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RESISTANCE OF MICE TO REINFECTION AFTER *E*-AMINOCAPROIC ACID TREATMENT OF PRIMARY INFLUENZA VIRUS INFECTION

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Summary. — The effect of proteolysis inhibitors on the formation of resistance to virus challenge has been studied in experimental influenza of mice. *E*-aminocaproic acid (E-ACA) when used in the treatment of influenza decreased the virus reproduction in lungs and also enhanced the humoral immune response. The antibody titre on days 14 to 21 post infection (p.i.) was significantly higher in the treated animals. On day 30 after challenge with the homologous strain (H3N2) the virus reproduced to low levels in the lungs of untreated convalescent mice, but no virus was detected in the lungs of mice which had been treated with E-ACA during primary infection. Marked increase of the antibody level was found in such mice. Upon challenge with lethal doses of the virulent strain (H1N1), the protection was significantly higher among animals treated with E-ACA during primary infection with a sublethal virus dose. We believe that the immunomodulatory action of E-ACA may play an important role in the increased resistance to challenge exhibited by such treatment.

Key words: experimental influenza; resistance; ε-aminocaproic acid

Introduction

Based on the importance of the proteolytic system in the development of influenza infection there was assumed that the therapeutic application of proteolysis inhibitors would provide a significant alleviation of the infection course under experimental (Lozitsky, Polyak, 1979; Lozitsky *et al.*, 1979) as well as clinical (Lozitsky *et al.*, 1980; Buiko, Lozitsky, 1984; Ursaki *et al.*, 1984) conditions. The drugs not only exerted an antiviral effect but also influenced the pathogenetic process (Lozitsky *et al.*, 1979). It has been shown that proteolysis inhibitors had no adverse effects on antibody production in experimental influenza (Degtyarenko *et al.*, 1978; Zhirnov *et al.*, 1983). Furthermore, single administration of ε-aminocaproic acid (E-ACA) or its hydrochloride had a prophylactic antiinfluenza effect persisting for over 10 days (Hrušková, Jarý, 1975; Lozitsky *et al.*, 1985); after a 5-day ad-

ministration schedule E-ACA appeared to act for at least 1 month (Puzis, Lozitsky, 1985; Puzis *et al.*, 1986).

However, the influence of proteolysis inhibitors on the formation of immune response during experimental influenza was insufficiently studied. The remote consequences of the therapeutic application of E-ACA on the course of reinfection are unknown. The present study has been devoted to these problems.

Materials and Methods

Viruses. Allantoic cultures of highly pathogenic influenza virus strains AO/32 (H1N1), A/PR/8/34 (H1N1) and strain A/Hong Kong/1/68 (H3N2) not adapted to mouse lungs have been used.

Experimental animals. Experiments were carried out on outbred albino mice of either sex weighing 15—17 g.

Proteolysis inhibitor. E-ACA was manufactured by the Lomonosov Chemico-Pharmaceutical company (Kiev, U.S.S.R.).

Effect of E-ACA on virus reproduction in the lungs and on the antibody formation. The mice were infected intranasally (i.n.) with influenza virus A/Hong Kong/1/68 at a dose of 10^4 EID_{50} in 0.05 ml. For 5 subsequent days p.i., the animals were administered subcutaneously E-ACA in 0.15 mol/l NaCl solution (90 mg/daily/per 1 mouse in 3 injections). The control animals were given 0.15 mol/l NaCl. By 30 days after primary infection, the mice were repeatedly infected with the homologous virus in the same dose. About 5 to 8 mice of each group were examined at given intervals. Blood was taken and the lungs were removed under ether anesthesia. Infectious virus in the lugs was determined by titration of 10% homogenates on the chorioallantoic membrane as described by Maltseva *et al.* (1973). Specific antibody titre with 2 HAU of inhibitor-resistant variant of influenza virus strain A/Hong Kong/1/68 was determined in individual samples of the serum heated at 56 °C for 30 min according to an earlier described procedure (Bichurina *et al.*, 1984; Puzis *et al.*, 1986).

Influence of E-ACA on protection of animals during lethal experimental influenza infection. For primary infection, the mice were given intranasally 0.5 LD_{50} of highly pathogenic influenza virus A/32. Treated animals were given E-ACA as described above. Challenge was carried out 30 days after primoinfection using 6 animals for each virus dilution within the range of 10^{-1} to 10^{-6}. Deaths of animals were recorded for 14 days.

Mathematical methods. With the use of a modification of Kaerber method suggested by Ashmarin a 50% tissue culture infective dose ($TCID_{50}$) was calculated for determination of infectious virus concentration and a 50% lethal dose (LD_{50}) was calculated for modelling of lethal infection. Statistical significance of the drug action was estimated (Ashmarin, Vorobiev, 1962). For the determination of specific antibody level in the blood of animals mean geometric titres were calculated and statistical significance of differences was determined (Voroshilova *et al.*, 1964).

Results and Discussion

On days 3, 5 and 7 p.i. with influenza virus A/Hong Kong/1/68 the virus content in the lungs of E-ACA treated mice was 10—100 times lower than in untreated mice (Fig. 1-I). This was indicative of marked therapeutic effect of the proteolysis inhibitor during sublethal infection caused by an influenza virus strain of low virulence for mice. This confirms the therapeutic efficiency of the drug previously demonstrated in experimental lethal influenza (Lozitsky *et al.*, 1979; Zhirnov *et al.*, 1983).

Suppression of virus reproduction in the lungs of mice treated with E-ACA was associated with gradual increase of specific antibody titres without significant differences from control on observation days 5—10. On day 14,

however, the antihaemagglutinin level in the blood of treated mice was significantly higher (Fig. 1-II).

Another two experiments were carried out following the same procedure. In one trial the animals were infected also with influenza virus A/Hong Kong/1/68 and in the other — with a sublethal dose of influenza virus strain A/PR/8/34 which is highly pathogenic for mice. The results of these additional experiments have confirmed the inhibitory effect of E-ACA on virus reproduction in the lungs. This effect was more marked on infection with strain A/Hong Kong/1/68. On observation day 21 the treated animals appeared to have a significantly higher antibody level (by 1.2 and 2.25 $\log_2$, respectively, when infected with viruses A/Hong Kong/1/68 and A/PR/8/34).

By challenging the mice with virus A-Hong Kong/1/68 (on day 30 after primary infection) no infectious virus was detected in the lungs within 1 hr p.i. Without E-ACA pretreatment the infectious virus was detected in the lungs for five subsequent days and the reproduction peak fell on day 3, i.e. day 33 of observation. In contrast, from animals pretreated with E-ACA the virus was isolated from the lungs of one mouse only on day 33 p.i. (Fig. 2-I).

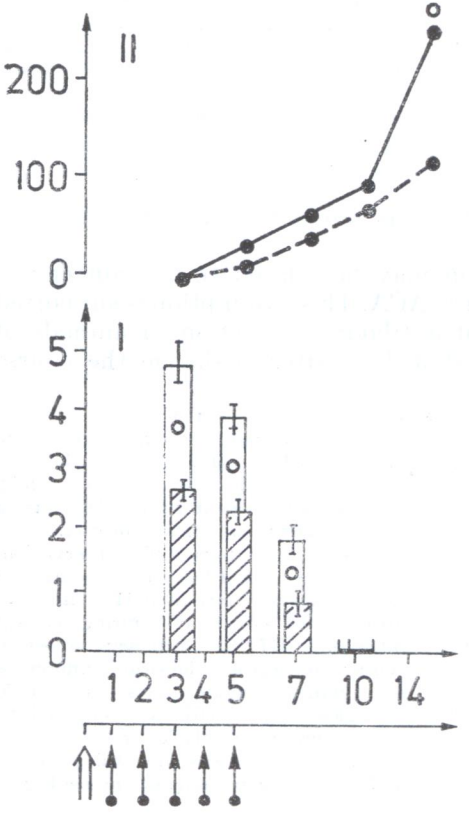

Fig. 1
Effect of E-ACA on the course of virus reproduction in the lungs (I) and accumulation of specific antibodies in blood (II) of mice infected with influenza virus A/Hong Kong/1/68. Abscissa — days of observation; ordinate — I: virus titre (log LD_{50}); II: mean geometric values of antibody titres (inverse values). Thick arrow: infection, thin arrows: E-ACA administration. Blank columns and dashed line — placebo shaded columns and solid line — mice after 5-day course treatment with E-ACA. ○ — significant difference ($p < 0.05$).

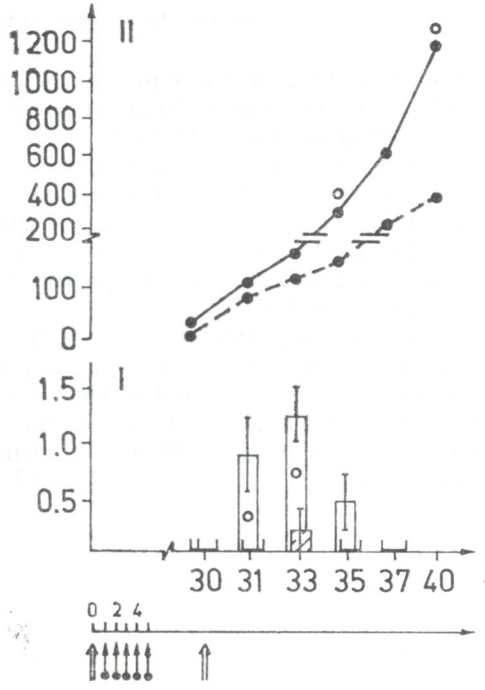

Fig. 2
Course of virus reproduction in the lungs (I) and accumulation of specific antibodies in blood (II) after repeated infection with influenza virus A/Hong Kong/1/68 of mice treated with E-ACA during primary infection with homologous virus.
30 – 60 min after challenge. Other notes as in Fig. 1.

At other times of observation the virus was not detected in the lungs of pretreated animals.

The mechanisms of this phenomenon may be related to a stronger immunologic memory in mice treated with E-ACA. This assumption is supported by comparative data on the course of antibody production in animals of both challenge groups. During the first 3 days after challenge the course

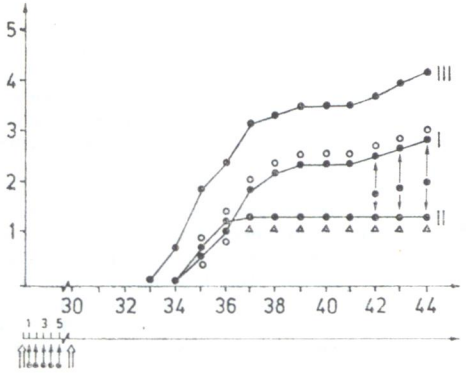

Fig. 3
Effect of repeated infection with influenza virus AO/32 as lethal doses on the time course fo death rate in mice which underwent treatment with E-ACA during primary sublethal infection.
Abscissa — days of observation; ordinate — log LD_{50}; I — reinfected mice, previously untreated, II — challenge mice treated with E-ACA during primary infection, III — primary infection (virulence control of the virus). Differences are significant: white circles — $P < 0.05$, triangles — $p < 0.01$ (as compared to control group 3), black circles — $P < 0.05$ (between experimental groups 1 and 2). For arrow designations see Fig. 1.

of secondary humoral response was similar in the mice of the compared groups. Later on, from day 5 after repeated infection, i.e. from day 35 to 44 (observation time) specific antibody titres in the serum of E-ACA-pretreated animals were significantly higher than in untreated mice (Fig. 2-II).

It has been found that mice treated with E-ACA during primary infection with highly pathogenic virus AO/32 at sublethal dose had an increased protection against challenge. Deaths of animals that had earlier been exposed to a homologous influenza were first registered on day 5 after challenge, i.e. one day later than in the group of mice that had not been previously exposed to infection (Fig. 3). On days 5 and 6 the death rate was similar in the groups of reinfected animals. However, even at these, as well as at subsequent intervals the death rate was significantly different from that of controls (group 3) in which the $\log_{10} LD_{50}$ was -4.18 ± 0.4 by the end of the observation period. Meanwhile, on day 6 post challenge the protection level of mice which had been treated with E-ACA during primary infection differed from untreated ones; on days 12 to 14 after challenge (days 42 to 44 of the experiment) these differences were statistically significant. By the end of the observation period the $\log_{10} LD_{50}$ in the group of untreated convalescent animals amounted to -2.83 ± 0.3 while in the group of animals treated with E-ACA during primary infection it reached $-1.33 \pm 0.\%$.

Thus, mice treated with E-ACA during primary infection seem better protected against challenge with the homologous virus than the untreated convalescents. Our findings indicate that therapeutic application of the proteolysis inhibitor E-ACA suppressed influenza virus reproduction in the lungs of infected mice and significantly enhanced the protection of treated animals against delayed (30 days) challenge. Moreover, it has been previously shown by means of biochemical and pathomorphological methods that protease inhibitors such as E-ACA had a marked effect on the pathogenesis of experimental influenza. This was evident from the suppression of enhanced proteolysis occuring in the lungs during influenza infection, from the decrease of microcirculation disturbances, from prevention of increased vascular permeability, unimpaired air-blood barrier and enhanced local protective responses (Lozitsky *et al.*, 1979).

It seems of special importance, therefore, that unlike other chemotherapeutic agents, E-ACA has been shown to stimulate the humoral immunity during primary and secondary immune responses. This provides additional argument for the application of proteolysis inhibitors as agents exerting etiotropic and pathogenetic influence during influenza, for they also showed an ability to enhance the resistance of the organism against reinfection. The causes of increased protection against reinfection in animals treated with E-ACA during primary infection are not yet clear. However, the results of this paper together with long-term prophylactic effect of E-ACA reported elsewhere (Puzis, Lozitsky, 1985; Puzis *et al.*, 1986) allow us to conclude that E-ACA can stimulate the mechanisms of specific and non-specific antiviral protection of the organism. This points to good prospects of E-ACA as an agent for influenza therapy and prophylaxis.

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