ARTICLE

IMMUNOGENIC AND ANTIGENIC PROPERTIES OF AGGLUTINOGENIC AND WEAKLY AGGLUTINOGENIC VACCINES AGAINST ANIMAL BRUCELLOSIS

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ABSTRACT

Background: *Brucellosis is a particularly dangerous zoonotic disease, widespread in the Russian Federation. The research was aimed at studying the immunogenic and antigenic properties of agglutinogenic and weakly agglutinogenic vaccines against animal brucellosis.* ***Methods:*** *A suspension of brucellae from the Brucella abortus 19 vaccine strain obtained from immune and intact animals had been injected into the tubes with various blood fractions until the final concentration of 1 billion microbial cells was reached. The concentration of brucellae in the bacterial mass was determined according to the optical standard of turbidity and monitored till incubation.* ***Results and Conclusions:*** *The agglutinating and complement-binding antibodies detected in the diagnosis of brucellosis are not essential for brucellosis immunity and mainly play the role of the "witness" of the immune system's contact with the pathogen. Brucellosis immunity is mainly of the cell type, but some precipitin antibodies show the phenomenon of opsonization and blocking the pathogenicity factor, and play a positive role in forming brucellosis immunity. Veracious assessment of the immunogenicity of agglutinogenic and weakly agglutinogenic vaccines against brucellosis is possible only with the use of the direct infestation method.*

KEY WORDS
brucellosis, brucellae, vaccine, strai, antibodies, immunogenicity, immunity, lymphocytes

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INTRODUCTION

Brucellosis is an infectious disease caused by bacteria of the *Brucella* genus, which infects almost all types of agricultural and many species of wild animals, as well as humans. The infectious agent, which easily migrates from animals to humans, causes acute febrile illness (*B. abortus* - undulating fever, or *B. melitensis* - Maltese fever) and can progress to a chronic form leading to serious complications affecting the musculoskeletal and other body systems.

In recent years, immunology has significantly advanced in studying the morphology and functions of various lymphocyte subpopulations and the mechanisms of their cooperation. As a consequence, the extreme titer of specific antibodies, or the number of antibody-forming cells, is sometimes considered as a key quantitative indicator of the immune status of the organism, which, however, does not always coincide with the results of experimental infestation.

In several experiments devoted to studying brucellosis immunity, the authors observed a clear lack of correlation between the titer of the antibodies detected in AT and CFT with the standard diagnostic and the state of immunity in guinea pigs, which was checked by experimental infestation. These data are consistent with the works of many researchers. Since the 40ies of the 20th century, reports have been published stating that high titer of antibodies does not guarantee protection for the animals from experimental infestation [1-3].

In veterinary medicine, therapeutic serums are widely and successfully used against many bacterial and viral infections. This also raises the possibility of the essential role of antibodies against the brucellosis infection. There is no such serum against brucellosis, although, in the 20th century, many authors tried to develop it and even obtained some positive results. In their studies with guinea pigs, the authors also injected the anti-brucellosis serum obtained by hyper immunization of rabbits with the brucellosis vaccine to the experimental animals immediately after infestation, and three more times at five to six days' intervals. The bacteriological study a month after infestation and treatment with the anti-brucellosis serum revealed 63 % of the experimental animals that were free from the brucellosis infection with 100 % infestation of the reference animals. Analyzing the materials of the developments in this area, it may be noted that in vivo, the preventive property of the immune anti-brucellosis serum was manifested, but only if the serum had been introduced before or simultaneously with the infecting culture. In the period of developed infection, the serum did not possess any preventive properties. Apparently, given the intracellular nature of the parasitism of *brucellae*, the cell membrane created a reliable barrier for the humoral bactericidal blood factors.

The body's defense against the brucellosis infection is determined by cellular immunity: after phagocytosis of the *brucellae*, macrophages transmit information to the T-lymphocytes responsible for cellular immunity.

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In this process, some antibodies, such as precipitin or bactericidal antibodies play a useful role. On the contrary, agglutinating antibodies (or agglutinins) are passive witnesses of the organism's contact with the *brucellae*. However, the authors note that a vaccine from a mutated *brucellae* strain in the R-form (of the Brucella abortus 45/20 type) induces exclusively cellular protection. In this case, a non agglutinogenic vaccine is obtained (of the Abortox type) [4, 5].

Thus, it seems to be convincingly proven that the genesis of antibodies upon vaccination against brucellosis does not correlate with immunity development. This is at least evidenced by the recent trend of developing weakly agglutinogenic and non agglutinogenic vaccines. The issue of the cellular type of immunity in the brucellosis infection seems to be beyond doubt. The experiments with the *Brucella abortus* RB51 vaccine show the cellular nature of the immunity induced by their vaccine [6]. This vaccine is used for the cattle aged four to eleven months at a dosage of 1 – 3 x 1010, and for the cattle aged 11 months — at a dosage of 1 – 3 x 109. In the experiments, the authors did not find antibodies at various times in 3,250 animals after vaccination using the standard methods, and did not find *brucellae* of the vaccine strain in the milk; therefore, a conclusion was made that this vaccine stimulated the cellular type of immunity. Recently, however, reports have reappeared about the positive role of the humoral immunity against the brucellosis infection. The experiments on the mice that were deficient in terms of the B-cells proved that antibodies played a limiting role in the infections with intracellular parasitism, brucellosis in particular [1, 7].

Understanding this issue is of great practical importance. At the present stage, the choice of the adjuvant is of significant importance in constructing killed vaccines, both corpuscular and from their fragments, including those obtained from purified protective antigens. To date, a significant number of immune stimulating drugs have been developed, and their correct selection allows purposeful stimulation of the immune system of either humoral or cellular type.

The purpose of the research was to study the immunogenic and antigenic properties of agglutinogenic and weakly agglutinogenic vaccines against animal brucellosis. This article presents the materials about studying the anti-brucellosis properties of various components (lymphocytes, neutrophils, plasma, and serum) of the blood of immune and intact animals. The authors hope that the results of this study will make a certain contribution to studying the role of antibodies in brucellosis.

MATERIALS AND METHODS

The research was conducted on the basis of the veterinary center of the Yakutsk Research Institute of Agriculture named after M.G. Safronov during 2014-2018 (the project code of the state task for research: 0821-2018-0003 of 04/15/2014).

For the study, the blood from immune and intact animals (cattle) (immune – 30 heads, intact – 30 heads) was separated into component fractions using the method of gradient centrifugation. At the time of the study, 1.5 months had passed after immunization of adult heifers with the vaccine obtained from the *Brucella abortus* 19 strain [1, 3, 8].

The blood from the heifers was taken into sterile tubes with heparin (25 – 50 U/ml). The density gradients were ficoll and verografin solutions (Preparation of density gradient: solutions of ficoll-400 and verografin are mixed. Using a hydrometer, the density of the resulting solution is measured, which should be 1.077 g/cm. If the density is more than necessary, then solution of ficoll-400 should be added, if it is less than necessary, then solution of verographin is added). The blood diluted to 1:3 with Hanks' solution (a ready-made solution is manufactured by Research and Production Enterprise PanEco, Moscow, comprised of inorganic salts and glucose solved in purified water and sterilized through 0.22 μm pore size filters; the solution contains salts of calcium and magnesium) was placed in layers on the density gradient and centrifuged in a horizontal rotor at 400 g for 30 minutes. After centrifugation, three layers formed in the centrifugal tube: the upper — blood plasma; the middle — the density gradient, and the lower — erythrocytes. At the interface of the blood plasma and the density gradient, the layer of lymphocytes (in the form of a light whitish cloud) was carefully sucked off with a sterile Pasteur pipette and transferred to another tube. The layer of polymorphonuclear leukocytes (in the form of a whitish film) was taken at the interface of the blood plasma and the density gradient, and was also transferred to another tube. The isolated blood cells were washed three times in Hanks' solution by centrifugation at 400 g for 10 minutes and re-suspended in Hanks' solution. Various populations of blood cells, serum, and blood plasma were isolated in strict sterility. Blood serum and plasma were used both in the native and heated forms. The plasma and serum were heated to 60 0C for half an hour for the inhibition of heat-labile bactericidal factors of the blood (lysozyme, the complement system, and M-class immunoglobulins).

A suspension of *brucellae* from the *Brucella abortus* 19 vaccine strain obtained from immune and intact animals had been injected into the tubes with various blood fractions until the final concentration of 1 billion microbial cells was reached. The concentration of *brucellae* in the bacterial mass was determined according to the optical standard of turbidity. The required concentration of microbial cells was obtained by a series of bacterial mass dilutions in sterile saline. For monitoring the concentration of *brucellae*, seeding was made into Petri dishes with meat-peptone glucose-glycerol agar (MPGGA) (five dishes for each sample) from the last and the penultimate dilutions of the culture, followed by counting the number of grown colonies.

IIOAB JOURNAL
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The concentration of microbial cells was monitored before incubation, immediately after injecting the suspension of *brucellae* into various blood fractions, and six to eight hours after incubation at 37 ºC.

Devices and equipment used in laboratory diagnosis of brucellosis complied with the Appendix 8 "The order of organization and conduct of laboratory diagnosis of brucellosis for laboratories at the territorial, regional and federal levels" of Methodological instructions MUK 4.2.3010-12.

RESULTS AND DISCUSSION

At the initial stage, the authors made a series of experiments for determining the possibility of the cytolytic effect of specific immunoglobulins on the *brucellae* culture in the absence of cells with killer effect, i.e., for determining the viability of *brucellae* under the effect of immune serum. After mixing the *brucellae* suspension with immune serum with an activity of 640 IU, the mixture was incubated at 37 ºC in a thermostat. The experiments were performed with *brucellae* from both the vaccine (*Brucella abortus* 19) and the virulent (*Brucella abortus* 54) strains. After 24 hours, the resulting agglutinate was sown on solid nutrient media. In the reference tubes, saline was used instead of immune serum. One to three days later, the plentiful growth of *brucellae* (in a lawn) started with no visible difference in the experiment and the reference. No significant differences were noted in the growth of the vaccine and the virulent cultures, either.

The experiments showed that *brucellae* could maintain viability even in the conditions of agglutination with their specific immunoglobulins. However, this technique did not allow to accurately determine the degree of the *brucellae* survival ability. Therefore, an experiment was made with the use of the method that involved seeding *brucellae* into Petri dishes and counting the number of grown colonies.

Table 1 shows the data of determining the bactericidal activity of various blood fractions from the intact animals and the animals immune to brucellosis.

Table 1: The bactericidal activity of various blood fractions from the intact animals and the animals immune to brucellosis

Object type			The number of microbial cells (billion)	
			Before incubation	After incubation
Animal blood samples	intact		0.99 ± 0.005	1.25 ± 0.008*
	vaccinated		0.98 ± 0.008	0.96 ± 0.012
Animal blood serum	intact	native	0.99 ± 0.012	0.79 ± 0.008*
		heated	1.01 ± 0.011	1.95±0.005*
	vaccinated	native	1.0 ± 0.012	1.35 ± 0.011*
		heated	0.98 ± 0.002	1.88 ± 0.008*
Animal blood plasma	intact	native	1.1 ± 0.005	0.99 ± 0.005
		heated	1.0 ± 0.012	1.75 ± 0.008*
	vaccinated	native	1.05 ± 0.008	1.2 ± 0.005*
		heated	0.99 ± 0.005	1.65 ± 0.005*
The population of segmented neutrophils from the animals	intact		1.06 ± 0.012	3.95 ± 0.013*
	vaccinated		0.97 ± 0.008	4.89 ± 0.017*
The population of lymphocytes from animals	intact		1.03 ± 0.005	0.99 ± 0.008
	vaccinated		1.1 ± 0.008	0.5 ± 0.009*

Note: * — statistically veracious difference (P < 0.05)

The Table 1 shows that the greatest bactericidal effect was observed in the population of lymphocytes obtained from the immune animals. The number of microbial cells after incubation decreased by more than half (0.5 ± 0.009 bln.m.c.). In the intact animals, these figures were lower (0.99 ± 0.008 bln.m.c.). That is, the number of *brucellae* remained at almost the initial level, although during such an incubation time, in otherwise favorable conditions, it could well have increased. The blood serum indicators were of interest. For *brucellae*, the bactericidal activity of the blood serum taken from an intact animal was higher (0.79 ± 0.008 bln.m.c.) compared to that of the blood serum taken from an immune one (1.35 ± 0.011 bln.m.c.). The blood serum taken from an immune animal not only did not inhibit the growth and reproduction of *brucellae* but even seemed to stimulate them. In any case, the bactericidal activity indicators of immune serum were approaching similar indicators of heated serum.

The decreased inhibitory ability of positive serum, compared to negative serum, was apparently due to a decrease in the levels of lysozyme and the complement, which occurred in the initial period after

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immunization with the live vaccine [5, 9], which, in turn, proved the nonspecific inhibitory ability of immune serum to *brucellae*.

Approximately the same was observed with the blood plasma obtained from intact and immune animals as with serum, but it was less pronounced.

The authors have found interesting the fact that after inactivation of the complement and other heat-labile factors, serum (both negative and positive) generally loses its ability to exert any inhibitory effect on the *brucellae*, which may multiply in it, using serum components as a nutritious substrate. By the way, this property of serum is widely used in making culture media for cultivating many microorganisms, including *brucellae*.

The bactericidal properties of the blood obtained from an immune animal (0.96 ± 0.012), compared to the properties of normal blood (1.25 ± 0.008), were more pronounced. Here, the cooperation of all parts of the immune system was observed. The somewhat lower bactericidal activity of the immune blood, compared to that of the lymphocyte population, in the opinion of the authors, was explained quite simply. With the introduction of a suspension of *brucellae* into an isolated population of lymphocytes, the pathogen encountered powerful cooperation of the cells that had a killer effect and nothing more. When the germ was introduced into the blood, the killer effect competed, and not always successfully, with the growth factors in the form of protein, carbohydrates, vitamins, etc. that were abundant in the blood. In this regard, the properties of the population of polymorphonuclear leukocytes were very obvious. It seemed like the *brucellae* had been introduced into an optimally chosen nutrient substrate. The number of *brucellae* grew four times and more both in the normal (3.95 ± 0.013) and in the immune (4.89 ± 0.017) population of neutrophils. However, the microscopy of smears stained by Romanovsky-Giemza showed that the phagocytosis was pronounced, especially in the immune population of neutrophils. Some neutrophils were so overfilled with *brucellae* that they threatened to rupture the membrane. This happened periodically, judging by some conglomerates of *brucellae* and nuclear material of polymorphonuclear leukocytes without cell membranes scattered in various fields of view. The question of whether the rupture of neutrophil shells occurred due to contamination with *brucellae* or as a result of mechanical damage by polished glass when smears kept open. However, the authors prefer the first brucellosis version of this phenomenon, given the significant increase in the number of *brucellae*.

In the experiment, only blood serum and the plasma could be used in a sufficiently pure form. The situation with the populations of cellular blood fractions was somewhat more complicated. Using the suggested method of blood fractionation, the authors failed to obtain a homogeneous population of the blood cells of both polymorphonuclear and mononuclear leukocytes. In the microscopy of the smears with polymorphonuclear leukocytes, the main part of the cells were segmented neutrophils. Basophils and eosinophils, and in studying several tens of visual fields, individual stab and even young neutrophils were found. The mononuclear fraction was presumably represented by various populations of T- and B-lymphocytes since the used method did not allow dividing them into subpopulations. Under smear microscopy, all cells represented an approximately homogeneous mass with some size variation (large and small lymphocytes). The authors were virtually unable to detect monocytes. Apparently, given the adhesion property, lymphocytes were lost during washing three times by centrifugation.

The obtained *in vitro* results cannot fully reflect the *in vivo* events. Yet, based on the results of the studies and the literature, some conclusions may be drawn. One cannot ignore the fact that the inculcated pathogen usually has tens and probably more antigenic determinants, rather than one. As a result of the biochemical structure heterogeneity, some of them may act as strong antigens (for example, polysaccharide antigens, such as lipopolysaccharides of the gram-negative bacteria), while the other induce less significant antigenic stimulus. All this leads to the fact that in many diseases, in particular, brucellosis, the antibodies complementary to the structural components that are not necessary for the life of the microorganism are detected in the highest titers with the standard diagnostic. Blocking of such antigenic macromolecules by the agglutinating and complement-binding antibodies had little effect on the viability of the pathogen. Therefore, it is said in such cases that there is no correlation between the titer of specific antibodies and the protective effect verified in acute experiments by the method of experimental infestation.

The literature that covers the immunology of brucellosis reports that specific antibodies have an opsonizing effect on the cultures of both immune and not immune macrophages, enhancing to some extent their phagocytic and bactericidal ability *in vitro* [3].

Today, several IgG subclasses are known (IgG1, IgG2, IgG3, IgG4), each being more pronounced in a certain serological reaction. Apparently, according to the old classification, the role of the antibodies that are significant in fighting the brucellosis infection and correlate with the immune system may be attributed to precipitins. This kind of antibodies has been detected with the soluble poly-B-antigen in the reaction of radial immunodiffusion (RRID) [6, 7, 10, 11] for differentiating the infected and vaccinated animals. Today, the immunodiffusion reaction with the O-polysaccharide antigen is officially approved in the Russian Federation.

Thus, the antibodies detected in the diagnosis of brucellosis, with the visible manifestations being the agglutinating and the complement-binding effects, most likely play only the role of witnesses of the

IIOAB JOURNAL
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immune system's contact with the antigen. The precipitating antibodies, which exhibit an opsonizing effect and can block epitopes that act as the pathogenicity factor, can play a positive role in brucellosis immunity, being, in essence, the protective antigens. Given the prevailing role of the cellular immunity in brucellosis, evaluating the efficacy of some vaccines using agglutinogenicity only is not justified, and does not show the true state of things [6, 10].

CONCLUSION

Based on the results of the studies, the following conclusions may be drawn. The agglutinating and complement-binding antibodies detected in the diagnosis of brucellosis are not essential for brucellosis immunity and play the role of mainly the "witness" of the immune system's contact with the pathogen. Brucellosis immunity is mainly of the cell type, but some precipitin antibodies show the phenomenon of opsonization and blocking the pathogenicity factor, and play a positive role in forming brucellosis immunity. Veracious assessment of the immunogenicity of agglutinogenic and weakly agglutinogenic vaccines against brucellosis is possible only with the use of the direct infestation method. In conclusion, it should be noted that these studies will be the basis in developing precision diagnostic and vaccines for treating brucellosis in animals, and will make it possible to improve the state of the farms affected by brucellosis.

CONFLICT OF INTEREST

The authors declare no competing interests in relation to the work.

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