Inhibitory Effect of a Protease Inhibitor, Leupeptin, on the Development of Influenza Pneumonia, Mediated by Concomitant Bacteria

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SUMMARY

The protease inhibitor leupeptin prevented multiple step replication of an influenza virus (A/swine/1976/31, H1N1) mediated by staphylococcal proteases. It also suppressed virus replication and development of fatal pneumonia in mice co-infected with the virus and Staphylococcus aureus.

With avian influenza viruses it could be demonstrated convincingly that post-translational proteolytic cleavage of the viral haemagglutinin glycoprotein by cellular proteases is essential for infectivity, spread of the virus in the host organism and pathogenicity (Rott & Klenk, 1986). In a host cell where an appropriate protease for cleavage is not present, and as a consequence infectious virus cannot be produced, infectivity can be recovered by treatment of non-infectious virus by trypsin in vitro (Klenk et al., 1975; Lazarowitz & Choppin, 1975). Recently it could be shown that Staphylococcus aureus, which is most frequently isolated from patients with influenza pneumonia (Stuart-Harris et al., 1985), secretes serine proteases that activate the infectivity of several influenza A viruses by proteolytic cleavage of the haemagglutinin (Tashiro et al., 1987a, b). The presence of the bacterial enzyme enables the virus to undergo multiple replication cycles in host cells that do not possess an activating protease. Correspondingly, co-infection of mice with influenza and S. aureus resulted in an enormous increase of virus titres in the lung with extensive lesions in the lung tissue and with death of the mice. It has also been found that plasmin-like alveolar proteases might promote cleavage of the haemagglutinin of virus strains susceptible to plasmin activation. Administration of protease inhibitors such as ε-amino caproic acid or aprotinins to mice infected with plasmin-sensitive influenza viruses reduced virus replication in the lung (Zhirnov et al., 1982, 1984).

In this communication we report that leupeptin suppresses cleavage of the haemagglutinin by S. aureus proteases and thereby inhibits spread of influenza virus in the respiratory tract of mice. Leupeptin is a small peptide (acetyl-L-leucyl-L-leucyl-L-argininal) produced by actinomycetes, which inhibits specifically trypsin, plasmin, papain, kallikrein and cathepsin B, all of which cleave a peptide bond on the carboxyl side of basic amino acids, arginine or lysine (Aoyagi & Umezawa, 1975). Since the haemagglutinin of influenza viruses has an arginine at its cleavage site, we expected that leupeptin would inhibit cleavage activation by the bacterial proteases.

For the combined viral–S. aureus (Wood 46 strain) infections (Tashiro et al., 1987a, b) the plasmin-resistant influenza virus strain A/swine/1976/31 (H1N1) was used to exclude interference by plasmin (Zhirnov et al., 1982).

Polyacrylamide gel electrophoresis revealed that proteolytic cleavage of the haemagglutinin by the staphylococcal enzymes was inhibited by leupeptin (data not shown). If chicken embryo (CE) cells were infected with the A/swine strain at an m.o.i. of $10^{-2}$ p.f.u./cell, progeny virus...
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Fig. 1. Inhibitory effect of leupeptin on activation of influenza A/swine/1976/31 (H1N1) by staphylococcal protease. Monolayer cultures of CE cells were infected with A/swine virus at an m.o.i. of 10^{-2} p.f.u./cell and incubated with MEM containing 200 µg/ml of protease from S. aureus strain Wood 46. Leupeptin was added to the medium at a concentration of 0 µg/ml (○), 10 µg/ml (△) or 100 µg/ml (■). Cells were infected with virus in the absence of bacterial protease and leupeptin (○). At intervals the culture medium was assayed for virus growth by haemagglutination (HA). Leupeptin alone did not have any inhibitory effect on haemagglutination.

Fig. 2. Appearance of lung lesions and growth of influenza virus and S. aureus after treatment with leupeptin in lungs of infected mice. Mice were infected intranasally (i.n.) with 10^2 p.f.u, of A/swine/1976/31 and 10^6 c.f.u, of S. aureus (Wood 46). One day after infection leupeptin (10 µg/mouse) was given i.n. several times as indicated by arrows. The animals were killed at the days indicated and 10% lung homogenates were prepared. Lung lesions (a) in mice treated (△) or not treated (○) with leupeptin were graded 1 to 4 according to the surface consolidation area and one point was added when the mouse died. Infectious virus (b) was determined by plaque assay on CE cells. Lung homogenates from leupeptin-treated (△, △) or untreated (○, ○) mice were supplemented with trypsin (●, ●) or left as such (○, △) (see Tashiro et al., 1987b). The number of bacteria (c) was determined by the colony counting method on nutrient agar plates. Each plot represents the mean of three mice.

Tashiro et al., 1987a, b). As shown in Fig. 1 leupeptin could prevent multistep replication of virus when added in addition to the staphylococcal proteases. The inhibitory effect clearly depended on the leupeptin dose. In addition to the proteases of the Wood 46 strain, the enzymes of other S. aureus strains were equally inhibited (data not shown).

To test whether leupeptin has a therapeutic effect, STU mice anaesthetized with ether and intranasally infected simultaneously with 10^2 p.f.u. of A/swine virus and 10^6 c.f.u. of S. aureus Wood 46 strain were treated intranasally with 10 µg leupeptin. When the inhibitor was given several times, starting from day 1 after infection (p.i.) up to day 6 p.i., the virus titre in the lung homogenates was significantly reduced when compared with the control animals (Fig. 2b). The infectivity in the lung tissue could be markedly enhanced by adding trypsin before the plaque test. This shows that most of the virus produced in the presence of leupeptin was non-infectious which is in contrast to the results obtained from mice not treated with the inhibitor. In this case high titres of virus were produced in the mouse lungs which could not be increased further by treatment in vitro with trypsin. Similarly, the development of lung lesion was significantly suppressed in leupeptin-treated mice (Fig. 2a) and the animals survived over a 12 day
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observation period. In contrast, mice infected with virus and bacteria but not treated with the protease inhibitor usually died between 5 and 7 days after infection, with extended lesions in the lung tissue. The number of staphylococci in mice treated or not treated with leupeptin was similar (Fig. 2c), which might indicate that the protease inhibitor had no influence on the growth of the bacteria. Finally, preliminary experiments confirmed that intranasal administration of leupeptin up to 100 μg per mouse did not show any toxic effect.

These results confirm our concept that a synergism exists between influenza virus and S. aureus in the development of pneumonia based on proteolytic activation of the haemagglutinin by bacterial proteases (Tashiro et al., 1987a, b). The data also show that leupeptin, which by itself has no toxic effect, prevents cleavage of the haemagglutinin and thereby inhibits the spread of the virus in the respiratory tract of mice. Prolonged administration of the protease inhibitor protected the mice from developing severe pneumonia and death. These observations might open a way for therapeutic treatment of influenza pneumonia by leupeptin or similar trypsin inhibitors, particularly in infection with bacteria which are resistant to antibiotics.

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REFERENCES


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