VIROLOGY

Structural and Functional Changes in Pulmonary Macrophages and Lungs of Mice Infected with Influenza Virus A/H5N1 A/goose/Krasnoozerskoye/627/05

A. V. Kovner, A. G. Anikina, O. V. Potapova, T. V. Sharkova, L. A. Cherdanceva, V. A. Shkurupy, and A. M. Shestopalov*

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 153, No. 2, pp. 196-199, February, 2012
Original article submitted November 9, 2010

C57Bl/6 mice were intranasally infected with influenza virus A/H5N1 A/goose/Krasnoozerskoye/627/05. The mortality rate of animals reached 70% on day 14 of the disease. The lungs of animals were characterized by necroses, destruction of vessels, hemorrhagic and thrombotic complications, edematous syndrome, and early fibrosis of the interstitium. On days 6-10 after infection, fibrosis was found in the zones of postnecrotic inflammatory infiltration. The expression of lysozyme and myeloperoxidase by pulmonary macrophages was initially increased, but decreased on day 10 of the study. The number of cathepsin D-expressing macrophages was elevated up to the 10th day of examination.

Key Words: *influenza virus A/H5N1 (A/goose/Krasnoozerskoye/627/05); lungs; macrophages; early fibrosis; immunomorphological study*

Mammals infected with influenza A are characterized by significant changes in the lungs and pulmonary compartment of the mononuclear phagocyte system (MPS) [1,3,4]. However, the pathogenesis of lung damage during infection of mice with influenza virus A/H5N1 A/goose/Krasnoozerskoye/627/05 is poorly understood. Here we studied the morphofunctional characteristics of pulmonary macrophages and type of destructive and reparative processes in the lungs of male C57Bl/6 mice in various periods after infection with a highly pathogenic strain of influenza virus A/H5N1 A/goose/Krasnoozerskoye/627/05.

Research Center of Clinical and Experimental Medicine, Siberian Division of the Russian Academy of Medical Sciences, Novosibirsk; *State Research Center "Vektor", Novosibirsk oblast, Kol'tsovo, Russia. ***Address for correspondence:*** potapova@soramn.ru. O. V. Potapova

MATERIALS AND METHODS

Experiments were performed on 2-month-old male C57Bl/6 mice (n=80) weighing 20-25 g and obtained from the nursery of the Institute of Clinical Immunology (Siberian Division of the Russian Academy of Medical Sciences).

The animals were maintained in a vivarium under standard conditions and had free access to water and food.

The mice were intranasally infected influenza virus A/H5N1 A/goose/Krasnoozerskoye/627/05 [1] in a single dose of 5 MLD_{50}.

Biological samples were obtained from animals of groups 1 (20 intact mice) and 2 (40 infected mice). The animals were killed by cervical dislocation on days 1, 3, 6, and 10 after infection (according to the rules of studies with experimental animals). Group 3

(n=20) was formed to evaluate the pathogenicity of study strain from the mortality rate (%) and average life span of animals (ALS, days).

Lung samples were fixed in 10% aqueous solution of neutral formalin. The sections (3-4 μ) were stained with hematoxylin and eosin, picrofuchsin (van Gieson method), orsein, and silver nitrate. The virus in lung tissue was visualized in an immunofluorescence study with a specific marker for the IAV antigen (Influenza A) labeled by FITC. Immunophenotyping of pulmonary macrophages was performed by the standard method with specific primary antibodies to markers for CD68 macrophages (DBS), proinflammatory cytokines TNF-α and IL-6 (Novocastra), inducible NP synthase (iNOS, Novocastra), lysosomal proteases lysozyme (DBS) and cathepsin D (DBS), and myeloperoxidase (DBS).

The study was conducted using an AxioImager A1 microscope and AxioCam MRc camera (CarlZeiss). A morphometric study was performed with an ocular grid of 100 points (area 3.64×10^5). We evaluated the numerical (Nai) and volume density (Vv) of MPS cells and infiltrates and cellular composition of these structures; Vv of hemorrhages and zones of edema and lung destruction; Nai and Vv of vessels and ratio of thrombotic vessels; and Vv of fibrous tissue (collagen, reticulin, and elastin). The mean values of these parameters were calculated by standard methods with Statistica software. The differences between the means were evaluated by Student's t test. The differences were significant at $p<0.05$.

RESULTS

The mortality rate and ALS of influenza virus-infected animals were 70% and 12.3 days, respectively.

The viral antigen was detected in alveolar cells, pulmonary macrophages, and endothelial cells of the pulmonary arteries and veins at various stages of the study.

The numerical density of pulmonary macrophages was elevated by 3.2 times on day 1 after infection. The numerical density of macrophages increased on days 1-6 and peaked on day 6 after infection (by 4.5 times; Fig. 1).

The numerical density of lysozyme-expressing macrophages was maximum on day 1, but decreased by the 10th day after infection (Table 1). The numerical density of myeloperoxidase-expressing macrophages was maximum on day 3, but slightly decreased by the 10th day of the study (Table 1).

The degree of destructive processes in the lungs increased sharply by the 6th day and remained high on day 10 after infection (Table 2). The impairment of vascular integrity was manifested in the appearance of massive hemorrhages, edematous syndrome, and thrombosis of the vessels. The area of edema was more than 60% of the area of the lung parenchyma and stroma. Thrombosis was typical of 15-25% vessels.

The signs of inflammation did not decrease in the dynamics of the study. The number of macrophages expressing TNF-α and IL-6 was high in all periods of the pathological process (Table 1).

TABLE 1. Numeral Density of Pulmonary Macrophages Expressing the Intracellular Enzymes and Proinflammatory Cytokines after Infection of C57Bl/6 Mice with Influenza Virus A/H5N1 A/goose/Krasnoozerskoye/627/05 ($M\pm m$)

Factors expressed by macrophages	Time after infection, days	Numerical density of macrophages (Nai)	
		intact mice	mice infected with influenza virus A/H5N1
Lysozyme	1	3.69±0.55	24.20±1.42*
	3		16.30±0.73*+
	6		6.67±0.71*+
	10		3.89±0.35+
Myeloperoxidase	1	3.56±0.63	20.8±2.0*
	3		25.60±0.63*+
	6		18.80±0.52*+
	10		9.22±0.70*+
Cathepsin D	1	4.87±0.84	14.61±1.02*
	3		6.76±0.92*+
	6		17.60±1.89*+
	10		14.11±0.42*+
iNOS	1	3.64±0.39	4.05±0.59
	3		9.40±0.81*+
	6		15.67±1.22*+
	10		27.94±2.78*+
IL-6	1	4.43±0.26	18.22±2.96*
	3		26.31±2.11*+
	6		21.11±2.35*+
	10		15.42±1.86*+
TNF-α	1	4.95±0.39	13.91±1.20*
	3		18.92±1.62*+
	6		25.26±1.50*+
	10		20.49±1.44*+

Note. Here and in Tables 2 and 3: $p<0.05$: *in comparison with the control, +in comparison with the previous stage of the study.

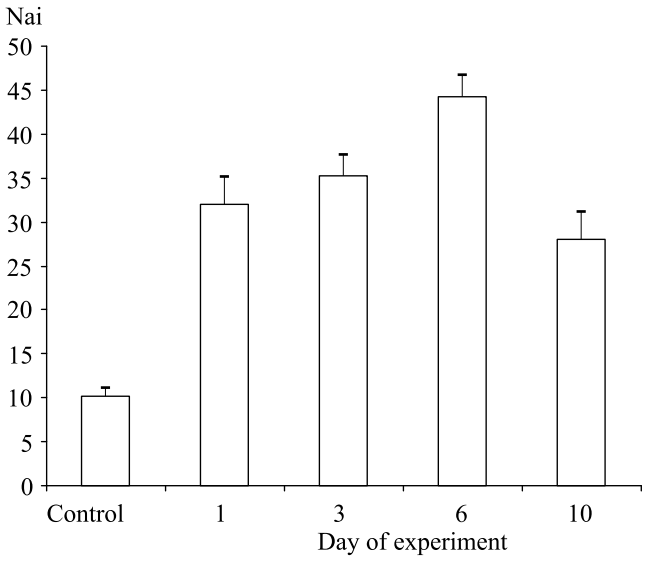

Fig. 1. Numerical density of pulmonary infiltrates in C57Bl/6 mice infected with influenza virus A/H5N1 A/goose/Krasnoozerskoye/627/05.

iNOS production by pulmonary macrophages increased by 6.9 times on days 1-10 after infection. By the end of the study, iNOS production by pulmonary macrophages was 7.7 times higher than the baseline (Table 1).

The increased secretion of proinflammatory cytokines and NO synthases by pulmonary macrophages was followed by vasodilation, endothelial damage, fibrinoid changes in the vascular wall, and hemorrhagic syndrome (Table 2).

Destructive changes in the lungs of animals on days 6-10 of inflammation were manifested in apoptosis of lung cells, which probably resulted from the increase in TNF-α expression and activation of the death domains TRAIL and FADD [4].

Another process that determines functional activity of the lungs and whole body after infection with influenza virus A/H5N1 was extremely early and massive fibrosis of the lung interstitium and sites of inflammatory infiltrates in the lungs. These data suggest

TABLE 2. Structural Changes in the Lungs of Mice after Infection with Influenza Virus A/H5N1 A/goose/Krasnoozerskoye/627/05 (*M*±*m*)

Parameter	Time after infection, days		
	C57Bl/6 mice	intact mice	mice infected with influenza virus A/H5N1
Volume density of destructive changes (Vv), %	1	0.34±0.12	16.56±1.44*
	3		20.34±2.03*+
	6		59.22±2.54*+
	10		59.94±1.58*
Volume density of hemorrhages (Vv), %	1	5.63±0.80	17.35±1.59*
	3		24.26±2.05*+
	6		31.68±2.52*+
	10		20.16±2.59*+
Numerical density of lung vessels (Nai), %	1	10.88±0.86	12.07±0.71
	3		13.45±0.51*+
	6		11.53±0.64+
	10		10.42±0.49
Ratio of thrombotic vessels, %	1	—	14.14±0.52
	3		20.03±0.61+
	6		24.72±0.84+
	10		18.9±1.03+
Volume density of edematous zones (Vv)	1	0.25±0.09	15.37±1.15*
	3		24.83±1.61*
	6		39.24±1.44*+
	10		63.47±0.85*+

TABLE 3. Results of Light Microscopy and Morphometry of Fibrous Connective Tissue in the Lungs of C57Bl/6 Mice Infected with Influenza Virus A/H5N1 A/goose/Krasnoozerskoye/627/05 (*M*±*m*)

Time after infection, days	Collagen fibers	Reticulin fibers	Elastic fibers
Control	0.91±0.58^{o}	6.10±1.41^{o}	1.60±0.45^{o}
1	4.65±1.29^{*o}	15.61±2.49^{*o}	7.45±0.71^{*o}
3	6.64±0.77^{*o}	20.13±2.76^{*o}	16.27±1.25^{*+o}
6	10.40±1.08^{*+o}	37.70±2.97^{*+o}	17.64±1.01^{*o}
10	12.65±0.86^{*+o}	34.30±3.36^{*o}	23.55±1.57^{*+o}

Note. $^{o}p<0.05$: differences between various types of fibers at various stages of the study.

that the process of lung fibrosis should not be considered only as postnecrotic incomplete regeneration. A histological and morphometric study during the early period of infection (days 1-3) showed that fibrous connective tissue is mainly present in the peribronchial and perivascular regions. The total volume (Vv) of reticulin and elastic fibers on day 3 of the pathological process was more than 35% of lung tissue volume. By contrast, the total volume of collagen fibers was only 7% (Table 3). Reticulin and elastic fibers were revealed in the interstitium and zones of inflammatory infiltration with an increase in the duration of the pathological process. These data indicate that the macrophageal component of the immediate early response system was adequate during infection. However, the production of lysozyme was reduced in the earlier period than that of myeloperoxidase. The high and constant level of cathepsin D expression (Table 1) was probably related to the elimination of not only viral proteins, but also of lung degradation products [2]. Moreover, the process of lung fibrosis after infection of animals with influenza virus A/H5N1 has not only the postnecrotic reparative function.

Our results indicate that the pathogenetic mechanisms of these events should be studied in details to form a basis for the prevention of destructive and reparative (fibrotic) components of study process and its complications.

This work was supported by the Federal Target Scientific-and-Technical Program "Scientific and Scientific-and-Pedagogical Staff of Innovative Russia for 2009-2013" (state contract No. 02.740.11.0709).

REFERENCES

1. O. V. Potapova, V. A. Shkurupy, T. V. Sharkova, *et al*., *Byull. Eksp. Biol. Med*., **150**, No. 12, 650-654 (2010).
2. T. Burster, T. Giffon, M. E. Dahl, *et al*., *Int. Immunol*., **19**, No. 5, 645-655 (2007).
3. K. F. To, P. K. Chan, K. F. Chan, *et al*., *J. Med. Virol*., **63**, No. 3, 242-246 (2001).
4. J. Zhou, H. K. Law, C. Y. Cheung, *et al*., *J. Infect. Dis*., **193**, No. 7, 945-953 (2006).