the interaction between the proteases and viral membrane proteins. Although the protease activities are predominant over the activities of inhibitory compounds under normal airway conditions, intranasal administration of inhibitors was able to significantly suppress multi-cycles of viral replication in the airway. In addition, we identified chemical agents that could act as defensive factors by up-regulating the levels of the natural inhibitors and immunoglobulin A (IgA) in airway fluids. One of these compounds, ambroxol, is a mucolytic and anti-oxidant agent that stimulates the release of secretory leukoprotease inhibitor and pulmonary surfactant in the early phase, and IgA in the late phase of infection at an optimal dose, i.e. a dose sufficient to inhibit virus proliferation and increase the survival rate of animals after treatment with a lethal dose of IAV. Another agent, clarithromycin, is a macrolide antibiotic that increases IgA levels through augmentation of interleukin-12 levels and mucosal immunization in the airway. In addition to the sialidase inhibitors, which prevent the release of IAV from infected cells, inhibitors of the processing proteases and chemical agents that augment mucosal immunity and/or levels of the relevant defensive compounds may also ultimately prove to be useful as new anti-influenza agents.

Keywords: ambroxol; clarithromycin; influenza virus; pulmonary surfactant; secretory leukoprotease inhibitor.

Introduction

In enveloped animal viruses, posttranslational proteolytic processing of the viral envelope glycoprotein precursors is a critical step for the activation of membrane fusion and thus is a critical step in viral infectivity (Homma and Ohuchi, 1973; Klenk and Rott, 1988). As is the case for almost all retroviruses and the highly virulent strains of Newcastle disease virus, paramyxovirus and myxovirus, endoproteolytic processing of the envelope fusion glycoprotein precursors takes place by means of ubiquitous intracellular processing proteases in the *cis* or medial cisternae of the rough endoplasmic reticulum-Golgi complex, or the late Golgi region, in a manner similar to prohormone processing (Klenk and Garten, 1994). These processing proteases cleave the carboxy-terminal side of the double basic consensus cleavage motif Lys/Arg-X-Lys/Arg-Arg in the envelope fusion glycoprotein precursors, and then induce the viral membrane fusion activity. Consequently, these viruses typically induce a serious systemic infection. There is also an extracellular processing system of the viral envelope glycoproteins for all human influenza A viruses (IAV) reported to date, as well as avirulent strains of Newcastle disease virus, paramyxovirus and myxovirus, infection by which is limited to the respiratory and/or digestive tracts. Most of the epidemic human IAVs are pneumotropic, and virus multiplication is restricted to the airway. The relative infectivity and pathogenicity of these viruses are primarily determined by the five different trypsin-type processing proteases identified to date (Yao et al., 2004), i.e. tryptase Clara (Kido et al., 1992), mini-plasmin (Murakami et al., 2001), ectopic anionic trypsin (Towatari et al., 2002), mast-cell tryptase (Chen et al., 2000) and tryptase TC30 (Sato et al., 2003). These proteases are found in the airway, and selectively cleave at the carboxyl side of the consensus cleavage motif Glu(Glu)-X-Arg of nearly all human IAV envelope-glycoprotein precursor hemagglutinins, and the fusion protein (F) of murine parainfluenza virus type 1. Therefore, the activity levels of these processing proteases, as well as their inhibitors, in the airway greatly determine the degree of infectious organ tropism and susceptibility to infection.

The activity of these processing proteases is strictly regulated by endogenous inhibitors, as well as host

defense compounds, in the airway, such as secretory leukoprotease inhibitor (SLPI) in the upper respiratory tract (Beppu et al., 1997) and pulmonary surfactant as an adsorbent molecule for the tryptase Clara in the lower respiratory tract (Kido et al., 1993; Tashiro et al., 1996). However, the levels of these inhibitory compounds are lower than the trypsin-type protease levels under conventional airway conditions, the ratio of which allows the efficient infection cycles of IAV seen after viral budding. In addition to these inhibitory compounds to virus multiplication, the mucosal immune system in the airway is the first line of immunological defense against pathogens on the mucosal surface. The levels of secretory immunoglobulin A (IgA) and IgG correlate with the level of protection against IAV infection (Wright et al., 1983; Liew et al., 1984; Tamura et al., 1996). The balance between the levels of trypsin-type proteases and these antiviral defensive compounds in the airway fluid is thought to influence the relative infectivity and pathogenicity of IAV.

Here we briefly review the natural compounds inhibitory to the IAV envelope glycoprotein-processing proteases in the airway, and also review several drugs, such as ambroxol and clarithromycin, which augment levels of host-defense compounds in the airway.

SLPI as a natural defense against IAV in the upper respiratory tract

We previously identified SLPI as an endogenous inhibitor of the tryptase Clara in the airway, with a K_i value of 9.7×10^{-8} M and which thus acts as a defensive compound against influenza virus infection (Beppu et al., 1997). SLPI is secreted from non-ciliated secretory airway epithelial cells, such as Clara and goblet cells (Mooren et al., 1983; Puchelle et al., 1985), and is found in bronchial lavage fluid and nasal and salivary secretions (Ohlsson et al., 1983), as well as in the walls of the alveoli (Willems et al., 1989). SLPI has been studied as an inhibitor of granulocyte elastase that can confer protection against the parenchymal destruction induced by granulocyte elastase in pulmonary emphysema and cystic fibrosis (Lucey et al., 1990; McElvaney et al., 1993; Stolk et al., 1994).

An investigation in our laboratory identified a new physiological role for SLPI as a defensive compound against IAV infection. Figure 1 shows the effect of intranasal administration of recombinant SLPI (rSLPI) at a dose of 6 μg 15 times every 8 h after infection of rats with 1×10^4 plaque-forming units (PFU) of influenza A/Asia/1/57 (H2N2) virus. Without treatment with rSLPI, progeny virus in the rat lung had increased by approximately 3000-fold on days 3–5 after virus inoculation, when it started to undergo termination. Under these conditions, over 95% of the progeny virus particles were proteolytically activated and became infectious in the lungs, indicating that trypsin-type proteases proteolytically activate the virus even in the presence of inhibitors in the airway. When rSLPI was administered intranasally, the lung viral titer was markedly reduced to less than 10% of that without rSLPI treatment, and almost all the progeny virus was in a non-infectious form with proteolytic

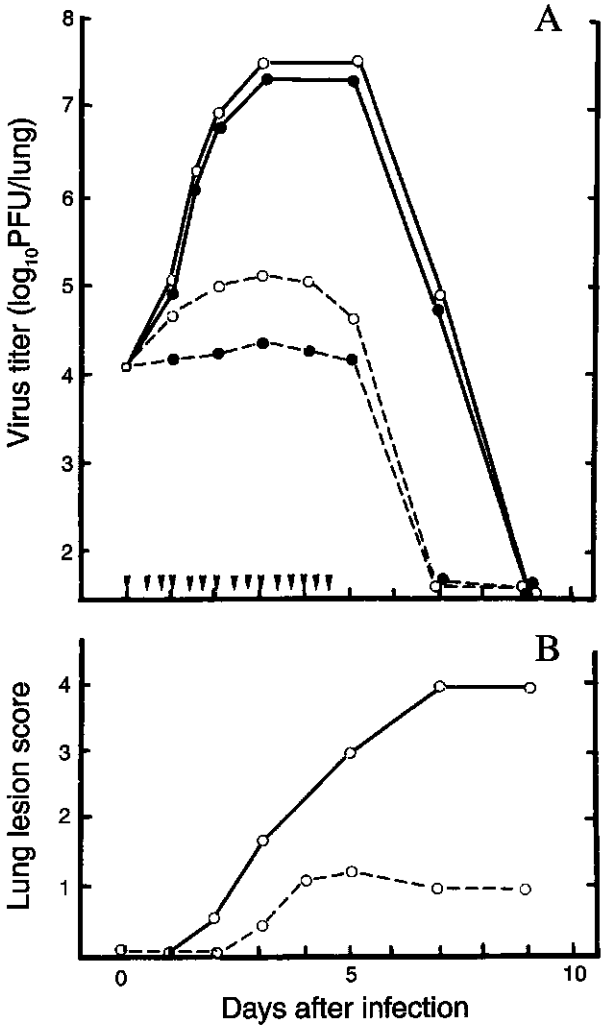

Figure 1 Effects of rSLPI on viral replication in the lungs (A) and pulmonary pathology of rats infected with mouse-adapted influenza A/Asia/1/57(H2N2) virus (B).
The influenza A/Asia/1/57(H2N2) virus was a gift from Dr R. Rott (Justus-Liebig University, Giessen, Germany). Three-week-old male rats of CD(SD) strain were infected intranasally with 1×10^4 PFU of influenza A Asia/1/57 (H2N2) virus (□). One group was administered 6 μg of rSLPI in 50 μl of phosphate-buffered saline (PBS) intranasally every 8 h (arrowheads) for a total of 15 administrations, and the other group received 50 μl of PBS as a control. (A) Virus titer in the lungs in the absence (solid lines) or presence (broken lines) of rSLPI. Lung homogenates were assayed for total yield (○) and proteolytically processed virus (●). Each plot represents the mean value for three animals. (B) Lung pathology of infected rats without (solid lines) or with (broken lines) the administration of rSLPI. Lung lesions were scored from 1 to 4, according to the extent of macroscopic consolidation of the lung surface (Beppu et al., 1997). Each plot represents the mean value of three animals.

activation absent. Lung lesions in the rSLPI-treated animals, which became manifest 1–2 days after virus proliferation and continued even after the termination of virus multiplication, were reduced as compared with the untreated animals. SLPI average levels in normal bronchial and nasal lavage fluids range between 11.0±1.2 nM (Vogelmeier et al., 1991) and 208.9±44.5 nM (Lee et al., 1993), respectively, and apparently are insufficient for protection from viral infection. These results suggest SLPI as a potential endogenous inhibitor of the multi-

cycles of viral replication in the airway, and show that intranasal administration of SLPI can efficiently prevent influenza virus infection.

SLPI is organized in two separate domains with an architecture similar to that of other serine protease inhibitors (Grütter et al., 1988), and the C- but not the N-terminal domain inhibits the tryptase Clara and trypsin activity (Van-Seuningen and Davril, 1991; Beppu et al., 1997). Studies on SLPI C-terminal deletion mutants revealed that the minimal size required for tryptase Clara inhibition is the peptide sequence Lys^{60}-Ala^{107}. Site-directed mutagenesis of the C-terminal domain of SLPI indicated that Leu^{72}-Met^{73} is the inhibitory site for the tryptase Clara, and mutants with residue Leu^{72} substituted with residues Arg^{72} and Lys^{72} had increased inhibitory activity against tryptase Clara (Kido et al., 1999).

Pulmonary surfactant as a defense against IAV in the lower respiratory tract

We found that pulmonary surfactant, a lipoprotein complex that coats the alveolar epithelium so as to lower the surface tension at the air-liquid interface and to increase phagocytosis by mononuclear cells and alveolar macrophages, efficiently adsorbed tryptase Clara and inhibited tryptase activity with a K_i value of 0.13 μM, although it did not inhibit ectopic anionic trypsin in the lungs (Kido et al., 1993). Figure 2A shows the inhibitory effects of pulmonary surfactant on the activation of the IAV Aichi/2/68(H3N2) *in vitro*. After activation of IAV for 20 min at 37°C by tryptase Clara pre-incubated with various concentrations of pulmonary surfactant for 20 min at 0°C, infectivity was measured by immunofluorescent cell-counting (Kido et al., 1993). Tryptase Clara caused up to a 100-fold increase in the infectivity of the inactive IAV, and the viral activation was inhibited by the surfactant in a dose-dependent manner from 5 to 500 μg/ml *in vitro*. Intranasal administration of pulmonary surfactant every 6–8 h shortly after infection of rats with Sendai virus, or murine parainfluenza virus type 1, suppressed virus multiplication in the lungs to approximately 10% of the viral titer in the lungs of control rats treated with vehicle (Tashiro et al., 1996). The cleavage of Sendai virus F_0 protein by the tryptase Clara, but not by trypsin, into its subunits F_1 and F_2 was inhibited in a dose-dependent manner by the pulmonary surfactant (Figure 2B). The major constituents of the pulmonary surfactant, phosphatidylcholine, phosphatidylglycerol, phosphatidylserine and pulmonary surfactant protein A (SP-A), by themselves did not show any inhibitory effects on tryptase activity. Surfactant reconstituted with these constituents in a ratio similar to the natural state exhibited adsorption of tryptase Clara, resulting in inhibition of the proteolytic activity.

Ambroxol, a mucolytic agent, and clarithromycin, a macrolide antibiotic, increase antiviral defense factors in the airway and protect against IAV infection

Since the activity levels of trypsin-like processing proteases for the IAV and parainfluenza virus envelope gly-

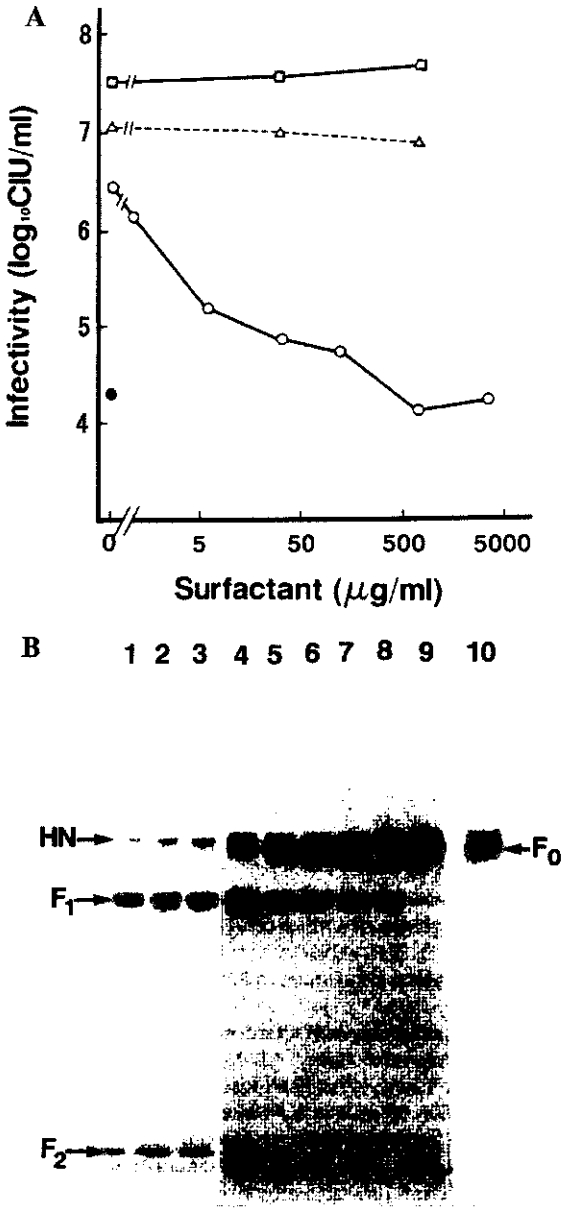

Figure 2 Inhibitory effects of pulmonary surfactant on (A) proteolytic activation by tryptase Clara and infection of influenza virus in MDCK cells and (B) cleavage of F_0 of the Sendai virus by tryptase Clara.
(A) Tryptase Clara purified from rat lungs (Kido et al., 1992) (50 μg/ml; ○) and bovine pancreatic trypsin (10 μg/ml; △) were preincubated with surfactant at the concentrations indicated at 0°C for 20 min. Then the mixtures were incubated with inactive progeny influenza A/Aichi/2/68(H3N2) virus (●) grown in MDCK cells for 20 min with tryptase Clara, or for 10 min with trypsin at 37°C. The infectivity was assayed in terms of cell infecting units by the immunofluorescent cell-counting method (Kido et al., 1993). The infectivity of active virus grown in embryonated chicken eggs (□) after treatment with surfactant was also measured. Concentrations of surfactant are expressed as those of the phospholipid levels. (B) Trypsin (10 μg/ml, lanes 1–3) and tryptase Clara (50 μg/ml, lanes 4–9) were incubated without surfactant (lanes 1 and 4) or with 0.26 mg/ml (lanes 2 and 5), 0.52 mg/ml (lane 6), 0.78 mg/ml (lane 7), 1 mg/ml (lanes 3 and 8), and 3 mg/ml (lane 9) of surfactant in 20 μl of 100 mM Tris-HCl buffer, pH 7.2, at 0°C for 5 min. Thereafter, [^{3}H]glucosamine-labeled inactive Sendai virus (lane 10) was incubated with the reaction mixture at 37°C for 10 min. The viral polypeptides were separated by SDS-PAGE under reducing conditions, followed by fluorography.

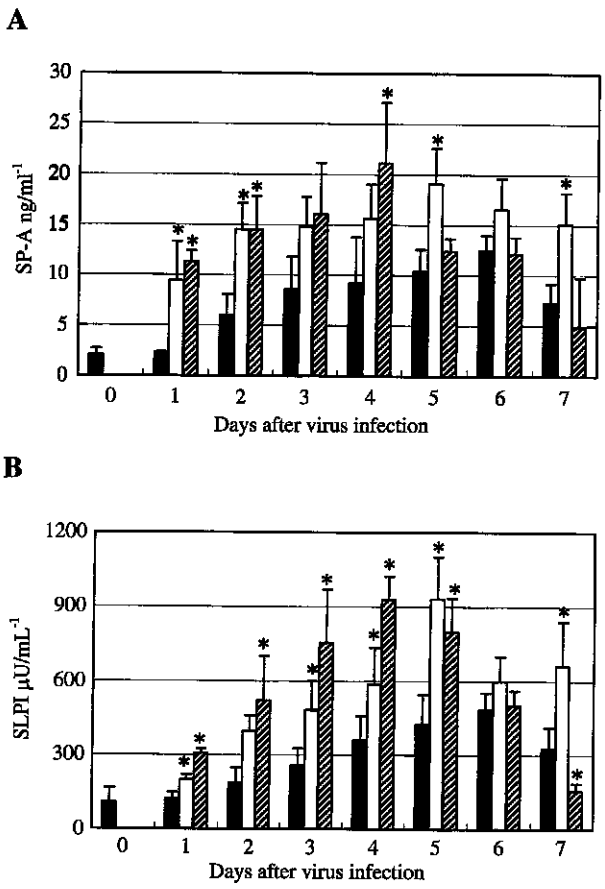

Figure 3 The effects of ambroxol on the levels of (A) pulmonary surfactant and (B) SLPI in bronchoalveolar lavage fluid (BALF) of mice infected with a lethal dose of influenza A/Aichi/2/68(H3N2) virus.
The levels of pulmonary surfactant were analyzed by enzyme-linked immunosorbent assay (ELISA) and are expressed as those of SP-A. The levels of SLPI were analyzed as reported by Yang et al. (2002). SP-A and SLPI levels in BALF of mice treated with saline (■), ambroxol at 10 mg/(kg day) (□) and ambroxol at 30 mg/kg/day (▨). Data are presented as mean values±SD (n=5). *$p<0.05$.

coproteins are higher than those of inhibitors in the airway, non-infectious progeny viruses are readily converted to infectious mature viruses by proteolytic activation. Therefore, the levels of SLPI and pulmonary surfactant may constrain the initial rate of viral entry and multiplication. Furthermore, in the late phase of infection, the levels of IgA and IgG against these viruses determine the severity of the pathology and prognosis of outcome. Along with an increase in the influenza viral titer in the airway fluids, increases in the secretion of trypsin-type protease, SLPI and pulmonary surfactant in the airway were observed, with peaks on days 4–5 in infected mice (Yang et al., 2002).

We found that ambroxol, known as a mucolytic agent with antioxidant (Gillissen et al., 1997) and anti-inflammatory properties, such as the ability to reduce the release of inflammatory cytokines (Pfeifer et al., 1997; Gibbs et al., 1999), stimulated a significant release of pulmonary surfactant on day 1 and onwards, even after IAV infection (Figure 3A). It also stimulated the release of SLPI (Figure 3B) and IgA (Yang et al., 2002) in the middle-late phase of infection after treatment for 4–5 days. An increase in the levels of these defense factors in the airway leads to significant suppression of virus multiplication in bronchial lavage fluid and an improved survival rate in mice (Figure 4A,B). When mice were treated with ambroxol at 10 mg/(kg day), one-half survived even a normally lethal dose of influenza virus, and viral proliferation was significantly suppressed. All of the effects of ambroxol reached a peak at a dose of 10 mg/(kg day), but a higher dose of 30 mg/(kg day) was less effective. Treatment with the higher dose of ambroxol rapidly increased the levels of these defense factors, with the peaks being reached earlier on days 4–5, but the levels could not be maintained, resulting in a rebound of virus titer and lethality in the late phase of infection. These findings suggest that ambroxol stimulates secretion rather than synthesis of these defense factors in target cells, and further, that maintenance of these defense compounds at high levels into the late phase may be necessary for improved survival. The stimulatory effect of

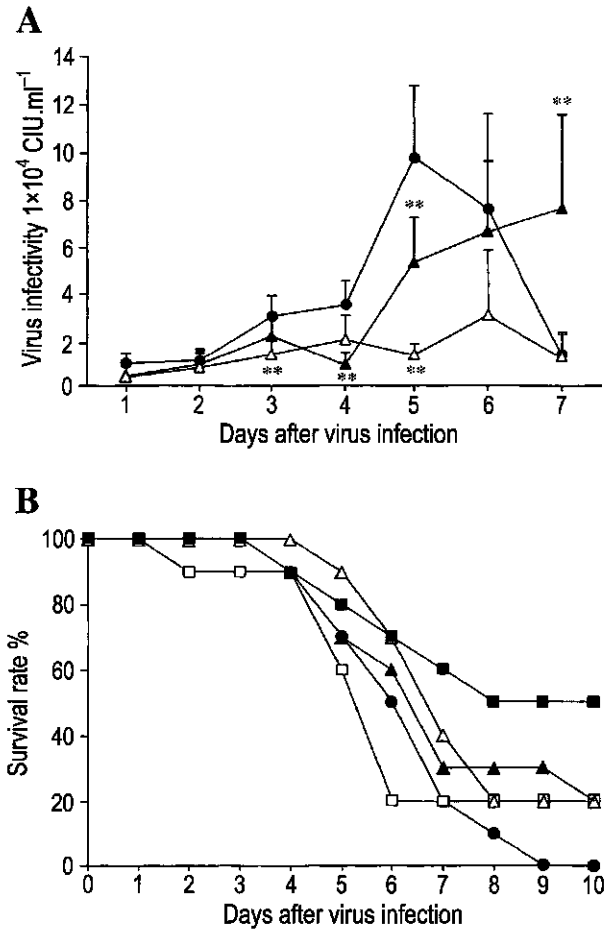

Figure 4 The effects of ambroxol on (A) virus proliferation in BALF and (B) the survival rate after infection of mice with influenza A/Aichi/2/68(H3N2) virus.
(A) Each group of mice (n=80) was infected with 6.6×10^{4} PFU of IAV and then treated with saline (●) or ambroxol at a total daily dose of 10 (△) and 30 (▲) mg/(kg day), respectively. CIU, cell infecting unit. Data are presented as mean values±SD. **$p<0.01$. (B) Each group of mice (n=10) was infected with 6.6×10^{4} PFU of IAV and then injected with saline (●) or ambroxol *i.p.* twice daily, at a total daily dose of 4 (▲), 10 (■), 20 (△) and 30 (□) mg/(kg day), respectively. Data are expressed as % survival rate.

ambroxol is not specific for secretion of the defense compounds in the airway, as it also stimulated the release of trypsin-like proteases. However, overall, ambroxol predominantly increased the defense compounds, resulting in suppression of virus multiplication in the airway.

Macrolide antibiotics have been used as bactericides in diffuse panbronchiolitis and have recently attracted attention as modulators of cytokine production and immune responses. Among the macrolides, FK-506 actually suppresses immune responses by interaction with FKBP-12, and erythromycin inhibits inflammatory responses by the suppression of interferon-γ induction. We could show that clarithromycin (CAM), a derivative of erythromycin, significantly enhances mucosal immunity and neutralizing IgA levels for IAV in the airway of IAV-infected mice. Oral administration of CAM at 10 mg/(kg day) immediately after infection with a lethal dose of influenza A Aichi/2/68(H3N2) virus significantly augmented the levels of interleukin (IL)-12 in airway fluids on day 4 in the late-early phase of infection, although IAV infection itself slightly induced IL-12 on days 1–2 as a mucosal immune response (Tsurita et al., 2001). CAM also suppressed the levels of inflammatory cytokines in the airway, such as tumor necrosis factor-α and interferon-γ, but did not affect the levels of the direct regulatory compounds for IAV multiplication, such as SLPI and the trypsin-type processing proteases. Furthermore, CAM enhanced the induction of IgA markedly and IgG moderately after infection for 6 days in the late phase of infection. Enhanced IgA and IgG induction by CAM may play a role in the suppression of virus multiplication from the late-early phase to the late phase of infection, thus increasing the survival rate. Since IL-12 is an effective adjuvant for the induction of mucosal immunity (Boyaka et al., 1999), augmentation of IL-12 by CAM may play a central role in mucosal immune responses to IAV infection (Ide et al., submitted for publication).

Conclusions

IAV and parainfluenza virus are exclusively pneumotropic, and the target of infection is further restricted to airway epithelial cells, although the virus receptor sialic acid is widely distributed across various cell types in the lungs and other organs. It has been postulated that the pneumotropism of these viruses is determined by a trypsin-like protease(s) present in the respiratory tract, which cleaves the precursors of the progeny virus envelope fusion glycoproteins to induce fusion activity, thereby enabling the viruses to undergo multiple cycles of replication. These proteases are strictly regulated by the activity of certain inhibitory compound(s) in the airway. One of these in the upper respiratory tract is SLPI. The inhibitory activity of SLPI resides in the C-terminal domain, and the Lys^{60}-Ala^{107} peptide constitutes the minimal efficient size of SLPI, in which the Leu^{72}-Met^{73} site has been identified as the molecule's inhibitory site by site-directed mutagenesis. The other inhibitory compound in the lower respiratory tract is pulmonary surfactant, which adsorbs the tryptase Clara but not trypsin, and inhibits the proteolytic activation of the virus envelope fusion activity. Despite the presence of these inhibitory compounds, the envelope glycoproteins of these progeny viruses are readily processed after budding in the airway under normal conditions. The intranasal administration of rSLPI and pulmonary surfactant to increase these natural inhibitory compounds to levels greater than those of the disease-enabling proteases significantly suppressed virus entry into cells, as well as multi-cycles of viral replication, *in vivo*.

The other strategy for prevention of IAV infection is up-regulation of levels of the host defense compounds, such as SLPI and pulmonary surfactant in the early phase and IgA in the late phase of infection. We found that at optimal doses, ambroxol, a mucolytic and anti-oxidant agent, stimulates the release of pulmonary surfactant and SLPI at pharmacological levels in the airway, levels sufficient to inhibit virus proliferation in the early phase and to stimulate IgA in the late phase of infection. In addition, CAM, a macrolide antibiotic, enhances mucosal immunization through augmentation of IL-12 levels in the airway, and increases IgA and IgG levels in the late phase of infection. Besides the well-known anti-influenza agents, the neuraminidase inhibitors, which selectively inhibit the release of virus from infected cells, natural inhibitors of the processing protease for viral envelope glycoproteins that inhibit viral entry, and/or chemical agents that up-regulate both these inhibitory compounds and IgA and IgG in the airway, are potentially useful candidates for treating the persistent health challenge of influenza virus infection.

Acknowledgments

Our studies were supported in part by Grants-in-Aid (13557014) and CLUSTER project from the Ministry of Education, Science and Culture, and Research on Brain Science (H12-Brain-015) from the Ministry of Health, Labor and Welfare of Japan. We appreciate the review of the manuscript by Pacific Edit prior to submission.

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