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The dynamics of the cytokine profile of the lungs and liver of BALB/C male mice infected with the H1N1 subtype avian influenza virus

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Abstract: The increasing number of reported cases of direct transmission of avian influenza viral strains to humans is obviously associated with the threat of a new pandemic and the need for a more detailed study of the pathogenesis of the viral infectious process. The aim of the research was to study the pathogenesis of influenza infection and in particular to establish the role of proinflammatory cytokines in the pathogenesis of the inectious process caused by influenza virus subtype A/WSN/1/33(H1N1). The experiment involved 21 male BALB/c mice aged 4-6 weeks weighing 16-18 g, which16were infected intranasally with influenza A/WSN/1/33(H1N1) virus under ether anesthesia by inoculation of 50 µl of allantoic fluid containing 10 LD50 of the virus. Histological studies were performed on samples of lung and liver tissues. It was evidenced that infection caused by the highly pathogenic for mice influenza virus A/WSN/1/33(H1N1) initiates the spreading of dystrophic and necrotic processes mainly in the lungs and liver. This can occur due the increase of proinflammatory cytokines leading to the violation of the circulatory system triggered by the active reproduction of the virus and toxic manifestations on behalf of cellular immunity. Structural changes in the lung and liver tissues of mice experimentally infected with the influenza A/WSN/1/33(H1N1) virus indicated the presence of a large amount of the virus at all stages of the experiment that requires further research.

Keywords: *Cytokines, Influenza virus, Liver, Lungs, Mice.*

1. Introduction

Influenza A viruses are considered to be one of the most significant pathogens of infectious diseases for mankind. The appearance of highly pathogenic subtypes of the virus in the poultry population and the increasing number of reported cases of direct transmission of avian influenza pathogens to humans are obviously associated with the threat of a new pandemic and the need for a more detailed study of the pathogenesis of the infectious process, the search for new means of prevention and treatment of the disease [1, 2]. The main risk factor is contact with infected poultry, which is more common in Asia and countries, where live bird markets are traditional and common [3]. People can become infected by inhalation or direct contact with saliva, mucus or faeces of sick birds [4]. The infection can also be transmitted with undercooked meat or eggs of infected birds [5]. Only one case of human-to-human transmission of the virus has been recorded, however, given the high rate of mutation of the virus, such a possibility cannot be ruled out in the future [6].

The avian influenza virus, like all influenza viruses, has a large antigenic variability — a change in surface proteins (antigens) during mutations. As a result, a new variant of the virus appears that can

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cause a pandemic. So, in 1918, the strain of the influenza A virus ("Spanish flu", H1N1) caused a pandemic that killed more than 50 million people. It is believed that the Spanish flu virus arose as a result of mixing the genes of human and avian influenza viruses [7]. The causative agents of avian influenza have particular research interest, since some parallels can be drawn between novel viruses and the Spanish flu virus (subtype H1N1), which caused the deaths of up to 40 million people during the 1918-1919 pandemic. Like the Spanish flu virus, the recently reported influenza A virus of the H5N1 subtype can be transmitted directly from infected birds to humans, has an unusually high pathogenicity and the ability to cause generalized infection in the absence of previously formed specific immunity in the human population [7, 8].

The avian influenza virus is quite resistant to environmental factors and persists outside the body for a long time, especially at low temperatures, so it can easily spread [9]. It can last for about 26 hours on plastic surfaces, and up to 4.5 hours on skin [10].

The recent experience of the outbreak of severe acute respiratory syndrome (SARS) serves as an instructive example in preparing for a potential influenza pandemic. Since the SARS coronavirus does not spread as quickly as influenza viruses, scientists are not sure whether the actions that led to the containment of SARS would have been as successful if the avian influenza virus had acquired the ability to effectively transmit from person to person [11].

It is not possible to determine with high accuracy when and where the next flu pandemic will occur, and whether it will be caused by a novel H1N1, current H5N1 or any unrelated virus. However, numerous researchers are sure that a new flu pandemic will eventually occur [5].

Along with virological studies that make it possible to identify the subtype of the circulating virus and its molecular genetic properties, it is necessary to study the features of pathological processes that occur in the body of animals as a result of exposure to a new viral agent. Due to the real danger of the spread of avian influenza virus in the human population, there is a need to study in depth the mechanisms of pathogenesis and morphogenesis of an infectious disease. To do this, it is necessary to investigate the features of the virus-cell relationship at both the light-optical and molecular levels. Of all infectious diseases, the most frequent observations of a "cytokine storm" occur in influenza. Thus, a "cytokine storm" often develops in patients with bird flu and there is a direct link between the severe and sometimes fatal course of bird flu and hypercytokinemia

The aim of the research was to study the pathogenesis of influenza infection, in particular, to establish the role of proinflammatory cytokines in the pathogenesis of the infectious process caused by influenza virus subtype A/WSN/1/33(H1N1) in mice.

2. Material and Methods

2.1. Material

The study was conducted on 21 male mice aged 4-6 weeks4of the BALB/c line weighing 16-18 g. The animals were divided into the following groups: a control group (C, 6 animals), which were orally administered isotonic sodium chloride solution (IR); a group infected with influenza A/WSN/1/33(H1N1) virus (V, 15 animals). The animals were infected intranasally with influenza A/WSN/1/33(H1N1) virus under ether anesthesia by injecting 50 μl of allantoic fluid containing 10 LD 50 of the virus [12, 13]. The initial version of the influenza virus (IV) was obtained from the Institute of Virology named after. D. I. Ivanovsky (Moscow, Russia). The animals were housed in standard animal house conditions in accordance with the norms and rules for the treatment of laboratory animals in accordance with the "Rules for carrying out work using experimental animals".

In order to determine the dynamics of the accumulation of influenza virus in the lung tissue of animals, some of the mice from the experimental groups were removed from the experiment and sacrificed under ether anesthesia on 1, 2, 3, 4, 5, 6 days after inoculation. The lungs of these mice were homogenized in a tenfold volume of a sterile phosphate-salt buffer containing 400 mcg/ml of gentamicin, and a series of tenfold dilutions were prepared from the homogenates on the same buffer. The tissue homogenate was incubated in a thermostat for 1 hour at 37 ^{0} C, then the liquid part was

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separated from the tissue sediment by centrifugation at 3000 rpm for 15 minutes and 10-fold dilutions of the supernatant were prepared, which were used to infect 10-day-old chicken embryos in order to detect the titer of the virus. The embryos were cultured in a thermostat at 37 ^{0} C for 48 hours, after which they were cooled and opened for the selection of allantoic fluid. The level of reproduction of the virus was assessed according to the hemagglutination reaction (HAR) of erythrocytes.

2.2. Histological Methods

The object of histological studies were samples of lung and liver tissues of experimental animals. For light-optical examination, tissue samples were taken from the right (middle and lower lobes) lung and the left lateral lobe of the liver.

The histological material was fixed in a 10% formalin solution, then dehydrated in a series of alcohols of increasing concentration (70°, 80°, 90°, 96°, absolute alcohol), butanol, xylene and were enclosed in a paraffin filling mixture HISTAMIX (Russia). Sections with a thickness of 2-5 microns were prepared on a rotary microtome HM 34036E (Carl Zeiss, Germany). Paraffin sections were stained with hematoxylin and eosin to perform a descriptive analysis of morphological transformations followed by morphometric assessment using the Aperio Image Scope program (Leica Biosystems, USA). Measurements were made at 200 magnification in 20 fields of view on an image area of 1 mm^{2}.

To analyze the data on the severity of the pathological process and the activity of proinflammatory cytokines, which is very important for assessing the specific immune response, specific monoclonal antibodies to IL-6, TNF-a (Novocastra) were used. To identify the corresponding antigens, an immunohistochemical (IHC) study was performed, including deparaffinization, rehydration, retrieval of paraffin sections, followed by blocking of endogenous peroxidase, as well as incubation with blocking serum. The incubation time was 1 hour at room temperature, after which paraffin sections with streptavidin peroxidase complex, DAB substrate were incubated, additionally staining the preparations with hematoxylin. Sample preparation was performed using a 700W microwave18 oven. The NovoLink detection system (Novocastra) was used for visualization.

Table 1 shows the gradation of the semi-quantitative IHC method used to determine the intensity of antigen-stimulated prod4uction of cytokines stimulating th4e inflammatory response (IL-6, TNF-a) in the tissues of the experimental group of animals.

Table 1.
Evaluation of the results of semi-quantitative immunohistochemical studies.

Designation	Number of positive structures observed in the visual fields
(- / 0)	No
(+/ 1)	Few
(++/ 2)	Moderate
(+++/ 3)	Many

2.3. Statistical Methods

Statistical processing of the obtained quantitative results was performed using Microsoft Excel and specialized software Statistica 10 (StatSoft Inc., USA). The statistical analysis included the construction of variation series of quantitative data, the determination of the normality of their distribution using the Kolmogorov-Smirnov criterion and the degree of uniformity of the variances of the compared samples, as well as the calculation of descriptive statistical parameters, including the mean (M) and the error of the mean (m). Due to the relatively small sample sizes, the reliability of the differences in the compared values was determined using the nonparametric Mann-Whitney U-test. The results were presented in the form of M±m. The differences between the comp14ared quantitative indicators were considered significant at a significance level of α =5% ($p < 0.05$).

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3. Results and Discussion

The lungs are considered to be the first and main target organ for influenza viruses, including influenza subtype H1N1. It is the main gate through which the virus enters the body and the biotope where its most intensive reproduction occurs, moreover, the functional state of the respiratory system determines the degree of oxygenation of all organs and tissues of the body and, consequently, the overall viability of the body.

The liver is the central organ of detoxification and immunity, also being an important barrier to the spread of viruses in the body which is often underestimated by researchers. This organ is responsible for maintaining chemical homeostasis, participates in the metabolism of many hormones, and plays a major role in the inactivation of various drugs. In addition, the liver, being the most important metabolic center in mammals, determines the level of trophism of the body as a whole and becomes affected during influenza.

During macroscopic examination of the lungs of animals removed from the experiment infected with the influenza A/WSN/1/33(H1N1) virus, pulmonary edema and spot hemorrhages were noted from the first day. Whereas liver enlargement, as a characteristic sign of destructive changes in the macroscopic assessment of the effect of the virus on the main organ of detoxification, was observed on the 3rd day of infection.

However, dystrophic changes on the part of hepatocytes, represented by dystrophy (*Fig. 1A*) and necrosis zones (*Fig. 1B*) during histological examination were detected in micro-preparations already from the first day after infection.

Figure 1. Sections of the liver of the BALB/c mice inoculated with the A/WSN/1/33(H1N1) virus. Hematoxylin and eosin staining.

A – dystrophic changes in hepatocytes are represented by the transition of hydropic dystrophy to the cell ballooning due to massive oedema, x 400;

B – centrolobular necrosis of hepatocytes, x400.

Histological examination of samples of affected lung areas obtained from infected animals revealed foci of interstitial and alveolar pulmonary edema against the background of pronounced hemodynamic disorders (*Fig. 2*A). Under the influence of the virus blood circulation is most often disrupted, which is accompanied by an increase in the permeability of the walls of blood vessels, which leads to moderate edema, combined with hemorrhages. The affected areas of the lung were characterized by accumulations of intraalveolar exudate associated with full blood vessels of the microcirculatory bed with the phenomena of stasis and microthrombosis. The respiratory epithelium of the bronchi was characterized by degenerative changes, the phenomena of hyperplasia and hypertrophy of cells were noted infection.

Figure 2. Sections of the lungs of the BALB/c mice inoculated with the A/WSN/1/33(H1N1) virus. Hematoxylin and eosin staining.

A – destruction and phenomena of serous hemorrhagic inflammation of the respiratory parts of the lung with foci of alveolar edema, ×200;

B – destructive changes in the form of hypertrophy and hyperplasia of the bronchial epithelium with accumulation of a significant amount of exudate in the lumen, × 400.

In this research the role of proinflammatory cytokines involved in the pathogenesis of the infectious process caused by the influenza virus subtype H1N1, such as TNF-a and IL-6, was studied. The high level of these cytokines in the samples reflects the activity and severity of the inflammatory process. In order to prove this fact the dynamics of the cytokine profile in the lungs of infected animals was evaluated on days 1, 2, 3, 4, 5, 6 of the lethal influenza infection by immunohistochemical staining of lung and liver tissue samples.

The results of the expression of proinflammatory cytokines in the lungs and liver of mice infected with influenza A/WSN/1/33(H1N1) virus are shown in *Table 2*.

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Table 2.
The results of the dynamics of the study of TNF-a and IL-6 in the lungs and liver of mice infected with influenza A/WSN/1/33(H1N1) virus.

Proinflammatory cytokines	Day after viral inoculation	Animal groups	
		H1N1 n=15	Healthy control n=6
Expression of TNF-α lung /Liver	1	++ / -	-
	2	+++ / +	-
	3	+++ / ++	-
	4	+++ / ++	-
	5	+++ / ++	-
	6	++ / +	-
Expression of IL-6 lung /Liver	1	- / -	-
	2	+ / +	-
	3	+++ / +++	-
	4	+++ / ++	-
	5	+++ / ++	-
	6	+++ / +	-

On the first day after infection of mice in samples infected with influenza virus type A subtype H1N1, a positive reaction with TNF-α was detected in lung and liver tissues. As early as 1 day after infection an increase in the level of this cytokine in alveolar cells was already observed (*Fig. 3A*), and expression of this cytokine in hepatocytes was detected on day 3 (Figure 4A).

This is considered to be typical for the acute phase of inflammation due to the replication of the virus in the lung and liver cells of experimental animals, whereas increased expression of IL-6 in samples of analyzed organs was observed mainly since the 3 day after infection (*Fig. 3B*, *Fig. 4B*). Starting from day 3 and on the subsequent 4th and 5th days of experimental influenza infection, alveolar macrophages and liver macrophages, Kupffer cells, acquired strong positive staining, which indicated an increase in the expression of the profile of the studied cytokines in immunocompetent cells. And by day 6 a significant increase in the studied cytokines in organ tissues was no longer observed (see *Table 2*). The obtained results indicate inadequate hyperstimulation of monocytic cells during the disease process with massive production of predominantly proinflammatory cytokines.

Figure 3. Sections of the lungs of experimental mice infected with influenza A/WSN/1/33(H1N1) virus. Immunohistochemical examination.
A – Expression of TNF-a in alveolocytes, day 1 after infection, x400;
B – Expression of IL-6 in alveolocytes, day 3 of the experiment, x400.

Figure 4. Sections of the liver of experimental mice infected with influenza A/WSN/1/33(H1N1) virus. Immunohistochemical examination.
A – Expression of TNF-a in hepatocytes, day 3 after infection, ×400;
B – Expression of IL-6 in hepatocytes, day 3 after infection, ×400.

The analysis of structural changes in two target organs during severe viral infection makes it possible to identify the ontogenetic features of the virus associated with the methods of its penetration into cells, reproduction and circulation in the animal's body, and, consequently, the causes of pathogenetic events leading to severe consequences for the virus-affected organism. The application of microscopic methods is a prerequisite for understanding the basic mechanisms of progress and the most important manifestations of the developing influenza infection.

The mechanism of action of elevated levels of TNF-α and IL-6 in severe cases of influenza is currently explained by the triggering of trypsin/MMP-9 cycle (MMP-9 – matrix metalloproteinases 9) in the cells of many organs and tissues, especially in endothelial cells, which leads to the destruction of the matrix around microvessels, an increase in vascular permeability with suppression of adenosine triphosphate production in cells, which causes an "energy crisis of cells" [13]. Other researchers

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associate the mechanism of the pathogenic action of an increased level of proinflammatory cytokines in severe cases of both influenza and other acute respiratory viral infections with a sharp change in the lipid and carbohydrate metabolism of cells, as well as with the effect on receptors that activate cell proliferation under the influence of peroxisomes (PPAR) [14].

To date, it remains unclear which of the studied cytokines play a leading, cardinal role in damage to most organs and systems. At the same time, the discovery of the main "culprits" of the syndrome would necessarily lead to the potential possibility of blocking their production by immunocompetent cells or to the possibility of neutralizing them in the blood even before binding to specific receptors of target organs. It is still necessary to investigate flu models on experimental animals (mammals), study the structure of virions and the features of their formation in mammalian cells in order to find new preventive measures aimed at reducing the risk of influenza in humans and its severe complications to develop new approaches in the fight against viral infections [15, 16].

It seems very difficult to predict or prevent natural phenomena in time that could contribute to the effective transmission of the pandemic bird or other animal influenza viruses among humans. However, virologists must be ready to respond quickly and decisively if such an event occurs [17, 18]. Careful surveillance of new viruses in both human and animal populations using appropriate molecular diagnostics is crucial to contain a potential influenza pandemic; viruses must be monitored for changes that may signal increased virulence or the possibility of their transmission from animals to humans. Equally important are immunological studies for the development and production of effective pharmaceutical countermeasures, such as vaccines and antiviral drugs.

4. Conclusion

A(H1N1) viruses are increasingly being used to study the effect of the virus on the host body and the subsequent sea20rch for new effective antiviral drugs for the treatment and prevention of influenza infection. The potential protective effect of the studied substances is assessed by the final s4urvival rate, average life expectancy, the onset of death and weight loss of animals, as well as according to pathohistological research. The pathogenesis of infection provoked by the highly pathogenic for mice influenza virus A/WSN/1/33(H1N1) causes the spread of dystrophic and necrotic processes in the lungs and liver. The violation of the circulatory system is provoked by the active reproduction of the virus and toxic manifestations on the part of cellular immunity.

Our results have revealed that destructive changes resulted in the pronounced structural change in the pulmonary tissues massively affected by the H1N1 influenza virus. Initially, under the influence of the virus, circulatory disorders occur in the tissues of the mentioned organs, manifested primarily by thrombosis and increased permeability of the walls of blood vessels. As a result, moderate edema develops, combined with hemorrhages and the formation of hyaline membranes. As a result, there is a progressive depletion of oxygenation and energy supply, so the destruction of tissue structures develops which in turn leads to organ dysfunction and the development of acute respiratory distress syndrome. Histological sections have shown that the morphogenesis of changes in the liver in viral infection caused by the influenza virus is dominated by destructive changes with inhibition of the cellular immune response. The production of proinflammatory cytokines, as a response to the penetration of the virus, leads to impaired blood circulation, followed by the deve5lopment of structural changes in the organs of mice, revealed during morphological studies. Structural changes in the lung16 and liver tissues of mice experimentally infected with the influenza A/WS4N/1/33(H1N1) virus revealed the presence of a large amount of the virus at all stages of the experiment.

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