

Резюме

РЕКОМЕНДАЦИИ ПО МЕДИКО-ПСИХОЛОГИЧЕСКОЙ ПРОФИЛАКТИКЕ И РЕАБИЛИТАЦИИ МОРЯКОВ В СВЯЗИ С ПРОБЛЕМОЙ ПИРАТСТВА

Оливер Брюер

В настоящее время пиратство стало серьёзной проблемой в безопасности морского судоходства. Угроза нападения, схватки с пиратами и особенно пребывание в плену вызывают психоэмоциональный стресс, приводят к нарушению психосоматического здоровья и являются угрозой для жизни моряков. Решению этих важных проблем посвящена настоящая статья.

Ключевые слова: психическое здоровье моряков, пираты, психоэмоциональный стресс

Резюме

РЕКОМЕНДАЦІЇ ЩОДО МЕДИКО-ПСИХОЛОГІЧНОЇ ПРОФІЛАКТИКИ ТА РЕАБІЛІТАЦІЇ МОРЯКІВ У ЗВ'ЯЗКУ З ПРОБЛЕМОЮ ПІРАТСТВА

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В даний час пиратство стало серйозною проблемою в безпеці морського судноплавства. Загроза нападу, сутички з пиратами і особливо перебування в полоні викликають психоемоційний стрес, призводять до порушення психосоматичного здоров'я і є загрозою для життя моряків. Вирішенню цих важливих проблем присвячена ця стаття.

Ключові слова: психичне здоров'я моряків, пірати, психоемоційний стрес

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PROTEOLYTIC THEORY OF PATHOGENESIS OF INFLUENZA AND IMPROVEMENT OF ITS TREATMENT

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A new theory of gripe pathogenesis with the use of proteinase-inhibitory system has been offered. It has been established that purification and concentration of gripe viruses by different methods did not release the virus from the cellular enzymes. When the experimental animals have been infected with gripe virus, we have observed misbalance in enzyme-inhibitory balance took place, especially during first hours after infection. Six isoforms of trypsin-like proteinases were obtained from the lungs of healthy mice. Antiproteinase immune sera have been isolated from these 6 isoforms which served to treat the experimental animals. It was antiserum to the third isoform that has prevented the experimental animals mortality. From the industrial wastes of gamma-globin manufacture the inhibitors of protein-like proteinases have been extracted which prevented the white mice's mortality in 80% of cases. Endogenous inhibitors of human blood proteinases are prospective in producing anti-influenza drugs for humans.

Key words: influenza, trypsin proteinase inhibitors, proteases, antiviral drugs.

In the pathogenesis of viral diseases the interaction of virus with the cell has not been sufficiently studied. The main point here is the virus intervention

into a healthy cell with a mandatory deproteinization of the virus. However, deproteinization of viruses is still poorly understood. This firstly refers to the

mechanism of influenza virus introducing into cells of mammals, including humans. In this regard, in 1983 we proposed a new theory of influenza pathogenesis with proteinase-inhibitory system participation [1, 2].

The difficulties in creating products with selective antiviral action are connected with biology of viral diseases pathogens. Recent achievements of biochemistry and molecular biology, revealing the features of virus reproduction, help to create new generations of drugs with directed intervention in the cycle of viral reproduction [3-6].

The purpose of the study - to examine the state and role of antiproteinase systems of virus and recipient in the development of influenza infection to obtain and use fundamentally new therapeutic drugs based on inhibitors of trypsin-like proteases.

Research objectives:

1. To clear flu virus till homogenous state.
2. To explore the nature of protease associated with influenza virus.
3. To examine the role of proteases and their inhibitors in different, especially at early stages, of influenza infection development.
4. To isolated and purified proteinase and its inhibitor from the lungs of healthy mice as well as from those infected with flu virus.
5. To obtain specific antibodies to isoforms of trypsin-like proteases and study their protective effect under the conditions of experimental influenza.
6. To study the protective effect of cellular inhibitors on infection of animals with a lethal dose of influenza virus.

Materials and methods

Materials for the study were:

1. Strains of influenza virus: A/PR/8/34 (H1N1), A/Aichi/2/68 (H3N2), A/USSR/90/77 (H1N1), A (Extra X-31),

A/WSN/33 (H1N1), A/Phil/2/82 (H3N2), AO/32 (NON1) grown on a 9-day chicken embryos, B strain PR-109, obtained by recombination of viruses V/Lee/40 and B / USSR / 100/83, and MDCK cells were obtained at the I.D. Ivanovsky Research Institute of Virology, Academy of Medical Sciences of Russia and strain AO/32 (HON1) - from the Influenza Research Institute of St. Petersburg, Russia;

2. 2358 items of white mice, line BALB/c and hybrids;
3. 3052 pcs of chicken embryos;
4. 1612 tubes of passaged MDCK cell culture;
5. 140 items of white rats, Wistar line.

We used the following techniques: virological, biochemical, immunological, molecular biological, radioisotopic.

To study the nature of proteolytic activity associated with influenza virus we used 10-11-day chicken embryos and influenza virus AO/32 (HON1) with infectious titers $7 \lg EID_{50/0.2}$ and HA-1: 256. 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 $+36^{\circ} C$. Then they were cooled for 18 hours at $+4^{\circ} C$ and then the virus-containing fluid was collected.

Own researches. In the early 80's, in purifying and concentrating of various strains of influenza virus to produce polyvalent anti flu vaccines, we faced the fact that we could not release the influenza virus from proteolytic activity (Fig. 1.) [7]. To resolve this problem we have improved the methods of purification, but nevertheless failed to release influenza virus from proteolytic activity.

We analysed purified preparations of influenza virus for the presence of proteolytic activity and revealed that the treatment of influenza virus by ultracentrifugation techniques does not relieve flu

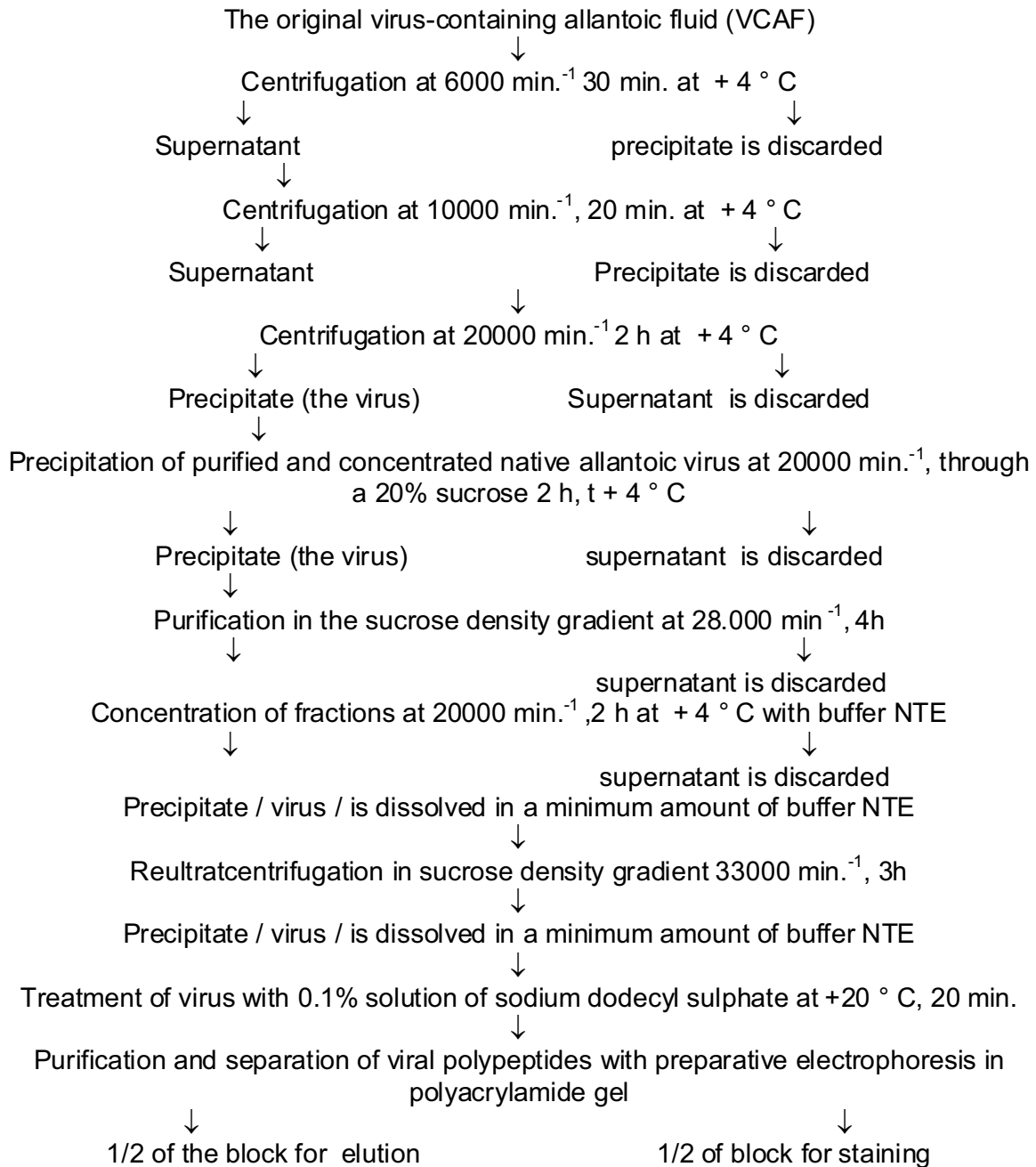


Figure 1. Scheme of purification of influenza virus.

virus from proteolytic activity. In saccharose gradient (15-60%) proteolytic activity was clearly separated into several isoforms (Table 1) [8].

The results obtained allowed us to conclude that flu virus is associated with serin-containing protease of trypsin-like type of cell origin, which has molecular heterogeneity. Our further studies (Table

2) showed that all commercial products contained both inhibitor, and trypsin-like protease, i.e. the today's drugs are not cleared completely of protein impurities, or it is impossible to separate the viral proteins from cellular components. Viral proteins are tightly associated with components of the cell, so the structure of influenza virus should be considered tak-

Table 1

 Purification of influenza virus AO/32 in succharose gradient and ultracentrifugation at 28000 min.⁻¹, 4 h

Fractions of saccharose gradient	Experiments											
	1 VCF *			2 VCF *			3 VCF *			4 OAF **		
	% saccharose	HR ¹	Proteinase, mg/ml	% saccharose	HR ¹	Proteinase, mg/ml	% saccharose	HR ¹	Proteinase, mg/ml	% saccharose	HR ¹	Proteinase, mg/ml
1	5	0	1,6	3	0	1,5	6	0	2,6	6	0	0,58
2	15	1:8	36	11	1:2	13,6	15	1:8	41,3	17	0	1,01
3	32	1:16	9,6	24	1:16	8,2	23	1:16	0,45	27	0	0,37
4	42	1:2048	33,6	24	1:16	10,2	30	1:64	0,9	29	0	0,59
5	49	1:2048	4,4	38	1:512	25,4	37	1:512	13,8	37	0	0,80
6	52	1:64	37	41,5	1:1024	26,2	44	1:16000	38	41	0	1,43
7	55	1:64	37,4	46,5	1:512	12,2	47	1:16000	3,29	49	0	0,64
8	57	1:62	6,8	53	1:16	7,9	51	1:256	0,3	53,5	0	1,28
9							57	1:512		56	0	1,08

Notes: * virus-containing fluid;

** original allantoic fluid; 1 – hemagglutination reaction

ing into account the interaction with cellular enzymes and their inhibitors.

In the body the system of proteinases and inhibitors is represented by a large group of proteins. Inhibitors of proteolytic enzymes counterbalance the corresponding enzymes in the body, and are in constant dynamic equilibrium with them [9-12]. Violation between enzymes and inhibitors is important for the development of pathological processes [13].

Our studies show that in the lungs and blood serum of uninfected animals and chicken embryos, the level of protease activity and protease inhibiting activity are in equilibrium, and the latter is violated at influenza virus A infection.

In the infectious process the most profound changes occur in the first hours

after infection. So, in 6 hours after infection the number of *proteinases* in the lungs and serum of infected animals is reduced and inhibitory activity is increased [14]. Influenza virus infected cells induce the appearance of the inhibitor both in the lung tissue and blood serum. Consequently, lung inhibitors are like the first line of an organ defense under the action of various strains of influenza virus.

When studying the dynamics of *proteinase* and inhibitory activity in the chicken embryos under the influence of large and small infecting doses of influenza virus A/RR/8/34 we have found that there occurred the same changes as in white mice organisms. During the period of maximal accumulation of infectious

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

and hemagglutinating activity (24 hours) they did not find either *proteinase* or inhibitory activity [15].

Six isoforms of trypsin-like proteinase have been isolated from the lungs of healthy mice by while 8 isoforms have been isolated from the lungs of infected mice. Their specific proteolytic activity increased sharply compared with the initial material (Table 2). *Proteinase* obtained isoforms had a broad substrate specificity and were capable to hydrolyze both natural and synthetic substrates [16]. *Antiproteinase* hyperimmune rat sera have been obtained to all isoforms of trypsin-like *proteinases*. When studying the protective properties of antiproteinase sera and sera of healthy rats on white mice infected intranasally with lethal dose of influenza virus A/PR/8/34 (IV passage), it was found that 100% mortality of control mice took place in 4-5 days (Table 3). The animals to which they administrated six times the healthy rats serum intranasally died on the 7th day. In the treatment of mice with the pools of immune sera I, II, IV, V and VI the animals mortality rate was reduced and lethality came much later than in the control group and 20% of the animals recovered [17].

The most effective was the fourth pool of immune serum to the III 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, although isoform III differed from isoform VI by only one protein with molecular mass 32 kDa [18].

From the lungs of healthy mice, we have isolated an inhibitor of trypsin-like proteases with molecular mass of 47.5 kDa, with a high degree of purity and small amount of impurities. We have developed and patented the method of obtaining and purification of trypsin proteinase inhibitor [19]. The inhibitor isolated is similar to the α_1 -proteinase inhibitor of human blood serum (48-55 kDa) and egg white trypsin inhibitor (49 kDa), but does not have likeness to trypsin inhibitor isolated from the lungs of cattle (an inhibitor of Northrop-Kunitz type), which had a molecular mass of 65 kDa. In the study of its effect on the proteolytic activity of trypsin-like *proteinase* isoforms by the test-tube method it has been shown that it inhibited the activity of almost all isoforms, except isoforms IV (41,8%) and VIII (28.3%). In our studies [20] we used a cellular inhibitor to suppress the development of influenza virus in chicken embryos. It has been revealed that it inhibited the development of infectious and hemagglutinating activity and the formation of total protein. At the same time, the inhibitor of trypsin-like proteinase isolated from the lungs of mice, previously infected with influenza virus, did not have this ability. In the further studies [21] for the treatment of in-

Table 3

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

N Gr	Isoform of proteinase	Sera group	Terms after infecting , days												survived	% of survive
			6 h	1	2	3	4	5	6	7	8	9	10	14		
1	I	I						2/10		2/10	2/10	2/10			2	20
2	I	II	2/10	2/10		4/10									2	20
3	II	III			2/10			2/10		2/10			2/10		2	20
4	III	IV						2/10			2/10				6	60
5	IV	V			2/10		2/10	2/14							2	20
6	V	VI			5/10	3/10	2/10									100
7	VI	VII				7/10	1/10	1/10							1	10
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	10	100
7	Serum-free virus						2/10	6/10							All died	0
8	Healthy rats serum						2/10	3/10		5/10					All died	0
9	Immune serum IVgr., 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	10	100

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

fluenza infection in animals, we used the inhibitor, which was isolated from the lungs of healthy mice.

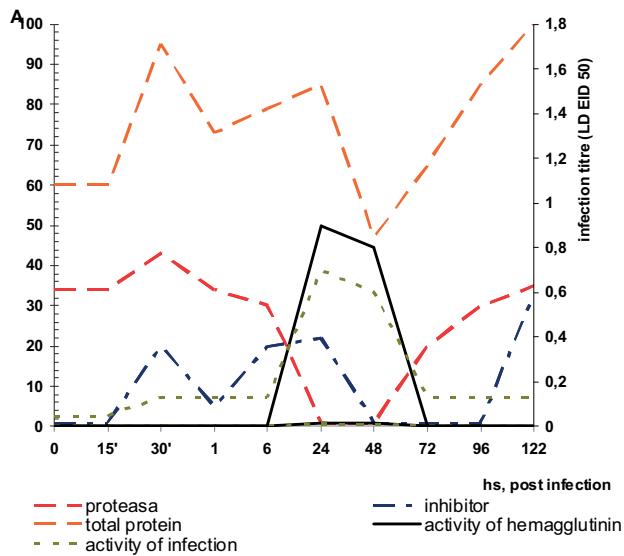
The introduction of this inhibitor to mice previously infected with a lethal dose of influenza virus reduced the death rate from this disease due to inhibition of HA splitting on reproduction of the virus in the lungs, stopping the generalization of the process, preventing the increase of proteolysis in the lungs, as well as preventing arohematic barrier and improved some of the reactions of local protection.

For an antiviral drug preparation that has the lowest allergic action on humans, we used wastes of donor blood

which is taken for isolation of gamma - globulin and albumin.

At the first stage of γ -globulin and albumin manufacture from fraction (II +III, by Cohn E.J.) fibrinogen is precipitated, which is later recycled. According to our data, the wastes contain 481.11 mg of trypsin-like proteinase inhibitor per kg of weight. This centrifugate contained α_1 - antitrypsin, which is the main inhibitor of serine proteases of human blood plasma. Its share in the norm is 90% of antitrypsin activity of human blood plasma [22].

In the second step of γ -globulin obtaining, precipitate containing prothrombin, α - and β -globulins and lipoids is utilized. This precipitate, according to our



A1 – 1 unit equals 1 mg crystalline trypsin
 A – 1 unit activity equals 1 mkg arginin / min.

Figure 2. Changes of proteinase and inhibiting activity in chicken embryos at a big infecting dose of influenza virus A/PR/8/34

data [23], contains 469.87 mg of trypsin proteinase inhibitor per kg of mass. Antithrombin-3 (AT-3) or heparin factor - a regulator of blood coagulation is included in this sediment. According to O.A Markova et al. [24], normally the content of AT-3 in donors ranged from 160 to 250 mcgr / ml. α_1 -antitrypsin and α_2 -macroglobulin [24, 25] are also in the area of α -globulins [24, 25].

In the third stage of γ -globulin obtaining the sediment containing plasminogen goes to waste. At this stage wastes, according to our data, the content of trypsin proteinase inhibitor was 137.40 mg/kg.

At the fourth stage at i-globulin sedimentation centrifugate N 3 is utilized. According to our records, the material of centrifuged № 3 contains 166.37 mg of trypsin – like proteinase inhibitor.

Thus, the waste product of the first and second stages of the technological process in which sediment (II +III) is washing and the allocation of prothrombin takes place may serve as the raw material for producing an inhibitor of trypsin-like proteinases. These wastes

contained the highest number of trypsin-like proteinase inhibitor.

To separate trypsin-like proteinase inhibitor we used the wastes of the Ith stage (II + III) of γ -globulin receipt from donor blood, which contained a significant amount of this inhibitor. We isolated trypsin-like proteinase inhibitor from the centrifugate (wastes) of fraction (II + III) of the first stage of the i-globulin by ion-exchange chromatography on DEAE-cellulose-53 (Watman, USA).

This method allowed to obtain five isoforms with inhibitory activity. The first two isoforms, in which there was a high content of trypsin-like

proteinase inhibitors, have been eluted from ion-exchange column with 0.1 M phosphate buffer, pH 7.5. The next three isoforms containing trypsin-like proteinase inhibitor have been eluted by stepwise NaCl gradient of different molarity: the third isoform - 0.1 M NaCl, the fourth isoform - 0.2 M NaCl, the fifth isoform - 0.5 M NaCl. The volumes of isoforms eluates were, respectively: Ist - 35 ml, IInd-195ml, IIIrd - 340 ml, IVth - 440 ml, Vth - 605ml.

The highest content of trypsin-like proteinase inhibitor has been registered in the fractions of the Vth isoform, which was the last eluted from the column 0.5 M NaCl, while the lowest content was in the IVth and IIInd isoforms eluted from the column 0,2 and 0,1 M NaCl, respectively.

Trypsin-like proteinase plays a key role in the development of pathological process in an organism. It splits the outer protein of the influenza virus - hemagglutinin into two subunits: HA₁ and HA₂. Only after the splitting of hemagglutinin with this protease, the virus enters the

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	Number of animals	Dose of influenza virus	Dose of inhibitor on mice per protein	Number of animals		% of animals protected from virus
				Dies	Survived	
1. Influenza virus	40	10 ⁻³	-	40	-	0
2. Influenza virus +trypsin, crystal.	40	10 ⁻³	18 mkg	40	-	0
3. Influenza virus + inhibitor from healthy lungs	40	10 ⁻³	18 mkg	7	33	82,5
4. Cellular inhibitor	40	10 ⁻³	18 mkg	-	40	100
5. Trypsin, crystal	10	-	18 mkg	-	10	100
6. Phosphate buffer	10	-	0,2 ml	-	10	100

cell and begins to multiply. Inhibitors block the process of cleavage of viral proteins by inhibiting the activity of cellular enzymes.

The viral progeny with non-split, functionally non-active viral proteins is formed in the presence of inhibitors of cellular trypsin-like proteinases after a single reproduction cycle of the primary virus with split proteins. Subsidiaries virions are not able to initiate the infectious process because of the block of the early stages of the reproduction cycle - adsorption and penetration of the virus [26, 27].

To study the protective action of trypsin-like proteinase inhibitor on the survival of mice infected with the lethal dose of influenza virus A/PR/8/34, we took 90 white mice of BALB/c line weighting 16-18 g and the fifth isoform of proteinase inhibitor isolated from the wastes of the first stage of i-globulin production, as it had the highest indexes of the inhibitor activity (132.52 g/l) and the lowest indexes of trypsin-like proteinase (0.0027 mmol in the sample).

The mice were divided into 7 groups

containing 15 items in each, with 10 items in control groups (Table 4). The animals of the first group received lethal dose of virus (virus control). The virus was administered intranasally in a volume of 0.05 ml under Rausch anesthesia. The second group of animals got a similar dose of virus, but they were treated with crystalline trypsin (control of medical properties of crystalline trypsin) in the same doses and terms as the animals of the 3rd group.

The third group of animals was infected with the same dose of virus and was treated with trypsin – like proteinase inhibitor obtained from the wastes of i-globulin.

The fourth group of animals got only an inhibitor of proteinases from the wastes of i-globulin production (control inhibitor's toxicity).

Crystalline trypsin (trypsin control) alone was introduced to the animals of the fifth group, the sixth group got phosphate buffer, with which we diluted virus inhibitor and trypsin. The seventh group was used as the control of intact animals. Both trypsin and inhibitor was adminis-

tered intranasally under light ether anesthesia during seven days. Each mouse got up to 140 mcg of the inhibitor for the course of treatment.

The results of our studies showed that the animals of the first and second groups died on the 6-7 post-infection day. In the third group 12 mice survived (80%). They remained alive for 14 days after infection (observation period). Animals of the 4th, 5th, 6th and 7th groups remained alive throughout the whole period of observation. In addition, newly acquired inhibitor of trypsin-like proteinases, did not cause toxicity, as white mice of the 4th group remained alive at the 14th day after introducing of the inhibitor.

Thus, the findings of our work indicate that trypsin-like proteinase inhibitor obtained from the wastes of the first stage of i – globulin production possessed antiviral properties. It is not excluded that it can be used not only for influenza treatment but for the treatment of other viral infections in which the splitting of viral precursor protein is produced by cellular trypsin-like proteinases [21].

Conclusions

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

Inhibitor of trypsin-like proteinases obtained from industrial wastes of i-globulin manufacture blocked the development of grippe infection in white mice preliminary infected with lethal dose of influenza virus A/PR/8/34 (A/H1N1).

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Резюме

**ПРОТЕОЛІТИЧНА ТЕОРІЯ
ПАТОГЕНЕЗУ ГРИПУ ТА
УДОСКОНАЛЕННЯ ЛІКУВАННЯ ГРИПУ**

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Запропонована нова теорія патогенеза грипу за участю протеїназно-інгібіторної системи. Встановлено, що очищення та концентрація вірусу грипу різними методами не звільняло вірус від клітинних ферментів. При інфікуванні тварин вірусом грипу відбувалось порушення ферментно-інгібіторної рівноваги, особливо у перші години після інфікування. З легенів дужих мишей одержано шість ізоформ трипсиноподібної протеїнази до яких отримані антипротеїназні імунні сироватки та проведено лікування ними тварин. Тільки антисироватка до третьої ізоформи захистила тварин від смерті. З промислових відходів одержання гамаглобуліну виділено інгібітор трипсиноподібних протеїназ, який захистив білих мишей від смерті на 80%. Ендогенні інгібітори протеїназ крові людини є перспективними антигрипозними препаратами для людини.

Ключові слова: грип, трипсиноподібні протеїнази, інгібітори протеїназ, очищення вірусу, противірусні препарати.

Резюме

**ПРОТЕОЛИТИЧЕСКАЯ ТЕОРИЯ
ПАТОГЕНЕЗА ГРИППА И
УСОВЕРШЕНСТВОВАНИЕ ЕГО
ЛЕЧЕНИЯ**

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Предложена новая теория патогенеза гриппа с участием протеиназно - ингибиторной системы. Установлено, что очистка и концентрация вируса гриппа разными методами не освобождало вирус от клеточных ферментов. При заражении животных вирусом гриппа происходило нарушение ферментно - ингибиторного равновесия, особенно в первые, часы после заражения. С легких здоровых мышей, получено шесть изоформ трипсиноподобной протеиназы к которым, получены антипротеиназные иммунные сыворотки и проведено лечение ими животных. Только антисыворотка к третьей изоформе защитила животных от смерти.

С промышленных отходов получения гаммаглобулина выделено ингибитор трипсиноподобных протеиназ, который защитил белых мышей от смерти на 80%. Эндогенные ингибиторы протеиназ крови человека являются перспективными антигрипозными препаратами для человека.

*Ключевые слова: грип,
трипсиноподобные протеиназы,
ингибиторы протеиназ,
противовирусные препараты.*

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