Pathogenesis of Infectious Disease of Mice Caused by H5N1 Avian Influenza Virus

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The pathogenesis of a disease caused by Qinghai-like H5N1 influenza virus in BALB/c mice was studied. Clinical, morphological, and immunological characteristics of the experimental infection caused by highly pathogenic A/duck/Tuva/01/06/ (H5N1) virus are described.

Key Words: *H5N1 infection; avian influenza; immunopathogenesis; cytokines*

In recent years, H5N1 subtype of avian influenza viruses (AIV) became a real threat for humans. Since 1997, when the first lethal case was recorded in a human who died from an AIV-induced disease, a total of 320 humans were infected and 193 died from it by August 14, 2007 [8]. Combination of such characteristics as adaptation to wild migrating birds and possible high pathogenicity for mammals makes the Qinghai-like viruses, first described in 2005 [1], a potentially pandemic agent. H5N1 avian influenza in humans can be characterized as primary viral pneumonia leading to the development of the respiratory distress syndrome, infectious toxic shock, and multiorgan failure. Systemic lesions are presumably caused by destruction of the target cells as a result of viremia and hypercytokinemia [3]. Several molecular markers can be used for selection of isolated strains highly pathogenic for mammals. One of these markers is the presence of six amino acids with positively charged polar radicals at the hemagglutinin proteolytic cleavage site. One more marker of potentially high pathogenicity of these viruses is lysine in position 627 in polymerase subunit PB2 [5].

We studied the pathogenesis of a disease caused by a Qinghai-like influenza A virus (subtype H5N1) in BALB/c mice.

MATERIALS AND METHODS

The study was carried out with AIV strain A/duck/Tuva/01/06 isolated in 2006 from great crested grebe *Podiceps cristatus* dead in epizooty on Uvs-Nuur Lake in the Republic of Tyva. Virological and serological methods recommended by WHO [7] were used in the study. Molecular biological studies were carried out using the methods described previously [2]. All manipulations with mice were carried out in accordance with Regulations for Handling Experimental Animals (Supplement to Order of the Ministry of Health of the USSR of August 12, 1977, No. 755).

RESULTS

Analysis of full-length nucleotide sequences of hemagglutinin, neuraminidase, and PB2 genes was carried out for molecular biological characterization of A/duck/Tuva/01/06 (H5N1) strain. We found that hemagglutinin contained a characteristic marker of

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TABLE 1. Results of Hemagglutination Inhibition Test

Strain	Polyclonal serum					
	Ck/Hidalgo/95	Gs/HK/99	HK/156/97	HK/213/03	VN/1203/04	Prachinbrr/6231/04
Tk/Suzdalka/1-12/05	80	160	10	80	80	20
Ck/Suzdalka/2-6/05	160	320	<10	80	40	10
Gs/Suzdalka/6-10/05	160	320	10	80	80	20
Ck/Omsk/108-14/05	80	160	10	160	80	20
Gray dk/Omsk/105-16/05 (wild)	160	160	10	160	80	20
mallrd/Dovolnoye/5-26/05 (wild)	10	10	10	640	320	10
A/duck/Tuva/01/06	320	640	40	160	160	40
A/chicken/krasnodar/06/06	320	1280	40	320	320	40
A/Chicken/Reshoty/2/06	160	640	40	320	320	80
Gs/Krasnoozerskoye/627/05	160	160	<10	80	80	10

Note. Antigens of H5N1 AIV isolated in Russia in 2005-2006 were used.

high pathogenic activity (PQGRRKKKR!GL) in the proteolytic cleavage site. According to previous findings [4,6], the receptor-binding domain of this strain is characteristic of influenza A viruses isolated from birds. Lysine was detected in PB2 gene position 627. All these facts attest to high pathogeneity of A/duck/Tuva/01/06 strain for mammals [5].

Analysis of M2 protein amino acid sequence revealed no mutations characteristic of remantadine-resistant viruses (in other words, the virus is sensitive to remantadine). Comparison of neuraminidase amino acid sequence of the reference oseltamivir-resistant A/Wuhan/359/95 (H3N2) strain and analogous sequence of this gene for A/duck/Tuva/01/06 (H5N1) revealed no substitutions characteristic of oseltamivir-resistant strains in A/duck/Tuva/01/06, which means that the strain is potentially sensitive to oseltamivir. Strain A/duck/Tuva/01/06 is characterized by wide cross-reactivity with

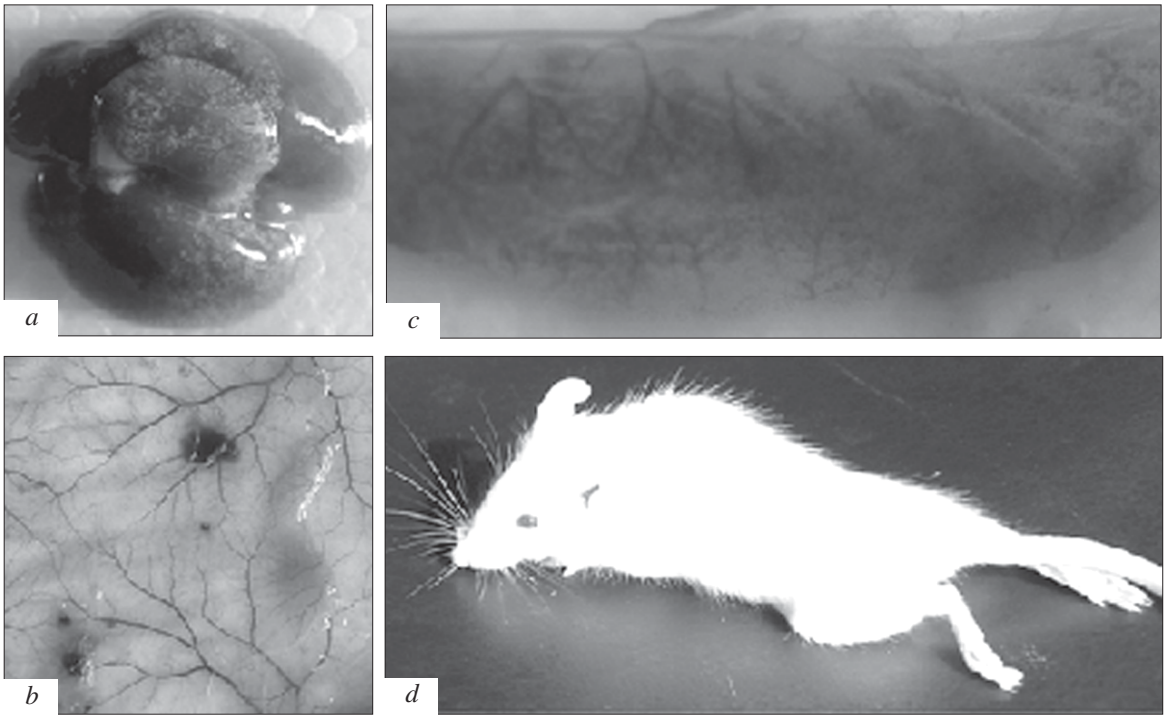

Fig. 1. Pathological changes in BALB/c mouse organs on days 8-9 of experimental infection.

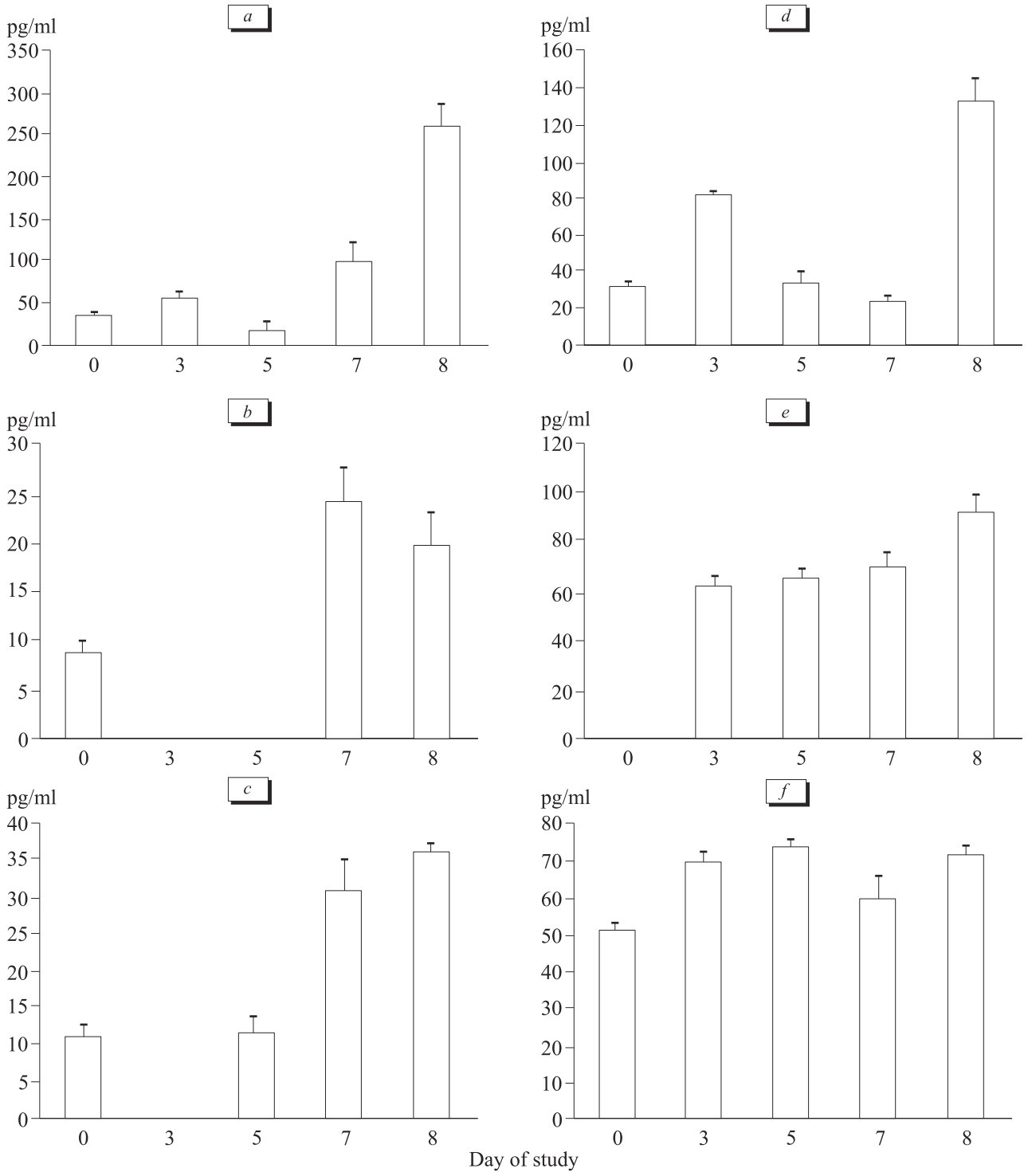

Fig. 2. Changes in total systems production of cytokines in experimental H5N1 influenza infection in BALB/c mice. *a*) IFN-γ; *b*) TNF-α; *c*) IL-1β; *d*) IL-6; *e*) IL-10; *f*) IL-12.

sera to reference AIV H5N1 strains isolated previously in South-East Asia (Table 1).

After infection, the animals developed acute infectious process. The mean life span was 8.19± 0.18 days. Rapid progress of the infectious process was noted from day 7, with development of paralysis and pareses during the terminal stage of the disease (Fig. 1, *d*). On days 8-9, the animals were in agony; autopsy showed hemorrhages in the lungs (Fig. 1, *a*), small intestine, and skin (Fig. 1, *b*, *c*), and significant dissemination of the virus in the viscera (Table 2).

TABLE 2. Characteristics of A/duck/Tuva/01/06 Strain, Virus Titers in Tissues (*M*±*m*)

Virus	Infective 50% dose for embryos, ml^{-1}	Minimum immunizing dose, ml	Median LD_{50}	Virus titer				
				lung	spleen	brain	liver	kidney
A/duck/Tuva/01/06	9.2	1.7±0.4	1.4±0.3	5.3±0.5	<1	3.4±0.3	<1	<1

We studied the involvement of some cytokines in the immunopathogenesis of experimental H5N1 avian influenza in mice. The results of EIA indicated hyperproduction of pro- and antiinflammatory cytokines (Fig. 2). However, the main changes in systemic production of cytokines were observed during the terminal stage.

Analysis of some genes of the A/duck/Tuva/01/06 strain genome showed that it belongs to the phylogenetic group of Qinghai-like viruses (clade 2.2). Its molecular markers (hemagglutinin proteolytic cleavage site, lysine in position 627 in PB2) characterize this virus as highly pathogenic for BALB/c mice. The results of molecular analysis are confirmed by tests for pathogenicity: IVPI=3.0, MLD_{50}=1.4 EID_{50}. Moreover, analysis of its genome structure showed potential sensitivity to the adamantane drugs and to oseltamivir (neuraminidase inhibitor). Serological analysis showed high cross-reactivity with sera to H5N1 avian influenza reference strains isolated previously in South-East Asia. The mean lifespan of mice infected with this strain is 8.19±0.18 days. In addition, we described for the first time the hemorrhagic syndrome in mouse infection by H5N1 AIV. Hypercytokinemia presented by hyperproduction of IFN-γ, IL-6, and IL-1β.

Hence, the results indicate that A/duck/Tuva/01/06 strain can be used as the basic strain for the development of therapeutic methods and evaluation of new vaccines for protection from H5N1 avian influenza A on BALB/c mice as model animals.

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