EXPERIMENTAL RESEARCH

STUDY OF THE ADJUVANT PROPERTIES OF CHITOSAN IN COMPLEX WITH RECOMBINANT OUTER MEMBRANE PROTEIN F AND RECOMBINANT TOXOID *PSEUDOMONAS AERUGINOSA*

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The aim of the study was to investigate adjuvant properties of chitosan in comparison with aluminum hydroxide during immunization with recombinant proteins of *Pseudomonas aeruginosa*. We used recombinant outer membrane protein F (OprF) and recombinant toxoid of *P. aeruginosa*, to which 0.5% chitosan preparation dissolved in glutamic acid with pH 5.0 or aluminum hydroxide gel were added. The ratios of aluminum hydroxide to protein of 3:1 and 1:1 were tested, and chitosan was added at 100 μg and 50 μg for one immunizing dose. The resulting preparations were administered to mice intraperitoneally twice with a two-week interval, and then two weeks later the animals were infected intraperitoneally with *P. aeruginosa* (PA-103). It was shown that double administration of the recombinant OprF protein at a dose of 25 μg and recombinant toxoid at a dose of 50 μg with both aluminum hydroxide and chitosan contributed to the development of equivalent protective properties. With the complex introduction of two recombinant proteins, an increase in protective properties was observed using both adjuvants. The possibility of using antigens without adjuvant and reducing their immunizing dose during booster immunization was investigated. It turned out that a two-fold reduction in the immunizing dose did not reduce the protective effect upon repeated administration, and in the case of chitosan, an increase in the immune response was observed during booster immunization with recombinant antigens without an adjuvant. Thus, for the recombinant proteins of *P. aeruginosa*, the adjuvant properties of chitosan were revealed. They are not inferior in the induction of protective properties to aluminum hydroxide.

Key words: *Pseudomonas aeruginosa*; chitosan; adjuvant; outer membrane protein F (OprF); exotoxin A; toxoid

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INTRODUCTION

Pseudomonas aeruginosa is one of the most serious pathogens of opportunistic infections; is characterized by a high ability to rapidly develop resistance to antibiotics. Active immunoprophylaxis of *P. aeruginosa* in patients at risk is considered as a possible way to control *P. aeruginosa* infection. At Mechnikov Research Institute of Vaccines and Sera, a number of recombinant proteins were obtained. Based on the results of studying their immunobiological properties the Recombinant Pseudomonas Vaccine (RPV) was created, and successfully passed preclinical studies [2].

It is known that recombinant proteins have low immunogenicity; therefore, when creating vaccines based on them, special attention is paid to the adjuvant selection. Aluminum hydroxide is used as an adjuvant in commercial vaccines currently used in healthcare practice to deposit antigens and stimulate the immune response [3]. In particular, the RPV vaccine, developed at Mechnikov Research Institute of Vaccines and Sera, is the complex of the recombinant outer membrane protein F (OprF) and the recombinant deletion form exotoxin A (toxoid) of *P. aeruginosa* adsorbed on an aluminum hydroxide gel [2]. Selection of traditional adjuvant is dictated by the long experience of its use as a component of various vaccines, which, however, is not without some drawbacks: it weakly stimulates cellular immunity, causes undesirable reactions in the form of skin irritation, pain, and inflammation in the injection area. In addition, increasing evidence exists that repeated use of aluminum hydroxide during a lifetime can lead to the development of allergic reactions and diseases of the central nervous system [4, 5].

The search and development of new classes of adjuvants is a promising direction in modern immunobiology [5]. In recent years, serious interest has arisen in studies of the adjuvant properties of a natural polysaccharide, chitosan, isolated from crustacean shells. It is known that chitosan has immunomodulatory properties necessary for adjuvants. This is confirmed by the fact that nanoparticles of chitosan and its derivatives associated with antigens are able to effectively activate the cellular and humoral components of the immune response [6]. The term “adjuvant based on chitosan” refers not only to a large group of substances that are chitosans that differ in their main characteristics (molecular weight, degree of deacetylation, and polydispersity index), but also preparations based on them in various physical states (gels, microparticles, and nanoparticles), as well as chitosan derivatives and complex adjuvants based on it [6-8]. The effectiveness of chitosan used as an adjuvant for parenteral administration of inactivated influenza vaccines has been established [9-11].

The aim of this study was to study the adjuvant properties of chitosan during immunization with recombinant *P. aeruginosa* proteins (OprF and the toxoid).

MATERIALS AND METHODS

Reagents

The following reagents were used in the work: antibiotics ampicillin and kanamycin sulfate (“Sintez”, Russia); bacto tryptone and bacto yeast extract (“Difco”, USA); isopropyl-β-D-thiogalactopyranoside (“Thermo Fisher Scientific”, USA); Ni-Sepharose (“GE Healthcare”, Sweden); aluminum hydroxide

gel ("Sigma-Aldrich", USA); the other chemical reagents were purchased from "Amresco" (USA); 0.5 % chitosan preparation dissolved in glutamic acid with pH 5.0 [11] was kindly provided by Dr. Yuri M. Vasiliev (Laboratory of Genetics of RNA-Containing Viruses of Mechnikov Research Institute of Vaccines and Sera).

Bacterial Strains

For synthesis of the recombinant proteins we used *Escherichia coli* strains producing of the recombinant protein OprF [12] and the recombinant toxoid [13] from the collection of the Laboratory of Protective Antigens (Mechnikov Research Institute of Vaccines and Sera). For the experimental infection of animals we used a virulent culture of *P. aeruginosa* PA-103 (ATCC29260).

Laboratory Animals

For immunization we used female white mice weighing 16-18 g obtained from the Andreevka Laboratory of the Scientific Center for Biomedical Technologies of the Federal Medical-Biological Agency (Russia).

Production of the Recombinant Protein OprF and the Recombinant Toxoid

The recombinant proteins were synthesized using the corresponding producer strains was carried out as described previously [2]. The recombinant proteins were isolated from the biomass using the previously developed original two-stage purification methods. In the first stage, in the case of the recombinant protein OprF, we obtained a "hydrophobic fraction" of bacterial proteins, and in the case of the recombinant toxoid, we isolated inclusion bodies. In the second stage, the recombinant proteins were purified by affinity chromatography on Ni-Sepharose in 8 M urea buffer followed by dialysis against 50 mM Tris-HCl buffer (pH 9.0) [2].

Protein Electrophoresis

The analysis of the obtained protein products was carried out in 12 % polyacrylamide gel by the method of Laemmli [14]

Immunization of Mice

Mice were immunized intraperitoneally with a volume of 0.5 ml of the administered preparations. The preparations of the recombinant proteins were diluted with physiological sodium chloride solution to the required concentration. The appropriate amounts of adjuvants were added to the preparations of the recombinant proteins beforehand. Chitosan preparation was used in doses of 100 μg and 50 μg, added immediately before immunization. Aluminum hydroxide, preparation was added to the recombinant proteins in a weight ratio of 3:1 and 1:1, and then the resulting preparations were incubated for 12-14 h 4°C for the absorption of the antigens on the adjuvant.

Two immunizations were carried out with an interval of two weeks. Non-immunized mice from the same batch were used as controls. Experimental infection with *P. aeruginosa* was induced two weeks after the second immunization.

Experimental infection with P. aeruginosa

In experimental infection, animals were treated intraperitoneally with various doses of a live virulent culture of *P.* aeruginosa administered in a volume of 0.5 ml as described previously [2]. Immunized mice were administered with 200, 50, and 12.5 million microbial cells (m.c.), while mice in the control groups were injected with 100, 25, and 6.25 million m.c.

The count of dead and surviving individuals was carried out for seven days, followed by the determination of LD_{50}. This value was calculated using the modified Kerber formula modified by Ashmarin-Vorobiev [15]. Ih these calculations, LD_{50} corresponds to the inverse logarithm [lgA – lg2×(B_1/C_1 + B_2/C_2 + B_3/C_3 – 0.5], where A is the maximum infectious dose in the experiment, B is the number of animals that died in the group, C is the initial number of animals in the group. Then, the indices of the effectiveness of protective properties were determined, which represented the ratio of the LD_{50} values for the experimental groups to the LD_{50} values for the control groups.

RESULTS

The adjuvant properties of chitosan in combination with 25 μg of the recombinant OprF protein and 50 μg of the recombinant toxoid were studied in this work. When immunizing animals, recombinant proteins were used both individually and in combination. Chitosan for experiments was dissolved up to 0.5 % in glutamic acid with pH 5.0 and tested at a dose of 100 μg per immunization. The scheme of immunization included two intraperitoneal injections with a two-week interval. Recombinant proteins (only OprF, only the toxoid and the complex of two proteins) were used as reference preparations, which were adsorbed on an aluminum hydroxide gel in a ratio of 1:3.

In the groups of animals immunized with the recombinant OprF protein, the LD_{50} values were: 61.6 million m.c. (mln m.c.) for the variant using aluminum hydroxide and 63.0 mln m.c. for the preparation with chitosan. The indexes of effectiveness of protective properties corresponded to 1.9 for both experimental groups. When immunized with the recombinant toxoid, the LD_{50} values were approximately the same: 72.4 mln m.c. in the group of mice that received the preparation with aluminum hydroxide and 83.1 mln m.c. in the group of mice treated with chitosan. The effectiveness indices of the protective properties of the preparations based on the recombinant toxoid corresponded to 2.2 and 2.5. When using the complex of two recombinant proteins, an increase in protective properties was observed: the efficiency indices increased to 3.5 when using aluminum hydroxide (LD_{50} – 114.9 mln m.c.) and up to 3.2 when using chitosan (LD_{50} – 104.7 mln m.c.) (Table 1).

At the next stage, various schemes of immunization with preparations were tested, in which the doses of chitosan and aluminum hydroxide were reduced. Chitosan was used at a dose of 50 μg per injection, and aluminum hydroxide gel was added in an equal weight ratio to the protein. For the first immunization, experimental animals were divided into four groups, which were injected with either the recombinant OprF protein at a dose of 25 μg, or the recombinant toxoid at a dose of 50 μg, using aluminum hydroxide or chitosan as part of the preparation. Thus, during the first immunization, experimental groups of mice were injected with: 1) the recombinant OprF protein with aluminum hydroxide; 2) the recombinant toxoid with aluminum

Table 1. Protective activity of the recombinant proteins of *P. aeruginosa* when used as an adjuvant of aluminum hydroxide and chitosan

Recombinant proteins and dose (μg)	Adjuvant and dose (μg)	Infection dose (mln m.c.)	Number of died/survived mice	LD_{50} (mln m.c.)	IE*
OprF 25	aluminum hydroxide 75	200	10/0	61.6	1.9
		50	3/9		
		12.5	1/9		
toxoid 50	aluminum hydroxide 150	200	9/1	72.4	2.2
		50	5/10		
		12.5	0/10		
OprF 25 toxoid 50	aluminum hydroxide 225	200	3/7	114.9	3.5
		50	3/12		
		12.5	0/10		
OprF 25	chitosan 100	200	8/2	63	1.9
		50	2/13		
		12.5	4/6		
toxoid 50	chitosan 100	200	2/13	83.1	2.5
		50	2/9		
		12.5	3/7		
OprF 25 toxoid 50	chitosan 100	200	7/3	104.7	3.2
		50	4/11		
		12.5	0/10		
non-immunized mice		100	9/1	33	-
		25	4/6		
		6.25	0/10		

Note. *IE – Indices of effectiveness of protective properties

hydroxide; 3) the recombinant OprF protein with chitosan; 4) the recombinant toxoid with chitosan. Two weeks after the first immunization, each experimental group was divided into three more subgroups that received: 1) the same preparations as in the first immunization; 2) the recombinant proteins without adjuvants; 3) the preparations used in the first immunization but containing halved doses. Thus, after the second immunization, the experimental animals were divided into twelve groups. It was found that the survival rate of mice immunized with different schemes for the administration of the recombinant antigens adsorbed on aluminum hydroxide was approximately the same. The effectiveness indices of the protective properties of these preparations ranged from 1.7 to 2.2. At the same time, the protective activity of the recombinant antigens administered in combination with chitosan was higher in groups that were the second (booster) immunized only with proteins. Indices of effectiveness of protective properties in this case were 2.6 and 3.2 for the recombinant OprF protein and recombinant toxoid, respectively. The effectiveness of schemes using chitosan at a dose of 50 μg turned out to be higher in comparison with similar preparations containing aluminum hydroxide (Table 2).

DISCUSSION

Mechnikov Research Institute of Vaccines and Sera developed the RPV vaccine for the prevention of *Pseudomonas aeruginosa* infection. The RPV vaccine is the complex of the recombinant porin protein OprF and the toxoid adsorbed on aluminum hydroxide [2]. In the present work, these proteins were used to study the adjuvant properties of chitosan. At the first stage, preparations were obtained and studied using the regimens and doses of antigens used in the RPV vaccine. The adjuvant properties of chitosan for recombinant *P. aeruginosa* antigens were as effective as aluminum hydroxide. Previously, a similar effect of chitosan, used as an adjuvant in the composition of influenza vaccines, was observed, as a result of immunization of animals with it, the accumulation of specific IgG antibodies occurred [10]. The conducted studies confirmed the additive effect of the use of two recombinant antigens during joint immunization, which was previously revealed in the study of the RPV vaccine [2].

The possibility of using adjuvants at lower concentrations and reducing the immunizing dose during the second immunization with antigens without adjuvant was further investigated. A decrease in the concentration of the adjuvant in preparations during booster immunization did not reduce its effectiveness, and in the case of chitosan, an increase in the immune response occurred when the recombinant antigens were used without an adjuvant. Thus, the possibility of reducing the concentration of adjuvants in preparations without reducing the effectiveness of the protective properties of immunization was revealed.

Based on the results obtained, the effectiveness of complex immunization with the recombinant antigens (OprF and the toxoid) of *P. aeruginosa* was confirmed. The possibility of using chitosan as an adjuvant was shown, since preparations of the recombinant proteins with its use were not inferior in the induction of protective properties to the preparations absorbed on aluminum hydroxide.

Table 2. Protective activity of the recombinant proteins of P. aeruginosa when using different amounts of the preparations at the second immunization.

Recombinant proteins and dose (μg) Adjuvant and dose (μg)		**Infection dose (mln m.c.)**	**Number of died/ survived mice**	**LD_{50} (mln m.c.)**	**IE***
First immunization	**Second immunization**				
OprF 25 aluminum hydroxide 25	OprF 25 aluminum hydroxide25	200	10/1	32.2	2
		50	6/5		
		12.5	4/7		
	OprF 25 -	200	10/2	35.4	2.2
		50	6/6		
		12.5	5/7		
	OprF 12.5 aluminum hydroxide 12.5	200	12/0	28.1	1.7
		50	8/4		
		12.5	3/9		
toxoid 50 aluminum hydroxide50	toxoid 50 aluminum hydroxide 50	200	10/1	36.5	2.2
		50	7/4		
		12.5	2/9		
	toxoid 50 -	200	9/2	36.5	2.2
		50	8/3		
		12.5	2/9		
	toxoid 25 aluminum hydroxide 25	200	11/1	35.4	2.2
		50	7/4		
		12.5	3/9		
OprF 25 chitosan 50	OprF 25 chitosan 50	200	9/2	36.5	2.2
		50	5/6		
		12.5	5/6		
	OprF 25 -	200	8/2	42.9	2.6
		50	7/3		
		12.5	1/8		
	OprF 12.5 chitosan 50	200	10/1	32.2	2
		50	8/3		
		12.5	2/9		
toxoid 50 chitosan 50	toxoid 50 chitosan 50	200	8/3	47	2.9
		50	7/4		
		12.5	2/9		
	toxoid 50 -	200	9/2	53.3	3.2
		50	4/7		
		12.5	3/8		
	toxoid 50 chitosan 50	200	8/3	47	2.9
		50	7/4		
		12.5	2/9		
non-immunized mice		100	10/0	16.5	-
		25	8/2		
		6.25	0/10		

Note. *IE – Indices of effectiveness of protective properties

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COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies involving humans; mice were immunized in accordance with all generally accepted standards for the humane treatment of laboratory animals. All the work with animals was carried out in accordance with the provisions of the GOST 33215-2014 "Guidelines for accommodation and care of animals. Environment, housing and management".

CONFLICT OF INTEREST

The authors declare they have no conflict of interests.

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ИССЛЕДОВАНИЕ АДЪЮВАНТНЫХ СВОЙСТВ ХИТОЗАНА В КОМПЛЕКСЕ С РЕКОМБИНАНТНЫМ БЕЛКОМ F НАРУЖНОЙ МЕМБРАНЫ И РЕКОМБИНАНТНЫМ АНАТОКСИНОМ *PSEUDOMONAS AERUGINOSA*

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Целью исследования было изучение адъювантных свойств хитозана в сравнении с гидроксидом алюминия при иммунизации рекомбинантными белками *Pseudomonas aeruginosa*. В работе использовали рекомбинантный белок F наружной мембраны (OprF) и рекомбинантный анатоксин *P. aeruginosa*, к которым добавляли 0.5% препарат хитозана, растворенный в глутаминовой кислоте с pH 5.0 или гель гидроксида алюминия. Испытали соотношение гидроксида алюминия к белку 3:1 и 1:1, а хитозан добавляли в количестве 100 мкг и 50 мкг для одной иммунизирующей дозы. При иммунизации препараты вводили мышам внутрибрюшинно двукратно с двухнедельным интервалом, а затем через две недели животных заражали внутрибрюшинно *P. aeruginosa* (PA-103), подсчитывая погибших и выживших животных. Иммунизация рекомбинантным белком OprF в дозе 25 мкг и рекомбинантным анатоксина в дозе 50 мкг как с гидроксидом алюминия, так и с хитозаном, способствовала развитию равнозначных протективных свойств. При комплексном введении двух рекомбинантных белков было выявлено усиление защитных свойств с использованием обоих адъювантов. Исследовали возможность применения рекомбинантных антигенов без адъюванта и уменьшения их иммунизирующей дозы при бустерной иммунизации. Снижение иммунизирующей дозы в два раза при повторном введении не уменьшило протективный эффект, а в случае хитозана, приводило к усилению иммунного ответа при бустерной иммунизации рекомбинантными антигенами без адъюванта. Таким образом, для рекомбинантных белков *P. aeruginosa* выявлены адъювантные свойства хитозана, не уступающие по индукции протективных свойств гидроксиду алюминия.

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Ключевые слова: *Pseudomonas aeruginosa*; хитозан; адъювант; белок F наружной мембраны (OprF); экзотоксин A; анатоксин

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