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ACTION OF ε-AMINOCAPROIC ACID ON THE PROTEOLYSIS SYSTEM DURING EXPERIMENTAL INFLUENZA IN MICE

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Summary. — Proteolysis system was examined in influenza-virus-infected mice after a 5-day course of therapeutic or preventive treatments with the proteolysis inhibitor ε-aminocaproic acid (E-ACA). The mice were infected with nonadapted influenza virus A/Hong Kong/1/68 (H3N2). E-ACA was shown to exert a pathogenetic action expressed by a marked tendency to normalization of elevated alkaline protease activity in damaged lung tissue and in the blood of infected animals. E-ACA induced a long-lasting high level activity of acidic proteases in the blood which correlated with increased protection of animals against influenza virus infection. It may be suggested that acidic proteases are involved in the preventive action of E-ACA and are a factor of resistance to virus infection.

Key words: ε-aminocaproic acid; experimental influenza; antiviral protection; decrease of alkaline protease activity; elevation of cathepsin level

Introduction

Inhibitors of preoteolytic enzymes seem to be the most promising among a wide variety of chemoagents used against diseases of virus aetiology. The inhibitors show both therapeutic (Lozitsky *et al.*, 1979; Lozitsky and Polyak, 1982; Zhirnov *et al.*, 1984) and preventive activities (Hrušková and Jarý, 1975; Puzis and Lozitsky, 1985; Puzis *et al.*, 1986). Basic mechanisms of etiotropic (Lozitsky and Polyak, 1982; Zhirnov *et al.*, 1984) and pathogenetic (Lozitsky *et al.*, 1979) therapeutic activities of some proteolysis inhibitors have already been studied. However, the mechanisms of action of proteolysis inhibitor E-ACA on the resistance of the treated animals against a delayed influenza virus infection are unclear.

Proteolysis inhibitor E-ACA is known to be rapidly eliminated from the organism (Mashkovsky, 1967). Therefore, an earlier reported phenomenon of prolonged preventive efficiency of the drug (Puzis *et al.*, 1986), as well as a higher level of protection against reinfection of animals treated with E-ACA during primary infection (Lozitsky *et al.*, 1988) cannot be explained by direct action of the inhibitor. Therefore, it seems likely that

E-ACA may exert a regulatory effect on the infection process by regulating the proteolysis system. This encouraged us to study the time course of the activity of some parameters characterizing the state of proteolysis system in the body. These studies aimed at the determination of probable mechanisms of the prolonged effect of E-ACA during experimental influenza infection.

Materials and Methods

Virus. Influenza virus A/Hong Kong/1/68 (H3N2) nonadapted to mouse lung was used in the form of allantoic fluid.

Proteolysis inhibitor. E-ACA (a commercial drug made in the U.S.S.R.) was used in the form of sterile 15 % solution in 0.15 mol/l NaCl.

Experimental animals. Outbred albino mice of either sex weighing 15—17 g were kept in the nursery under conventional conditions. The mice were intranasally infected with influenza virus A/Hong Kong/1/68 at a dose of 10^4 EID_{50} in 0.5 ml. The E-ACA solution was injected subcutaneously for 5 days at the dose of 90 mg per mouse daily, divided in 3 injections by 0.2 ml. The solution or placebo were injected starting one day after primoinfection. Altogether 360 mice were used. They were divided into 5 experimental groups as indicated in the protocol (Table 1).

Five to 8 mice were taken in the experiment at various times. Their blood was totally collected under ether anesthesia, the lungs were then removed and homogenized in 0.15 mol/l NaCl, 2 ml of the solution per lungs of mouse. The activity of proteolytic enzymes was determined in lung homogenates clarified by centrifugation (1000 rev/min, 10 min) and in the blood serum.

Analysis of enzymes. Activity of acidic proteases was assessed in the blood serum (Vovchuk, 1979); 2% hemoglobin solution (Reanal, Hungary) in 0.2 mol/l acetate buffer, pH 5.0, denatured by heating in a boiling water bath for 5 min was used as a substrate. Reaction mixture was incubated at 37 °C for 20 hr in the presence of preservative (toluene). The activity of alkaline proteases (pH 7.6) was determined in the blood serum and in clarified lung tissue homogenates (Vovchuk, 1979) using as substrates 1 % solution of protamine sulphate (Merck, F.R.G.) and 2 % solution of casein (Biokhimreaktiv, U.S.S.R.), respectively. Reaction mixture was incubated at 37 °C for 30 min or 4 hr for determination of protaminase or caseinolytic activity, respectively. Quantity of enzyme causing the formation of 1 μmol/l of arginine (in the case of protamine digestion) or tyrosine (at hemoglobin or casein digestion) during 1 min of incubation was taken as the unit of enzyme activity (units/ml).

Statistical significance of the differences was determined as described elsewhere (Ashmarin and Vorobiev, 1962).

Results

After the administration of E-ACA on the proposed schedule a therapeutic and long-lasting preventive effect was observed (Puzis *et al.*, 1986; Lozitsky *et al.*, 1988).

Table 1. The experimental protocol

Animal group	Number of animals	Infection on day 0 of the experiment	E-ACA treatment	Infection on day 30 of the experiment
1	80	+	—	+
2	80	+	+	+
3	40	—	—	+
4	80	—	+	+
5	80	—	—	—

Enzymatic activities characterizing the state of protoelysis system indicated that in untreated animals (groups 1 and 3) the virus infection was associated with a significant rise in the lungs of the caseinolytic activity of alkaline proteases (Fig. 1) since day 7 p.i. (for group 3 on day 37 of the experiment). The same enzyme activity was significantly increased (3.5

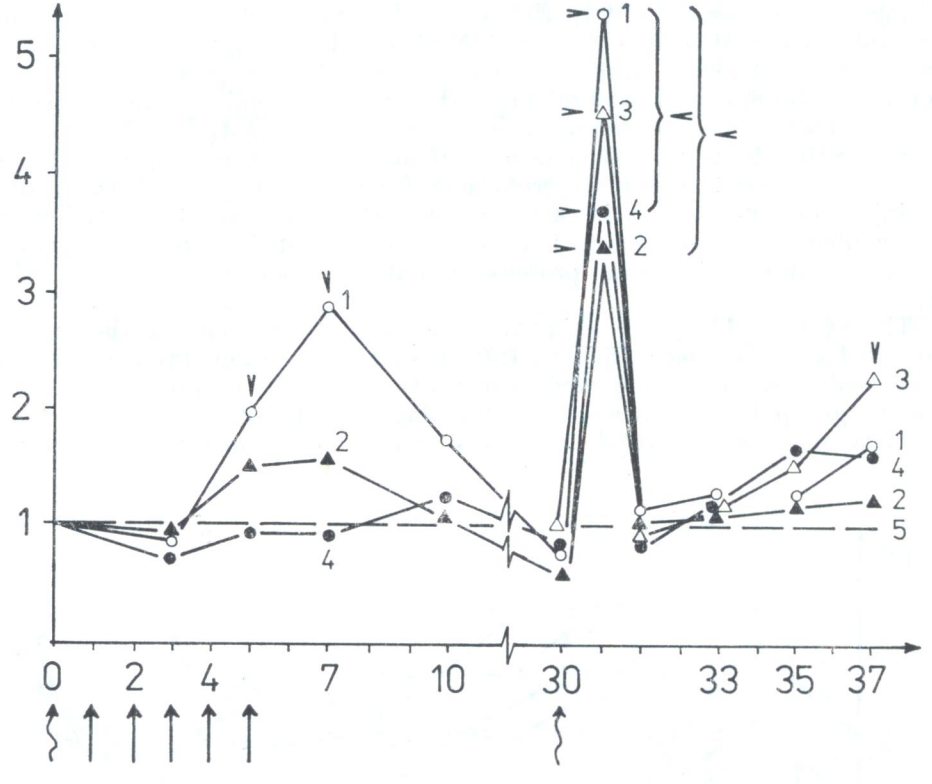

Fig. 1

Therapeutic or preventive action of E-ACA on the course of alkaline protease activity (pH 7.6) in the lungs of mice infected with influenza virus

Abscissa: time (days); ordinate: activity of the enzyme (relative arbitrary units: RAU). At each observation interval the level of enzymatic activity was determined in placebo-treated mice and regarded for 1.

Group 1 (o) — mice untreated during primary infection and reinfected on day 30 of the experiment. Group 2 (▲) — mice treated with E-ACA after primary infection and reinfected on day 30. Group 3 (△) — placebo-treated mice infected on day 30 of the experiment. Group 4 (●) — mice which were exposed to preventive E-ACA treatment and then infected on day 30 of the experiment. Interrupted line (group 5) — uninfected placebo-treated mice.

Curved arrow — intranasal infection with nonadapted influenza virus A/Hong Kong/1/68 (H3N2) at a dose of 10^4 EID_{50}, straight arrow — E-ACA injection (90 mg/mouse/day). 30^+ = = 60 min postinfection.

Triangles — statistically significant differences ($p < 0.05-0.01$).

up to 5.5-fold) by 1 hr p.i. on day 30 of the experiment in animals of groups 1 through 4. It should be noted that the observed changes were much less marked in infected animals after therapeutic (group 2) or preventive (group 4) E-ACA treatment. In the latter case the administration of the drug before infection was essentially without effect on the activity of alkaline proteases in the lung tissue.

Protaminolytic activity of alkaline proteases of the blood serum also changed in the course of experimental influenza infection. In animals untreated with E-ACA (groups 1 and 3) this activity appeared to rise with peaks on days 3 and 7. In mice treated with E-ACA during primary infection (group 2) the first peak was missing, whereas the second one was preserved (on day 7 when the compound was no longer administered) (Fig. 2).

Preventive E-ACA treatment of experimental influenza (group 4) first caused a certain decrease of protaminase activity in blood in the course of injection but later on its activity gradually resumed the normal level. After infection of mice groups 1, 2 and 4 on day 30 of the experiment similar slight variations of alkaline protease activity in blood were observed (Fig. 2).

The level of acidic protease in the serum showed the following: the changes revealed a similar time course regardless whether mice were infected without treatment (group 1), subjected therapeutic (group 2) or preventive treatments (group 4). After a certain fall lasting until day 3, the acidic protease activity increased to reach a level similar to the control and remained

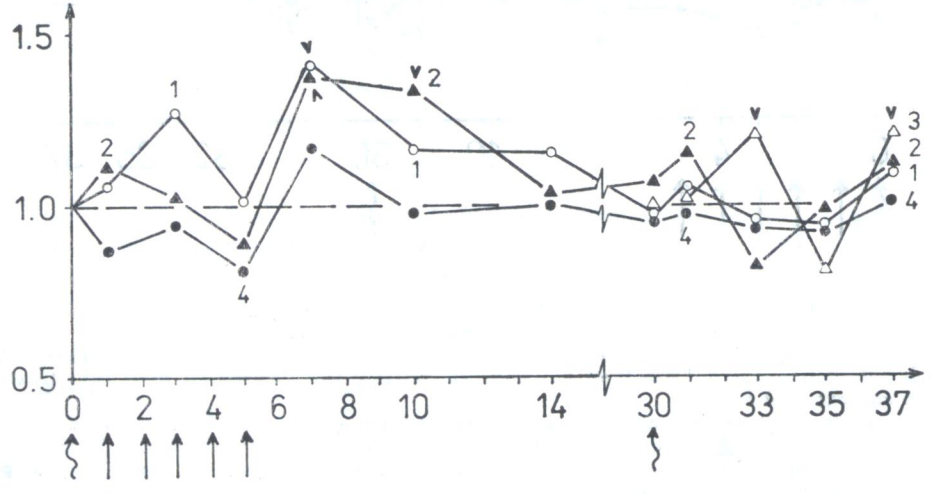

Fig. 2

Action of E-ACA therapeutic and preventive treatment on the time course of alkaline protease activity (pH 7.6) in the blood serum of mice infected with influenza virus
For explanations see Legend to Fig. 1.

at that level within observation days 5 through day 10. On day 14, however, the activity of acidic proteases in the blood of above-mentioned mice was as high as the control level (Fig. 3). On day 30 significant differences were observed. In infected animals treated with E-ACA (group 2) the activity of acidic proteases appeared to be the highest (about 2.6 times as high as in control group), while in those which received a preventive treatment (group 4) the enzyme activity remained at an unchanged level (2 times as high as in controls) and in those previously infected untreated animals (group 1) it decreased to reach the control level. After infection of mice on day 30 of the experiment, the high level of acidic proteases activity dramatically decreased in groups 2 and 4 reaching a control level on day 31; later on it did not change even in groups 1 and 3. Hence, the increased activity of acidic proteases was readily resumed a normal level in response to virus infection.

Discussion

The present study has confirmed in the lungs of mice the previously reported rise of the activity of serum alkaline proteases in response to infection with a highly pathogenic influenza virus strain and a significant leveling of these

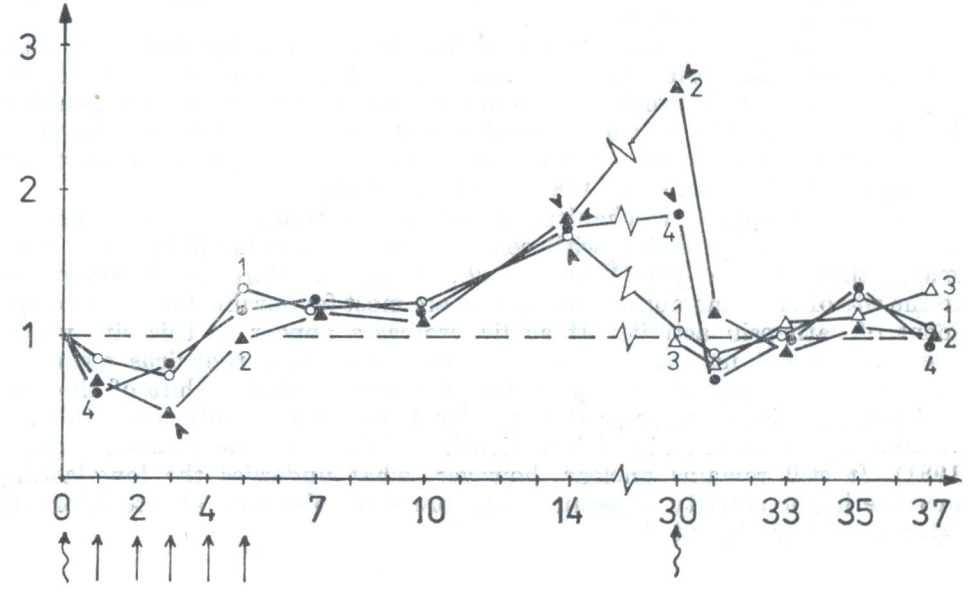

Fig. 3

Therapeutic and preventive E-ACA action on the course of activity of acidic proteases (pH 5.0) in the blood serum of mice infected with influenza virus

For explanations see Legend to Fig. 1.

changes after therapeutic administration of proteolysis inhibitors (Lozitsky *el al.*, 1979). Moreover, similar changes were observed in delayed infection after preventive treatment and also up on reinfection of convalescent animals that had been previously treated with E-ACA. The comparison of the results obtained with the data reported earlier (Puzis *et al.*, 1986; Lozitsky *et al.*, 1988) suggests the following: activation of alkaline proteases in the lungs in response to infection is lower in those groups of animals which developed increased protection against infection. This seems to be quite natural as trypsin and trypsin-like proteases are known to provide proteolytic cleavage at realization of infectious potential of the virus as well as to participate in the pathogenesis of infection. It still remains unclear, however, which mechanism underlies elimination of proteolysis enhancement in response to delayed infection (25 days after the last administration of the agent). Probably, this is due to the abatement of the infection course associated with E-ACA treatment.

The results obtained also indicate that E-ACA injection induces changes characterized by a prolonged rise in activity of acidic proteases in the blood at late intervals after the administration which correlates with increased protection of mice against influenza infection. This suggests that acidic proteases participate in the effectuation of preventive antiviral action of E-ACA, probably, as factors of antiviral resistance of the host. Their high content in blood may probably be regarded as a sign of increased resistance against infection.

This suggestion is also supported by data indicating that the high activity of cathepsin D correlates with high resistance of mouse cell line L_R against vesicular stomatitis virus. Also, it is strengthened by the fact that the loss of biologic activity of vesicular stomatitis virus in HeLa cells results from the enhancement of endopeptidase activity of cathepsin D (Tutelyan *et al.*, 1981; Tutelyan, 1984).

We suggest a likely mechanism of cathepsin antiviral effect. It is known that influenza virus fusion with cell membranes is induced by virus haemagglutinin at acid pH (White *et al.*, 1983). Therefore, local conditions in the site of virus-membrane interaction are most favourable for the development of cathepsin activity. If acidic proteases appear at this site where they should not normally occur, they may inactivate the virus owing to proteolytic cleavage of viral proteins. It is known that a shift of the site of haemagglutinin precursor cleavage by 1 amino acid only leads to irreversible inactivation and not to activation of the molecule (Garten *et al.*, 1981). It still remains unclear, however, what underlies the long-lasting increase in the activity of serum acidic proteases observed at late intervals after E-ACA treatment.

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