Antiproteinase therapy of influenza virus

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Abstract
A new theory of grippe pathogenesis with participation of proteinase-inhibitory system has been offered. It has been established that purification and concentration of grippe viruses by different methods did not release the virus from cellular enzymes. At the experimental animals infecting with the virus of grippe, disturbance of enzyme-inhibitory balance took place, especially during first hours after the animals being infected. From the lungs of healthy mice, they have got six isoforms of trypsin-like proteinases. To all of them they got antiproteinase immune sera and have treated the experimental animals. It was antiserum to the third isoform that has prevented the experimental animals fatality.

Introduction
In the pathogenesis of viral diseases the interaction of virus and cell is under-investigated. Penetration of a virus into a healthy cell with virus’s obligatory deproteination is of top importance here. While viruses deproteinization is studied insufficiently. First of all it refers the mechanisms of grippe virus’s penetration into the cell of mammals, including a human being. In this respect we had offered a new theory of grippe pathogenesis with proteinase-inhibitory system (PIS) participation [1,2]. The difficulties of new antiviral drugs creation rise from specific biological properties of viral diseases pathogens. The recent findings of biochemistry and molecular biology have highlighted some peculiarities of virus’ reproduction and provided new approaches for a directed interference into the cycle of viral reproduction [3,4].

The problem statement: to examine the state and role of antiproteinase systems of virus and recipient in the development of influenza infection and obtained fundamentally new medicinal preparations based on of trypsin – like proteinases inhibitors.

The objectives: to examine the state and role of antiproteinase systems of virus and recipient in the development of influenza infection and obtained fundamentally new medicinal preparations based on of trypsin – like proteinases inhibitors.

The tasks:
1. To learn the proteinases’ and their inhibitors role at different, especially early stages, of grippe development.
2. To eliminate and purify proteinase and its inhibitor from the lungs of healthy and infected by grippe mice.
3. To investigate the protective properties of cellular inhibitor at infection of the animals with lethal dose of grippe virus.

Results
At early 1980 when purified and concentrated different races of grippe viruses to obtain polyvalent anti-gripe vaccines we met with impossibility to release influenza’s virus from protolithic activity. To solve this problem we have improved the methods of purification but nevertheless we failed to purify influenza virus from protolithic activity [5].

Analysis of purified preparations of influenza virus showed that ultracentrifugation does not release influenza’s virus from protolithic activity and in saccharose’s gradient (15-60%) it distinctly got into several isoforms (Table 1).

The results obtained allowed us to conclude that serine-containing proteinase of trypsin-like type of cellular origin with molecular heterogeneity is associated with influenza virus [6,7].

System of proteinases and inhibitors is presented in the body by a vast group of proteins. Inhibitors of protolitich enzymes have a role of the constant level of corresponding enzymes and are in a constant dynamic equilibrium with the latter. Disturbance between enzymes and inhibitors is of importance for the development of pathological processes.

Our researches show that the level of proteinase activity and activity which inhibits proteinase is in equilibrium in the lungs and blood serum of uninfected animals and the latter disturbances at infection by the virus of influenza A [8].

At communicable process the deepest changes have place during first hours after introduction of infection. Thus, in 6 hours after contamination the amount of proteinase in lungs and blood serum of the infected animals reduces while inhibiting activity increases. The cells contaminated by influenza virus induce appearance of inhibitor both in lung tissue and in blood serum. So, lung inhibitors are an organ “first line of resistance” at the action of various flu strains [9].

6 isoforms of trypsin-like proteinase were extracted from the lungs of the healthy mice and 8 isoforms – from the infected animals by ion-exchange chromatography. Their specific proteolytic activity vastly increased compared to the source material. The proteinase isoforms’ obtained had a wide range of substrate specificity and were able to hydrolyse substrates of both natural and synthetic origin (Table 2).

Antiproteinase hyper immune rat sera were obtained to both

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isofoms of trypsin-like proteinases [10]. In studies of the protective properties of antiproteinase sera and serum of healthy rats on the white mice infected intranasal with the lethal dose of influenza virus A/PR/8/34 (IV passage) it was found that 100% of control mice fatality took place in 4-5 days. The animals to which they injected healthy rats serum intranasal 6 times, died on the 7th day. In the treatment of mice with the pools of immune sera I, II, IV, V and VI groups the animals mortality rate was reduced and lethality came much later than in the control group. 20% of the animals recovered (Table 3).

The most effective was the fourth pool of immune serum to the IIIrd isoform. In its presence 60% of infected mice survived and on the 14th post-infection day in the blood serum and in the lungs we did not detect either hemagglutinin, no infectious virus. Immune serum to isoform VI did not protect mice from death at all, although isoform III differed from isoform VI by only one protein with molecular mass 32 kDa.

From the lungs of healthy mice, we have isolated an inhibitor of trypsin-like proteinases with molecular mass 47.5 kDa, with a high degree of purity and small amount of impurities. We have designed and patented a technique of procedure and decontamination of trypsin-like proteinases [11,12]. The inhibitor isolated is a like α1-inhibitor of human blood serum (m.m. 48-55 kDa) and egg-yolk trypsin (m.m.

### Table 1. Protamine-shifting and hemagglutinating activity of influenza virus in fractions of sugar gradient to neutralization and after neutralization by immune (CAM) rat sera.

| Expire-ments | No of sugar gradient fraction | % of sucrose | Proteinase before neutralization, mkg/arg per min in 1 ml | Proteinase after neutralization, mkg/arg per min in 1 ml | HA before neutralization in 0.1 ml | HA after neutralization in 0.1 ml |
|---------------|-------------------------------|--------------|--------------------------------------------------------|--------------------------------------------------------|---------------------------------|---------------------------------|
| 1. Influenza Virus | 1 | 5 | 1.42 | 0 | 0 | 1:2 |
| | 2 | 15 | 32.0 | 0 | 1:8 | 1:2 |
| | 3 | 32 | 8.5 | 0 | 1:16 | 1:2 |
| | 4 | 42 | 29.8 | 0 | 1:2048 | 1:2 |
| | 5 | 49 | 3.9 | 0 | 1:1024 | 1:12 |
| | 6 | 52 | 32.9 | 0 | 1:512 | 1:64 |
| | 7 | 55 | 32.2 | 0 | 1:64 | 1:64 |
| | 8 | 57 | 6.04 | 0 | 1:2 | 1:2 |
| 2. Influenza Virus | 1 | 3 | 13 | 0 | 0 | 1:2 |
| | 2 | 14 | 12.0 | 0 | 1:2 | 1:2 |
| | 3 | 24 | 7.28 | 0 | 1:16 | 1:16 |
| | 4 | 24 | 9.42 | 0 | 1:16 | 1:16 |
| | 5 | 38 | 84.6 | 0 | 1:512 | 1:2048 |
| | 6 | 42 | 23.28 | 0 | 1:1024 | 1:12 |
| | 7 | 46 | 10.8 | 0 | 1:2 | 1:2 |
| | 8 | 48 | 7.02 | 0 | 1:2 | 1:2 |
| 3. CAM | 1 | 1 | 0.05 | 0 | 0 | 0 |
| | 2 | 17 | 0.101 | 0 | 0 | 0 |
| | 3 | 27 | 0.037 | 0 | 0 | 0 |
| | 4 | 29 | 0.059 | 0 | 0 | 0 |
| | 5 | 37 | 0.080 | 0 | 0 | 0 |
| | 6 | 41 | 0.143 | 0 | 0 | 0 |
| | 7 | 49 | 0.064 | 0 | 0 | 0 |
| | 8 | 53 | 0.128 | 0 | 0 | 0 |
| | 9 | 56 | 0.108 | 0 | 0 | 0 |

CAM – chorionallantoic membrane; HA – hemagglutinating activity.

### Table 2. Purification DEAE-cellulose-53 of lung trypsin-like proteinase of healthy mice.

| N of fraction | N of isoform | Specific proteolytic activity per mg of protein | % of proteinase outcome | % of purification by protein |
|---------------|--------------|---------------------------------------------|------------------------|----------------------------|
| 33 | I | 4.285 | 2.09 | 96.8 |
| 53 | II | 83.75 | 5.84 | 99.07 |
| 65 | III | 22.42 | 2.703 | 98.38 |
| 75 | IV | 40.00 | 6.279 | 97.92 |
| 121-130 | V | 32.6 | 136.74 | 99.98 |
| 161-189 | VI | 0.787 | 421.74 | 64.90 |
Discussion

One of the most important stages of the development of many viruses in the host organism is their introduction in the cell after preliminary deproteinization. Regulation of this development of virus is one fundamental principles of their reproduction. Induction or intake of inhibitor of virus proteolytic activation is one of the promising ways of viral diseases treatment, including influenza.

Methods

Strains of influenza virus: A/PR/8/34 (H1N1), grown on a 9-day chicken embryos, were obtained at the D.I. Ivanovsky Research Institute of Virology, Academy of Medical Sciences of Russia and strain AO/32 (H1N1) - from the Influenza Research Institute of St. Petersburg, Russia; white mice and hybrids; chicken embryos; white rats; Wistar line.

Virological analysis

Infection and accumulation of influenza virus A/PR/8/34 (H1N1) on chicken’s embryos. Adaptation of influenza virus A/PR/34 to white mouse. Four passages of influenza virus has been done. A fatal dose of influenza virus equals to 5 LD50 has been obtained. The choice of the dose of trypsin-like proteinase inhibitor for the treatment of white mice infected with IAV.

Concentration and purification of influenza virus

Influenza virus AO/32 (H1N1) with infectious titers 7.0 log EID50/mI and hemagglutinin (HA) -1:256. To obtain influenza virus preparations we used 10-11-day chicken embryos. The virus was accumulated by infecting chicken embryos in a volume of 0.2 ml, diluted to 10⁻³ with infectious material. Infected chicken embryos were incubated for 48 hours at +36°C. Then they were cooled for 18 hours

Table 3. Influence of antiproteinase immune sera on survival of mice under infection with lethal dose of influenza virus A/H1N1/PR/8/34.

| N of gr of mice | Isoform of proteinase | Sera group | 6 h | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 14 | % of survival |
|----------------|----------------------|------------|-----|---|---|---|---|---|---|---|---|---|---|---|-------------|
| 1. I           | I                    | 0/10       | 0/10| 0/10| 0/10| 2/8| 0/8| 2/6| 2/4| 2/2| 0/2| 0/2| 2/20| 60 |
| 2. I           | II                   | 2/8        | 2/8 | 0/6 | 4/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 2/0 | 2/20 | 60 |
| 3. II          | III                  | 0/10       | 0/10| 2/8 | 0/8 | 0/8 | 2/6 | 0/6 | 2/4 | 0/4 | 0/4 | 2/2 | 0/2 | 2/20 | 60 |
| 4. III         | IV                   | 0/10       | 0/10| 0/10| 0/10| 0/10| 2/8 | 0/8 | 2/6 | 0/6 | 2/6 | 0/6 | 6/6 | 0/6 | 60 |
| 5. IV          | V                    | 0/10       | 0/10| 0/10| 0/10| 2/8 | 2/8 | 2/6 | 2/4 | 2/2 | 0/2 | 0/2 | 0/2 | 2/20 | 60 |
| 6. V           | VI                   | 0/10       | 0/10| 0/10| 5/5 | 3/2 | 2/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 100|
| 7. VI          | VII                  | 0/10       | 0/10| 0/10| 7/3 | 1/2 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | 1/10| 1/10 | 60 |
| 6. Sodium chloride solution |          | 0/10       | 0/10| 0/10| 0/10| 0/10| 0/10| 0/10| 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 50 |
| 7. Serum-free virus |             | 0/10       | 0/10| 0/10| 0/10| 0/10| 2/8 | 2/8 | 2/6 | 2/4 | 0/4 | 0/4 | 2/20 | 60 |
| 8. Healthy rats serum |           | 0/10       | 0/10| 0/10| 0/10| 0/10| 2/8 | 3/5 | 3/5 | 5/10| 5/10| 0/10| 0/10 | 0/10 | 50 |
| 9. Immune serum IV gr., virus-free (toxicity) |          | 0/10       | 0/10| 0/10| 0/10| 0/10| 0/10| 0/10| 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 100 | 100|

Note: 1. numerator – number of mice died; 2 – denominator – number of mice in the experiment.

Table 4. Action of cellular inhibitor of trypsin-like proteinase of the survival rate of mice infected with lethal dose of influenza virus A/PR/8/34.

| N and name of group of animals (white mice) | Number of animals | Dose of influenza virus 5 LD50 | Dose of inhibitor, µg on a mice, one injection | Number of animals | % of animals protected from virus |
|--------------------------------------|------------------|-----------------------------|---------------------------------------------|------------------|----------------------------------|
| 1. I influenza virus                  | 40               | 10⁻³                       | -                                           | 40               | 0                               |
| 2. Influenza virus + trypsin, crystal.| 40               | 10⁻³                       | 18 µg                                       | 40               | 0                               |
| 3. Influenza virus + inhibitor from healthy lungs | 40               | 10⁻³                       | 18 µg                                       | 7                | 33                              |
| 4. Cellular inhibitor                 | 40               | 10⁻³                       | 18 µg                                       | -                | 40                              |
| 5. Trypsin, crystal                   | 10               | -                          | 18 µg                                       | -                | 10                              |
| 6. Phosphate buffer                   | 10               | -                          | 0.2 ml                                      | -                | 10                              |

Note: 1. N and name of group of animals (white mice) - 40 mice, 2 – dose of influenza virus 5 LD50 on a mice, one injection.
at +4°C and then the virus-containing fluid was collected, purified and concentrated with centrifugation.

Concentration and purification of influenza virus was done in as follows: virus-containing liquid was consecutively centrifuged with 6 000 R.P.M. during one hour at +4°C on centrifuge CLR-1 (Ukraine) for purification from components of tissue and erythrocytes. Then 30 min at 10,000 R.P.M. at +4°C on centrifuge CLR-1 (Ukraine) to remove the main mass of cellular proteins. After that virus was precipitated at 2000 R.P.M. during an hour on centrifuge CLR-1 (Ukraine) through 20% layer of sucrose for more complete purification of virus. Purified and concentrated virus was additionally purified with speed centrifugation in sucrose density gradient (15-60%) on centrifuge VAK-602 in backet-rotar at 28 000 R.P.M. during four hours. Influenza virus which was in the zone 37-45% of sucrose was reultacentrifugated at 33 000 R.P.M. in rotor SV-40, ultracentrifuged Spinko, three hours.

In the preparations obtained and eluates of fractions the content of proteins in O.H. Lowry [15] method was controlled after dialysis at all stages. As well as content of HA in routine method with 1% solution of chickens embryo erythrocytes and the presence of proteolitic activity in K. N. Veremeyenko method [16] in S. V. Vovchuck's [17] modification.

Study of trypsin-like proteinas role in the development of viral infection in mice

In the present work we use influenza virus A/PR/8/34 (H1N1) adopted to lung tissue of white mice; virus’ infectious titer was 7.0 log EID₃₀₉₀₆ ml and HA titer 1:128. Outbred white mice with mass of 16-17 gr were taken in the experiment. Contamination of the animals was done at a light ether intranasal anesthesia, volume 0.05 ml at the dilution 10⁻⁶, which corresponded to 2,5 -2 LD₅₀ and a small one (at the dilution 10⁻⁶, that corresponded 1 LD₅₀). Outbred white mice with mass of 16-17 gr were taken in the experiment. Contamination of the animals was done at a light ether intranasal anesthesia, volume 0.05 ml at the dilution 10⁻⁶, which corresponded to 2,5 -2 LD₅₀ and a small one (at the dilution 10⁻⁶, that corresponded 1 LD₅₀).

The lungs were shredded with scissors, then homogenized with ultrasound in phosphate buffer (1 ml for one lung), homogenized with ultrasound in phosphate buffer pH 7.5 twice, grinded in a cold mortar, suspended in 72 hours after contamination. Three embryos for each term) and learnt the same indexes as in contaminated material. In the material selected we learnt the presence of proteinase, inhibiting and infectious activity, HA and protein.

Biochemical analysis

We used chromatographic methods for extraction and purification of inhibitor from the wastes of sera-manufacturing industry. Protein determinations was done in O. Lowry method, determination of trypsin inhibitor activity by casein’s hydrolyse in K. M. Veremeyenko method, modified by A. P. Levitsky. Electrophoretic analysis was done in U.K. Laemmli method [19].

Extraction and purification of trypsin – like proteinases

Trypsin – like proteinases have been extracted from the lungs of the healthy mice (100 items for an experiment) and also from the lungs in 72 hours after contamination with influenza virus A/H1N1/PR/8/34 (100 items for an experiment). The experiment was performed with the mice of the line «Balb/ », weighted 16-18 gr. Contamination of the animals with the virus of influenza was performed under light ether anesthesia in the volume 0.05 ml in the dilution 10⁻⁶ which corresponds virus infectious dose 1 LD₅₀.

In 72 hours after the animals’ contamination the animals were killed and their lungs were extracted. Parallels with it blood was taken. The lungs were shredded with sissors, then homogenized in a mortar, processed with ultrasound at 18 kHz, 75 ° on the device Soniprep 150 MSE. The whole work was done at the temperature +4°C. Homogenate was centrifuged at 10,000 R.P.M. during 1 h at centrifuge RC, temperature +4°C. Supernatant I was frozen, 1% Triton X-100 was added to the precipitate and left in the refrigerator till morning.

Next day the precipitate was processed with ultrasound once more and centrifugated at the same regimes. Supernatant II was taken. Both supernatants (I and II) were joined later on for purification.

Purification of the material dialyzed was done by ion-exchange chromatography on DEAE-cellulose-32, device ZKB - 2023, Minicolob (Broma). Column’s height was 18 cm, diameter 1.75 cm. The extent of purification was controlled by proteins and protease’s activity. Protein was determined by O. Lowry’s method and protamine-splitting activity by K. Veremeyenko method.

Fractions with high indexes of protease were purified additionally by dialysis, then dried by liophilization on Hetocite Heto (Denmark). Extracted and purified isoforms were used for immunization of the animals. At all stages of isoforms of trypsin-like proteinase purification, in all fractions determination of proteinase, inhibiting, hemagglutination activity and total protein was carried out, 19 biological repetitions were done.

Production of hyperimmune antiproteinase sera

To obtain sera we used white rats of Wistar line weighed 170-200 gr.

White rats were immunized four times, once per week with each isoform of trypsin-like proteinase extracted from the healthy and infected lungs of mice with a complete Friend’s adjuvant. Each rat got 560 units of protein and 890 units of proteinase. Total pick up of

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hyperimmune sera was done in 7 days after the last immunization.

**Extraction and purification of trypsin inhibitor from lungs of mice**

Extraction of inhibitor was done by the method used for proteases extraction. Trypsin inhibitor purification was done by ion-exchange chromatography on DEAE–cellulose, gel-filtration – on Sephadexes G-15 and G-50, by affinity chromatography on trypsin – sepharose 4B. In the last-mentioned case trypsin was covalently perfected in 0.05 M tris-HCl buffer pH 7.6. Desorption was done consequently with buffer solutions, containing 1.0 M NaCl, 8 М urea and 0.2 M KC1-HCl pH 2.0 solution. Trypsin’s inhibitor in fractions was determined by deceleration of benzoyl-arginine-p-nitroanilide hydrolysis by crystalline trypsin (A.P. Levitsky, 1979).

**Determination of trypsin-like proteinases activity by proteamine hydrolysis**

Qualitative reaction to arginine, forming at the hydrolysis of proteamine and histones, and which does not precipitate with 20% ClCl3 COOH, forms the basis the method.

Reagents.
1. 1.0% solution of protamine sulphate on 0.1 M phosphate buffer pH 7.5.
2. 20% solution of ClCl3 COOH.
3. 0.004% solution of oxychinoline. 100 mg of oxychinoline was diluted in 500 ml of spirit of wine. The working solution was prepared by 50 times soluted with aqua distillate initial solution just before determination.
4. 10.0% NaOH.
5. 10.0% solution of NaBrO. 1.0 gr. Br (0.3 ml) was brought up to 100 ml with cooled to 0°C 5.0% solution NaOH.
6. 40.0% solution of urea.

**Procedure of determination.** To the mixture, containing 0.2 ml of proteamine sulphate and 0.5 ml 0.1 M phosphate buffer pH 7.5, 1 ml of enzymatic solution was added. The samples were incubated 150 min on the water bath at +38 °C. The reaction was stopped by 0.9 ml of 20.0% ClCl3 COOH, forms the basis the method.

To add 2 ml of reagent A and 2 ml of Folin’s reagent. Contact – 30 min at room temperature. Analyze at spectrophotometer.

**Calculate of inhibiting activity (IA)**

Serum: $\text{IA} = \frac{(\Delta E_n - \Delta E_0) \cdot 0.2 \cdot n}{\Delta E}$

Tissue: $\text{IA} = \frac{(\Delta E_n - \Delta E_0) \cdot 200 \cdot 21 \cdot m}{\Delta E_0 \cdot 1000}$

where: $\Delta E_n$ - extinction of the sample with trypsin; n - dilution of the solution with serum; 0.2 – trypsin’s concentration, mg/ml; m – dilution of inhibitor’s solution; 200 – the amount of trypsin in 1 ml (200 mgk); 21 – ratio of tissue’s charge to extrangent, weight 100 mg. per 2 ml; 1000 – recalculation coefficient against 1 gr of tissue; I - content of inhibitor per 100 ml; 1 - a unit corresponding to 1 gr of crystalline trypsin; $F_{ts}$ - extinction of the sample with the mixture trypsin + inhibitor.

**Statistical analysis**

The results of the investigations carried out have been processed with the programme «Microsoft Excel».

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