Pathogenicity assessment of wild-type and mouse-adapted influenza A(H1N1) pdm09 viruses in comparison with highly pathogenic influenza A(H5N1) virus

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Summary. Here we compare the results of pathological and virological examinations of mice experimentally infected with either wild-type or mouse-adapted pandemic A(H1N1) pdm09 viruses and highly pathogenic avian influenza (HPAI) virus A(H5N1). Mice were sacrificed on days 1, 3, 6, and 10 post infection or whenever morbidity was severe enough to justify euthanasia. Morbidity rates were calculated on the basis of clinical signs (weight loss, poor hair coat, hunched posture and paresis); virus-induced disease was characterised by the histopathology of lung; virus dissemination was determined by virus isolation on organ samples of lung, brain, liver, kidney and spleen. All mice infected with mouse-adapted A(H1N1) pdm09 died in the course of the experiment, whereas 20% of animals survived the infection with A(H5N1). Echinocyte formation changed the rheological properties of blood in animals infected with either mouse-adapted A(H1N1) pdm09 or A(H5N1). To sum up, the adaptation of pandemic A(H1N1) pdm09 virus can confer an enhanced virulence similar to or even exceeding that of HPAI A(H5N1) virus.

Key words A(H1N1) pdm09, A(H5N1), Mouse-adaptation, Virulence, Echinocyte

Introduction

According to the World Health Organization, influenza viruses infect 5-15% of global population, cause 3-5 million cases of severe illness and about 250,000 to 500,000 reported deaths annually. Highly pathogenic avian influenza (HPAI) A(H5N1) and pandemic influenza A(H1N1) pdm09 are newly emerging viruses that recently posed a significant threat to public health in many countries. The emergence of influenza A(H1N1) pdm09 virus in 2009 caused the first pandemic in the 21st century, which quickly spread across the globe to more than 214 countries, causing 151,700 - 575,400 deaths worldwide (WHO, 2010; Dawood et al., 2012). The pandemic influenza A(H1N1) pdm09 virus had a high rate of transmission, but its virulence and lethality were relatively low compared with those of the HPAI A(H5N1) virus. The mortality from the HPAI A(H5N1) virus is more than 60%, and that virus can seriously damage the human respiratory system, but cannot be effectively transmitted from person to person (Korteweg and Gu, 2010).

The HPAI A(H5N1) virus preferentially binds to $SA\alpha2,3Gal$ receptors (avian type) and the pandemic influenza A(H1N1) pdm09 virus preferentially recognizes $SA\alpha2,6Gal$ receptors (human-type) (Rogers et al., 1983; Paulson, 1985; Ito et al., 1998; Connor et al., 1994; Shinya et al., 2006; van Riel et al., 2006). Both types of receptors are present in human lung. The $SA\alpha2,6Gal$ receptors are predominantly expressed on the surface of ciliated cells of the mucous membranes of nose, sinuses, pharynx, trachea, extrapulmonary and

pulmonary bronchi, terminal bronchioles and the surface of brush cells in the small and terminal bronchioles (Lu et al., 1999; Kuiken and Taubenberger, 2008). According to recent reports, the SAα2,3Gal receptors are present in lower respiratory tract of the lung, where they predominate in type II pneumocytes but are rarely detected on the surface of brush cells (Shinya et al., 2006; van Riel et al., 2006; Korteweg and Gu, 2010).

High viremia leads to the spread of virions by bloodstream with subsequent infection of non-respiratory organs (brain, spleen, thymus, intestines, heart, liver, kidneys, adrenal glands, etc.) in the case of severe hypertoxic form of disease caused by influenza A(H5N1) virus (Lu et al., 1999; Rimmelzwaan et al., 2006; Joseph et al., 2007; Belser et al., 2009). A(H1N1) pdm09 virus dissemination to non-respiratory organs such as brain, spleen, and blood is possible only under the influence of mouse-adapted variant of virus (Ilyushina et al., 2010).

The emergence of new influenza viruses will continue to present challenges for public health and research. A detailed understanding of the mechanisms underlying the pathogenicity and interspecies transmission of influenza viruses is important both for predicting new virus emergence and for the development of effective prevention and treatment means and medical interventions essential to control the influenza infections.

The aim of the study was to compare the pathogenicity the nature of pathological and virological changes in inner organs and the mortality in acute phase of BALB/c mice experimental infection for wild-type pandemic influenza A(H1N1) pdm09 *A/Russia/01/2009* virus, the mouse-adapted pandemic influenza A(H1N1) pdm09 *A/Russia/01 MA/2009* virus, and HPAI A(H5N1) (*A/goose/Krasnozerskoye/627/05*) virus.

Materials and methods

Animals

Six-to-eight-week-old male BALB/c mice weighting 20-25 g were obtained from the Federal Budgetary Research Institution State Research Center of Virology and Biotechnology VECTOR (Novosibirsk, Russia). Challenge studies were conducted under Biosafety Level 3 laboratory conditions. All procedures were performed in accordance with a study protocol approved by the VECTOR Bioethical Board. The mice were housed in microisolator cages ventilated under negative pressure with HEPA-filtered air. The light/dark cycle was 12/12 h, and the animals were allowed free access to food and water.

Viruses

Three strains of influenza A viruses were employed in this study: wild-type pandemic influenza A(H1N1)pdm09 *A/Russia/01/2009* (*Rus/09*) (GenBank accession numbers GU211221.1 GU211234.1), mouse adapted pandemic influenza A(H1N1)pdm09 *A/Russia/01 MA/2009* (*MA-Rus/09*) (GenBank accession numbers HQ661364 HQ661371), and HPAI A(H5N1) (*A/goose/Krasnozerskoye/627/05*) (*Krasn/05*) viruses (GenBank accession numbers EF205206.1, EF205199.1, EF205192.1, EF205157.1, EF205178.1, EF205171.1, EF205164.1, and EF205185.1).

The *Rus/09* strain was isolated from pathological material (lung, trachea, and bronchi) obtained from a woman who died during the 2009 pandemic in Russia. A working stock with a titer of 7.25 ± 0.24 lgTCID$_{50}$/ml (tissue culture infectious dose per 1 ml) was obtained after a single passage in Madin-Darby canine kidney (MDCK) cells.

To increase the virulence of the wild-type *Rus/09*, the virus was adapted by passaging in the lungs of BALB/c laboratory mice. When the lethality of the virus reached 100% in the mice (total of 7 passages), the mouse-adapted A(H1N1) pdm09 variant was isolated and named *MA-Rus/09* (Romanovskaya et al., 2010), and the 50% lethal dose (LD$_{50}$) was determined (WHO, 2011). The complete genome sequencing of the wild-type *Rus/09* virus and *MA-Rus/09* virus revealed 7 amino acid substitutions: HA (I183T, R222K), NA (V106I; N248D; R257K), PB1 (V656A), PB2 (V111F).

The highly pathogenic avian influenza strain *Krasn/05* was isolated from sick birds during an epidemic in 2005 in Novosibirsk Region (Russia). The isolated strain was passaged five times in embryonating chicken eggs to obtain a working stock virus with titer of 9.2 lgEID$_{50}$/ml (50% egg infectious dose per 1 ml) (Shestopalov et al., 2008).

Study design

The mice were separated into four groups. The 1st group (n=17) was intranasally (i.n.) infected with 50 μl of physiological saline containing 10 median infectious dose (ID$_{50}$) of the strain *Rus/09* with 10 median infectious dose (ID$_{50}$), because this virus is not lethal for experimental animals. The 2nd (n=34) and 3rd (n=34) groups were i.n. infected with strain *MA-Rus/09* and strain *Krasn/05*, respectively, with each mouse receiving a total volume of 50 μl physiological saline containing 5-10 median lethal dose (LD$_{50}$) of the virus. And the 4th group (n=3) included uninfected controls, used to determine background histopathological lesions in mouse lungs. The survival and changes in body weight of the mice were recorded. Mice demonstrating $\geq$25% loss of body weight were sacrificed and considered dead. To evaluate the replication tropism of the selected viruses in the mice and to conduct the histopathology analysis, mouse organs (lungs, brains, livers, spleens, and kidneys) were collected from each infected group (n=3) according to a pre-designated schedule at 1, 3, 6 and 10 days post infection (d.p.i.). The uninfected control mice were humanely sacrificed on the 10th d.p.i. by cervical dislocation. All procedures were performed

in accordance with the study protocol approved by the Vector Bioethical Board (registration number IRB00001360; FWA00014113).

Virology

Fresh organ fragments from lungs, brains, livers, spleens, and kidneys were ground by mortar and pestle, and 10% w/v homogenates in phosphate-buffered saline were prepared. The homogenates were centrifuged at 3000 rpm in an OPN-3 (Russia) centrifuge. The resulting supernatant was used to prepare ten-fold dilutions in Hank's solution. Strains *Rus/09* and *MA-Rus/09* were titrated in MDCK cells. Flat-bottomed 96-well microtiter plates were seeded with MDCK cell suspension (10^6 cells per plate). Strain *Krasn/05* was titrated according to a conventional protocol using developing chick embryos and subsequent virus confirmation was performed using a hemagglutination assay. An uninfected cell control was present on each plate. The plates were incubated at 37°C in a carbon dioxide incubator and examined for cytopathic effects after 5 days. The 50 percent Embryo Infectious Dose (EID_{50}) values were calculated using Reed and Muench method (Reed and Muench, 1938) and and 50 percent Tissue Culture Infectious Dose ($TCID_{50}$) values were calculated using the Kerber technique with the Ashmarin Vorobyov modification (Ashmarin and Vorobyov, 1962) according to the following formula: $lgTCID50/ml = lgDn-\delta(\Sigma Li -0.5)$ where: Dn, maximum effect of dose; Li, killed-to-Dn-survived animals ratio

Histopathology

After euthanasia, fragments of lungs of each mouse were fixed in 4% neutral-buffered formalin, routinely processed, and embedded in paraffin for histopathologic evaluation. Lung sections (4-5 μm) were cut, placed onto slides, and stained with haematoxylin and eosin (HE) for visualization. The light microscopic examination and microphotography were performed using an Axioskop 40 Carl Zeiss (Oberkochen, Germany).

Results

Clinical signs, pathogenicity, and lethality of the H1N1pdm09 and H5N1 viruses in vivo

In our study the 1st group of BALB/c mice was infected i.n. with 10 ID_{50} of strain *Rus/09*, because this virus is not lethal for experimental animals. Some of the infected mice showed signs of illness, such as ruffed fur and stooped posture from 3 to 6 d.p.i. None of the mice in the 1st group died during the experiment.

In the 2nd group of BALB/c mice, infected i.n. with 5-10 LD_{50} of strain *MA Rus/09*, and in the 3rd group, which were infected i.n. with 5-10 LD_{50} of strain *Krasn/05*, the first signs of illness such as ruffed fur, stooped posture, altered gait, inactivity and conjunctivitis developed at day 2 and 3 p.i. Furthermore, in most cases the body weight of most the mice significantly decreased (Fig. 1). By days 4 6 p.i. most animals in both groups presented more severe clinical signs of respiratory disease, including accelerated abdominal breathing and labored respiration with almost all mice huddling together. The first cases of 10-15% of initial body weight loss were detected in *MA-Rus/09* group by 6 d.p.i., and all of the mice in that group died between days 6 and 11 p.i. (Fig. 2). For comparison, in the 3rd group, the first mortality was detected later, at 9 d.p.i. and 20% of the infected mice survived (Fig. 2).

Viral shedding

To determine the virulence among the three viral strains (*Rus/09, MA Rus/09,* and *Krasn/05*) we assessed their replication in the different organs (lung, brain, liver, spleen, and kidney) of the infected mice at 1, 3, 6 and 10
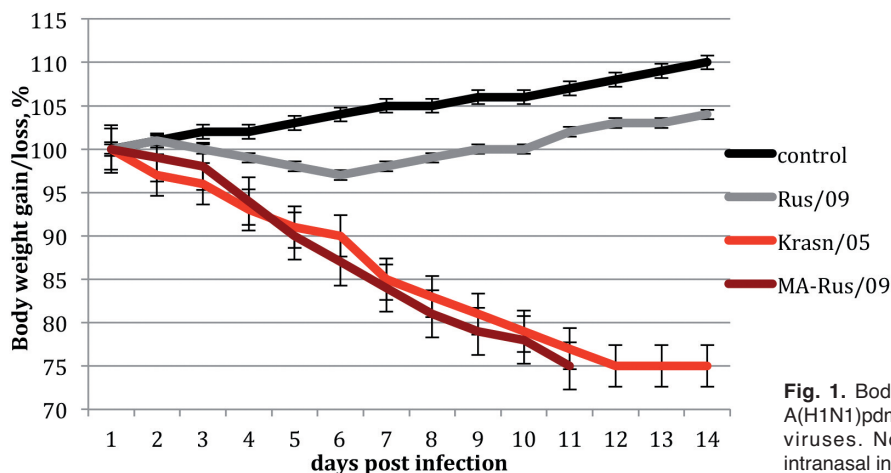
Fig. 1. Body weight of BALB/c mice infected with pandemic A(H1N1)pdm09 and highly pathogenic avian influenza A(H5N1) viruses. Note: Strain on body weight gain or loss after intranasal inoculation of viruses on day 0.

d.p.i. Strain *Rus/09* replicated efficiently in the mouse lungs without prior host adaptation during 6 days p.i. and did not spread to the other organs tested (Table 1). In contrast, strains *MA-Rus/09* and *Krasn/05* had a longer period of replication in lungs and were detected in brain, liver, spleen, and kidney of mice at 6 and 10 d.p.i. (Tables 1, 2).

Histopathology of the lung

The first lesions in mice lungs were detected in all experimental groups on 1st d.p.i., and were accompanied by increased capillary hyperemia and secretion by the mucin-producing cells of bronchial epithelium. The degree of cell secretory activity from the 1st to the 3rd d.p.i. was significantly higher in lung of the mice infected with strains *Rus/09* and *MA-Rus/09* (Fig. 3A,B) than in the lungs of mice infected with HPAI strain

Krasn/05 (Fig. 3C).

Atelectasis, focal vacuolar degeneration and desquamation of the bronchial epithelium in medium and small bronchi were observed from the 3rd to the 6th day in lungs of mice from all experimental groups. Hemolysis in the small vessels of the lungs, alveolar hemorrhages, fibrin masses, and acute hyperemia, were also observed. Due to *MA-Rus/09* and *Krasn/05* virus infection, the initially normal discoid shape of the erythrocyte underwent outward undulation of the membrane to yield echinocytes (Fig. 3F) and the exudation of plasma proteins with formation of blood sludge were observed (Fig. 3E), such a violation of blood rheology was not observed in the mice infected with strain *Rus/09* at any time during the entire period of observation (Fig. 3D).

At 10 d.p.i. the mice infected with strain *MA-Rus/09* or strain *Krasn/05* displayed interstitial pneumonia (Fig. 3H,I), and those infected with strain *Rus/09* displayed bronchitis with localized interstitial inflammation (Fig. 3G).

**Table 1.** Viral titers of Rus/09 and MA-Rus/09 viruses in mouse organs.

A(H1N1)pdm09	Virus titer (lgTCID50/ml, mean ± S.E.)			
	1 d.p.i	3 d.p.i	6 d.p.i	10 d.p.i
MA-Rus/09				
lung	6.11±0.5	5.55±0.2	5.63±0.9	2.38±1.5
brain	-	3.75±0.5	4.25±0.2	4.75±0.6
liver	-	2.52±0.3	2.71±0.5	2.91±0.7
spleen	-	1.22±0.5	1.54±0.5	1.72±0.4
kidney	-	0.74±0.5	1.25±0.2	2.20±0.5
Rus/09				
lung	4.21±0.5	4.31±0.5	3.56±0.1	-

Note: viral titers are expressed in lgTCID50 in 1 ml of studied sample as M±CI95, where M is an arithmetic mean value, CI is a confidence interval.

Table 2. Viral titers of Krasn/05 virus in mouse organs.

A(H5N1)	Virus titer (lgEID50/ml, mean ± S.E.)			
	1 d.p.i	3 d.p.i	6 d.p.i	10 d.p.i
Krasn/05				
lung	2.41±0.4	3.31±0.6	3.38±1.9	3.33±3.1
brain	-	-	1.83±1.7	3.20±3.0
liver	-	1.19±0.9	1.51±1.4	2.21±2.1
spleen	-	-	1.63±1.6	1.63±1.6
kidney	-	0.48±0.4	0.35±0.3	0.75±0.6

Note: EID50 values were calculated by the method of Reed and Muench.
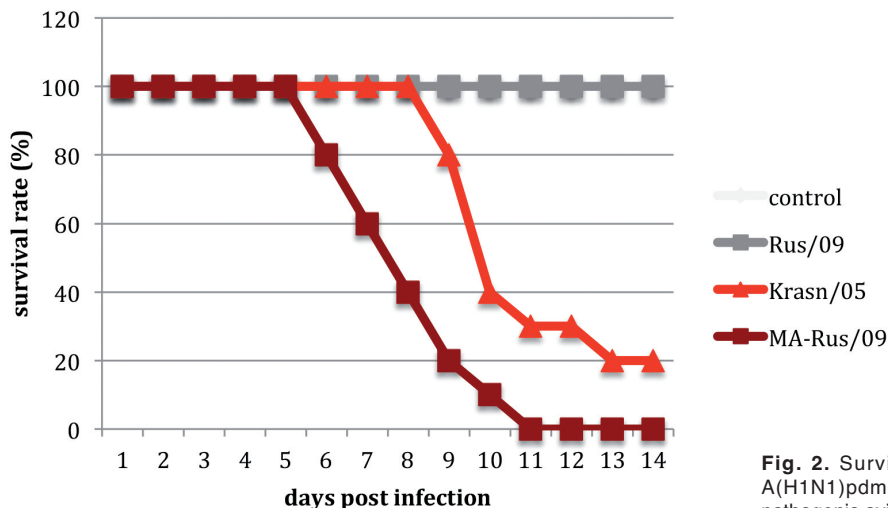
Fig. 2. Survival of BALB/c mice infected with pandemic A(H1N1)pdm09 (wild-type and mouse-adapted) and highly pathogenic avian influenza A(H5N1) viruses.

Discussion

There is a concern that if A(H1N1) pdm09 virus retains its transmissibility and becomes more virulent due to further adaptation to human, it will have a potential to pose a serious public health risk worldwide (WHO, 2010). The objective of the study was to compare disease severity associated with influenza A(H1N1) pdm09 (wild type and mouse-adapted) and HPAI A(H5N1) viruses in a mouse model. It is known that the pandemic influenza A(H1N1) pdm09 virus could not cause lethal disease in mice without prior adaptation (Ilyushina et al., 2010; Prokopyeva et al., 2016). That is why wild-type influenza A(H1N1) pdm09 virus (*Rus/09*) caused relatively mild disease, whereas mouse adapted variant (*MA-Rus/09*) caused fatal outcome in mice similar to those of HPAI A(H5N1) virus (*Krasn/05*).

Probably due to a high titer of *Rus/09* and *MA-Rus/09* viruses in the initial stage of lung inflammation and bronchial epithelial cell-specific tropism of pandemic A(H1N1)pdm09 virus, the secretory activity of bronchial epithelium from the 1st to the 3rd day was different for influenza A(H1N1)pdm09 viruses (*Rus/09* and *MA Rus/09*) and *Krasn/05* virus. Probably the increased secretion by the mucin-producing cells in the case of infection with *Rus/09* and *MA-Rus/09* viruses could be explained by preferentially binding of the
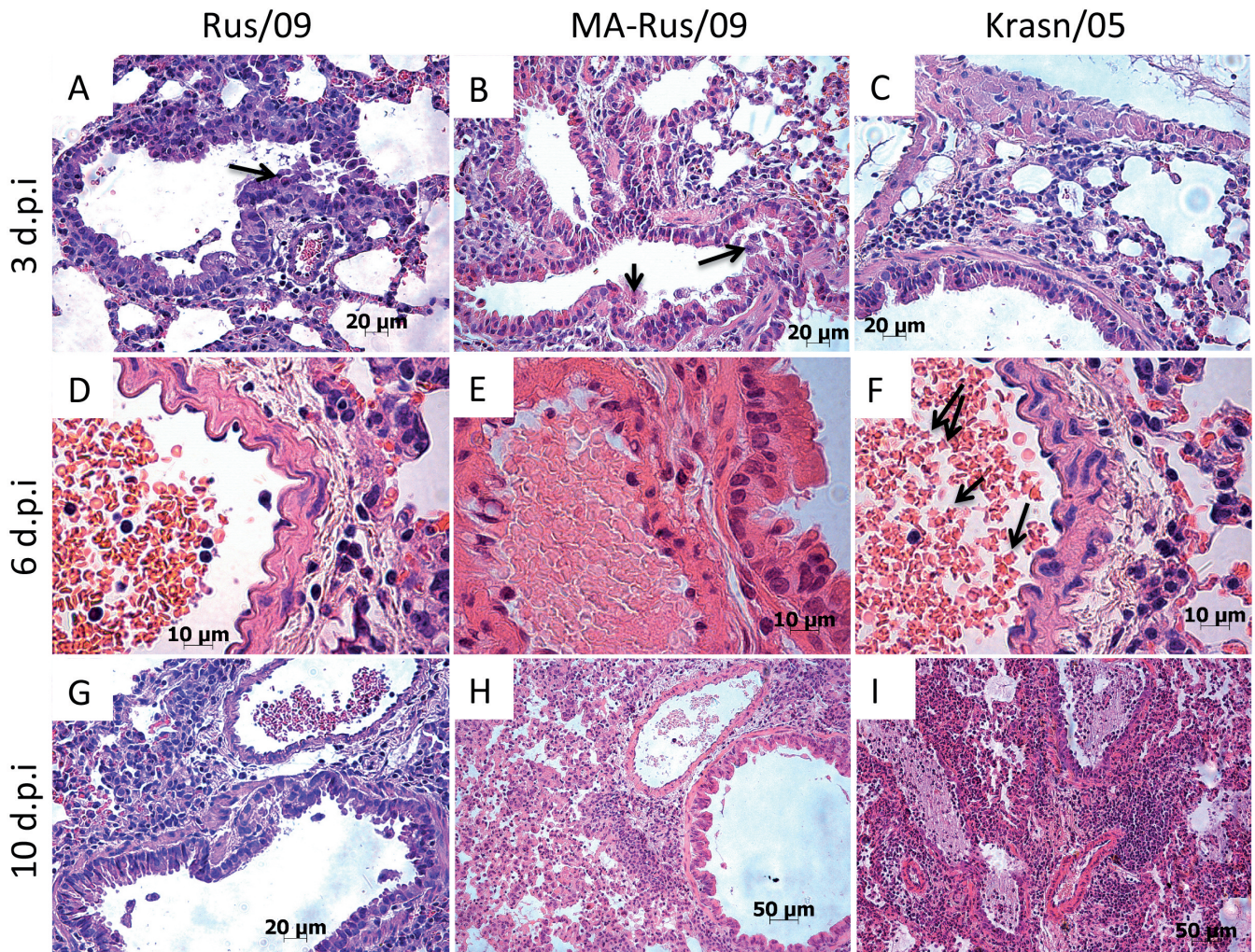
Fig. 3. Pathological changes in lungs of BALB/c mice infected with pandemic A(H1N1)pdm09 (wild-type and mouse-adapted) and highly pathogenic avian influenza A(H5N1) viruses. Note. 3rd day post infection: **A, B, C** - the degree of cell secretory activity by the mucin-producing cells in lung of the mice infected with strains Rus/09 and MA Rus/09 were higher than in the lung of mice infected with strain Krasn/05. 6th day post infection: **D, E, F** - large vessels of lungs of the mice infected with strains Rus/09, MA Rus/09 and Krasn/05. **E** - sludge of erythrocytes in large vessels, exudation of plasma proteins; **F** - echinocytosis. 10th day post infection: **G** - bronchitis in mice infected with strain Rus/09. **H, I** - interstitial pneumonia in mice infected with strais MA-Rus/09 and Krasn/05. HE staining.

pandemic influenza A(H1N1) pdm09 viruses with SAα2,6Gal receptors which are predominantly expressed on the surface of the epithelium of the upper respiratory tract (Shinya et al., 2006; Kuiken and Taubenberger, 2008). The common signs of influenza disease from the 3rd d.p.i to the 6th d.p.i. were acute hyperemia, vascular edema, fibrin masses and hemorrhage. On day 6 p.i. the pulmonary vein microthrombosis due to echinocytosis, pulmonary vascular endothelium damage, and blood sludge caused by influenza virus was detected in groups of *Krasn/05* and the *MA-Rus/09*-infected mice. Possible echino-cytosis and microvascular endothelial cells support productive replication of mouse-adapted pandemic A(H1N1) pdm09 virus and highly pathogenic avian influenza virus A(H5N1) and develop the pathogenesis of virus infections (Shestopalova et al., 2008; Zeng, 2012). It is known that red blood cells (erythrocytes) are involved in the pathological process of inflammatory diseases and undergo major structural and functional changes. Erythrocyte shape changes may have diagnostic significance. Normally, most erythrocytes of peripheral blood are discocytes (normocytes). Due to cytoskeleton damage, red blood cells turn into echinocytes under pathological conditions. Erythrocyte deformation and aggregation resulted in micro-circulatory failure, abnormal blood rheology, and subsequent disseminated intravascular coagulation and functional failure of vital organs (brain, liver, kidney, spleen) (Fourrier et al., 1992; Levi and Ten, 1999).

Conclusions

Mouse-adapted pandemic A(H1N1) pdm09 virus infection leads to lethal disease comparable with HPAI A(H5N1). But the rate of lethality and dynamic of inflammation are more severe in the case of infection with mouse-adapted pandemic A(H1N1) pdm09 virus. Alteration of the red blood cells membrane with the formation of echinocytes can be considered as the universal marker of intoxication. The data obtained can be used in clinical practice to improve the assessment of A(H1N1) pdm09 and A(H5N1) virus-induced disease severity and for monitoring of the treatment efficiency.

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