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Preclinical studies of safety, immunogenicity and protective activity of attenuated *Bordetella pertussis* bacteria on the *Macaca mulatta* model

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Introduction. An increasing incidence of pertussis among different groups of population and shortcomings of the existing preventive solutions pinpoint urgency of development of new safe vaccines suitable for immunization of infants and for booster immunization of adolescents and adults.
The purpose of this study is evaluation of safety, immunogenicity and protective activity of the new constructed attenuated *Bordetella pertussis* bacteria 4MKS by infecting immunized *Macaca mulatta* monkeys intranasally with virulent bacteria of the pertussis pathogen.
Materials and methods. Five adult, clinically healthy *Macaca mulatta* monkeys aged 3–4 years were used for immunization and experimental infection. The re-immunization was performed in 6 months. Three non-immunized animals of the same age were used as controls.
Results. The intranasal single-dose inoculation and re-inoculation of attenuated *B. pertussis* bacteria did not cause any nasopharyngeal inflammation in the *Macaca mulatta* monkeys and any changes in the blood lab test values after the nonhuman primates had been infected with virulent bacteria. No elevation of total IgE was detected in blood serum of the rhesus monkeys after the single-dose and double-dose immunization. When the rhesus monkeys were intranasally immunized with attenuated and virulent *B. pertussis* bacteria, they developed a defensive reaction to re-infection, namely suppression of the bacterial growth, increased rates of elimination of bacteria from the animals' nasopharynxes and development of a humoral immune response to the infection. The development of immunity against pertussis re-infection is accompanied by a pronounced booster effect.
Discussion. The obtained results suggest common mechanisms of development both of post-vaccination immunity after intranasal vaccination of animals and infection-acquired immunity against pertussis. Both of them provide protection against re-infection with *B. pertussis* bacteria and prevent development of clinical symptoms of pertussis.

Keywords: *pertussis; live intranasal vaccine; protective activity; attenuated Bordetella pertussis bacteria; nonhuman primates.*

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Доклинические исследования безопасности, иммуногенности и защитной активности аттенуированных бактерий *Bordetella pertussis* на экспериментальной модели *Macaca mulatta*

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Введение. Рост заболеваемости коклюшем среди разных групп населения и несовершенство существующих профилактических препаратов требуют разработки новых безопасных вакцин, удобных для иммунизации детей раннего младенческого возраста, реиммунизации подростков и взрослых.
Целью настоящей работы является характеристика безопасности, иммуногенности и защитной активности сконструированных нами аттенуированных бактерий *Bordetella pertussis* 4MKS в тесте интраназального заражения иммунизированных обезьян *Macaca mulatta* вирулентными бактериями возбудителя коклюша.
Материалы и методы. Для иммунизации и экспериментальной инфекции использованы 5 половозрелых, клинически здоровых обезьян *Macaca mulatta* в возрасте 3–4 лет. Реиммунизацию проводили через 6 мес. В качестве контроля использовали 3 неиммунизированных животных того же возраста.
Результаты. Интраназальная однократная и повторная инокуляции аттенуированных бактерий *B. pertussis* не вызывали воспалительных процессов в носоглотке обезьян *Macaca mulatta* и изменений лабораторных показателей крови, наблюдаемых после экспериментальной инфекции нечеловекообразных приматов вирулентными бактериями. Не зарегистрировано увеличения количества общих IgE в сыворотке крови обезьян макака резус после однократной и двукратной иммунизации. Интраназальная иммунизация обезьян макака резус аттенуированными и вирулентными бактериями *B. pertussis* приводит к формированию защитной реакции организма на повторную инфекцию, проявляющейся в подавлении размножения бактерий, ускорении темпов их элиминации из носоглотки животных и развитии гуморального иммунного ответа на инфекцию. Развитие иммунитета к повторной коклюшной инфекции сопровождается выраженным бустерным эффектом.
Обсуждение. Представленные результаты указывают на общие механизмы формирования поствакцинального иммунитета в результате интраназальной вакцинации животных и постинфекционного противококлюшного иммунитета, обеспечивающих защиту от повторного инфицирования бактериями *B. pertussis* и развития клинических симптомов коклюша.

Ключевые слова: *коклюш; живая вакцина интраназального применения; защитная активность; аттенуированные бактерии Bordetella pertussis; нечеловекообразные обезьяны.*

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Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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Introduction

Despite wide-scale vaccination against pertussis in many countries since the early 1950s, pertussis-causing bacteria have not been eliminated so far. Even with many underdiagnosed pertussis cases, there are annually reported more than 16 million cases of pertussis of different severity and around 200 thousand deaths worldwide [1]. Laboratory-confirmed pertussis cases among adolescents and adults have been relentlessly increasing over the past decade [2, 3]; the number of subclinical and asymptomatic cases of *Bordetella pertussis (BP)* carriage is also climbing [2, 4, 5]. In the United States, where pertussis vaccination (PV) coverage in children is 95%, the pertussis incidence has been dramatically increasing since the early 2000s, nearly reaching the before-vaccination levels [6, 7]. An increase in incidence has been reported by Italy and England [8, 9]. In Russia, pertussis cases more than doubled from 2017 to 2018. The upward trend continued both in 2019 and at the beginning of 2020. [10]. In previous years, the increased incidence was mainly recorded in Moscow and St. Petersburg, which, most likely, can be explained by the high quality of diagnostics [11].

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At present, pertussis prevention is built on vaccines containing a corpuscular pertussis component (whole-cell pertussis vaccines (wPV)) or an acellular pertussis component (acellular pertussis vaccine (aPV)) combined with inactivated diphtheria and tetanus anatoxins. Sometimes, wPV or aPV are used as monovaccines. Although aPVs are believed to be less reactogenic, the direct studies on primates showed that these vaccines do not provide antibacterial immunity and do not protect the animals from experimental pertussis infection [12]. The comparative assessment of the incidence in the vaccinated and unvaccinated groups demonstrated low efficacy in adolescents and adults revaccinated with aPV [13, 14].

The short duration of vaccine-acquired immunity is another significant shortcoming of the currently available PVs. The evaluation of efficacy of different types of PVs shows that the duration of post-vaccination immunity does not exceed 5 years. The infection-acquired immunity wanes after 10 to 15 years [15].

All contemporary PVs are administered to children over 2 months of age as a three-dose series. Therefore, the full vaccination series is completed when children reach the age of 6 months, which implies a high risk of infection during the first few months of their life when they are most vulnerable to pertussis.

An increasing number of pertussis cases, including older children and adults, culminated in understanding the importance of revaccination of adolescents and adults. Maternal vaccination leading to building of "family immunity" is placed on the agenda [3, 4, 16, 17]. The only recommended vaccine is aPV [4], which, as mentioned above, does not provide children and adults with protection against infection acquisition and transmission. Thus, we have to admit that although there are solid grounds for revaccination of adolescents and adults as well as for building of family immunity, none of the currently available vaccines can be used for these purposes. As for wPVs, they are not recommended by WHO for using in adults, and contemporary aPVs are most likely inefficient. The aPV demonstrated its efficacy and safety as an alternative to the wPV in vaccination of infants. Such vaccination controls mortality and disease severity in infants who are most vulnerable to pertussis. However, similar to the wPV, it is administered as a 3–4-dose series vaccination, which, if not completed, results in substantially decreased vaccine protective efficacy in children.

Based on preclinical studies, we demonstrated safety of the intranasal vaccination of laboratory animals with attenuated *BP* 4MKS bacteria and the protective effect produced by the mice vaccination providing protection in their intracerebral and intranasal infection with virulent *BP* bacteria [18]. The recent studies have demonstrated the viability of using the experimental model of nonhuman primates in studying of immunobiological characteristics of the pertussis pathogen and PV immunogenicity [19–23]. The studies have shown that the experimental infection of monkeys results in laboratory test presentation of pertussis infection, nasopharyngeal hyperemia, long-term *BP* persistence and an increased titer of specific immunoglobulins in blood serum of animals. The studies on hamadryas baboons proved that infection can be transmitted from a human to a primate and among primates [23].

The purpose of this study is evaluation of safety, immunogenicity and protective activity of the new constructed attenuated *BP* bacteria 4MKS by infecting immunized *Macaca mulatta* monkeys (rhesus macaque; RM) intranasally with virulent bacteria of the pertussis pathogen.

Materials and methods

BPs were grown in solid casein-charcoal agar (CCA) media supplemented with 10% defibrinated sheep blood, at 36°C. Attenuated *BPs 4MKS* collected from nasopharyngeal washings were seeded in the CCA medium containing 200 µg/ml of streptomycin.

To identify the serotype composition of the culture, we used diagnostic pertussis sera for agglutinogens of *BP 1, 2, 3* bacteria, adsorbed, for agglutination test, dry (Gamaleya Federal Research Center for Epidemiology and Microbiology, Federal State Budgetary Institution) in accordance with the manufacturer's recommendations.

Five adult, clinically healthy RMs aged 3–4 years were used for immunization and experimental infection. The re-immunization was performed in 6 months. Three non-immunized RMs of the same age were used as controls. The tests were conducted with animals from the Sukhumi monkey breeding farm (The Research Institute of Experimental Pathology and Therapy of the Academy of Sciences of Abkhazia). The studies involving animals were conducted in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, and in compliance with the applicable Russian regulations.

Protective activity of the attenuated bacteria was assessed by comparing rates of elimination of virulent *BP 475* bacteria from the nasopharynxes of the vaccinated and non-immunized, control RMs, by comparing the development of immune responses, clinical symptoms and laboratory presentation of pertussis.

Prior to the procedures (immunization, experimental infection, collection of nasopharyngeal swabs), RMs were anesthetized with intramuscular injections of 0.03–0.04 ml of Zoletil (Virbac, France) at a concentration of 100 mg/ml (after the premedication with *xylazine hydrochloride*, 20 mg/ml). The experimental infection and vaccination (10^7–10^{10} bacteria) were performed by inoculation of 0.5 ml of suspension containing virulent or attenuated bacteria into each nostril o the animals in a supine position.

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Blood samples were taken from non-anesthetized RMs in squeeze cages.

To identify *BP* bacteria DNA, we used nasopharyngeal swabs washed with 500 µl of saline. After centrifugation, DNA was isolated through standard treatment with *guanidine thiocyanate solution and subsequent sorption by using the magnetic sorbent* (Promega). We used the real-time PCR test designed by us for identification of *BP* bacteria DNA [24, 25].

RM blood sera were examined for presence of pertussis toxin and *filamentous hemagglutinin*-specific IgG and IgM as well as non-specific IgE after the single-dose and double-dose intranasal vaccination by using Ridascreen test systems. Vector-Best test was used in identification of non-specific IgE.

The obtained results were analyzed by using Student's t-test. The differences are significant at $p \leq 0.05$.

Results

The RMs' overall condition and blood test results after the immunization and infection with virulent BPs 475

After the first and second intranasal immunization, RMs did not show any abnormalities in their behavior, overall condition, blood count, white blood cell count and glucose levels, any changes in the activities of the *aspartate* and *alanine aminotransferase*, and any signs of inflammation or any other reactions in the nasopharynx (**Table 1**).

The experimental infection of the immunized monkeys with virulent *BP* bacteria 12 months after the re-immunization also did not demonstrate any deviations from the normal values (Table 1). At the same time, in the control group, the infection of native monkeys with virulent bacteria resulted in significantly increased white blood cell counts and decreased glucose levels (Table 1), nasal mucus and nasopharyngeal inflammation developed on the 3rd to 10th day of the infection. No cough was observed in the control and vaccinated monkeys.

The measurement results for non-specific IgE in blood sera of the immunized and infected animals are given in **Table 2**.

Table 2 shows that out of all the immunized animals, only monkey # 31883, and out of all the control animals, only monkey # 31927 has the IgE levels close to the negative control values in humans (20–30 IU/ml). In all the other monkeys, the IgE values range from 130 to 250 in # 31882 and from 390 to 720 in # 31901.

No steady increase in IgE levels was observed after the immunization and re-immunization. On the contrary, monkey # 31882 demonstrated a decreasing tendency in its IgE concentration immediately after the re-immunization; all the animals had significantly decreased IgE levels after they were infected with virulent *BP 475* bacteria.

The BP bacterial load in the monkeys' nasopharynx after the immunization and infection with virulent BP 475

The bacteriological technique is the "gold standard" in pertussis diagnostics. The washings of the nasopharyngeal swabs collected from the posterior wall of the monkeys' nasopharynxes were seeded on the blood-containing CCA medium with or without streptomycin. The swabs were taken one hour after the immunization or the experimental infection and then in 3, 7,

Table 1. RMs' blood biochemistry after their intranasal immunization with attenuated *BP* bacteria and experimental infection with virulent *BP* 475 bacteria

Intranasal inoculation	Days	Alanine aminotransferase, U/ml	Aspartate aminotransferase, U/ml	Glucose, Mm/l	White blood cells, ×10³
First immunization	Background	34,5 ± 4,4	36,5 ± 12,5	5,4 ± 1,1	10,1 ± 2,8
	3	38,8 ± 10,2	36,2 ± 10,4	4,7 ± 0,3	8,2 ± 1,9
	7	42,1 ± 6,1	33,1 ± 2,6	5,5 ± 0,5	9,8 ± 2,7
	14	46,1 ± 6,7	41,1 ± 10,6	4,7 ± 0,7	8,8 ± 2,3
Re-immunization	Background	39,1 ± 4,6	43,5 ± 11,5	4,4 ± 0,6	9,1 ± 2,6
	3	37,2 ± 3,9	38,2 ± 8,4	4,7 ± 0,4	7,2 ± 1,9
	7	40,0 ± 3,1	34,1 ± 5,6	5,3 ± 0,5	8,0 ± 3,1
	14	41,8 ± 4,0	39,1 ± 7,1	4,7 ± 0,4	8,3 ± 2,5
Infection of native monkeys with *BP* 475 bacteria	Background	42,2 ± 6,4	42,0 ± 6,5	6,2 ± 0,3	12,1 ± 1,4
	3	44,2 ± 4,9	39,2 ± 7,4	5,7 ± 0,7	17,8 ± 2,9
	7	40,4 ± 4,1	41,1 ± 5,5	4,4 ± 0,6*	19,3 ± 2,9*
	14	39,8 ± 3,8	40,1 ± 5,1	4,7 ± 0,4*	19,3 ± 2,9*

Note. *$p < 0.05$ as compared to the background.

Table 2. Values of IgE in blood sera of RMs vaccinated and re-vaccinated with attenuated *BP* bacteria

Day after infection	Monkey identification number									
	31881	31882	31883	31901	31908	31926	31927	31843	31870	31888
First immunization										
Background	320	250	66	680	678					
7	290	190	73	–	–					
14	285	220	56	845	693					
24	427	350	48	690	578					
64	469	191	53	662	750					
180	668	117	63	609	555					
Re-immunization in 6 months										
Background	668	117	63	609	555					
7	720	141	31	745	449					
14	518	187	43	722	458					
24	562	203	62	648	–					
64	669	177	47	393	524					
180	580	258	–	–	180					
Infection with virulent *BP* bacteria										
Background	219	22	45	58	55	656	48	559	376	257
7	73	13	15	45	32	732	37	635	281	201
14	48	13	17	10	14	651	25	759	351	224
28	43	7	9	23	9	515	52	569	390	356

10, 14 days, etc. The growth of bacteria in the CCA medium was measured on the 4th–5th day after the seeding. The grown colonies were typified by using agglutinogen 1, 2 and 3 specific sera. The growth of *BP* bacteria on plates was recorded during the first 2 weeks and, in rare cases, 3–4 weeks after the inoculation. The presence of foreign microflora posed an additional problem, especially for the analysis of the bacteria grown in the antibiotic-free CCA medium. Taking this fact into account and considering the low effectiveness of the bacteriologic culture technique, we used our real-time PCR method to diagnose pertussis and to measure the number of bacteria in the nasopharynx of the animals.

Fig. 1 and **Fig. 2** demonstrate changes in the number of bacteria genome-equivalents in a conventional milliliter of washings from the nasopharyngeal swabs (aspirate) of the monkeys one-time and two-time infected with virulent and attenuated *BP* bacteria.

The repeated inoculation with attenuated *BP* bacteria was performed 6 months after the 1st dose and the experimental infection with virulent *BP* bacteria was performed 12 months after the re-immunization.

To clarify the relationship between the elimination and bacterial strains as well as the infecting dose,

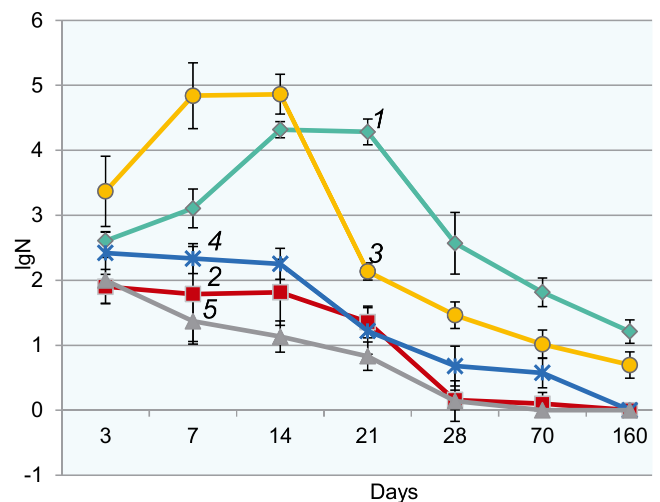

Fig. 1. Changes in the number of *BP* genome-equivalents in the RMs' nasopharynx after the first and repeated intranasal vaccination with *BP* bacteria.

N — the number of *BP* genome-equivalents in 1 ml of the nasopharyngeal aspirate. *1* — the first vaccination with virulent *BP* bacteria; *2* — the repeated vaccination with virulent *BP* bacteria; *3* — the first vaccination with attenuated *BP* bacteria; *4* — the repeated vaccination with attenuated *BP* bacteria; *5* — the infection with virulent *BP* bacteria 12 months after the vaccination with attenuated *BP* bacteria.

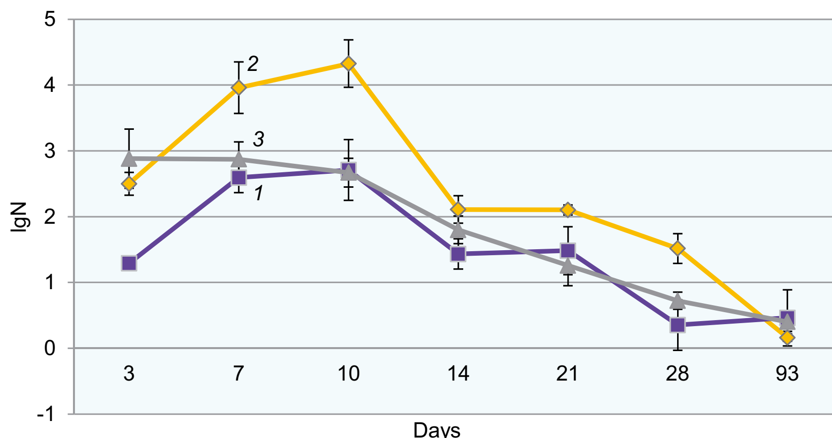

Fig. 2. Changes in the number of *BP* 18323 genome-equivalents after the 1st and 2nd experimental intranasal infection of RMs.
1 — the 1st experimental intranasal infection at a dose of 10^7 CFU; *2* — the 1st experimental intranasal infection at a dose of 10^9 CFU;
3 — repeated experimental infection with *BP* 18323 at a dose of 10^9–10^{10} CFU.

adult RMs were infected with two doses (10^7 CFU and 10^9–10^{10} CFU) of virulent *BP 18323* bacteria. Each of the doses was administered intranasally to 3 RMs. All the RMs were infected for a second time with one dose of 10^9–10^{10} bacteria. The repeated experimental infection with virulent *BP 475* or *BP 18323* was performed 4–6 months after the 1st inoculation. Fig. 1 and 2 show that the number of bacteria in the RMs' nasopharynxes reaches its maximum 7–14 days after the experimental infection with attenuated *BP 4MKS* bacteria (t_{max} = 7–14 days), 14–21 days after the infection with isogenic virulent *BP 475* bacteria and 7–10 days after the intranasal inoculation with virulent *BP 18323* bacteria.

The seeding and PCR analysis of the washings from the nasopharyngeal swabs from the control animals did not reveal any growth of colonies and DNAs of the pertussis pathogen.

Titer of specific IgG in the blood serum of the vaccinated RMs after the intranasal infection with BP

All the tests described in the previous section were accompanied by the analysis of the changes in the levels of *BP*-specific IgG in the blood serum of the infected animals. After the 1st experimental infection, the levels of specific IgG in the blood serum of the animals were increasing (Fig. 3, 4), starting from the 10th to 14th day, and reached its maximum by the 28th day in the monkeys infected with virulent and attenuated *BP 475* bacteria, and by the 35th – 48th day after the infection with *BP 18323***.** After the repeated infection with *BP 18323* bacteria, the IgG levels reached their maximum values by the 14th day after the inoculation with bacteria of any strain.

Discussion

The preclinical studies of acute toxicity on infant rates and mice, *leukocytosis*-promoting and *histamine-sensitizing activity of the* pertussis toxin and weight toxicity of the suspension of attenuated *BP* bacteria in classical tests on linear mice, activity of dermonecrotic endotoxin and hypoallergenicity in tests on rabbits and guinea pigs demonstrated the safety of intranasal administration of the new constructed live recombinant pertussis vaccine (LPV) [18].

The performed studies on adult nonhuman primates showed that experimental intranasal infection of monkeys with virulent *BP* bacteria results in development of laboratory presentation of pertussis infection in RMs, hamadryas baboons, Javanese macaques and Japanese macaques [22]. Juvenile Anubis baboons developed a whooping cough typical of pertussis [19]. The immunization of Anubis baboons with LPV based on attenuated *BP BPZE1* bacteria demonstrated safety and immunogenicity of the vaccine [21]. The results pointed at viability of using the experimental model of

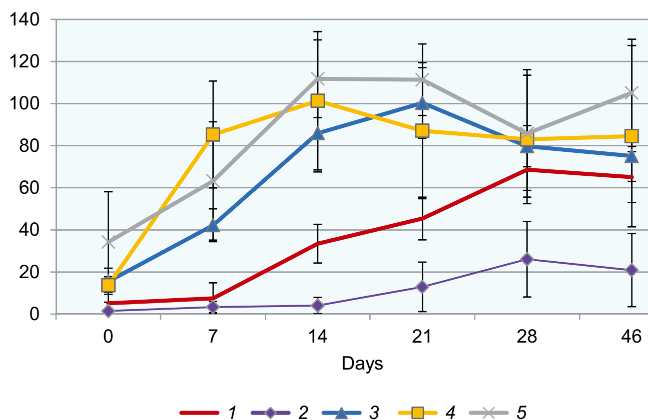

Fig. 3. Changes in the IgG level in the RMs' blood serum after the 1st and repeated intranasal vaccination with *BP* bacteria.

On the vertical axis — the relative value of IgG levels (%): OD_{C+}/OD_i, where OD_{C+} — optical density of the positive control, OD_i — optical density in the well with the studied serum.
1 — the 1st vaccination with attenuated *BP* bacteria; *2* — the 1st vaccination with virulent *BP* bacteria; *3* — the infection with virulent *BP* bacteria 12 months after the vaccination with attenuated *BP* bacteria; *4* — the repeated vaccination with virulent *BP* bacteria; *5* — the repeated vaccination with attenuated *BP* bacteria.

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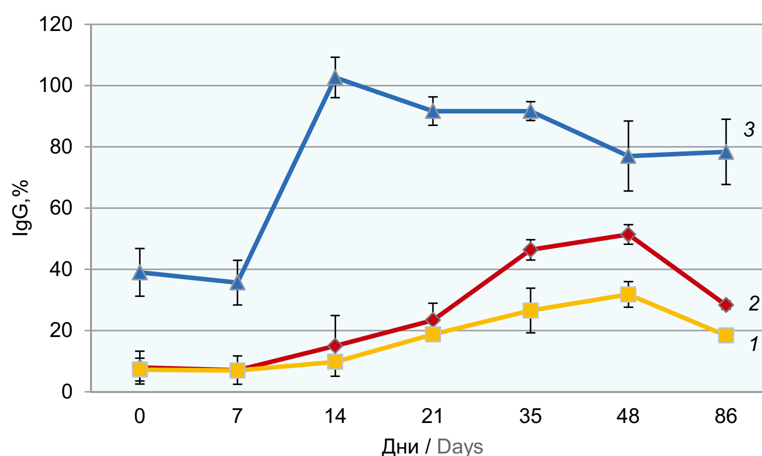

Fig. 4. Changes in the IgG relative levels in the RMs' blood serum after the 1st and repeated intranasal infection of the animals with *BP* 18323 bacteria.

1 — the 1st experimental intranasal infection at a dose of 10^7 CFU; *2* — the 1st experimental intranasal infection at a dose of 10^9 CFU; *3* — the repeated experimental infection with *BP* 18323 at a dose of 10^9 CFU.

nonhuman primates in the studies of immunobiological characteristics of the pertussis pathogen and immunogenicity of pertussis vaccines.

The obtained results showed absence of any changes in blood counts and site responses in RMs after the intranasal inoculation with attenuated *BP* bacteria, thus fully corresponding to the results of the tests on smaller laboratory animals. No inflammatory processes were detected in the nasopharynxes of the animals monitored after the experimental infection with virulent bacteria after the first and repeated vaccination. No increase in total IgE was found in the monkeys' blood serum after the vaccination and re-vaccination. All the immunized animals demonstrated a decrease in IgE after being infected with virulent *BP 475* bacteria. These findings are consistent with the results obtained by R. Li et al. [26] who showed that intranasal vaccination of mice with attenuated *BP BPZE1* bacteria not only causes no allergic reactions, but also protects animals from experimental allergic inflammation and decreases IgE levels in blood sera.

It should be noted that the IgE Ridascreen test designed for human blood serum testing did not detect any IgE in the monkeys' sera. The IgE levels in the monkeys' blood were measured with the IgE Vector-Best test and the results are shown in Table 3. This test had never been used for evaluation of monkeys' IgE levels; therefore, the obtained values cannot be used in quantitative measurements, though they can be used for qualitative assessment of changes in IgE levels after the animals' immunization.

The growth of virulent *BP* bacteria in the nasopharynx and their persistence in a human body are critical characteristics of pertussis infection. Based on the available data, the immunization of monkeys with wPVs and attenuated bacteria, as opposed to immunization with aPVs, results in development of mucosal immunity preventing the growth of virulent *BP* bacteria when they enter the body of a human or a monkey. The charts shown in Fig. 1 and 2 demonstrate the highly similar dynamics of the growth/eradication of attenuated and virulent bacteria of different strains in the nasopharynx of RMs. Individual *BP* genome-equivalents are detected in nasopharyngeal isolates during 6 months by using the real-time PCR. We received similar results during examination of children of different age, recovering from pertussis, where in 15–20% cases, the pertussis pathogen was detected by the real-time PCR 6 months after the diagnosis [27].

The similar picture was observed after the repeated inoculation of monkeys with attenuated and virulent bacteria and after the experimental infection of the immunized animals with virulent bacteria. The curves of growth/eradication after the repeated inoculation of bacteria (Fig. 1 and 2) do not demonstrate any qualitative differences, though they fundamentally differ from the picture observed after the 1st inoculation by absent accumulation and higher rates of bacteria eradication. For example, by the 28th day after the repeated infection (immunization), bacteria were not detected or were detected only individual cases, while during the same period after the 1st infection, the number of *BP* genome-equivalents could reach several dozen. It should be noted that the sensitivity of our test is 0.01–0.1 genome-equivalent in 5 μl of the solution and is based on using multi-copy sequence of IS481 as the amplification target [24, 25].

To analyze the relationship between the changes in the bacterial growth and development of an immune response from the infecting dose and the bacteria strains, along with isogenic virulent and attenuated *BP 475* bacteria and *4MKS* bacteria, we used virulent *BP 18323* bacteria, which are used for evaluation of protective activity of wPVs. The bacteria were vaccinated to 3 RMs at a dose of 10^9–10^{10} CFU and to 3 RMs at a dose of 10^7 CFU. The number of bacteria and changes

in growth/eradication after the experimental infection of RMs vaccinated with 10^9 bacteria did not show any statistically significant differences as compared to the results obtained from infection of animals with other *BP* strains. The t_{max} values we measure for *BP 475, 18323* and *4MKS* are similar and correlate with the values given in the work of J.M. Warfel et al. [19] for *BP D420* bacteria; the latter values were obtained after infecting of Anubis infant baboons.

During the entire development of the 1st infection, the monkey infected with *BP* bacteria at a dose of 10^7 CFU demonstrated significantly lower amounts of *BP* genome-equivalents in the animals' nasopharynxes (Fig. 2). This circumstance was taken into account in our decision on the vaccination dose for ongoing clinical trials of LPV on healthy volunteers.

It should be noted that our previous studies showed that the phase composition of the population undergoes changes during the persistence of pertussis bacteria in the monkeys' bodies. While during the first hours after the infection, most of the *BP* bacteria are in a virulent state characterized by the native structure of the *bvgAS* operon, during the infection development, the heterogeneity of the *BP* bacteria population increases due to the increasing levels of non-virulent mutants of the pertussis pathogen, which carry the *IS481* insertion in the *bvgAS BP* operon. The process is especially pronounced after repeated inoculations when the number of insertion mutants of *BP* bacteria in the persisting bacteria population can reach 50% of the total number of the recorded bacteria [28]. This observation suggests the possible existence of the mechanism responsible for forming long-term persisting bacteria that secure the survival of the pathogen and its transmission to a new host.

Fig. 3 and 4 show that the changes in the titer of specific IgG in the blood sera of the vaccinated monkeys experimentally infected with virulent *BP* bacteria show resemblance with the respective curve obtained after the repeated infection of RMs and are significantly different from the changes in the IgG levels after the 1st experimental infection with virulent and attenuated bacteria. All the animals re-immunized and infected after the immunization with virulent *BP* bacteria demonstrated a rapid increase in the levels of antibodies, which reached maximum values by the 10th – 14th day. These results are totally consistent with the results for IgG levels after the repeated infection with virulent *BP 475* bacteria and correlate with the above results capturing changes in the accumulation of bacteria in the nasopharynxes of the infected animals. The rapidly increasing levels of specific antibodies after the repeated exposure to infection contribute to suppression of the bacterial growth and bacteria elimination from the nasopharynxes of the animals.

The similar dynamics was observed during 2 and 3-time experimental infections of different species of monkeys of the Old World monkeys with virulent *BP* bacteria, including infection at a dose of 10^{10}–10^{11} CFU. All the cases demonstrated a pronounced boosted humoral immune response and rapid elimination of the pathogen after the repeated infection [22]. The presence of the booster effect produced by the repeated vaccination at low or even zero levels of IgG in some animals after the 1st vaccination as well as the rapid elimination of bacteria in all the animals after the repeated vaccination suggest that even one-time intranasal vaccination with attenuated bacteria can be sufficient and can provide protection against experimental infection.

Thus, the experimental intranasal infection of RMs with attenuated and virulent *BP* bacteria results in developing of a protective reaction to repeated infection, including suppression of the bacterial growth, increased rates of bacteria elimination from nasopharynxes of the animals and development of a humoral immune response to infection. The development of immunity against repeated pertussis infection is accompanies by a pronounced booster effect regardless of the bacteria strain. The obtained results imply common mechanisms of development of post-vaccination immunity after intranasal immunization of the animals with LPV and post-infection pertussis immunity, both of them providing protection against re-infection with *BP* bacteria and against development of clinical symptoms of pertussis. The obtained data are consistent with the results obtained by C. Locht at al. [22], who demonstrated the presence of a pronounced protective effect from the experimental infection with virulent *BP D420* bacteria through the example of Anubis baboons immunized with live attenuated *BP* bacteria. Inactivated bacteria of the pathogen (wPVs) have a less pronounced protective effect, while aPVs do not provide protection against the bacterial growth after the repeated infection [20, 21].

The absence of monkey-specific enzyme-linked immunoassay (ELISA) tests makes quantitative measurement of pertussis antibodies in these animals highly challenging. While the Ridascreen human IgG enzyme immunoassay kit was suitable for measurement of IgG levels in RMs, hamadryas baboons, Javanese macaques and Japanese macaques, the test from the same manufacturer for measurement of human IgA levels was totally insensitive to simian IgA. We were able to measure IgM levels in different periods of the experimental pertussis infection of the monkeys by using Ridascreen ELISA test for human IgM, though low absolute values and widely scattered results make their discussion unproductive. Most likely, the observed picture can be explained by the insufficient efficiency of the commercial tests for monkeys' sera. The obtained results emphasize the urgency of designing ELISA tests specific for immunoglobulins of nonhuman primates, so that they could be further used as an experimental model.

Conclusion

The single-dose intranasal inoculation and reinoculation with attenuated *BP* bacteria did not cause any inflammatory processes in the RMs' nasopharynxes and any changes in the blood counts after the experimental infection of nonhuman primates with virulent bacteria.

No increase in the levels of total IgE in the monkeys' blood serum was detected after single-dose and double-dose immunization.

Experimental infection of monkeys with attenuated and virulent *BP* bacteria induces the development of protective reaction to repeated infection, including suppression of the bacterial growth, increased rates of bacteria elimination from animals' nasopharynxes and development of a humoral immune response to infection. The development of immunity against the repeated pertussis infection is accompanied by a pronounced booster effect regardless of the bacteria strain.

The obtained results suggest common mechanisms of development of post-vaccination immunity after intranasal vaccination of animals and post-infection pertussis immunity, which provide protection against repeated infection with *BP* bacteria and development of clinical symptoms of pertussis.

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