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IMMUNO-MORPHOLOGICAL AND SEROLOGICAL INDICES IN IMMUNIZATION  
AGAINST ORNITHOSIS WITH AEROSOLS OF LIQUID VACCINE

Following is the translation of an article by I. I. Terskikh, B. S. Gusman, and A. I. Danilov, Institute of Virology imeni D. I. Ivanovskogo, AMN USSR, and the Institute of Human Morphology AMN USSR, Moscow, published in the Russian-language periodical Voprosy Virusologii (Problems of Virology), No 2, 1968, pages 192-199. It was submitted on 6 Dec 1965.

Up till the present time methods of vaccination against ornithosis have not been worked out conclusively. It has been shown by experimental investigations, conducted with vaccines from yolk sacs, allantoic membrane, and allantoic fluid which had been inactivated by various methods (with formalin, phenol, ultraviolet rays, and methylene blue), that in animals which had been vaccinated parenterally a resistance was developed to the intraperitoneal infection with virus, but it was almost completely lacking or was weakly expressed during infection through the respiratory tract [6, 11, 12, 16].

In the development of a method of vaccination against ornithosis it is necessary to take into consideration the peculiarities of the pathogenesis of this infection, in which the lungs are the site of primary localization of the causative agent, and the most sensitive cells are the cells of the respiratory bronchioles and the alveolar epithelium [4, 7, 10, 14]. Taking into consideration that with ornithosis the resistance of the organism to infection is conditioned mainly by cellular local immunity, we decided to develop a method for the administration of live vaccine (against ornithosis) in the form of an aerosol. A necessary condition here was the creation of a finely-divided (1-3 microns) phase of aerosols, since it is easier for aerosols of such dimension to reach the respiratory bronchioles and alveoli [9].

During the aerosol method of immunization the vaccines should meet certain requirements. Previously we published data on the preparation and checking of a tissue vaccine against ornithosis and the conditions necessary for carrying out the immunization with finely-dispersed aerosols [3-5].

In the present report we are presenting the results of a study of the immuno-morphological reaction in the organs of the reticulo-endothelial system of monkeys which reflect the development of immunity following immunization with aerosols of tissue vaccine against ornithosis. Simultaneously a determination was made of the virus-neutralizing antibodies in the organs and tissues. The results

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obtained make it possible to evaluate the immunological effectiveness of the tissue vaccine and the method of vaccination with finely-dispersed aerosols of liquid vaccine.

#### Materials and Methods

Monkeys (rhesus) were immunized with tissue killed vaccine against ornithosis which was prepared by a method which was described earlier [2, 3]. For obtaining a finely-dispersed aerosol a volume of 4.5-5 ml of liquid vaccine was sprayed for 20 minutes with the help of a metallic (jet) sprayer (design of A. I. Uromyko and A. V. Kashin) in an LVK<sub>2</sub> chamber [6]. The arithmetical mean radius ( $r_{50}$ ) of particles was equal to 0.8 microns (with a spread from 0.5 down to 1.2 microns with a predominance of the finer fractions). The concentration of aerosol particles in 1 ml of air at the moment of conclusion of spraying reached  $1.0 \times 10^5$ , and by the time of conclusion of vaccination of the animals -  $8 \times 10^4$ . The monkeys, 2-2½ years of age and weighing 2.3-2.5 kg, were found in the chamber and inhaled the aerosol of vaccine for an hour. Immunization was performed 3 times with an interval of a day for the simultaneous clearing up of the reactogenicity of the vaccine. Preliminarily we established the absence of a temperature reaction and X-ray changes in the lungs of the immunized monkeys [3].

The dose of inhaled vaccine was determined by the formula:

$$D = C \cdot V \cdot P \cdot t,$$

where C - concentration of vaccine (in g/ml) in the aerosol chamber; V - respiratory capacity (in ml/min for 1 g of weight) of the monkey; P - weight of animal (in g); t - time of contact with aerosol (in min). The concentration of vaccine (C) we calculated by multiplying the number of aerosol particles in 1 ml of air by the weight of one aerosol particle, the mean radius ( $r_{50}$ ) of which comprised 1.4 microns ( $1.1 \cdot 10^{-11}$  g). We assumed that the density of an aerosol particle equals a unit,  $V = 0.29$ ,  $P = 2400$ ,  $t = 60$ , and that the whole mass of the absorbed aerosol was retained in the respiratory organs. The inhalation dose of vaccine during one sitting of immunization comprised  $5.5 \cdot 10^{-2}$  g, and during triple -  $1.6 \cdot 10^{-1}$  g.

In 1, 4, 7, 14, and 21 days and 2-2½ months after completion of immunization the animals were exsanguinated, cut open, and organs selected for the determination of virus-neutralizing antibodies (lungs, bifurcate, axillary, and inguinal lymph nodes, bone marrow, spleen). Blood serum and 10% tissue suspensions were used in the reaction of complement fixation and the neutralization reaction with the ornithosis virus (strain No 15). These were set up in the generally accepted manner; the complement fixation reaction was set up

in the cold, using the serum and supernatant portion of the suspension as the antibodies; the neutralization reaction was performed by intracerebral administration to white mice weighing 6-7 g. The results were considered in 21 days; processing was carried out by a modified method of Kerber 17.

Histological investigation was performed on the lymph nodes (bifurcate, axillary, and inguinal), tonsils, spleen, bone marrow, lungs, liver, and heart of 10 vaccinated and 6 control monkeys (4 inhaled aerosols of vaccine without viral antigen, and 2 "fresh" monkeys were not subjected to vaccination). The material for histological investigation was fixed in formalin and in acetone in the cold and sealed in paraffin-celloidin. Sections 5-7 microns thick were stained for histological and histochemical investigation with hematoxylin-eosin, azan 18, TN. This word has not been identified in available dictionaries, and toluidine blue, for RNA in the Brash reaction, for DNA in the Feulgen reaction, and for impregnation of argyrophil fibers and metallocytes in the Avtsyn modification. Also the PAS reaction was set up and the Gomori reaction for oxidase and alkaline phosphatase.

### Results

During setting up of the reaction of neutralization of virus with sera and suspensions of organs from monkeys which were immunized with aerosols of vaccine, virus-neutralizing antibodies were clearly revealed in the bifurcate lymph nodes and in bone marrow while they were absent in the serum from the 1st day after the last vaccination, i. e., on the 6th day from the beginning of vaccination (Table 1). Complement-fixing antibodies were not detected in tissue extracts from the lungs, bone marrow, lymph nodes, and spleen, and in the serum were revealed in a very low titer and then not constantly (Table 2).

As is known, virus-neutralizing antibodies in titratable amounts could not be detected in man and monkeys which had endured ornithosis, and in the latter, in particular, even after the parenteral administration of live virus 13, 15. Complement-fixing antibodies may be revealed, however; their presence in the blood does not stipulate and does not reflect a condition of nonsusceptibility of the animal organism to infection.

Inhalation of a finely-dispersed aerosol of vaccine in a comparatively short time guarantees the participation of the entire reticulo-endothelial system in immunogenesis. This is testified to by data from histological investigations.

In 24 hours after completion of the vaccination no particular changes were detected in the lymphoid organs, with the exception of a small increase in the centers of multiplication of follicles of the lymph nodes and spleen.

Table 1

Virus-neutralizing antibodies in the serum and suspensions of organs of monkeys, immunized with aerosols of vaccine against ornithosis

№ обезьяны	Срок работы после иммунизации	Индекс нейтрализации (в 1%)						Примечания
		сыворотка	серум	бифуркатные лимф. узлы	подмышечные и паховые лимф. узлы	костный мозг	селезенка	
39	1-е сутки	0	0,25	2,0+	0	1,25+	1,25	Опытная группа
39	4-е "	0	0,25	—	—	—	1,25	
20	7-е "	0,25	—	—	—	—	1,75+	
21	7-е "	0	0	—	—	—	0	
21	7-е "	0,75	0,75	—	—	—	0	
33	14-е "	0	0,25	—	0,25	4,0+	0,25	
37	21-е "	0	2,0+	2,25+	—	—	—	
38	21-е "	0,25	0,75	2,75+	—	—	—	
48	1½ месяца	—	1,0	0,5	1,0	1,5+	0,25	
46	2½ "	0	—	0,75	0,75	1,5+	—	
49	5 месяцев "	0,25	0,75	0,75	—	0,7	0	
39	7-е сутки	0,25	0,25	0,75	—	0,75	0,5	Контрольная среда (без вирусного антигена)
45	7-е "	0,25	0,25	0,5	0,5	1,0	0,25	
40	14-е "	0,25	—	0,75	0,25	0,5	—	
52	14-е "	0,25	0,25	0,5	0	0	0,25	
55		0	0,75	—	0	0,25	—	«Чистый» контроль (свежие животные)
56		0	0,25	—	0,5	—	0,25	

Legend: - not investigated; 0 absence of differences with control; + reliability of difference significant, remaining cases insignificant. Key: (a) No. of monkey; (b) Period of sacrifice after immunization; (c) indices of neutralization (in 1%); (d) serum; (e) lungs; (f) bifurcate lymph nodes; (g) axillary and inguinal lymph nodes; (h) bone marrow; (i) spleen; (j) notes; (k) Test group; (l) Control medium (without virus antigen); (m) "Pure" control ("fresh" animals).

On the 4th day the picture was changed sharply; in all the investigated lymph nodes and tonsils an increase was revealed in the centers of multiplication of follicles and a large number of microbes in them, there was a microphagal reaction and many reticular and immature plasma cells with an expressed pyconinophilic cytoplasm; in this period mature plasma cells were revealed in small quantities (Fig. 1). The Feulgen reaction for DNA in the nuclei of lymphoid cells was sharply positive, and less intensive in the nuclei of reticular cells. An accumulation of PAS-positive substances was noted in the center of the follicles and in the cytoplasm of reticular cells. The endothelium of vessels and sinuses was swollen. It is necessary to stress that all these changes were expressed with the same intensity in all the groups of lymph nodes, but considerably less in the spleen.

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Table 2

Complement-fixing antibodies in the sera of monkeys which were immunized with aerosols of vaccine against ornithosis

№	Титры в различные сроки после вакцинации						Примечания
	4-й день		7-8 дней		15-16 дней		
	1:8	1:16	1:8	1:16	1:8	1:16	
34	2+	0	+	0	-	-	Опытная группа
35	+	0	+	±	+	0	
37	2+	+	-	-	-	-	
38	+	0	-	-	-	-	
39	0	0	0	0	-	-	Контрольная группа (среда без вирусного антигена)
40	0	0	-	-	-	-	

Legend: (a) No. of monkey; (b) Titers in various periods after vaccination; (c) 4th day; (d) 7-8th day; (e) 15-16th day; (f) Notes; (g) Test group; (h) Control group (medium without virus antigen).

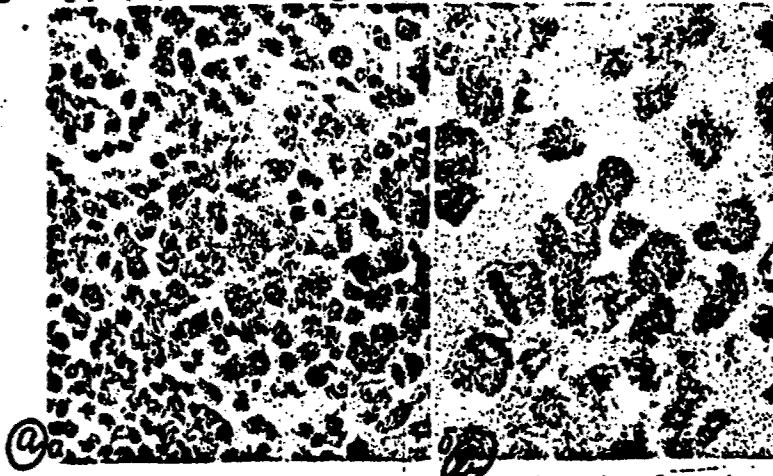


Figure 1. Immunomorphological changes in the lymph nodes. a - monkey No 20: macrophages, mitoses, reticular and plasma cells in various stage of maturity in the center of a follicle of the popliteal lymph node on the 7th day after aerosol immunization (Brash stain, X500); b - monkey No 33: mitosis, reticular and plasma cells in the center of a follicle of the bifurcate lymph node on the 14th day after aerosol immunization (Brash stain, immersion).

These changes reached their greatest intensity by the 7th day after vaccination. A large number of mature plasma cells appeared. In this period RNA was revealed both in the cytoplasm and the nucleoli of the reticular and plasma cells. These phenomena took place on a

background of hyperemia of the organs. Metallocytes were characterized by an increased argyrophil state. There was a decrease in the amount of PAS-positive substance in the reticular cells. On the 7th day the described changes were also clearly expressed in the spleen. The reaction for oxidase revealed a considerable amount of oxidase-positive elements in the spleen. An expressed picture of myelosis in the spleen was preserved up to 2½ months based on the termination of immunization. On the 14th day after vaccination the described changes were somewhat attenuated, but subsequently they remained at the same level in monkeys which were sacrificed on the 21st day and after 2½ months (period of observation; Fig. 2).

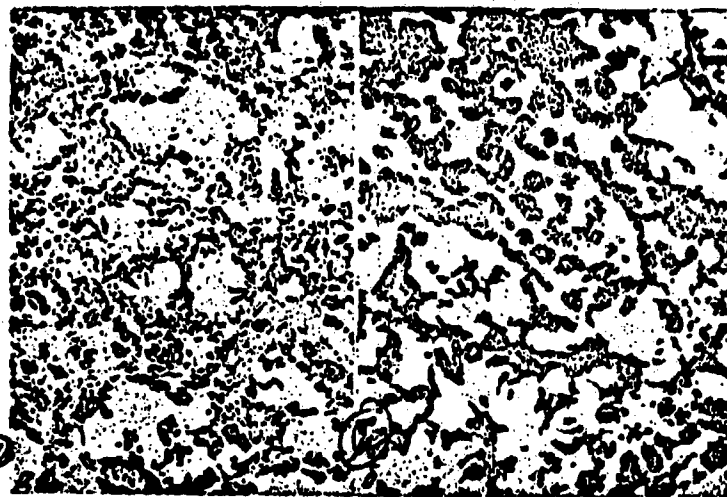


Figure 2. Immunomorphological changes in the spleen. a - monkey No 21: increased argyrophilia of metallocytes in the spleen on the 7th day after immunization (impregnation with silver base on Avtsyn, X500); b - monkey No 37: hypertrophied metallocytes in the spleen on the 21st day after immunization (impregnation with silver based on Avtsyn, X500).

Changes in the bone marrow were revealed sharply on the 4th day after immunization: hyperplasia of cellular elements was noted, there was a picture of mitosis, and there was a large number of reticular and immature plasma cells with expressed pyroninophilia of the cytoplasm and a positive PAS-reaction (Fig. 3). Also a considerable number of mature plasma cells was detected. The reaction for DNA in the nuclei was sharply positive. Blood vessels were expanded and filled with erythrocytes and the endothelium was swollen sharply. Metallocytes with thickened processes were argyrophilic. On the 7th day after vaccination the described changes reached their greatest level, weakened somewhat on the 14th day, but remained expressed up to 2½ months (period of observation).



Figure 3. Monkey No 20: focal thickening of the interalveolar partition of the lungs on the 7th day after immunization due to proliferation of lymphohistiocytic elements. Staining with hematoxylin-eosin. X120.

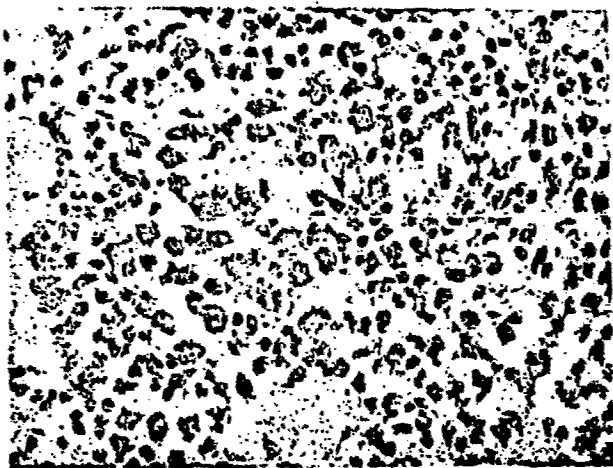


Figure 4. Monkey No 21: immature plasma cells in the center of the peribronchial follicle on the 7th day after immunization. Brash stain. X500.

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Changes were not detected in the lungs on the 1st day after vaccination. On the 4th day expressed hyperemia was revealed (in one monkey hemorrhages were detected in the alveolar cavity), there was swelling of the endothelium of vessels, focal thickening of the interalveolar partitions due to proliferation of lymphohistiocytic elements, and figures of mitosis in the reticular cells. In the lumen of the alveoli there was a small number of alveolocyttes with a weakly pyroninophilic protoplasm. On the 7th day the immunological reaction was sharply expressed: diffuse interstitial reaction, sharp swelling of the endothelium of the vessels, profuse desquamation of the alveolocyttes, many of which transformed into macrophages; there was an increase in the number of lymphoid follicles with wide centers of multiplication. Following staining with methyl green pyronin a large number of reticular and plasma cells of various stages of maturity was determined in the peribronchial follicles and the thickened interalveolar partitions. The reaction for DNA was sharply positive in the nuclei of lymphoid cells. The PAS-reaction in the reticular and plasma cells was expressed less intensively than on the 4th day after vaccination. During staining for oxidase according to Goldman a large number of oxidase-positive elements was revealed in the peribronchial follicles. The reaction for phosphatase was sharply positive. There were metallocytes with an increased argyrophilic condition (Figure 4, A). Argyrophilic stroma was not changed. On the 14th day after vaccination the changes were less intensive, and on the 21st day an evident abatement was noted. During silvering in these periods the metallocytes were close to normal and the argyrophilic stroma remained unchanged.

In the liver first and foremost was the onset of phenomena of edema and small dystrophic changes of hepatic cells. On the 4-7th day after vaccination a weak swelling of Kupffer cells was noted as well as the appearance of plasma cells in small perivascular infiltrates, RNA in the nuclei of hepatic cells, and a positive PAS-reaction.

#### Discussion

Killed vaccines against ornithosis (proposed by a number of authors) which were prepared from vitelline sacs of chick embryos and from the lungs or spleen of white mice when administered by the parenteral route did not create a resistance in animals which were infected by the respiratory route or they conditioned a weakly expressed immunity. These failures may be connected with the insufficient immunogenicity of killed vaccines, but depended mainly on the method of its application (parenteral route) which did not ensure the development of resistance in sensitive cells.

In developing a method of vaccination against ornithosis we consider it necessary to cause in the animal the development of a local immunological reaction of sensitive tissue (in the lungs)

against a background of a general immunological response on the part of organs of the reticulo-endothelial system, which is responsible for immunogenesis. This response, recorded based on morphological transformation, is compared with the results of an investigation of tissues of these organs for the presence of virus-neutralizing antibodies. It is necessary to note that virus-neutralizing antibodies were revealed in a high titer in tissues with more intensive morphological manifestations of immunogenesis (bone marrow, lungs, and lymphatic nodes). In sera virus-neutralizing antibodies were determined in an insignificant titer.

In monkeys which were vaccinated with aerosols of liquid tissue vaccine a resistance was established to infection even during contamination with aerosols of virus, as was reported earlier [7]. Thus, the method developed for the preparation of vaccine and the method of its application in the form of a finely-dispersed aerosol in the absence of an expressed reactogenicity create in monkeys an immunity which is recorded serologically, morphologically, and during testing of the resistance of the animal to infection.

The question of the harmlessness of immunization with aerosols of liquid vaccine can be resolved positively on the basis of clinical-laboratory and histological investigations of pulmonary tissue from monkeys which were used in the experiments described. The results of additional investigations for determination of the harmlessness of the vaccine will be presented in the next report.

#### Conclusions

1. Killed tissue vaccine against ornithosis during aerosol immunization with a finely-dispersed fraction is areactogenic and in monkeys creates an expressed immunological response:

a) morphological changes, reflecting the development of immunity, are revealed beginning with the 4th day after vaccination, reach a maximum by the 7th day, and abate, though they still remain clearly expressed up to 2½ months (period of observation);

b) a widespread immunomorphological reaction is characteristic in all the organs of the reticulo-endothelial system. The same intensity of reactions is noted in all the groups of lymph nodes (both regional and distal).

2. The exposure of myelosis in the spleen of vaccinated monkeys, particularly in later periods, testifies in favor of the proposal of myelosis as an index of immunogenic activity of an organ.

3. The presence of diffuse interstitial reactions in the lungs of vaccinated monkeys without symptoms of pneumonia testifies to the protective nature of this reaction.

4. Virus-neutralizing antibodies were revealed beginning with the 4th day and in higher titers in the tissues of those organs where there was a more intensively expressed immunomorphological reaction (bone marrow, lungs, and lymph nodes).

5. Immunization with liquid vaccine with the use of a finely-dispersed aerosol is an effective method of specific prophylaxis of ornithosis as a respiratory infection.

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