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High Protection of Animals Lethally Infected With Influenza Virus by Aprotinin-Rimantadine Combination

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The successful therapeutic synergism of aprotinin and rimantadine, which are known to attack different viral targets, was demonstrated in influenza-virus-infected animals. Combined treatment with these drugs of mice infected with a highly lethal dose of mouse-adapted influenza virus prevented the development of fatal haemorrhagic pneumonia and protected about 75% of animals; whereas the separate administration of aprotinin and rimantadine induced 35% and 15% protection, respectively.

Key words: **Influenza, antiviral compounds, combined antivirals**

INTRODUCTION

Rimantadine is known to induce protection against influenza-virus-infected mice [Grunert et al, 1965]. This antiviral drug was found to block internalization of virus into the host cell [Skehel et al, 1977; Koff and Knight 1979; Bukrinskaya et al, 1982] and seemed to damage the assembly of virions in infected cells [Hay et al, 1985]. It has been reported also that protection of mice lethally infected with influenza virus could be performed by aprotinin [Zhirnov et al, 1982a, 1984a], a polypeptide antiproteinase [Trautschold et al, 1967]. This compound was demonstrated to inhibit proteolytic cleavage of virus haemagglutinin [Zhirnov et al, 1982b, 1985], and thus to prevent activation of progeny virions and to reduce the spread of infection [Zhirnov et al, 1984a, 1985]. Unlike rimantadine [Grunert et al, 1965], aprotinin has been observed to induce marked protection of animals infected with influenza virus not only when it has been administered early on but also following administration later on in infection [Zhirnov et al, 1982a]. It is shown here that these drugs, which affect the different stages of virus replication, are able to induce a synergistic therapeutic effect in animals infected with influenza virus.

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MATERIALS AND METHODS

Antiviral Drugs

In this study N,methyl-adamantane hydrochloride (rimantadine) obtained from Institute of Organic Synthesis, Latvian Academy of Sciences (U.S.S.R.) and aprotinin, a commercial solution (Gordox®, produced by Gedeon Richter, Hungary), were used. Aprotinin is a disulfide-linked polypeptide dimer of molecular weight of about 12,000 that inhibits a wide spectrum of proteinases: trypsin, chymotrypsin, cathepsin, plasmin, etc [Trautschold et al, 1967]. It is obtained from animal organs and is widely used in medical practice to prevent hyperfibrinolysis and to treat pancreatitis [Amris 1966; Trapnell et al, 1974; Müller et al, 1980]. With respect to their antiproteinase specificity, this compound is analogous to Trasylol® produced by Bayer AG [Malis et al, 1979]. It was possible to suppress influenza and paramyxovirus infection in chicken embryos and mice by Gordox® [Zhirnov et al, 1984a, 1985].

Virus

The Mouse-adapted influenza virus A/Aichi 2/68 (H3N2) was propagated in the allantoic sac of 9-day-old embryonated chicken eggs by passaging at low multiplicity. The titre of virus in the allantoic fluid was usually 5×10^8 PFU/ml (~1,000 haemagglutinating units/ml.) The virus infectivity was titrated in chicken fibroblasts by standard plaque assay, with trypsin in the agar overlayer [Zhirnov et al, 1982c].

Experimental Animals

Mice (8–10 g) were infected intranasally (~50 μl/mouse) under light ether narcosis with the mouse-adapted virus A/Aichi 2/68 at a multiplicity of about 400 50% mouse lethal doses, MLD_{50}, (~5×10^5 PFU) per mouse. After infection either physiological saline (placebo), gordox (2,000 kallikrein inhibition units/mouse), rimantadine (100 μg/mouse) or the same doses of both drugs were injected intraperitoneally in 0.2 ml physiological saline at 4–6-h intervals for 7.5 days. Each group usually contained 29–33 animals. The doses of the compounds examined did not induce any visible toxicity or death of animals under the conditions of administration.

RESULTS

The protection effect of the anti-influenzal drugs examined was studied on mice infected with the influenza A/Aichi 2/68 (H3N2) virus. This virus strain was adapted for mouse lungs by mouse-to-mouse intranasal passage, and after adaptation it replicated effectively in the mouse lungs and induced fatal haemorrhagic pneumonia [Zhirnov et al, 1982a]. Experiments designed to study a combined aprotinin-rimantadine therapy were carried out on mice infected with about 400 MLD_{50}. This highly lethal dose of virus was chosen in order to estimate a therapeutic synergism of aprotinin and rimantadine since its separate administration under these conditions was found to protect less than 50% of animals (see below). After infection, aprotinin and rimantadine were injected intraperitoneally at 4–6-h intervals for 7.5 days. A single injection dose of aprotinin and rimantadin was 2,000 KIU and 100 μg per mouse, respectively. These doses were chosen in accordance with the previous data, which had indicated marked antiviral activity of aprotinin [Zhirnov et al, 1984a] and rimantadine [Grunert et al, 1965; Galegov et al, 1981] in influenza-infected mice.

The results of a typical experiment, showing the survival of mice treated with aprotinin and rimantadine, are represented in Figure 1. It is seen that in a group of placebo-treated animals 100% lethality was registered on the 7th day after infection. Protection that was due to rimantadine and aprotinin treatment was about 15% and 35%, respectively. Moreover, the death of mice was delayed by aprotinin and rimantadine for 3–4 and 1–2 days, respectively (see Fig. 1). Combined chemotherapy with both drugs protected about 75% of animals, and death occurred 5–6 days later in the rimantadine-aprotinin-treated animals than in the placebo-treated ones.

The successful therapeutic effect of an aprotinin-rimantadine combination in influenza-infected mice was also demonstrated by examination of pulmonary pathology. As seen in Figure 2 there was fatal confluent haemorrhagic inflammation of the lung tissue in the placebo-treated mice, and a few loci of haemorrhagic or mild haemorrhagic inflammation in the lungs of aprotinin and rimantadine-treated mice. In the mice treated with both drugs, lung pathology was minimal, and only sole loci of nonhaemorrhagic pneumonia were registered (see Fig. 2).

DISCUSSION

The data of this report show that aprotinin and rimantadine produce synergistic protection of mice lethally infected with influenza virus. The therapeutic synergism revealed is most probably a result of different antiviral targeting of these compounds. It has been previously proposed that pathways of influenza and other viruses, which undergo proteolytic activation, have developed via a vicious circle [Zhirnov, 1983]. This idea has been illustrated in Figure 3. It is known that virus replication in cells is

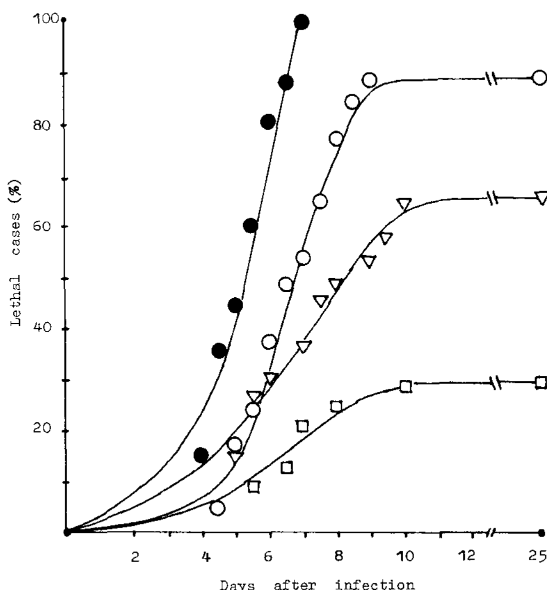

Fig. 1. Protection of mice lethally infected with influenza virus by aprotinin and rimantadine. Mice were infected with about 400 MLD_{50}/mouse of mouse-adapted influenza A/Aichi 2/68 virus. After infection, physiological saline (placebo, ●), aprotinin (▽), rimantadine (○), or both drugs in combination (□) were injected intraperitoneally for 7.5 days. Deaths were recorded at 6–12-h intervals.

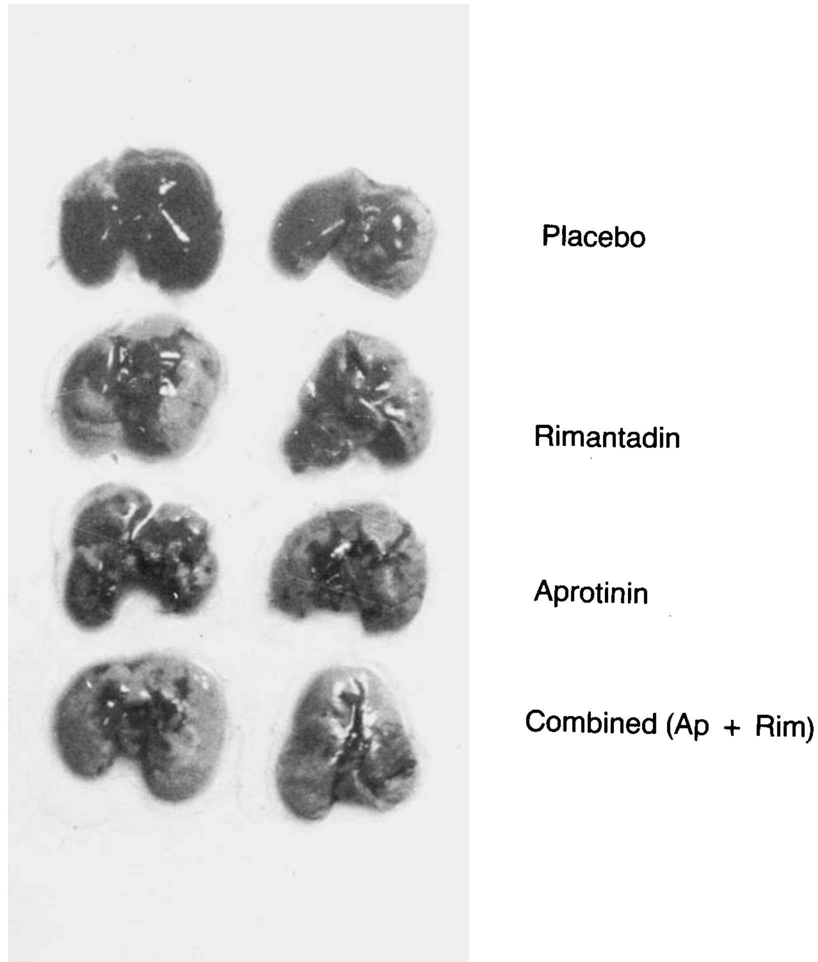

Fig. 2. Pulmonary pathology in influenza-virus-infected mice treated with aprotinin and rimantadine. Infection and treatment of mice are described in the legend to Figure 1. On day 5 after infection, the lungs (from two animals in each group) were removed and photographed.

followed by activation of host proteinases [Goldberg and Lazarowitz 1974; Lazarowitz et al, 1973; Degtyarenko et al, 1977]. The action of these proteinases is most probably one of the triggering factors of inflammatory reaction and fibrinolytic-clotting system defects, which are often observed during influenza [Davison et al, 1973; Lindsay et al, 1970; Noble et al, 1973; Louria et al, 1959]. On the other hand, host proteinases cleave the virus haemagglutinin glycopolypeptide HA (molecular weight 75 kD) into two disulfide-linked fragments HA1 (50 kD) and HA2 (25 kD) to activate virion infectivity [Klenk et al, 1975; Lazarowitz and Choppin 1975] and realize virus virulence in the host organism [Bosch et al, 1979; Rott et al, 1980; Vallbracht et al, 1980]. To summarise, higher induction by the virus strain of the host proteinase activity leads to a more intensive virus proteolytic activation, and virus spreading develops in the host organism [for review, see Zhirnov, 1983]. It follows from this supposition that proteinase inhibitors, including aprotinin, may normalize proteolytic balance to limit inflammatory process and development of a syndrome of

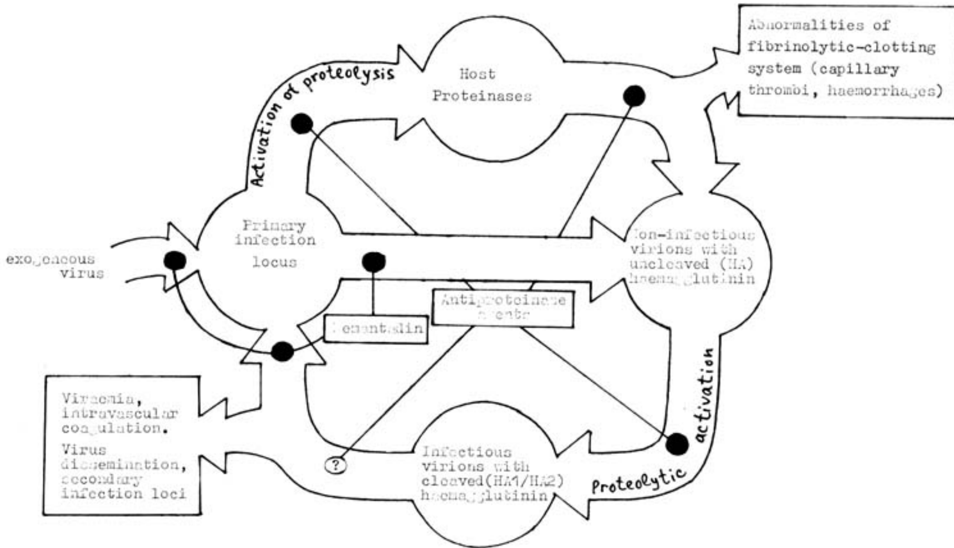

Fig. 3. Pathogenetic pathways of influenza virus infection and targets of antiviral drugs. Antiproteinase agents (?) can directly block the fusion and/or deproteinization of virions during internalization into the host cell. An early stage in the retrovirus- and coronavirus-host cell interaction can be inhibited by leupeptin, a tripeptide proteinase inhibitor [Andersen, 1983: Appleyard and Tisdale, 1985]. On the other hand, antiproteinases, such as TPCK, TLCK, PMSF, and aprotinin failed to block penetration of measles virus and coronavirus into the host cells [Appleyard and Tisdale, 1985; Richardson and Choppin, 1983].

haemorrage and coagulation defects, and that they may suppress proteolytic activation of virions to reduce virus multicycle replication and spread (see Fig. 3). It also follows that rimantadine attacks other targets in virus replication to prevent penetration of infectious virions into the host cell and disturb the assembly of virions. Targeting, for different stages of influenza virus pathogenic pathway, is thought to be the main mechanism of synergistic anti-influenzal therapeutic action of the combined aprotinin-rimantadine treatment.

Aprotinin and rimantadine are medicinal compounds. The synergistic anti-influenzal effect reported here suggests that combined aprotinin-rimantadine anti-influenza therapy might be used for clinical trails. In man, influenza virus infection is primarily localized in the upper respiratory tract. Therefore, it seems expedient to use aerosol inhalations of these compounds. The first clinical observations during an outbreak of influenza have demonstrated the antiviral and therapeutic efficasy of aerosol inhalations of antiproteinases [Zhirnov et al, 1984b] and rimantadine [Hayden et al, 1982]. Based on these findings it seems feasible to design manual inhalers with antiproteinase agents and rimantadine. Such inhalers are thought to make earlier prophylactic and therapeutic applications of both compounds possible, especially during influenza outbreaks and epidemics.

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