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Alphavirus replication in cultured cells and infected animals is inhibited by antiproteinase agents

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Summary

The influence of different antiproteinase agents on alphavirus replication was examined. Sindbis virus multicycle replication in cultured cells was suppressed by *N*-tosyl-phenylalanine chloromethyl ketone (TPCK), an inhibitor of chymotrypsin-like proteinases, and by aprotinin, an inhibitor of a wide spectrum of proteinases. Antiviral activity of TPCK was also demonstrated in Sindbis virus-infected animals. Parenteral injections of TPCK in infected mice reduced virus titers in brain and blood. The possible mechanism(s) of antiviral action of the antiproteinase agents are discussed.

alphaviruses; replication; antiproteinase agents; antiviral agents

Introduction

It is well known that virus-specific proteins of many viruses (including alphaviruses [8]) are proteolytically cleaved during virus replication and this proteolytic processing is necessary for the development of the infection. Based on these observations it has been suggested that inhibition of proteolysis of the viral proteins would result in an inhibition of virus replication, and that antiproteinase agents could be used for this purpose [for review see 12,29]. In exploring this chemotherapeutic approach, we were able to suppress influenza and paramyxovirus infections through blockage of virus glycoprotein cleavage by antiproteinases [27,28,30,33], and beneficial results were obtained in clinical trials with antiproteinase aerosol inhalations during an influenza

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outbreak [32]. In this communication we present experimental data on the proposed chemotherapeutic approach against alphavirus infection.

We studied the ability of different proteinase inhibitors to suppress Sindbis virus, (an alphavirus) multicycle replication in cultured cells and infected animals. The following proteinase inhibitors were chosen to examine the antiviral effect. *N*-tosyl-phenylalanine chloromethyl ketone (TPCK), an inhibitor of chymotrypsin-like proteinases [20], *N*,α-tosyl-L-lysine chloromethyl ketone (TLCK), an inhibitor of trypsin-like proteinases [21], and aprotinin, an inhibitor of a wide spectrum of proteinases, such as trypsin, chymotrypsin, cathepsin, plasmin, etc. [24]. Two aprotinin compounds, Gordox® kallikrein-trypsin inhibitor (lyophilized form) produced by Gedeon Richter (Hungary) (designated aprotinin-G), and Contrycal® produced by VEB Arzneimittelwerk (GDR) (termed aprotinin-C) were used. With respect to their antiproteinase specificity, these compounds are analogous to Trasylol® produced by Bayer AG [14]. Aprotinin is a disulfide-linked polypeptide dimer of about 12 000 molecular weight [24]. It is obtained from animal organs and widely used in medical practice to prevent hyperfibrinolysis and to treat pancreatitis [2,7,14].

Materials and Methods

Virus

Sindbis AR/339 virus was propagated in brain of suckling mice and an appropriate dilution of 10% brain suspension was used to infect cultured cells and experimental mice.

Culture cell experiments

Two-day-old primary cultures of chicken embryo cells (CEC) were infected with virus at a multiplicity of about 10^{-4} plaque forming units (PFU) per cell. After 45 min the virus inoculum was removed and cells were incubated with medium 199 supplemented with 20 mM Hepes (pH 7.7) and antiproteinase agent. In all experiments 100 mM TPCK in DMSO was used as stock solution which was diluted in warm medium before it was added to the cell monolayer; TLCK was prepared in aqueous solution. Alkaline medium (pH 7.7.) used permitted a more stable replication of alphaviruses in cultured CEC than neutral (pH 6.9–7.2) medium (Zhirnov et al., unpublished data). After 13.5 h incubation, the culture medium was removed and assayed for infectivity. The cells were washed with medium lacking antiproteinase, and to study cellular polypeptide synthesis, cell monolayers were incubated for an additional 30 min with ^{14}C-labelled algal hydrolysate dissolved in Hank's salt solution without proteinase inhibitors as previously described [31]. Cellular polypeptides were dissolved in 2% SDS, 0.01 M dithiothreitol, 0.2 M Tris-HCl (pH 6.8) and equal aliquots were put on electrophoretic gels.

Experimental animals

Mice (8–10 g) were infected intramuscularly with Sindbis AR/339 virus at a multiplicity of 10^6–10^7 PFU/mouse. After infection either physiological saline (placebo), or Gordox®, a commercial solution (2000 Kallikrein Inhibitor Units (KIU)/mouse), or

TPCK (0.2 ml of 60 μl solution/mouse) were injected intraperitoneally. The injections were repeated at 4–6 h intervals for 6 days. At different times after infection, 5 animals (3 animals on day 1) from each group were killed and 10% homogenates of brain and blood were prepared. The tissue homogenates were assayed for infectivity by the plaque method in cultured CECs.

Plaque assay

CECs grown in 24-cm^2 flask were infected with 0.2 ml/flask of 10-fold virus dilutions in medium 199. After 45 min adsorption the inoculum was removed and the cells were overlaid with 4.5 ml of 1% bactoagar (DIFCO) prepared in medium 199 supplemented with 25 μg/ml DEAE-dextran and 20 mM Hepes (pH 7.7). Plaques were counted 40–48 h postinfection by neutral red staining.

Polyacrylamide gel electrophoresis

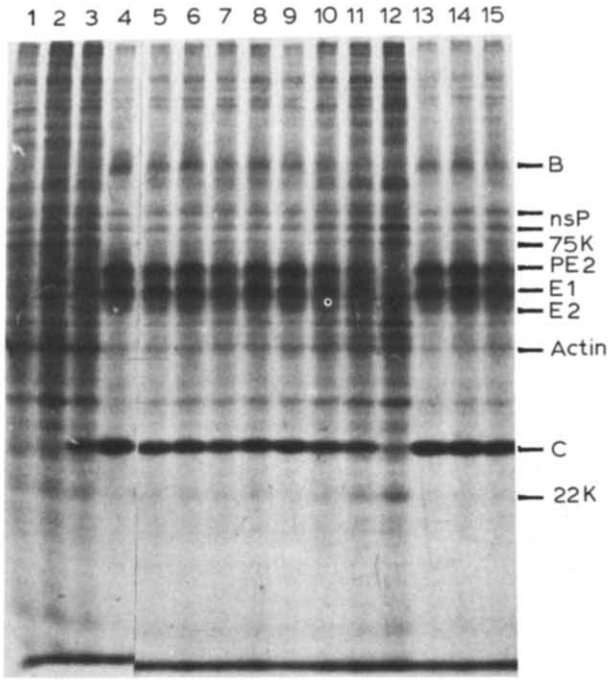
Cellular polypeptides were submitted to electrophoresis in a 10–15% gradient polyacrylamide gel using Tris-glycine–SDS buffer followed by gel autoradiography as described [31]. Molecular weights of viral polypeptides were evaluated according to Weber and Osborn [25] with influenza virus proteins as markers.

Results

The ability of different proteinase inhibitors to suppress multicycle Sindbis virus replication in cultured cells was studied in preliminary experiments. For this purpose cell monolayers were primarily infected with a low virus dose (10^{-4} PFU/cell) and incubated with antiproteinase-containing medium. After incubation, the medium was removed and infected cells were processed for analysis of protein synthesis. Three patterns of polypeptide synthesis could be expected from such experiments. First, the virus replication might fail to be suppressed by the proteinase inhibitor; the monolayer, therefore, would be totally infected due to an effective multicycle virus replication, and the synthesis of virus proteins would be revealed. Second, the antiproteinase agent may suffer virus replication without concomitant toxicity for the host cells; host cell protein synthesis would be detected in these samples. Third, cell monolayer might be damaged by toxic concentrations of the proteinase inhibitors and in this case protein synthesis would be markedly reduced or even abolished.

The results of a typical experiment are represented in Fig. 1. Lines 2–4 (Fig. 1A) show virus protein synthesis during the course of infection. Host protein synthesis was virtually shut off and the synthesis of virus non-structural B (M_w 110 kDa), nsPs (85 kDa and 78 kDa) and structural E1 (59 kDa), PE2 (62 kDa), E2 (55 kDa) and C (28 kDa) polypeptides dominated in cell culture up to 13.5 h postinfection (Fig. 1A, line 4). This result indicates that multicycle replication of Sindbis virus in CEC leads to a total infection of the monolayer. It is also seen that TLCK and aprotinin-C did not induce visible antiviral activity and synthesis of the main virus proteins was clearly detected in cell monolayers incubated with high concentrations of these inhibitors (Fig. 1A; lines 5–7, 13–15). In contrast, aprotinin-G at a concentration of 1000 KIU/ml or higher

A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B
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E1
E2
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C
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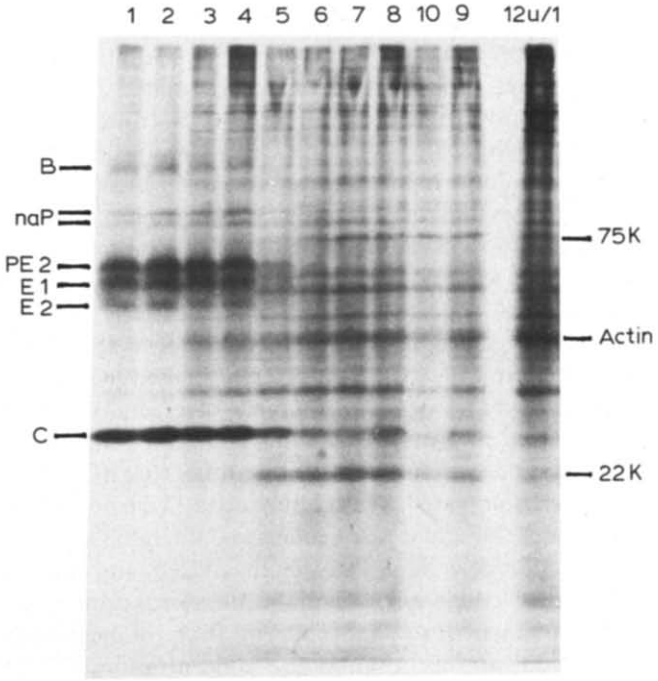
B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demonstrated a marked reduction of virus-specific protein synthesis (Fig. 1A, lines 11,12). Aprotinin-G had no toxicity for the cells and did not suppress host protein synthesis at a concentration up to 10^4 KIU/ml (data not shown).

More detailed experiments were designed to study the antiviral activity of TPCK which has been reported to have alkylating activity [17,22], thus could be toxic for cells. The results of these experiments are demonstrated in Fig. 1B,C. It can be seen that TPCK concentrations up to 2-5–5.0 μM did not cause antiviral activity; at concentrations of 7.5–12.5 μM, TPCK markedly reduced virus polypeptide synthesis without affecting host cell synthesis. Initial toxicity resulting in a decrease of protein synthesis was observed at 22.5 μM TPCK and extensive cell damage was seen at concentrations of 25 μM or more (Fig. 1B, line 11; Fig. 1C, lines 10–12). It is also seen, that in samples where synthesis of virus polypeptides was reduced the synthesis of 75 kDa and 22 kDa host polypeptides was enhanced (Fig. 1B, lines 5–7). Similar parallelism was denoted in cells treated by high concentrations of aprotinin-G (Fig. 1A, lines

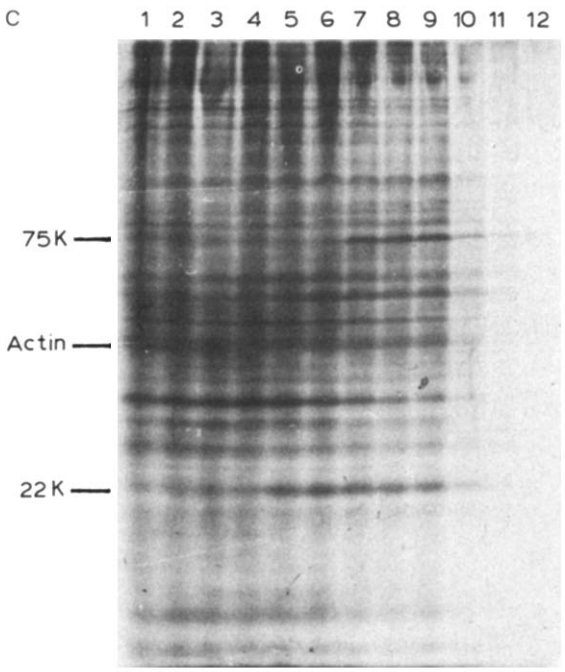

Fig. 1. Protein synthesis in Sindbis virus-infected cells incubated with antiproteinase agents. CEC cultures were infected with Sindbis AR/339 virus and incubated with medium 199 supplemented with antiproteinase agents. After incubation the culture fluid was removed and assayed for infectivity (see Table 1) and cells were processed to examine polypeptide synthesis. Cellular polypeptides synthesized were analyzed by electrophoresis in 10–15% gradient polyacrylamide gel. (A) Line 1; control uninfected cells; 2,3,4: infected cells incubated in medium without antiproteinases for 7, 10 and 13.5 h, respectively; infected cells incubated for 13.5 h with medium containing 50 (line 5), 100 (6), and 150 (7) μM TLCK; 100 (8), 250 (9), 500 (10), 1000 (11), and 2500 (12) KIU/ml of aprotinin-G; 500 (13), 1000 (14), and 2500 (15) trypsin inhibitor units/ml of aprotinin-C. (B) Infected and (C) uninfected cells incubated for 13.5 h with medium 199 alone (line 1) or medium containing 2 (2), 5 (3), 7.5 (4), 10 (5), 12.5 (6), 15 (7), 17.5 (8), 20 (9), 22.5 (10), 25.0 (11), and 30.0 (12) μM TPCK.

11,12). Enhancement of 75 kDa and 22 kDa proteins appeared to be independent of virus and was detected in non-infected cells treated by corresponding concentrations of TPCK (Fig. 1C, lines 6–9) and aprotinin-G (data not shown). Virus-ineffective antiproteinases (TLCK, aprotinin-C) did not induce the 75 kDa and 22 kDa proteins (Fig. 1A, lines 5–7, 13–15).

The data on the inhibition of virus protein synthesis by TPCK and aprotinin-G fully correlated with the results indicating a decrease of virus yield in culture fluid of antiproteinase-treated cells (see Table 1). It is important to emphasize that aprotinins and TPCK had no direct virucidal effect on virions and did not reduce virus infectivity during incubation in vitro for at least 5–7 h at 37°C (data not shown). These observations support the conclusion that TPCK and aprotinin-G specifically suppress virus replication in cultured cells in the range of non-toxic concentrations.

The experiments in cultured cells prompted evaluation of the antiviral activity of TPCK and aprotinin-G in infected animals. For this study, Sindbis virus AR/339 was chosen because this strain multiplicates effectively in mouse organs without lethal effects. Mice were inoculated intramuscularly (or intraperitoneally) with Sindbis virus and then treated with proteinase inhibitors by the intraperitoneal route. Virus replication in animals was estimated by infectivity assay of mouse brain and blood during the course of infection (see Fig. 2). In placebo-treated mice the virus was clearly shown in blood on days 1–3 while virus titers of 10^3–10^5 PFU were detected in brain from day 3 to 7 after infection. Virus titers in brain were virtually decreased to zero on day 10–15

TABLE 1

Virus yield in Sindbis virus-infected cells incubated with antiproteinase agents

Treatment of cells	Virus titer (PFU/ml of culture fluid)*
Control (untreated)	3.4×10^7
TLCK-treated	
50 μM	1.6×10^7
100 μM	2.1×10^7
150 μM	0.9×10^7
TPCK-treated	
2 μM	1.2×10^7
5 μM	5.3×10^6
10 μM	1.8×10^5
15 μM	1.6×10^4
Aprotinin-C-treated	
1000 TIU	3.2×10^7
2500 TIU	2.1×10^7
Aprotinin-G-treated	
500 KIU	0.5×10^7
1000 KIU	1.4×10^6
2500 KIU	2.3×10^5

* Infection and incubation of cultured cells are as in the legend to Fig. 1. The virus titer of culture fluid was determined by a plaque assay on CEC.

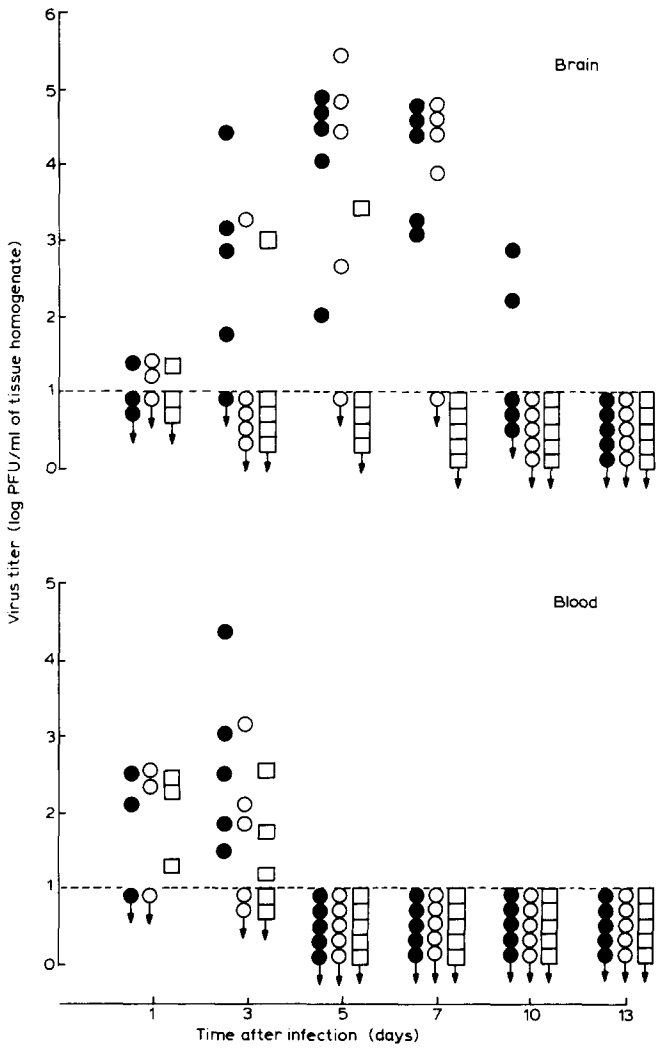

Fig. 2. Sindbis virus replication in mice treated with TPCK or aprotinin-G. Mice were infected intramuscularly with AR/339 virus and treated intraperitoneally with physiological saline (●), Gordox® (○) or TPCK (□) at 4–6 h intervals for 6 days. At different times after infection, infectious virus titers in brain and blood for 5 animals per group were assayed.

of infection. These data confirmed that Sindbis virus replicated effectively in placebo-treated mice. Almost complete inhibition of virus replication in brain and decrease of virus in blood of a majority of TPCK-treated mice was observed. On the third day after infection mean virus titers (log PFU/ml) in blood of placebo, aprotinin G, and TPCK-treated mice were 2.0 ± 0.32, 1.2 ± 0.16, and 0.95 ± 0.21, respectively. Statistical significance of virus titer reductions in blood of TPCK-treated mice is $P > 0.95$. Mean virus titer in brain of control and TPCK-treated mice on day 5 of

infection was 4.5 ± 0.58 and 1.2 ± 0.53, respectively (mean values for three independent experiments); and this decrease was statistically significant ($P > 0.999$). A clear antiviral effect was observed at a TPCK dose of about 20 μg/mouse per day (Fig. 2), whereas a less prominent effect was seen with TPCK at 5 μg/mouse per day (data not shown). The primary non-lethal toxic symptoms (such as hypothermia, reduced weight gain, etc.) were found to occur at TPCK doses of 80–100 μg/mouse per day (data not shown). Aprotinin-G did not cause reduction in the overall virus yield in mouse brain. However, aprotinin-G delayed virus replication in brain and brought about a slight reduction of viraemia (see Fig. 2).

Discussion

Data are presented showing that alphavirus multicycle multiplication in cultured cells can be suppressed by antiproteinase agents at concentrations that do not cause toxicity to the host cells. One-cycle growth of alphavirus in cell culture has been reported previously to be inhibited by high concentrations (more than 100 μM) of TPCK [15,16]. Such concentrations are in agreement with our data and were found to be toxic for CEC, and within 2 h of incubation this concentration of inhibitor induced more than 50% reduction in cellular protein synthesis [16]. Therefore, virus inhibition achieved by high TPCK concentration may result from its toxicity to the host cells [16].

The exact mechanism of antiviral activity of antiproteinase agents is not clear and awaits further study. Inhibition of virus replication by TPCK is most likely due to an inhibition of the proteinases responsible for virus protein cleavage. High concentrations (> 100 μM) of TPCK have been reported to suppress proteolysis of the structural C/PE2/E1 polypeptides in Sindbis virus-infected cells [16] and block proteolytic processing of 270 kDa polyprotein, a precursor of a non-structural polymerase-associated nsP1–nsP4 protein of Sindsbis virus [5]. Partial inhibition of proteolytic formation of viral proteins C, PE2 and E1 and appearance of high molecular weight precursors have been noted when cells infected with another alphavirus, Semliki Forest virus, were exposed to high concentrations (> 300 μM) of TPCK [15]. During examination of the antiproteolytical activity of TPCK in alphavirus-infected cells we did not see a reduction of C and E1 proteolytic formation and only partial inhibition of PE2→E2 cleavage was obtained at non-toxic concentrations (10–20 μM) of TPCK (Zhirnov et al., unpublished results). On the other hand, TPCK has been reported to interact selectively with structural proteins P and M of measles virus [9,26]. A similar direct interaction of TPCK with alphavirus-specific protein(s) may affect their intracellular conversions.

The antiviral activity of aprotinin is most probably due to the antiproteinase action of the drug. Aprotinin-G had been shown to penetrate into influenza-infected cells and inhibit intracellular proteolysis of viral nucleocapsid protein [31]. There is another precedent for the blockage of intracellular proteolytic processing of virus proteins by the exogenuous antiproteinase. Cystatin, a 12 000 molecular weight proteinase inhibitor prepared from chicken eggs, added to poliovirus infected cells, has been reported to alter the proteolysis of virus polyproteins and to reduce the virus yield [13].

Although virus-specific proteolysis seems to be a plausible target for antiproteinase agents, other explanations for the antiviral activity of antiproteinase agents can not be excluded. For instance, antiproteinase agents may directly block the fusion and/or deproteinization of virions during internalization into host cell. Indeed, an early stage in retrovirus and coronavirus-host cell interaction can be inhibited by leupeptin, a tripeptide proteinase inhibitor [1,3]. On the other hand, antiproteinases, such as TPCK, TLCK, PMSF, and aprotinin fail to block penetration of measles virus and coronavirus into the cells [3,19].

The antiviral activity of antiproteinases may be mediated by the host cell. Alphavirus replication is well known to interrelate with host cell metabolism [4,6,11]. Imbalance of the host cell metabolic factors, which could be provoked by antiproteinase agents, may lead to inhibition of virus development in the aprotinin- and TPCK-treated cells. It is tempting to speculate that superproduction of 75 kDa and 22 kDa host proteins is an effector, which determines the inability of cells to maintain virus replication. Similar resistance of interferon-treated mouse cells to influenza virus was shown to be mediated by a 75 000 molecular weight host protein (Mx), the intracellular production of which appeared to be enhanced by interferon [10,23].

We have reported here that TPCK and aprotinin inhibit multiplication of alphavirus in cultured cells. Only TPCK was found to suppress virus replication in infected animals. These results can be explained by a differential transport of the antiproteinase agents towards their viral targets in animals. TPCK is a low molecular weight phenylalanine analogue which is well known to penetrate effectively through the cell membrane. Thus TPCK distribution in the host organism is possibly adequate for antiviral targetting. Aprotinin is a 12 000 molecular weight polypeptide and its distribution in the organism therefore could be insufficient for inhibition of alphavirus replication. The antiviral activity of TPCK in animals as shown here is mainly of theoretical significance. Much more needs to be known about the toxicity and exact mode of action of the antiproteinase agents in the whole organism. Elucidation of these factors may allow the design of effective non-toxic antivirals with appropriate delivery to the viral targets.

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