VIROLOGY

Studying the Pathogenicity of Avian Influenza Virus Subtype H5N1 Strains from the Russian Federation Using Mouse Model

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 146, No. 9, pp. 317-319, September, 2008
Original article submitted July 28, 2008

The pathogenicity of strains of avian influenza virus subtype H5N1 from the Russian Federation (A/turkey/Suzdalka/12/05, A/goose/Krasnoozerskoye/627/05, A/duck/Tuva/01/06, A/chicken/Reshoty/02/06, and A/chicken/Krasnodar/123/060) was studied in mice. Morphological and immunological characteristics of experimental viral infection with avian influenza virus subtype H5N1 were evaluated.

Key Words: *H5N1 influenza viruses; pathogenicity; cytokines; immunomorphology*

The appearance of highly pathogenic virus subtypes in the population of domestic poultry and increase in the incidence of direct transmission of avian influenza viruses to humans contribute to high risk of new pandemics [10]. Influenza A virus subtype H5N1 is of particular significance in this respect. Epidemiological and epizootic studies show that influenza A virus subtype H5N1 is the most probable cause of a future influenza pandemic. The concept of limited range of host organisms for avian influenza viruses lacks support from observations of infection with A/H9N2 (Hong Kong) [7] and A/H7N7 (Netherlands, 2003) [3].

Study of circulating H5N1 strains revealed continuing evolution of this virus and increase in species range of host organisms. These data indicate that influenza A virus subtype H5N1 is the most probable cause of future influenza pandemic. Subtype H5N1 virus caused high mortality in domestic birds in Suzdalka (Novosibirsk region) in 2005. The viruses spread rapidly over Western Siberia, central regions, and southwest area in Russia [1]. During this period, highly pathogenic avian influenza virus subtype H5N1 was first revealed in Russia. Therefore, it was important to evaluate phenotypic characteristics of the virus (pathogenicity for birds and mammals, phylogenetic position, and serological properties). Mammalian models for avian influenza subtype H5N1 were developed to study the pathogenetic mechanisms and to develop new vaccines and medicinal drugs.

Here we studied the pathogenicity of avian influenza H5N1 strains from Russia in laboratory mice.

MATERIALS AND METHODS

Experiments were performed with 5 strains of avian influenza virus subtype H5N1 (A/turkey/Suzdalka/

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0007-4888/08/1463-0341

12/05, A/goose/Krasnoozerskoye/627/05, A/duck/Tuva/01/06, A/chicken/Reshoty/02/06, and A/chicken/Krasnodar/123/06). These strains were isolated during an outbreak of avian influenza in the following regions of Russia (2005-2006): Suzdalka village (Novosibirsk oblast, June 2005); Krasnoozerskoye village (Novosibirsk oblast, September 2005); Reshoty village (Dovolensk region of the Novosibirsk oblast, July 2006); and Krasnyi Komissar village (Krasnodar Krai, 2006). The isolates were also obtained from the lungs of *Podiceps cristatus* who died during an outbreak of influenza in June 2006 (Ubs-Nuur Lake, Republic Tyva). Virological, serological, and biomolecular studies were approved by the World Health Organization [8]. Experiments were conducted on outbred albino mice aging 6-8 weeks and obtained from the nursery of laboratory animals (State Research Center of Virology and Biotechnology "Vector"). The animals were infected intranasally.

For immunohistochemical study of proinflammatory and antiinflammatory cytokines, paraffin sections were treated as follows: deparaffinization, rehydration, unmasking of antigens in a microwave oven (700 W), blockade of endogenous peroxidase, incubation with blocking serum, and incubation in the presence of specific monoclonal antibodies against interleukin-2 (IL-2), IL-6, and tumor necrosis factor-α (TNF-α) (Novocastra). Incubation was performed at room temperature for 1 h. Further incubation was conducted with streptavidin-peroxidase complex (diaminobenzidine substrate). The samples were stained with hematoxylin. A NovoLink detection system (Novocastra) was used for visualization.

RESULTS

Biomolecular analysis of all strains showed that the amino acid sequence of hemagglutinin contains a typical marker of high pathogenicity (PQGRRK KKR↓GL) in the proteolytic cleavage site [6,8]. Table 1 illustrates the major virological characteristics of experimental infection with test strains of avian influenza virus subtype H5N1 (Table 1).

The viral titers were measured in brain tissue, spleen, kidneys, liver, and lungs of dead mice (Table 2).

Only strain A/goose/Krasnoozerskoye/627/05 was efficiently replicated in the spleen, liver, and kidneys. The kidneys included considerable amounts of this strain, which is untypical of mouse model for avian influenza virus subtype H5N1. By contrast, replication in the brain was characteristic of all viruses. The brain of dead mice was characterized by high titer of the A/turkey/Suzdalka/12/05 isolate. This isolate is classified as low pathogenic for mice. Strains A/chicken/Krasnodar/123/060 and A/chicken/Reshoty/02/06 exhibited high neurotropism. All viruses were efficiently replicated in the lungs (high viral titers). Viral titers in the lungs after infection with A/chicken/Reshoty/02/06 and A/goose/Krasnoozerskoye/627/05 were much higher compared to other highly pathogenic and low pathogenic viruses. These values were high and statistically indistinguishable for A/chicken/Krasnodar/123/060, A/duck/Tuva/01/06, and A/turkey/Suzdalka/12/05 viruses (Table 2).

Our results indicate that these viruses are characterized by different pathogenicity for mice. The pathogenicity depends on viral properties, which determines neurotropism and replication in the lungs and other internal organs [2,4]. The A/goose/Krasnoozerskoye/627/05 isolate was used to study the pathogenesis of influenza infection. As differentiated from other viruses, this isolate was efficiently replicated in all organs (lungs, spleen, liver, and kidneys).

Previous studies showed that the major pathogenetic stage of avian influenza H5N1 infection is related to not direct effect of this agent on cells, but systemic immune response associated with blockade of the interferon reaction and dysregulation of proinflammatory and antiinflammatory cytokine

TABLE 1. Main Virological Characteristics of Infection with Strains of Influenza A Virus Subtype H5N1

Strain	$EID_{50/ml}/ELD_{50/ml}$	MID_{50}	MLD_{50}
A/goose/Krasnoozerskoye/627/05	9.2±0.1	2.2±0.1	2.3±0.2
A/turkey/Suzdalka/12/05	9.3±0.2	5.3±0.4	6.3±0.2
A/duck/Tuva/01/06	9.2±0.4	1.4±0.3	1.4±0.2
A/chicken/Krasnodar/123/06	8.4±0.3	2.1±0.4	2.3±0.4
A/chicken/Reshoty/02/06	8.6±0.3	1.2±0.3	1.3±0.2

Note. EID, embryonic infection dose; ELD, embryonic lethal dose; MID, mouse infection dose; MLD, mouse lethal dose. Data are expressed as Lg $EID_{50/ml}$. MID_{50} and MLD_{50} were determined in 3 independent experiments.

TABLE 2. Viral Titers in Organs of Outbred Albino Mice during Experimental Infection with Strains of Influenza A Virus Subtype H5N1

Strain	Organ				
	lungs	spleen	brain	liver	kidney
A/goose/Krasnoozerskoye/627/05	6.1±0.3	1.6±0.5	5.2±0.2	1.6±0.3	2.6±0.2
A/turkey/Suzdalka/12/05	4.1±0.5	<1	2.3±0.5	<1	<1
A/duck/Tuva/01/06	5.3±0.4	<1	3.4±0.3	<1	<1
A/chicken/Krasnodar/123/06	4.7±0.6	<1	3.1±0.7	<1	<1
A/chicken/Reshoty/02/06	6.7±0.4	<1	4.2±0.5	<1	<1

Note. Data are expressed as lg $EID_{50/ml}$.

production by mononuclear phagocyte system, NK cells, and activated infected macrophages [9].

Immunohistochemical study of the cytokine profile was performed with A/goose/Krasnoozerskoye/627/05-infected mouse cells. A positive reaction for TNF-α was revealed in samples of the lungs, liver, brain, kidneys, and spleen on day 1 after infection. The expression was most significant in the lungs and liver. It should be emphasized that cytokine expression was detected in alveolar cells and hepatocytes. Our findings illustrate viral replication in these cells and induction of the acute inflammatory phase. The expression of proinflammatory cytokines IL-2, IL-6, and TNF-α increased on day 3 due to positive staining of immunocompetent cells and, particularly, of macrophage cells (Kupffer cells, alveolar macrophages, and glial cells of the brain). Cytokine content in these tissues did not increase on days 6-10.

Our results indicate that high pathogenicity of strain A/goose/Krasnoozerskoye/627/05 with 85% mortality is related to long-term overproduction of proinflammatory cytokines in response to infection. This process is followed by circulatory disorders, hypercoagulation, and increase in cytotoxic activity of the mononuclear phagocyte system, which results in the development of irreversible changes in organs and death of animals.

This work was supported by the Federal Target Program "Research and development in the priority areas of scientific and technological complex of Russia for 2007-2012" (State Contract No. 02.512. 11.2193), Bio Industry Initiative, and International Scientific and Technical Center (grant No. 3436).

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