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THE METHOD FOR OBTAINING OF THE PROTECTIVE PERTUSSIS ANTIGEN BY LOW-FREQUENCY ULTRASOUND

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This article describes the physical technology for obtaining surface antigens with protective properties from Bordetella pertussis microbial cells in the absence of additional use of chemical and synthetic substances. The mechanical destruction of cell membranes of microorganisms was carried out under mild conditions using low power low-frequency ultrasound, it allowed not to damage the protective bioactive substances. Ultracentrifugation of microbial ultrasound desintegrates and the subsequent gel – chromatographic separation allowed to obtain a protective antigen with the molecular weight of ~ 8.1 kDa and significantly enhance its specific weight from (62.5±9.1%) to (86.2±4.6%) (P<0.05). The study of toxicity in the test of the mice weight changes showed the presence of pathogenicity factors in the entire antigenic complex and in the fractionated component with the weight of ~ 3.0 kDa. And the purified native antigen with the weight of ~ 8.1 kDa in the calculated dose (160 mg) did not contain toxic compounds. The antigen with the molecular weight of ~ 8.1 kDa showed no histamine-sensitizing and dermonecrotic properties. While studying immunogenicity of the fraction with the weight of ~ 8.1 kDa a strong direct correlation was found between increase of vaccination antigen doses and the corresponding increase of the immunity intensity of vaccinated animals. It indicates the specificity of the results obtained and the protective activity of this fraction. The percentage of survived mice vaccinated by the native antigen with the molecular weight of ~ 8.1 kDa 1.9 times exceeded the percentage of animals vaccinated by the standard industry sample of pertussis vaccine.

Nowadays commercial drugs of acellular pertussis vaccines manufactured by various countries (Japan, France, UK, etc.) are usually obtained using chemical methods that have significant drawbacks: difficult set-up because of the necessity to carry out multistage operations of isolation and purification of bacteria cell structures, and the chemical structure of the isolated antigens can change under the influence of reagents.

Physical methods of antigens obtaining are considered as possible alternative technologies. They are attractive, first of all, by the fact that their implementation is achieved under standard conditions and avoids the need to remove extracting agents from the isolated composite structures of pathogenic bacteria. This opens the prospect of obtaining permanent components with a high protective activity. Ultrasound disintegration plays the major role in the mechanical destruction of the microorganism cell walls for extracting protective bioactive substances; it also allows to break microbial cells while keeping their intercellular content [5].

The aim is to create the technology for obtaining surface antigens with protective properties from pertussis pathogen microbial cells by ultrasound in the absence of additional use of chemical and synthetic substances.

Materials and Methods

Isolation of native pertussis antigens was carried out from the production strain of *Bordetella pertussis* No.267 provided by "Biolik" CJSC (now "Pharmstandard – Biolik" JSC) by the physical method. Destruction of microbial cells was performed by low-frequency ultrasound, followed by ultracentrifugation of desintegrates, filtration, concentration and fractionation of the antigenic complex by gel-chromatography (the equipment of the company LKB, Sweden).

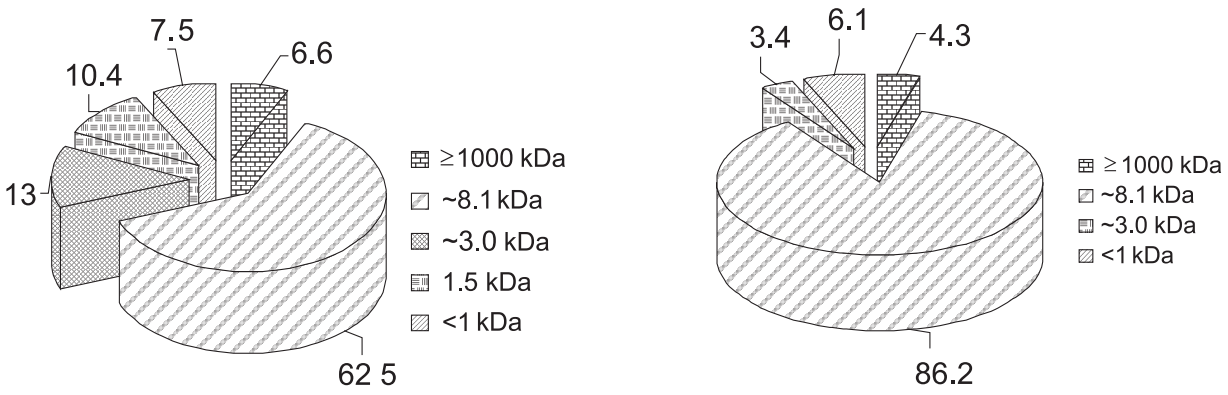
The degree of disintegration of *B. perertussis* cells was controlled by the following parameters: decrease of the optical density of the microbial suspension was measured by Densi-La-Meter, and the total protein concentration was measured by Lowry (a set by "Simko LTD", Lviv) [6].

The biochemical analysis of the antigenic drugs obtained from pertussis pathogen microbial cells was carried out at the Department of Biochemistry of the Kharkiv National Medical University. In the filtrate the amount of total protein was determined by Lowry method. Then precipitation and hydrolysis of protein were performed. In the hydrolysate the content of carbohydrate compounds, lipids and nucleic acids was determined.

Toxicity of the antigen substances studied was evaluated according to the WHO recommendations and "Analytical documentation for the pertussis suspension" using the following tests:

1) the test of weight changes in mice with calculation of the absolute and relative increase of the body weight [1, 7-8];

2) the test on the presence of the dermonecrotic effect determined by intradermal introduction of the drugs studied to guinea pigs weighing 300 g and rabbits weighing 2 kg in the dose of 0.2 ml. The end result of



A B

Fig. The specific weight of fractions with a different molecular weight by low-frequency ultrasound.
A – in the absence of additional ultracentrifugation of microbial disintegrates.
B – after ultracentrifugation of microbial disintegrates.

the experiment was assessed in 96 hours by the presence or absence of necrosis at the injection site [1];

3) the histamine-sensitizing test of purified antigens with calculation of HSD (the histamine-sensitizing dose) and HSD_{50} (the estimated dose sensitizing 50% of the animals) indices [1, 7-8].

Immunogenicity of the antigenic drugs under research was studied according to the WHO recommendations in intracerebral infection of animals with a virulent pertussis culture (strain No.18323) [7].

Experiments on animals were conducted in compliance with the requirements of the Directive of the European Parliament "On the protection of animals used for scientific purposes" (2010/63/EU), in accordance with the Declaration of Helsinki of the World Medical Association (WMA), recommendations of the code of ethics (1985) in the section "International Guiding Principles For Biomedical Research Involving Animals" and the Law of Ukraine "On protection of animals from cruel treatment" No.3447-IV (2006).

All experiments were repeated 3-4 times. Statistical processing of the experimental results was performed using the programme packages Excel 2003 and "Biostat-4". To characterize the reliability of the results obtained the parametric criteria were used with determination of the mean value (M) and its standard deviation (±m). To assess the reliability of differences between figures of the control and experimental groups the Student t-test correlation method was used [2-3].

Results and Discussion

The treatment of the *B.pertussis* microbial mass when using low-frequency ultrasound was 7 hours since exactly this exposure was considered to be enough to provide a significant result of disintegration of pertussis pathogen microbial cells: within the first 6 hours disintegration of the industrial strain did not occur, but longer treatment (8 hours) allowed to statistically unreliable increase the number of the isolated intracellular complexes.

Chromatographic fractionation of the disintegrated pertussis pathogen microbial mass allowed to isolate both high- and low-molecular complexes, among which antigens with the molecular weights of ~ 8.1 kDa dominated, and high-molecular antigens with the molecular weights over 1.000 kDa were present in small amount (Fig.).

Purification of the desintegrate by ultracentrifugation significantly affect the ratio of the isolated fractions. The fraction specific weight of ~ 8.1 kDa increased by 1.4 times (from 62.5±9.1 to 86.2±4.6, $P<0.05$), and the total percentage of fractions with the lower molecular weight reduced by 3.3 times, i.e. the ratio of fractions (≥1000 kDa, ~ 8.1 kDa, ≤3.0 kDa) before ultracentrifugation in relation to decrease of the molecular weight was 1:9.5:4.7, and after it – 1:20:2.2.

The chemical structure of the desintegrate, supernatant after centrifugation of the samples, as well as the corresponding samples of antigenic fractions with the molecular weights over 1000 kDa, ~ 8.1 kDa, ~ 3.0 kDa are represented as mixtures where proteins dominate; carbohydrates and lipids are found in much lower concentrations, and nucleic acids are present only in the hundredths parts of microgram. The antigenic fraction with the molecular weight of ~ 8.1 kDa contains 85% of protein, 7.9% of carbohydrates and 7.1% of lipids. Ultracentrifugation of the desintegrate affects their ratio in the faction. Thus, antigens with the molecular weight of ~ 8.1 kDa obtained by ultracentrifugation contained 1.3 times less carbohydrates and 7.5 times less nucleic acids. When comparing indicators of separate purified components some differences were found: in the fraction with the weight of ~ 8.1 kDa there were more protein structures (1.8 times), as well as lipids and carbohydrates (1.6-1.8 times) than in a high-molecular antigen with the weight of more than 1000 kDa.

When studying reactogenicity of the entire unfractionated antigen complex in the test of weight changes in mice with calculation of absolute and relative increase in the body mass there was the death of mice within 3 days of observation. It can indicate the presence of pathogenicity factors in this complex drug. After vaccination of laboratory animals with antigenic fractions with the molecular weight of ~ 8.1 kDa in the calculated dose (160 mcg) the increase in mice weight was (66.6±4.2) % of the weight increase of animals in the control group, i.e. within the requirements of nor-

mative documents for vaccine drugs [1]. The study of toxicity of the compound with the molecular mass of ~ 3.0 kDa showed that this fraction significantly inhibited the animal mass growth. It is assumed that during fractionation of the entire antigenic complex its toxic compounds remain in the low-molecular fractions (~ 3.0 kDa).

When studying the dermonecrotic activity of the fraction with the molecular weight of ~ 8.1 kDa, in the dose being 1.7 times higher than the calculated one the necrotic skin injuries of animals were not observed. It indicates the absence of the dermonecrotic factor in the given drug.

According to the WHO recommendations the histamine-sensitizing test reflects reactogenicity of acellular vaccines most accurately [7-8]. To compare the results the corpuscular pertussis vaccine (produced by "Biolik" CJSC) was used. As a control ISS-5 (the industry standard sample: pertussis vaccine calibrated by L.A.Tarasevich`s DISC according to the international standard) and saline solution (placebo) were used. The histamine-sensitizing of antigens with the molecular weight of ~ 8.1 kDa in the vaccinated dose containing 160 mcg of protein showed no sensitizing effect in mice: the average HSD index values were 16.7 times less 0.5 (control value of HSD index corresponding to the low-toxic level). These fractions showed a significantly lower histamine-sensitizing activity compared to other antigens and a corpuscular pertussis vaccine.

The methodical peculiarity of the study of immunogenic properties of antigenic fractions is introduction of the virulent culture of *Bordetella pertussis* No.18323 in the infected dose of 476.2 LD_{50} and increase of the number of mice in each experimental group from 16 to 20 according to the recommendations [4]. As a control ISSS-42-28-89-01 P (the industry standard sample) was used.

According to the results of studying immunogenicity of the fraction with the molecular weight of ~ 8.1 kDa it has been found that increase of the antigen dose leads to increase of the immunity intensity of the vaccinated animals. Thus, mice vaccination with the specified fraction in amounts of 25 mcg/dose, 18 mcg/dose and 12 mcg/dose causes the protective effect in (71.7±5.8)%, (26.7±5.7)% and (18.3±5.0)% cases, respectively (ρ – the rank correlation coefficient – 1). A strong direct correlation indicates the specificity of the results obtained and the protective activity of the faction specified. The percentage of survived mice vaccinated by the native antigen with the molecular weight of ~ 8.1 kDa 1.9 times exceeded the percentage of animals vaccinated by the standard industry sample of pertussis vaccine.

CONCLUSIONS

1. Disintegration of *B.pertussis* microbial cells when using low power low-frequency ultrasound has allowed identifying native chemically unmodified biologically-active substances. By chromatographic separation of the disintegrate the protective antigen with the molecular weight of ~ 8.1 kDa has been obtained in a dominant amount of (62.5±9.1%), and an additional ultracentrifugation has increased its specific weight by 1.4 times (86.2±4.6%), $P<0.05$.

2. The purified fraction with the weight of ~ 8.1 kDa has no toxic, dermonecrotic and histamine-sensitizing properties.

3. The antigenic component with the molecular weight of ~ 8.1 kDa in the dose of 25.0 mcg provides the survival of mice after their infection with virulent culture of *Bordetella pertussis* No.18323 in (71.7±5.8) % cases. It proves its protective properties.

4. Application of physical factors without chemicals while isolating surface antigens of *B. pertussis* microbial cells can serve as a benchmark in developing technologies for obtaining native prototypes of candidate-vaccines, which are not modified by extractants.

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СПОСІБ ОТРИМАННЯ НАТИВНОГО ПРОТЕКТИВНОГО КАШЛЮКОВОГО АНТИГЕНУ ЗА ДОПОМОГОЮ НИЗЬКОЧАСТОТНОГО УЛЬТРАЗВУКОВОГО ЧИННИКА

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Ключові слова: *ультразвук; фракція; антиген; вакцина; імуногенність; Bordetella pertussis*

Описана фізична технологія одержання поверхневих антигенів з протективними властивостями з мікробних клітин збудника кашлюка при відсутності додаткового застосування хімічних і синтетичних речовин. Механічне руйнування клітинних оболонок мікроорганізму здійснювалось в щадному режимі за допомогою низькочастотного ультразвуку малої потужності, що дозволило не пошкодити протективні біологічно активні речовини. Ультрацентрифугування мікробних ультразвукових дезінтеграторів та наступне гель – хроматографічне розділення дозволили отримати протективний антиген з молекулярною масою ~ 8,1 кДа та достовірно збільшити його питому вагу з (62,5±9,1%) до (86,2±4,6%) (P<0,05). Вивчення токсичності в тесті зміни маси мишей показало наявність факторів патогенності в цілому антигенному комплексі та у фракціонованому компоненті з масою ~ 3,0 кДа, а очищений нативний антиген з масою ~ 8,1 кДа в розрахованій дозі (160 мкг) не містив токсичних сполук. Антигенний компонент з молекулярною масою ~ 8,1 кДа не проявляв гістамін-сенсибілізуючих та дермонекротичних властивостей. При вивченні імуногенності фракції з масою ~ 8,1 кДа встановлено сильний прямий кореляційний зв'язок між збільшенням щеплювальної дози антигену та відповідним збільшенням напруженості імунітету у вакцинованих тварин, що вказує на специфічність одержаних результатів та протективну активність зазначеної фракції. Відсоток мишей, що вижили, яких вакцинували нативним антигеном з молекулярною масою ~ 8,1 кДа перебільшив відсоток тварин, щеплених галузевим стандартним зразком кашлюкової вакцини, в 1,9 рази.

СПОСОБ ПОЛУЧЕНИЯ НАТИВНОГО ПРОТЕКТИВНОГО КОКЛЮШНОГО АНТИГЕНА С ПОМОЩЬЮ НИЗКОЧАСТОТНОГО УЛЬТРАЗВУКОВОГО ФАКТОРА

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Ключевые слова: *ультразвук; фракция; антиген; вакцина; иммуногенность; Bordetella pertussis*

Описана физическая технология получения поверхностных антигенов с протективными свойствами из микробных клеток возбудителя коклюша при отсутствии дополнительного применения химических и синтетических веществ. Механическое разрушение клеточных оболочек микроорганизма осуществлялось в щадящем режиме с помощью низкочастотного ультразвука малой мощности, что позволило не повредить протективные биологически активные вещества. Ультрацентрифугирование микробных ультразвуковых дезинтеграторов и последующее гель-хроматографическое разделение позволило получить протективный антиген с молекулярной массой ~ 8,1 кДа и достоверно увеличить его удельный вес с (62,5±9,1%) до (86,2±4,6%) (P<0,05). Изучение токсичности в тесте изменения массы мышей показало наличие факторов патогенности в целом антигенном комплексе и во фракционированном компоненте с массой ~ 3,0 кДа, а очищенный нативный антиген с массой ~ 8,1 кДа в расчетной дозе (160 мкг) не содержал токсических соединений. Антиген с молекулярной массой ~ 8,1 кДа не проявлял гистамин-сенсибилизирующих и дермонекротических свойств. При изучении иммуногенности фракции с массой ~ 8,1 кДа установлена сильная прямая корреляционная связь между увеличением прививочной дозы антигена и соответствующим увеличением напряженности иммунитета у вакцинированных животных, что указывает на специфичность полученных результатов и протективную активность указанной фракции. Процент выживших мышей, которых вакцинировали нативным антигеном с молекулярной массой ~ 8,1 кДа превысил процент животных, привитых отраслевым стандартным образцом коклюшной вакцины, в 1,9 раза.