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Aprotinin aerosol treatment of influenza and paramyxovirus bronchopneumonia of mice

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Summary

The therapeutic efficacy of aerosolized aprotinin, a natural proteinase inhibitor, against influenza and paramyxovirus bronchopneumonia of mice is shown. Small-particle aerosol of aprotinin solution was generated by a Collison type nebulizer and infected mice were exposed to aerosol atmosphere by four 30–40 min incubations per day for 6 days. This regimen provided an inhalation aprotinin dosage of approx. 6 μg/mouse/day. With such treatment more than 50% of mice infected with lethal doses of either influenza virus or paramyxovirus were protected from death. A suppression of the development of fatal hemorrhagic bronchopneumonia and a normalization of the body weight gain were observed in infected mice treated with aerosolized aprotinin. These data suggest that low doses of aerosolized proteinase inhibitors could be successfully applied against respiratory influenza-like virus diseases.

Influenza virus; Paramyxovirus; Bronchopneumonia; Aerosol treatment; Antivirals; Aprotinin

Introduction

The influenza viruses and paramyxoviruses, like other enveloped viruses, contain glycoproteins which are located outside the virion lipid bilayer as 'spikes'. The spike glycoproteins, HA of influenza viruses and F of paramyxoviruses, undergo post-translational proteolytic cleavage. Glycoproteins synthesized as precursor proteins with m.w. approx. 75 kD are cleaved to

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yield two disulphide-linked subunits with m.w. 55 and 20 kD, respectively (Skehel and Waterfield, 1975; Scheid and Choppin, 1977; Lambert and Pons, 1983). The cleavage of these viral glycoproteins is accomplished by host trypsin-like proteinases (Lazarowitz et al., 1973; Silver et al., 1978; Rott et al., 1980; Muramatsu and Homma, 1980; Zhirnov et al., 1982b; Appleyard and Davis, 1983; Tashiro et al., 1990; Gotoh et al., 1991; Kido et al., 1992). This cleavage is a prerequisite for the infectivity of the virions (Scheid and Choppin, 1974; Lazarowitz and Choppin, 1975; Klenk et al., 1975; Ohichi and Homma, 1976) and thus for the spread of the virus in the host organism (Nagai et al., 1979; Bosch et al., 1980; Itoh et al., 1990; Tashiro et al., 1991; Ogasawara et al., 1992).

It has been reported previously that aprotinin, a natural proteinase inhibitor (Fritz and Wunderer, 1983), suppresses the cleavage of the orthomyxo- and paramyxovirus fusion protein and prevents the activation of virion infectivity in chicken eggs (Zhirnov et al., 1985) and in mouse lungs (Zhirnov et al., 1984; Hayashi et al., 1991). Intraperitoneal and intranasal instillations of relatively high aprotinin doses (1500–2500 TIU/mouse/day) in influenza or paramyxovirus-infected mice were shown to interfere with virus replication and to protect mice from lethal virus pneumonia (Zhirnov et al., 1982a; Zhirnov 1987; Hayashi et al., 1991). Noteworthy, these administration routes are probably inadequate against respiratory infections because: (i) only 0.5–2.0% of the polypeptide drug infused intravenously appears to reach the lungs (Wewers et al., 1987; Koscar et al., 1972); (ii) intranasal infusions obviously lead to preferential deposition of substance into the upper airways and do not deliver it onto the lower regions. We have suggested that exposition of infected mice to small-particle aerosol atmosphere would permit inspiration of air-aprotinin mixture and its targeting to the respiratory infection loci. Here we show that infected mice were protected from death and lungs showed a therapeutic effect when infected mice had inhaled a small-particle aerosol. With such treatment only approx. 6 μg of aprotinin per mouse per day were received through inhalation.

Materials and Methods

Viruses. Influenza virus A/Aichi/2/68 (H3N2) (pneumotropic for mice) and paramyxovirus Sendai/960 were propagated in 9-day-old embryonated eggs, as described previously (Zhirnov et al., 1985).

Aerosol-generating system. To optimize the delivery of aprotinin to the respiratory organs, a Collison type ultrasonic nebulizer was used that maximized the generation of small-particle aerosol. The aerosol was directed into the exposition chamber and the droplets of aerosol flow were analysed by a Laser Malvern Controller (Malvern Instruments, LTD). This measurement showed that approx. 90% of particles had an aerodynamic mass diameter of

1.3–3.0 μm. This particle size is known to be appropriate for deposition throughout the respiratory airways including the alveolar regions (Schlesinger, 1985; Newmann et al., 1986). To accomplish this, a solution of aprotinin (either Gordox®–Gedeon Richter, Hungary (Malis et al., 1979); or Contrycal®–AWD, Germany) at 5000–10 000 Kallikrein-Inhibiting Units (KIU)/ml (*Ca*) in distilled water containing 0.5% glycerol, was placed into the reservoir of the nebulizer and was aerosolized at a rate of 0.1 ml/min (*Qa*). The aerosol thus produced was mixed with compressed air, which was compulsorily introduced into the 0.015 m^3 exposition chamber at a flow rate of 9 l/min(*Vc*). Aerosol flow directed into the chamber contained 0.06–0.11 KIU of aprotinin per ml of air (*Cc*), that was calculated by the equation $Cc = Ca*Qa/Vc$.

To evaluate the possible effect of aerosolation on the function of aprotinin, the chamber aprotinin aerosol flow was collected by bubbling through buffer saline (pH 7.4). The resultant fluid was concentrated by pressure filtration and was then tested for ability to inhibit trypsin. It was found that the aerosolation process used had no significant effect on aprotinin inhibitory function (not shown).

Evaluation of the aprotinin dosage. The 6-day course of treatment starting after infection included four 30–40 min expositions a day of infected mice to aprotinin aerosol atmosphere in the chamber. The aprotinin deposition in the respiratory tract was calculated by the Guyton's equation $Dp = Cc*k*V*4t*R*P$ (Guyton, 1947). $R = 0.75$ (estimated fraction of inhaled aerosol deposited in the respiratory tract); t, period of one inhalation (35 min); P, mouse body weight (9–10 g); V, mouse respiratory rate (1.2 ml/g/min); Cc, aprotinin concentration in the aerosol flow directed into the exposition chamber; k, was assumed as 1, because input and output compulsory aerosol flows were equal during period of inhalation. The calculated aprotinin dosage was 50–120 KIU/mouse/day. It should be noted that 1 KIU corresponds to approx. 0.33 Trypsin-Inhibiting Unit (TIU) and 0.14 μg of aprotinin protein (Trautschold et al., 1967; Fritz and Wunderer, 1983).

Experimental mice. Male outbred mice (6–8 g) were infected intranasally (approx. 50 μl/mouse) under light ether narcosis with the influenza virus or paramyxovirus at a multiplicity of 10–100 50% Mouse Lethal Doses (MLD50) per mouse. 1 h after infection either placebo (0.5% glycerol in distilled water), or aprotinin aerosol expositions were began. Each group usually contained 25–30 animals. The aprotinin aerosol was highly tolerated by animals and the inhalations did not change the behavior of the animals. No significant changes occurred in hematologic and biochemical values of blood; histologic analysis of animal organs; the functional examination of CNS and psychometer tests. The inhalation of aerosolized aprotinin failed to cause local irritation or (immediate or retarded) allergic reactions (data submitted for publication).

Histological examination. Lungs of mice were removed aseptically on day 4 after infection. Lung tissues were fixed in a solution consisting of 25%

formaldehyde and 5% acetic acid in a solution of saturated picric acid in water, embedded in paraffin, and stained with hematoxylin and eosin.

Results

In our experiments mice were infected intranasally with influenza A/Aichi/2/68 virus and paramyxovirus Sendai/960. With this route of virus inoculation, trachea, bronchi, and lungs are involved in the infection process (Yetter et al., 1980; Tashiro and Homma, 1983). Therefore, to optimize the deposition of aprotinin throughout the respiratory tract, 1.3–3.0 μm droplet aerosol was applied for breathing of infected mice. This particle size is known to be appropriate for deposition throughout the respiratory airways including the alveolar regions (Schlisinger, 1985; Newman et al., 1986). The successful therapeutic effect of aprotinin aerosol inhalations was primarily demonstrated by examination of macroscopic and histologic pulmonary pathology. Such examinations of lung lesions were performed on day 4, when virus yields in the lung of infected mice peaked (Tashiro and Homma, 1983; Zhirnov et al., 1984). In control non-infected mice (treated with placebo or aprotinin aerosol), neither lung infiltration nor histological lesions were found and only loci of non-specific inflammation were occasionally identified (Fig. 1A/3, 1B/3, 2A). In contrast, as can be seen in Fig. 1A/1, there was a fatal confluent hemorrhagic inflammation of the lung tissue in the placebo-treated mice infected with Aichi/2/68 influenza virus. A few loci (30–50% of lung surface) of hemorrhagic inflammation in the lungs of aprotinin-treated mice were observed. Similar hemorrhagic pneumonia and reduction of the lung lesions under the aprotinin aerosol treatment were revealed in mice infected with Sendai virus (see Fig. 1B).

Histological evaluation revealed marked degenerative changes and sloughing of the bronchial and alveolar epithelium with concomitant lymphocytic and polymorphonuclear cell infiltration of bronchial walls and the lung interstitium. Venous congestion and bleeding into the interstitium were frequently seen. Many bronchi and alveoli become clogged and collapsed. Aprotinin-treated mice had much-reduced inflammatory response and morphologic lesions in lungs (see Fig. 2B). A characteristic finding in aerosol-treated mice was a process of intensive regeneration of bronchial and alveolar epithelium cells. Peribronchial and perivascular cell infiltration was observed to be less intense. Alveoli become free and extended. Blood microcirculation return to normal. Similar reduction of lung lesions in Sendai virus-infected mice were observed when aerosolized aprotinin was applied (data not shown). These findings additionally have shown a therapeutic efficacy of aprotinin aerosol treatment.

As have been shown previously, mice infected with Sendai virus lost body weight (Tashiro and Homma, 1983). Therefore, this parameter was also checked to evaluate the therapeutic activity of aprotinin inhalations. Each group of approx. 30 animals was monitored until 21 days after inoculation of

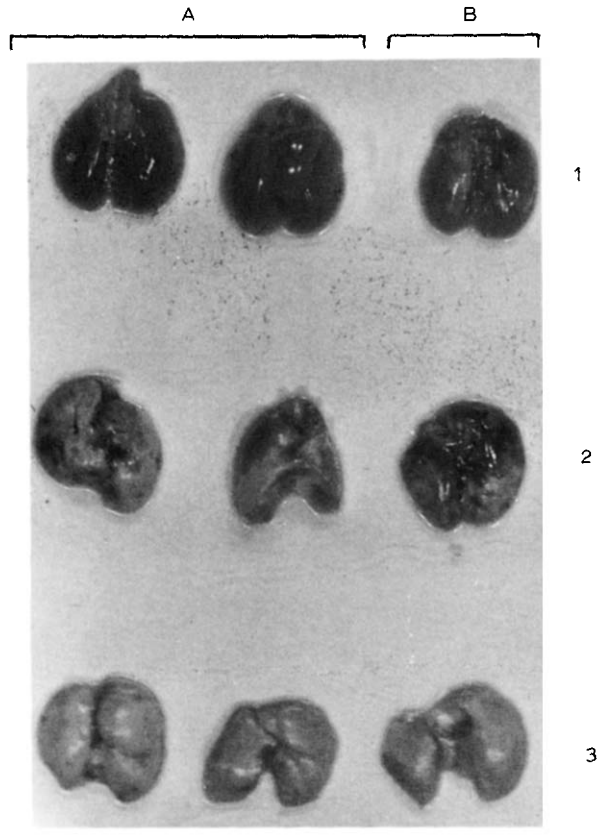

Fig. 1. Lung pathology of infected mice treated with aprotinin inhalations. Mice were infected with A/Aichi/2/68 (A) and Sendai/960 (B) viruses at a multiplicity of approx. 50 MLD50/mouse. After infection the mice were treated with either aerosolized placebo (1) or aprotinin (2), four 30–40 min expositions daily for 6 days. On day 4 after infection, the lungs were removed and photographed. (3) Lungs of uninfected mice treated with placebo.

the virus. The results of a typical experiment are shown in Fig. 3. The uninfected mice gained regularly in weight, approx. 1 g per day (line 1), whereas those challenged with influenza virus at a nultiplicity approx. 50 MLD50/mouse did not increase in body weight and began to loose weight from the 3rd day up to the time of death (line 3). Conversely, influenza-infected mice inhalating the aprotinin aerosol (line 2) lowered the rate of the body weight gain only at the first days and then began to gain in weight at the normal rate (approx. 1 g per day). Similar kinetics of body eight gain were revealed in experiments with aerosol aprotinin treatment of Sendai-infected mice (data not shown).

Body weight gain of uninfected mice given the aerosolized aprotinin was similar to that of uninfected-nontreated mice (see Fig. 3; line 1). These results strengthen the conclusion regarding the beneficial therapy of respiratory

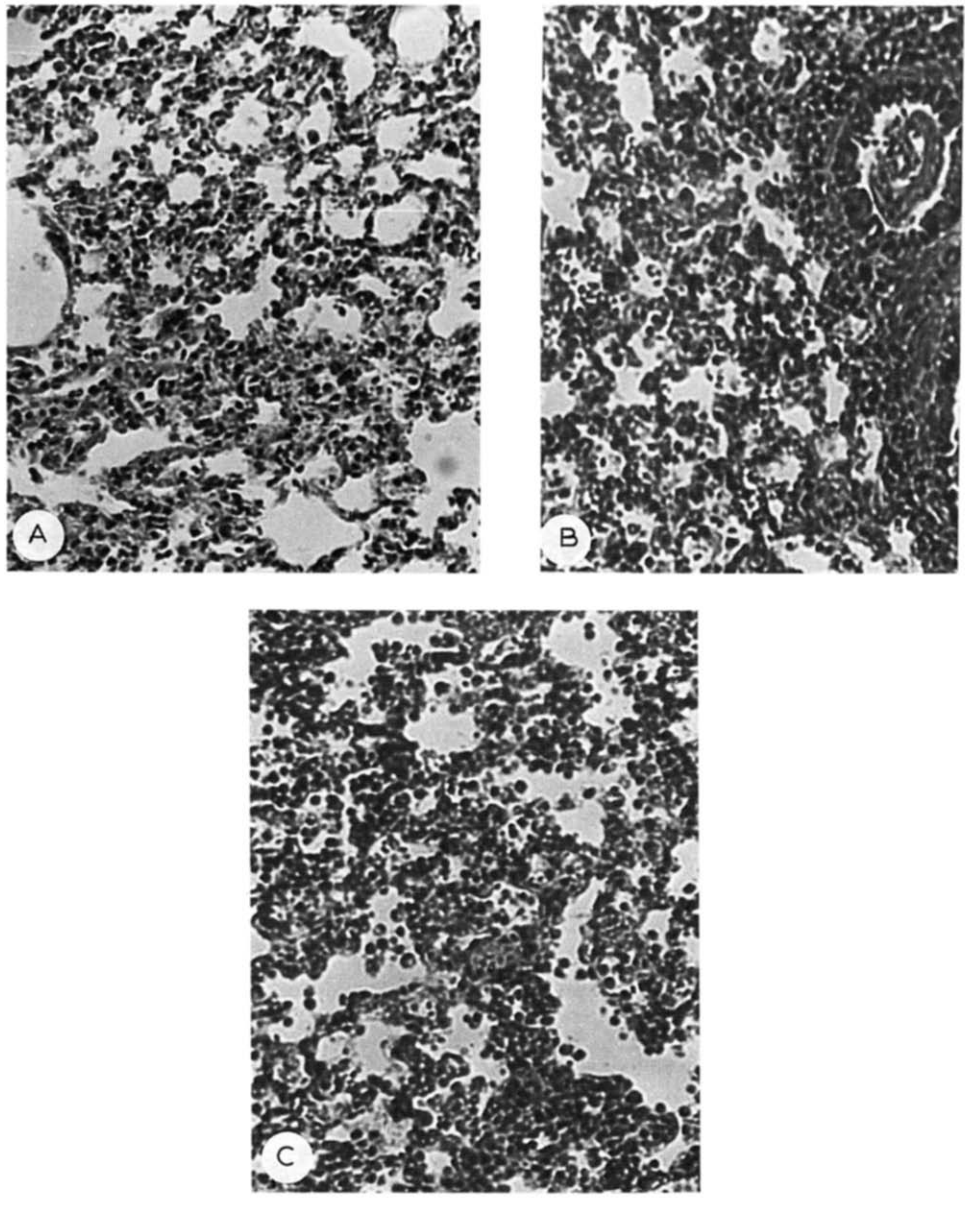

Fig. 2. Histology of infected mice treated with aprotinin inhalations. Mice were infected with A/Aichi/2/68 virus and treated with aprotinin inhalations (see legend to Fig. 1). On day 4 the lung sections were stained with hematoxylin and eosin. (A) Placebo-uninfected mice; (B) aprotinin-treated infected mice; (C) placebo-infected mice (original magnification × 440).

orthomyxo- and paramyxovirus infections with the aerosolized aprotinin.

In the final set of experiments the protective effect of aprotinin aerosol

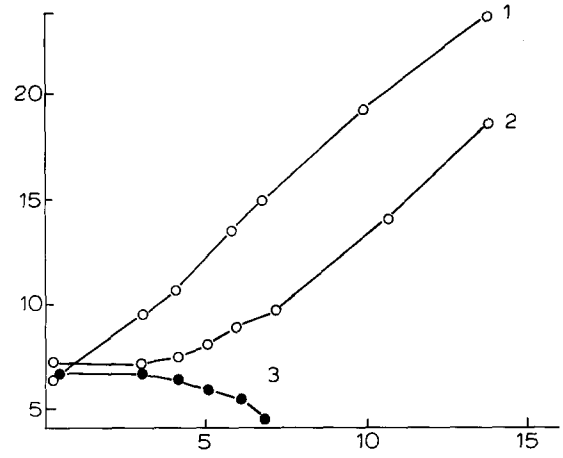

Fig. 3. Time course of the body weight in influenza-infected mice treated with aprotinin aerosol. Infection (approx. 50 MLD50/mouse) and treatment of mice described in the legend to Fig. 1. The surviving mice were examined for body weight on day 1, 3, 4, 5, 6, 7, 10, 15, and mean values (g) are indicated on ordinate. Line 1, placebo-treated uninfected mice; 2, infected, aprotinin-treated mice; 3, placebo-treated infected mice. Abscissa, days after infection.

treatment in mice infected with lethal doses of the viruses was studied. The typical results, showing the protective effects of aprotinin aerosol inhalations in mice infected with approx. 50 MLD50 per mouse of influenza A/Aichi/2/68 virus (A) and Sendai virus (B) are presented in Fig. 4. In the control non-treated group 100% lethality was registered on the 7th day after infection, whereas about 40–50% of animals survived on the 15th day due to 6.5 day long-course of treatment. These protective effects increased up to 70–90% when the challenge virus dose was lowered to approx. 10 MLD50 (data not shown).

Discussion

Proteinase inhibitors, including aprotinin, are potential antiviral agents (Korant, 1989; Zhirnov, 1988). In this communication we report that inhalation of aprotinin aerosol causes beneficially therapeutic effects in mice with ortho- and paramyxovirus bronchopneumonia. The therapeutic course included four short expositions in aprotinin aerosol atmosphere a day for 6 days. As calculated by Guyton's equation (Guyton, 1947), 15–30 TIU of aprotinin per day were inhaled by one mouse. These doses were approx. 100 times lower then those required for parenteral treatment of mouse influenza and paramyxovirus bronchopneumonia. It has been shown, that aprotinin protects against lethal Influenza and Sendai virus pneumonia of mice when it is

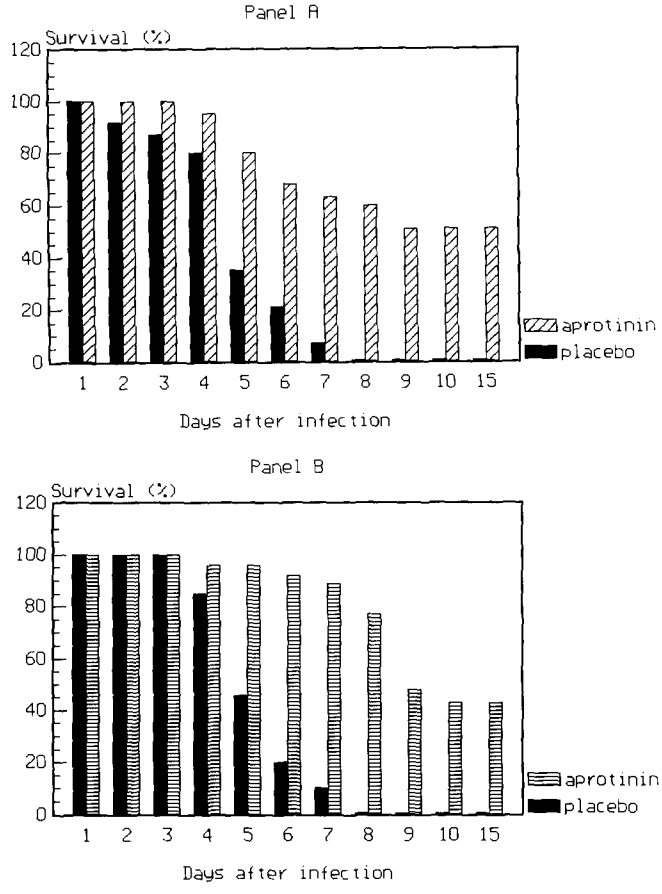

Fig. 4. Protection of orthomyxo- and paramyxovirus-infected mice by aprotinin aerosol. Mice (27–30 animals in each group) were infected with either influenza A/Aichi/2/68 or Sendai virus (B) viruses at a multiplicity of approx. 50 MLD50/mouse and, after infection treated with four 40-min expositions daily in either placebo (■) or aprotinin (▤) aerosol atmosphere for 6.5 days. Ordinate, survival rate (%); abscissa, days after infection.

administered through intraperitoneal or intranasal instillations at a dose of 1500–2500 TIU/mouse/day (Zhirnov et al., 1982a; Zhirnov, 1987; Hayashi et al., 1991). Thus, this comparison clearly demonstrates that aerosol aprotinin inhalations appear to be effective in the treatment of respiratory influenza and parainfluenza virus diseases.

It is well known that aerosol particles of 1.3–3.0 μm deliver significant quantities of inhaled compounds onto the middle and distal respiratory regions, the bronchi and the alveoli (Morrow, 1986). Moreover, 0.5–3.0 μm particles are sufficiently small to avoid obstructions at airway branch points (Schlesinger, 1985; Morrow, 1986) and are generally retained in the respiratory

tract and do not leave the lung with the expired air (Schlesinger, 1985; Newman et al., 1986). Notably, in our experiments infected mice were exposed in the chamber to aerosol atmosphere. It has been reported that under these conditions the small-droplet aerosol may appreciably dry up (Plyushch et al., 1990) and thus a mixture of dried and humid particles is inhaled by the mice.

The therapeutic effect of aprotinin is most probably the result of combined antiviral and antipathological actions. It is known that virus replication in cells is followed by the release of host proteases (Goldberg and Lazarowitz, 1974; Lazarowitz et al., 1973; Akaike et al., 1989; Tashiro et al., 1993b) which are one of the triggering factors of inflammation (Ratnoff, 1969; Furie, 1988; Barnett et al., 1990; Pround et al., 1990). On the other hand, host proteinases cleave the virus fusion glycopolypeptide to activate virion infectivity and afford virus virulence in the macroorganism (Klenk et al., 1975; Scheid and Choppin, 1974; Lazarowitz and Choppin, 1975). In short, influenza and influenza-like pathology evolvs via a vicious circle: the virus unleashes the host proteinases, which leads to an increased activation of virions and thus virus spread, which, again, enhances the release of host proteinases (for review see Zhirnov, 1983; 1987). It follows that aprotinin may normalize proteolytic balance, limit the inflammatory process and may suppress virus multicycle replication. Notably, virus replication is reduced in the lungs of aerosol-treated mice (unpublished data).

It is not clear whether the cleavage of the orthomyxo- and paramyxovirus fusion proteins is performed inside the cells or on the cell surface of the respiratory epithelium. There is evidence that at least a portion of virions is activated extracellularly by proteases in the bronchial secretions (Tashiro et al., 1993a). The epithelial-lining fluid was shown to contain a trypsin-like proteinase that effectively activates Sendai virus and influenza virus glycoproteins (Kido et al., 1992). This proteinase was found to be specifically sensitive to aprotinin. Moreover, both intra- and extracellular sites of such proteolytic cleavage seem to be vulnerable to exogenous aprotinin because the drug has been found to act both extracellularly (Zhirnov et al., 1982) and intracellularly (Zhirnov et al., 1984b; 1986). These data support our suggestion that direct targeting of aerosol aprotinin to the respiratory tract promotes efficient inhibition of the virus-activating proteinase.

The results reported here point to the therapeutic efficacy of aprotinin aerosol inhalations against virus bronchopneumonia. A manual inhalation of aprotinin may represent an ideal approach for prophylactic and therapeutic application of aprotinin especially during influenza outbreaks. In this context, we believe that a manual propellent-containing inhaler with dried micronized aprotinin will be suitable to generate the properly sized aerosol. Clinical trials are being planned. In the light of these trials aprotinin seems to have valuable characteristics. (i) Aprotinin is well known officinal compound lacking toxic side effects (Fritz and Wunderer, 1983; Muller et al., 1980; Freidenberg et al., 1981). (ii) Due to a low air-to-blood permeability of the lower respiratory tract to external polypeptides (Matthay et al., 1985; Gorin and Stewart, 1979;

Hubbard et al., 1989), the respiratory epithelial surface may serve as an 'accumulator' to prolong the aprotinin action. (iii) To augment the deposition of the compound in the respiratory area, absorption enhancers such as bile salts, surfactants, or acrylic resins may be included in the aerosol mixture (Gordon et al., 1985).

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