VIROLOGY

Structural Changes in the Brain of Mice Infected with Influenza A/H5N1 Virus

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Structural changes in the brain of outbred mice were studied after infection with influenza A/H5N1 strain isolated in the Novosibirsk region. High mortality was observed after intranasal infection. Examination of brain specimens revealed vasculopathies with thrombosis of the microcirculatory vessels, pericellular and perivascular edema with multifocal ischemic necrosis, hyperplasia of glial cells, caspase-dependent apoptosis of neurons caused by the cytopathic effect of the virus, and hypercytokinemia.

Key Words: *influenza A/H5N1 virus; neurotropism; brain; hypercytokinemia; immunomorphology*

Numerous outbreaks of avian influenza A/H5N1 recorded over the recent decade in 58 countries of the world are historically unprecedented: evolutionally stable influenza viruses circulating in its natural host have acquired the capacity to overcome the barrier between different species and be directly transmitted from infected birds to humans causing a generalized infection with mortality over 60% [12], which is caused by effective replication of avian influenza virus in cells of many organs, primarily the lungs and CNS [4,8-10]. Pathogenic activity of various influenza A virus strains largely depends on their neurotropism. However, modern studies of the pathomorphosis of viral involvement of CNS in avian influenza H5N1 are rare and they do not fully describe the pathogenetic aspects of the disease, particularly for new recombinant strains.

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We studied the structural changes in mouse brain after infection with avian influenza H5N1 virus isolated in the Novosibirsk region.

MATERIALS AND METHODS

Two series of experiments were carried out on outbred 6-week-old male mice (20-25 g; Breeding Center, Vector Center), due to which the animal genotype determination of the immune response peculiarities could be ruled out. All studies on mice were carried out in accordance with Regulations for Studies on Experimental Animals (Supplement to the Order of the Ministry of Health of the USSR of August 12, 1977, No. 755) and with consideration for the international principles of the Helsinki Declaration on humane handling of animals. The animals were intranasally infected in a dose of 10 MLD_{50} (mouse lethal dose, causing the death of 50% animals).

In experimental series I, we studied neurotropism of five influenza A/H5N1 virus strains isolated in the Russian Federation in 2005-2006: A/chicken/

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Krasnodar/123/06, A/turkey/Suzdalka/12/05, A/duck/Tuva/01/06, A/goose/Krasnoozerskoye/627/05, and A/chicken/Reshoty/02/06.

In series II, we studied the morphogenesis of viral involvement of the CNS in outbred mice infected with influenza virus A/H5N1 strain isolated in the Novosibirsk region and characterized by the highest pathogenic activity and neurotropism. Two groups of animals were formed. Controls (n=30) received 50 µl sterile phosphate buffer (pH 7.2), 25 µl into each nasal passage. Experimental mice (n=60) were intranasally infected with avian influenza A/H5N1 virus in a dose of 10 MLD_{50} in 50 µl phosphate buffer (pH 7.2), 25 µ into each noistril. The type and severity of pathological changes in the brain over the course of viral infection were evaluated in an immunomorphological study. Brain tissue specimens were collected 1, 2, 3, 6, and 10 days after infection (10 animals per term) after sacrifice by cervical dislocation under ether narcosis. Organ specimens for light microscopy were fixed in 10% formalin, dehydrated in ascending alcohols, and embedded in paraffin. The sections (4-5 µ) were stained with hematoxylin and eosin, 0.5% cresyl violet and thionin after Nissl. In order to detect fibrin, the preparations were stained by Weigert's method. Paraffin sections for immunohistochemical study were deparaffinated, rehydrated, the target antigens were decamouflaged in a microwave oven at 700 W, endogenous peroxidase was blocked, the sections were incubated with blocking serum and with specific monoclonal antibodies to CD34, IL-6, and TNF-α (Novocastra). The mechanisms of apoptosis were studied using markers caspase-3 and TRAIL (TNF-related apoptosis-inducing ligand; Novocastra). The preparations were incubated with the antibodies for 1 h at ambient temperature, which was followed by incubation with streptavidin peroxidase complex, DAB substrate, and poststaining of the preparations with Meyer's hematoxylin. The NovoLink detection system (Novocastra) was used for visualization. The means were calculated by methods of variation statistics. The significance of differences between the means was evaluated by Student's test. The differences were considered significant at p<0.05.

RESULTS

Biomolecular analysis of the epizootic strains isolated from domestic and wild birds in 2005-2006 showed their high pathogenic activity determined by the amino acid sequence of hemagglutinin containing a characteristic high pathogenicity marker (PQGRRKKKR↓GL) in the proteolytic cleavage site [11]. Evaluation of neurotropism of A/H5N1 strains A/turkey/Suzdalka/12/05, A/duck/Tuva/01/06, A/chicken/Krasnodar/123/06, A/goose/Krasnoozerskoye/627/05, and A/chicken/Reshoty/02/06 showed that replication in the brain is characteristic of all the studied strains. This was proven by high titers of the virus in the brain tissue of experimental animals (Fig. 1). Thus, high pathogenic activity of influenza A/H5N1 strains isolated in Russia is determined by characteristics of the viruses responsible for their neurotropism and efficiency of replication in mammalian lungs and CNS [3,4].

Neuromorphogenesis of viral infection was studied in series II on A/goose/Krasnoozerskoye/627/05 strain, characterized by the highest neurotropism, high pathogenic activity, and causing generalized infection with mortality reaching 85% in experimental animals [1-3].

Histological study of the brain from mice infected with avian influenza virus H5N1 subtype A/goose/Krasnoozerskoye/627/05 showed signs of serous leptomeningitis presenting as edema and groups of solitary lymphocytes in the pia mater. One of the leading pathomorphological signs of viral involvement of the CNS in avian influenza A/H5N1 is edematous destructive syndrome associated with early hemocirculatory disorders. Plethoric vessels with fibrinoid changes in the basal membrane, endothelial necrosis, signs of hypercoagulation, and progressive thrombosis of the microcirculatory bed were detected in the brain of experimental mice starting from day 1 postinfection. The percentage of clotted vessels increased 2.5 times from day 1 to day 6 (percent of total numerical density of vessels, Table 1). Small infiltration foci, consisting mainly of lymphocytes, formed in perivascular spaces.

Starting from day 3, neoangiogenesis was observed, the numerical density of the vessels increasing

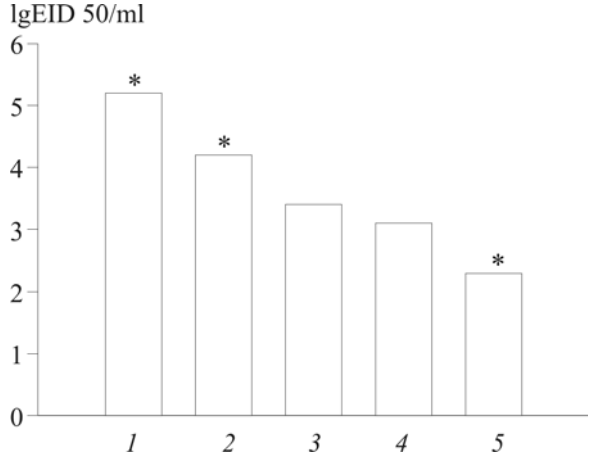

Fig. 1. Influenza virus A/H5N1 titers in the brain of mice infected with strains isolated in Russia. *1*) A/goose/Krasnoozerskoye/627/05; *2*) A/chicken/Reshoty/02/06; *3*) A/duck/Tuva/01/06; *4*) A/chicken/Krasnodar/123/06; *5*) A/turkey/Suzdalka/12/05. EID: embryonal infective dose. *Values are significant at p<0.05.

TABLE 1. Morphometric Parameters of Structural Changes in the Brain of Mice Infected with Influenza A/goose/Krasnoozerskoye/627/05 Virus (*M*±*m*, %)

Parameter	Control	Infected animals				
		Day postinfection				
		1	2	3	6	10
Numerical density of vessels (Nai) 4.2×10^4 μ^2	6.42±0.29	7.02±0.31	7.52±0.26*	8.10±0.41*	8.26±0.50*	10.20±0.34*+
% of thrombosed vessels	—	22.25	28.71	43.25	56.2	52.67
Volume density of destructive changes (Vv), %	0.40±0.06	15.10±0.62*	27.48±0.51*+	46.00±0.33*+	53.20±0.04*+	47.30±0.73*+
Numerical density of astrocytes (Nai) 4.2×10^4 μ^2	3.20±0.06	5.61±0.60*	5.82±0.28*	8.64±0.20*+	10.2±0.4*+	11.60±0.52*+
Volume density of perivascular infiltration (Vv), %	0.25±0.06	0.36±0.05*	0.48±0.08*+	0.50±0.07*	0.68±0.16*+	1.53±0.30*+

Note. $p<0.05$ compared to the parameter: *in control, +at the previous term of observation.

by day 10 of the disease by 25.6% (Table 1) at the expense of the capillaries with expression of the CD34 endothelial marker. The development of vascular ischemia was paralleled by augmenting pericellular and perivascular edema and multifocal ischemic necroses in brain tissue. The volume density of destructive changes increased 3.5 times from day 1 to day 6 postinfection (Table 1).

One of the causes of hemodynamic disorders in A/H5N1 influenza can be hyperproduction of proinflammatory cytokines [6,7], whose serum levels are elevated in infected mice starting from day 1 [2,3]. Immunohistochemical study of the cytokine profile in the brain of mice infected with A/goose/Krasnoozerskoye/627/05 revealed expression of TNF-α and IL-6 as early as on day 1 postinfection. Later the expression increased, manifesting by increasing counts of positively stained endothelial and glial (astrocytes and microglia) cells and solitary neurons. The levels of these cytokines virtually did not increase from day 6 to day 10.

Hypercytokinemia can also be the cause of neurocyte apoptosis detected outside the ischemic necrotic foci and confirmed during all periods of the study by the TRAIL immunopositive staining of apoptotically modified neurons with the cell death domain. This indicated the proapoptotic TNF cascade [13]. In parallel, neurons and vascular endothelial cells with expression of caspase (effector enzyme cleaving cellular proteins [5]) were detected in the brain tissue of infected mice on days 1-3 of disease. Presumably, the realization of procaspase apoptosis could be due to the cytopathic characteristics of A/goose/Krasnoozerskoye/627/05 strain during its effective replication in brain cells [8-10].

Destructive changes in brain tissue of experimental mice were paralleled by hyperplasia of glie elements (Table 1). Differences in the topographic location of microglia and astrocytes were seen. The majority of microglial cells functioning as phagocytes were located outside the necrotic zones. Astrocyte hyperplasia was observed mainly perivascularly around paretically dilated or new vessels in the form of chain accumulation, which could indicate the defense role of astrocytes in the restoration and support of the structural and functional integrity of the blood-brain barrier.

Hence, hypersecretion of proinflammatory cytokines in response to effective replication of A/H5N1 virus in cells of different histogenesis, causing hemocirculatory disorders with development of edema and ischemic necrosis and triggering the TNF-induced apoptosis of neurons, which is conjugated with reactive hyperplasia of glial elements, seems to be the triggering mechanism in the pathomorphogenesis of CNS involvement in mammals infected with this virus.

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