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Protection of mice by a protease inhibitor, aprotinin, against lethal Sendai virus pneumonia

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Proteolytic activation of Sendai virus in the lungs of mice is necessary to cause pneumopathogenicity. Using Sendai virus-infected lung block cultures, protease inhibitors were tested for their antiviral effect by examining inhibition of proteolytic activation. Among the inhibitors tested, a serine protease, aprotinin, was shown to be most effective. *In vivo* protection experiments demonstrated that aprotinin, when administered intranasally, could confer protection on mice against lethal Sendai virus pneumonia through the same mechanism as observed in the *in vitro* system. The present study provides an experimental basis for the use of protease inhibitors as antiviral drugs.

Sendai virus, a member of the paramyxoviruses, causes bronchopneumonia in mice and has been studied as a model of respiratory viral infections (Ishida & Homma, 1978). Sendai virus has two glycoproteins, HANA and F, on its envelope (Mountcastle *et al.*, 1971; Homma *et al.*, 1975). The HANA protein binds to cellular receptors and the F protein, after being cleaved into subunits F_1 and F_2 by a proteolytic enzyme, confers the envelope fusion activity on the virus by which the viral genome enters the host cell (Homma, 1971, 1975; Homma & Ohuchi, 1973; Homma & Tamagawa, 1973; Scheid & Choppin, 1974; Ohuchi & Homma, 1976). Thus, the cleavage of the F protein is a prerequisite for activation of virus infectivity. Mouse bronchial epithelium possesses a trypsin-like protease(s) and, with the aid of the enzyme, Sendai virus is activated to exert pulmonary pathogenicity (Tashiro & Homma, 1983*a*, 1985). On the other hand, when cells lacking such a protease are infected with Sendai virus, they produce inactive progeny virus with uncleaved F protein which can no longer infect neighbouring target cells because of its inability to fuse to the cell membrane (Homma, 1971; Homma & Tamagawa, 1973). It is likely therefore that, if the protease in the bronchial epithelium is inhibited, the progeny virus will remain non-infectious. Consequently, spread of the virus in the lung would be suppressed and the severity of disease would diminish. In the present paper we report that a protease inhibitor, aprotinin, conferred protection on mice against lethal Sendai virus pneumonia through suppression of the proteolytic activation of the virus.

Sendai virus-infected mouse lung block culture mimics *in vivo* pulmonary infection; the target of the virus is largely bronchial epithelium which possesses a protease(s) which cleaves the F protein and the virus can then replicate in a multiple step manner (Tashiro & Homma, 1983*b*). This *in vitro* system was used to determine potential antiviral activities of protease inhibitors. The inhibitors tested were aprotinin [Trasylol; 10000 units (U)/ml] obtained from Bayer Corporation, and leupeptin, soybean trypsin inhibitor and tosyllysylchloromethylketone (TLCK) from Sigma. Three-week-old male ICR/CRJ (CD-1) mice (Charles River Japan) were inoculated intranasally with the Fushimi strain of Sendai virus for 2 h, and the lungs excised aseptically. The lungs were minced with scissors into small pieces (2 mm in size), washed with Eagle's MEM to remove the residual virus and incubated in serum-free MEM in 5% CO_2 at 34 °C in either the presence or absence of the protease inhibitors. A portion of the culture medium was taken every day and virus contents were measured in two different ways; one was adapted for the measurement of whole virus including both active and inactive viruses and the other for only active virus (Tashiro & Homma, 1983*b*; Itoh *et al.*, 1990).

As shown in Fig. 1, almost all of the progeny virus was infectious in the control culture, and the virus titres reached 2×10^4 cell-infecting units (CIU)/ml 3 days after infection. When cultures were treated with aprotinin, however, production of both whole and infectious virus was suppressed; the titres 3 days after infection were 3×10^3 CIU/ml and 1×10^2 CIU/ml, respectively. The decrease in the infectious virus titres was greater than that of whole virus, indicating that aprotinin suppressed proteolytic activation of the virus. A number of protease inhibitors other than aprotinin were also tested and representative results are shown in Table 1.

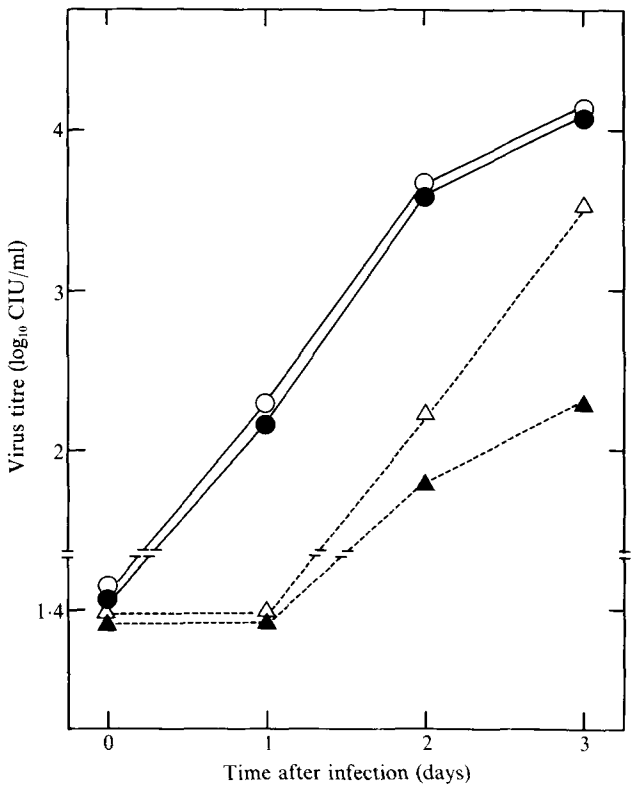

Fig. 1. *In vitro* suppressive effect of aprotinin on Sendai virus replication in mouse lung block cultures. Sendai virus-infected mouse lung blocks were cultured either in the presence (△, ▲) or absence (○, ●) of aprotinin at a concentration of 2500 U/ml. Amounts of whole (open symbols) and infectious virus (closed symbols) in the culture medium were determined.

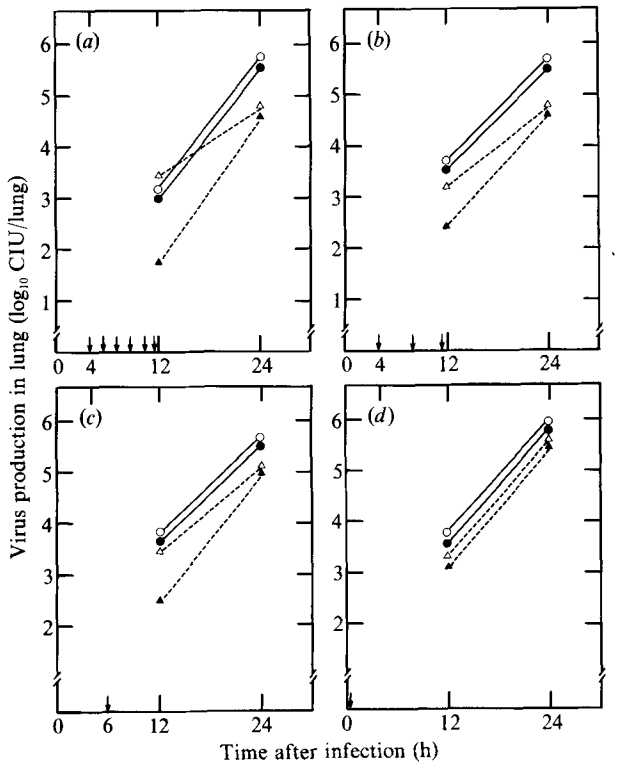

Fig. 2. *In vivo* suppressive effect of aprotinin on Sendai virus replication in mouse lung. Mice received intranasal administrations of aprotinin (△, ▲) or PBS (○, ●) six times (*a*) and three times (*b*) within 12 h of virus inoculation, and once 6 h after (*c*) and at the time of (*d*) virus inoculation. Open and closed symbols indicate amounts of whole and infectious virus in the lung, respectively. Each point represents the mean value of three independent samples. Arrows indicate the time of intranasal administration of aprotinin or PBS.

Among the inhibitors, leupeptin (200 μg/ml) was as effective as aprotinin but no inhibitory effect was observed with either soybean trypsin inhibitor (1000 and 2000 μg/ml) or TLCK (20 μg/ml).

We also tested the *in vivo* effect of aprotinin. When aprotinin was administered intranasally six times after virus inoculation, production of infectious virus in the lung was reduced markedly by 12 h after infection (Fig. 2*a*). Whole virus titres at this time, however, were the same as those in the control mice, suggesting that the suppressive mechanism of aprotinin observed *in vitro*

Table 1. *Effects of various protease inhibitors on Sendai virus production in mouse lung block cultures*

Protease inhibitors	Concentration (μg/ml)	Virus production (CIU/ml)*		Ratio of Infectious/whole virus
		Infectious	Whole	
None	0	$4{\cdot}1 \times 10^4$	$4{\cdot}4 \times 10^4$	0·91
Aprotinin	2500†	$3{\cdot}2 \times 10^3$	$4{\cdot}8 \times 10^4$	0·07
Leupeptin	200	$1{\cdot}8 \times 10^3$	$1{\cdot}8 \times 10^4$	0·09
Soybean trypsin inhibitor	1000	$9{\cdot}3 \times 10^3$	$1{\cdot}3 \times 10^4$	0·71
	2000	$8{\cdot}6 \times 10^3$	$9{\cdot}0 \times 10^3$	0·95
TLCK	20	$3{\cdot}2 \times 10^4$	$5{\cdot}1 \times 10^4$	0·63
	200	−‡	–	–

* Virus titres in the culture fluids 3 days after infection.
† Units/ml, instead of μg/ml.
‡ Unable to be determined because of the severe cytotoxicity of the reagent.

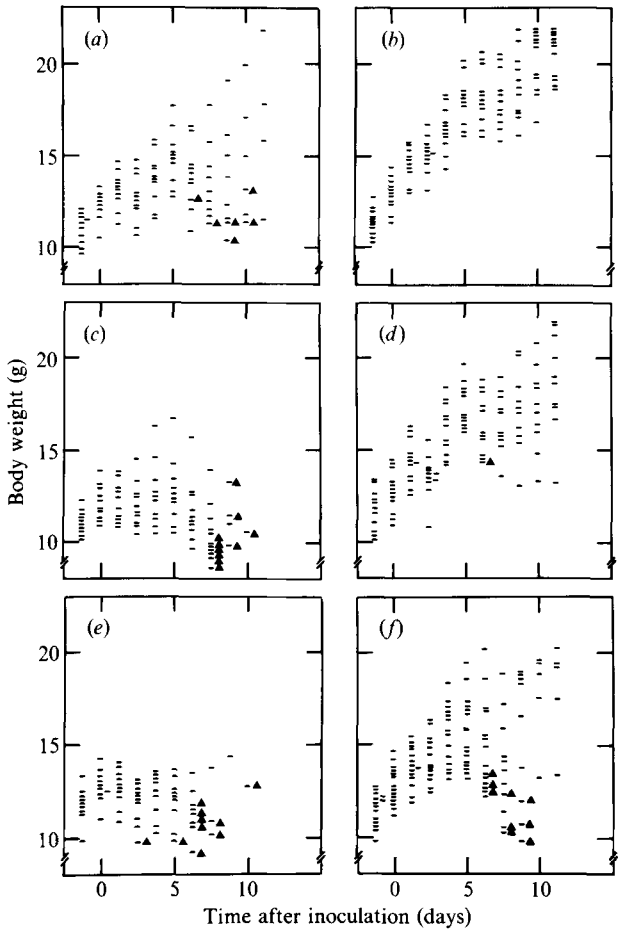

Fig. 3. Protection of mice against lethal Sendai virus pneumonia with aprotinin. Mice infected with $2{\cdot}5 \times 10^4$ CIU (*a* and *b*), $2{\cdot}5 \times 10^5$ CIU (*c* and *d*) and $1{\cdot}3 \times 10^6$ CIU (*e* and *f*) received intranasal administrations of either aprotinin (*b*, *d* and *f*) or PBS as a control (*a*, *c* and *e*) six times within 12 h of virus inoculation. The body weight of each mouse was plotted daily. Closed triangles indicate dead mice.

was also operating *in vivo*. Since production of infectious virus was suppressed at 12 h, viral spread in the lung was limited thereafter and, as a consequence, virus production 24 h after infection was significantly lower in the aprotinin-treated mice than in the control mice. Intranasal administration of aprotinin three times after virus inoculation also exerted a suppressive effect, with the titre of infectious virus being 10-fold lower than that of whole virus 12 h after infection (Fig. 2*b*). A single administration of the drug 6 h after, but not at the time of, virus inoculation still appeared to be effective (Fig. 2*c* and *d*). Intraperitoneal injection of aprotinin six times during the 12 h period after virus inoculation exerted no suppressive effect (data not shown).

In order to see whether aprotinin could confer protection on mice against lethal Sendai virus pneumonia, the following experiments were conducted. Test mice received an intranasal administration of 250 U of aprotinin six times within 12 h of virus inoculation while the control mice received phosphate-buffered saline (PBS) instead of aprotinin. When infected with $2{\cdot}5 \times 10^4$ CIU per mouse, all of the 10 aprotinin-treated mice survived, whereas six out of 10 mice in the control group died of viral pneumonia (Fig. 3*b* and *a*, respectively). Similarly, when infected with $2{\cdot}5 \times 10^5$ CIU per mouse, 10 out of 11 aprotinin-treated mice, but none of the 10 control mice, survived the infection (Fig. 3*d* and *c*). The protective effect of aprotinin was still observed when the mice were infected with $1{\cdot}3 \times 10^6$ CIU per mouse (Fig. 3*e* and *f*). The differences in the mortality ratios between the aprotinin-treated and the control mice were statistically significant ($P < 0{\cdot}005$, $P < 0{\cdot}001$ and $P < 0{\cdot}05$ for the mice infected with $2{\cdot}5 \times 10^4$ CIU, $2{\cdot}5 \times 10^5$ CIU and $1{\cdot}3 \times 10^6$ CIU per mouse, respectively).

The present results clearly demonstrate that intranasal administration of aprotinin suppressed the activation of Sendai virus in mouse lung and thereby conferred protection against lethal viral pneumonia. This phenomenon is thought to be a direct reflection of the inhibitory effect of aprotinin on the proteolytic cleavage of the F glycoprotein in the bronchial epithelium. It has been reported previously that aprotinin suppressed the cleavage of Sendai virus F protein in embryonated eggs (Zhirnov *et al.*, 1985). In influenza virus-infected mice, aprotinin suppressed proteolytic cleavage of virus haemagglutinin and interfered with multiple step replication of the virus in mouse lungs (Zhirnov *et al.*, 1984). Tashiro *et al.* (1987) also reported that another protease inhibitor, leupeptin, suppressed a bacterial protease(s) which mediates cleavage of influenza virus haemagglutinin and that consequently the mice were protected from lethal virus pneumonia. Many other viruses also require proteolytic cleavage of their structural proteins for viral maturation. Replication of poliovirus and some retroviruses was inhibited by a cysteine protease inhibitor, cystatin (Korant *et al.*, 1985) and an aspartyl protease inhibitor, pepstatin A (Yuasa *et al.*, 1975; Katoh *et al.*, 1987), respectively, and both of these suppressed the virus-encoded proteases.

Aprotinin is a well known serine protease inhibitor, consisting of 58 amino acids with an M_r of about 6500. It has a broad inhibitory spectrum including trypsin, chymotrypsin, plasmin and kallikrein, and is widely used for therapeutic purposes in patients with acute pancreatitis. The present study showed that aprotinin needs to be administered at relatively short intervals to exhibit its inhibitory effect because of its rapid clearance or inactivation in the lung. However, frequent intranasal administration of the drug rather decreased its protective effect (data not shown), probably by injuring physical

protective mechanisms such as ciliary movement of the bronchial epithelium. A directly toxic effect of aprotinin was not likely to be the reason because no toxicity was observed in mouse lung block cultures.

It was shown in the present study that higher concentrations of aprotinin were required to inhibit the cleavage of Sendai virus F protein both *in vitro* and *in vivo*. This is not surprising, because aprotinin probably needs to be taken up by the bronchial epithelium in an active form to interact with the protease. The rationale for this statement originated from the observation by Tashiro & Homma (1983*a*) that non-infectious Sendai virus with uncleaved F protein did not become infectious when inoculated into mouse respiratory tracts. The result suggests that the F protein-cleaving enzyme was present inside the bronchial epithelium but not on the cell surface. It may be reasonable, therefore, to assume that a protease inhibitor with higher accessibility to the intracellular target would be more effective. To that end, we are currently screening synthetic protease inhibitors with lower M_rs which may be taken up more easily by the cells. Our present study provides an experimental basis for the use of protease inhibitors as antiviral drugs.

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