Suppression of Influenza Virus Replication in Infected Mice by Protease Inhibitors

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SUMMARY

Administration of the protease inhibitors, e-aminocaproic acid or aprotinins, to mice infected with mouse-adapted influenza virus strain A/PR/8/34 (H1N1) and A/Aichi/2/68 (H3N2) reduced virus replication in the lungs. Up to 100-fold reduction of virus titre and virus-induced neuraminidase activity were revealed in mouse lungs under protease inhibitor treatment. As a result, drug-treated mice rapidly cleared the virus from their lungs. The predominant synthesis was of non-infectious virions with uncleaved haemagglutinin in the lungs of drug-treated mice, in contrast to the production of highly infectious virions with proteolytically cleaved haemagglutinin in untreated mice. These observations suggest that protease inhibitors suppress influenza virus replication in mouse lungs due to prevention of haemagglutinin cleavage and virus proteolytic activation.

The influenza virus haemagglutinin consists either of the precursor HA (75 000 mol. wt.) or of the disulphide-bonded fragments HA1 and HA2, 50 000 and 25 000 mol. wt., respectively (Laver, 1971; Stanley & Haslam, 1971; Lazarowitz et al., 1971). Virions with cleaved haemagglutinin are essentially more infectious than virions with uncleaved haemagglutinin (Klenk et al., 1975; Lazarowitz & Choppin, 1975). Proteolytic cleavage of HA into HA1 and HA2 varies with virus strain and host system (Klenk et al., 1972, 1975, 1977; Lazarowitz et al., 1973a, b; Stanley et al., 1973; Zhirnov et al., 1982c). Host dependence is mainly determined by the participation of host proteases in haemagglutinin cleavage, together with the virus strain-specific susceptibility of HA to proteolysis. The spread of virus in the infected organism appears to be determined by the rate of haemagglutinin cleavage: the strains possessing highly protease-sensitive HA induce generalized virus infection and have high pathogenicity (Bosch et al., 1979, 1981; Kilbourne et al., 1979; Nakajima & Sugiuara, 1980; Rott et al., 1980; Vallbracht et al., 1980). It follows that proteolytic activation of the virus by host proteases is one of the key mechanisms in influenza virus infection. Based on these findings, we suggest that protease inhibitors suppressing HA cleavage should inhibit influenza multiplication and limit the spread of infection in the host organism.

To test this hypothesis, physiological protease inhibitors, e-aminocaproic acid (e-AcA; Alkjaersig et al., 1959; Brockway & Castellino, 1971) and aprotinins (Gordox, Contrycal), were chosen. The drugs Gordox® (Gedeon Richter, Hungary) and Contrycal® (GERMED, G.D.R.) are polypeptides of about 6000 mol. wt. obtained from bovine organs. These compounds are analogues of Trasylol and inhibit a wide spectrum of proteases (trypsin, chymotrypsin, kallikrein, plasmin, cathepsin, etc.). These anti-protease agents are widely used in medical practice to prevent bleeding, hyperfibrinolysis, and in the treatment of pancreatitis, ulcers, burns, arthrosis (Mashkowsky, 1977). The compounds (e-AcA, Gordox, Contrycal) did not induce animal death under the conditions of administration.

Previously, we have described that these compounds prevented virus haemagglutinin cleavage in cultured cells infected with the WSN strain of influenza virus. As a result, non-infectious virions with uncleaved HA were predominantly synthesized by the cells (Zhirnov et

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Fig. 1. Infectivity titre and neuraminidase activity in the lungs of influenza virus-infected mice and the effect of protease inhibitors. Mice (8 to 10 g) were infected intranasally (approx. 50 µl/mouse) under light ether narcosis with mouse-adapted influenza A virus strain Aichi/2/68. After infection either physiological saline (control), Contrycal (200 trypsin inhibition units/mouse), Gordox (2000 kallikrein inhibition units/mouse), or ε-AcA (30 mg/mouse) was injected intraperitoneally in 0.2 ml physiological saline at intervals for 6 days. At different times after infection, three animals from each group were killed, the lungs were removed and homogenized in 9 vol. (3 ml) of Medium 199. Two ml of suspension was centrifuged for 15 min at 2000 rev/min. The supernatants were assayed for infectivity (a). Cell pellets were dissolved in 0.015 M-sodium phosphate buffer containing 10⁻³ M-CaCl₂, 0.5% Triton X-100, and neuraminidase activity was determined with ovomucoid (final concentration 10 mg/ml) as substrate (b). The amount of N-acetylneuraminic acid (NANA) released after incubation for 16 h at 37 °C was measured by the colour reaction of Warren (1959) and is expressed as µmol NANA formed per sample after 16 h. The colour reaction value for uninfected mouse lung suspension was subtracted as background. ●, Untreated; ○, ε-AcA-treated; □, Contrycal-treated; Δ, Gordox-treated.
The antiviral and therapeutic activities of the inhibitors were also demonstrated with influenza virus-infected animals. Administration of these compounds to mice and chickens infected with lethal doses of influenza virus protected more than 75% of animals and prevented dissemination of virus throughout the host organism (Zhirnov et al., 1982b, 1983). The present work was designated to study the mechanism of the antiviral therapeutic action of protease inhibitors in animals. We show here that protease inhibitors injected into infected animals reduce virus proteolytic activation and thereby suppress virus replication and development of infection.

The influenza virus strains A/PR/8/34 (H0N1) and A/Aichi/2/68 (H3N2) adapted for mouse lungs were used in all experiments. These strains induced fatal haemorrhagic pneumonia when inoculated intranasally with a challenge dose of about 10^4 p.f.u./mouse. To induce non-lethal multicycle virus infection in mouse lungs the mice were intranasally infected with a challenge dose of 10 to 100 p.f.u./mouse. After infection, one of the protease inhibitors, e-aminocaproic acid or aprotinin, was injected intraperitoneally at 4 to 6 hourly intervals over 6 days. The rate of virus replication in mouse lungs was estimated by titration of lung suspension infectivity using a standard plaque assay method on chick embryo fibroblasts. Previously, we have shown that this method measured the overall virus yield (including virions with cleaved and uncleaved haemagglutinin) in the host system used (Zhirnov et al., 1982a). The results of typical experiments are shown in Fig. 1 (a). It can be seen that in the lungs of control mice (not treated with inhibitors) the virus yield markedly increased during the first days after virus inoculation and remained detectable for 15 days after infection. These data show that extensive multicycle virus replication occurred in the lungs of untreated mice. Meanwhile, in the lungs of drug-treated mice the overall virus yield was 20 to 50 times lower and rapidly decreased (virtually to zero level) on the 7th day after infection. Similar results were obtained with the A/PR/8/34 strain (not shown). These observations indicated that treatment with protease inhibitors reduced multicycle virus replication in the lungs of infected mice.

The protease inhibitors which reduced virus multiplication should decrease the involvement of lung cells in virus-specific synthesis. To evaluate the amount of virus-specific material in infected lung cells, we measured virus-induced neuraminidase activity in lung cells of untreated and treated animals (Fig. 1 b). It can be seen that neuraminidase activity in the lung cells of drug-treated mice was about 100-fold lower and decreased more rapidly during the course of infection than the neuraminidase activity in the lungs of untreated animals. These results demonstrate additionally that protease inhibitor treatment of influenza virus-infected mice reduces the spread of virus infection in lungs.

The most probable mechanism of the antiviral action of protease inhibitors, as pointed out above, is a suppression of haemagglutinin cleavage which is a prerequisite of virus infectivity. To test this suggestion, the specific infectivity of the virus produced in the lungs of mice was estimated by two different plaque assay methods. These methods were developed to distinguish between viruses containing cleaved or uncleaved haemagglutinin (Zhirnov et al., 1982a, c). A modified plaque assay method allows the determination of the real infectivity of the virion population, i.e. virions with cleaved haemagglutinin, whereas the potential infectivity, i.e. that provided by the combined effect of virions with cleaved haemagglutinin and virions with uncleaved haemagglutinin, is measured by the standard method. Consequently, if the virus sample contains highly infectious virus with cleaved haemagglutinin the titres determined by both these methods are the same. On the other hand, if the preparation contains potentially infectious virus with uncleaved haemagglutinin, the titre determined by the modified method will be lower. The infectivity titres of the virus isolated from the lungs of infected, control (not treated with inhibitors) mice were similar in both plaque assay methods (Table 1), whereas the infectivity titre of the virus isolated from the lungs of mice treated with inhibitors was 10 to 100-fold lower in the modified plaque assay method in comparison with the standard method. It follows from these data that highly infectious virus with cleaved haemagglutinin was predominant in the lungs of the infected, control mice whereas non-infectious virions with uncleaved haemagglutinin were predominant in the lungs of infected mice treated with inhibitors.
Table 1. Effect of protease inhibitors on the infectivity of influenza virus in the lungs of infected mice*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Modified method</th>
<th>Standard method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>9.10 × 10⁵</td>
<td>1.25 × 10⁶</td>
</tr>
<tr>
<td>Gordon</td>
<td>6.05 × 10⁵</td>
<td>8.20 × 10⁵</td>
</tr>
<tr>
<td>ε-AcA</td>
<td>1.25 × 10⁴</td>
<td>2.20 × 10⁴</td>
</tr>
<tr>
<td>Contrycal</td>
<td>0.50 × 10⁴</td>
<td>2.20 × 10⁴</td>
</tr>
<tr>
<td>Contrycal</td>
<td>2.68 × 10⁴</td>
<td>2.24 × 10⁴</td>
</tr>
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* Infection and protease inhibitor treatment of mice were performed as described in Fig. 1. Lung suspensions from untreated (control) and treated mice were prepared on the 4th day after infection and infectivity titres were measured by two plaque assay methods, the standard and the modified. The protease inhibitor phenylmethylsulphonyl fluoride (final concn. 50 μM) was added to the suspension medium to exclude artificial activation of virus by tissue proteases during preparation of the lung samples. Plaque titration was performed on chick embryo fibroblasts which did not cleave either the PR/8/34 or the Aichi/2/68 virus haemagglutinin and did not activate the virus during plaque assay (Zhirnov et al., 1982a). The modified plaque assay method included a double agar overlay, trypsin being included in the second layer added 24 h after infection to permit plaque formation. In the standard method, trypsin was included in the agar from the beginning (Zhirnov et al., 1982a).

These data demonstrate that protease inhibitors preventing the haemagglutinin cleavage and proteolytic activation of virus reduce influenza virus replication and the development of infection in mouse lungs. The aprotinin Gordon had the highest antiviral activity (Fig. 1; Table 1). The limitation of virus infection possibly contributed to an effective immune response and rapid elimination of virus from the lungs of drug-treated mice. These observations suggest that anti-protease compounds have therapeutic potential against influenza (for review, see Zhirnov, 1983). Our previous findings that the protease inhibitors, ε-aminocaproic acid and aprotinins, effectively protect animals infected with lethal doses of influenza virus and prevent dissemination of virus throughout the host organism reinforce this conclusion (Zhirnov et al., 1982b, 1983). Clinical investigations on children during an outbreak of influenza H3N2 virus in February and March of 1983 have also demonstrated the antiviral efficacy of protease inhibitor treatment (O. P. Zhirnov et al., unpublished results).

Treatment of virus infection by this approach has considerable advantages. Since host-dependent proteolytic activation of virus infectivity is a universal mechanism in infection induced by influenza viruses (Klenk et al., 1975; Lazarowitz & Choppin, 1975; Bosch et al., 1979; Sugawara et al., 1981) and paramyxoviruses (Homma & Ohuchi, 1973; Scheid & Choppin, 1974; Nagai et al., 1976; Nagai & Klenk, 1977), protease inhibitors may be used for treatment of infection induced by most myxoviruses. Moreover, this approach may be valuable in therapy of a wide spectrum of viral diseases because many other viruses, including poxviruses (Ichihashi & Oie, 1982), reoviruses (Wallis et al., 1966; Rubin & Fields, 1980), retroviruses (Chang & Friedman, 1977; Pitka et al., 1978; 1980; Van der Hoorn et al., 1983), rotaviruses (Estes et al., 1981) and coronaviruses (Otsuki & Tsubokura, 1981; Stroz et al., 1981), require proteolytic activation for their replication and pathogenicity. Thus, antiprotease compounds seem to be potential universal antiviral drugs.

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REFERENCES


Short communication


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