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Histopathological And Clinical Analysis Of Balb/C Mice Infected With Mouse-Adapted Pandemic Influenza A/H1N1 Virus Using Different Viral Doses.

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Abstract: Influenza A is a serious respiratory illness that can be debilitating and may cause complications leading to hospitalization and death. Pathology has been associated with severe immune response in the lung and is the main cause of death among patients with viral pneumonia caused by pandemic (H1N1) 2009, producing acute respiratory distress syndrome (ARDS) with diffuse alveolar damage (DAD). Here, we examined serial pathological changes in the lungs of the mice infected with mouse-adapted pandemic influenza 2009 A/H1N1 virus using different viral doses. Correlating the clinical signs and the histopathological changes in the different experimental groups, we identified specific viral doses causing from a slight pneumonia to ARDS/DAD. In the group infected with 8 hemagglutinating unit (HA), we found edema, lymphocytic infiltrates, acute interstitial pneumonitis and fibrosis. However, in the group infected with 16 HA, severe interstitial pneumonitis, alveolar collapse, hemorrhage, septal congestion and dense fibrosis was developed as reported in humans. The infection with 16 HA is an ideal, fast and efficient model to evaluate immunomodulatory drugs that diminished the ARDS and DAD.

Keywords: Influenza A, ARDS, DAD, inflammation, fibrosis.

INTRODUCTION

Influenza A is an infectious disease characterized by an aggressive immune response due the recruitment of inflammatory leucocytes and exaggerated levels of cytokines, causing high mortality[1-3]. The main cause of death among patients with viral pneumonia caused by pandemic (H1N1) 2009 is acute respiratory distress syndrome (ARDS), which is clinically defined as acute respiratory failure, diffused alveolar damage and other pathological changes[2,4,5]. Necropsies of patients with ARDS have also showed diffuse alveolar damage (DAD), which is defined by the formation of a hyaline membrane lining the alveoli and alveolar ducts, inflammatory cell accumulation in the lungs, and pulmonary edema[2, 6]. Although effective anti-influenza virus drugs are currently available, the mortality rate of ARDS caused by influenza virus remains high. The mouse model constitutes a useful model for the investigation of the immune response against influenza virus[7]. Particularly, to the understanding of ARDS/DAD in order to develop new immunomodulatory and anti-inflammatory drugs, in this work, we performed serial pathological analysis of lungs from mice infected with mouse-adapted influenza A/H1N1 pandemic virus using different viral doses. By this process we identified specific viral doses causing from a slight pneumonia to ARDS/DAD to evaluate the inflammatory response and the effect of different immunomodulatory therapies.

METHODS

Virus and cells: Pandemic influenza A/H1N1 viruses were kindly provided by the Virology laboratory of the National Institute of Respiratory Diseases (INER, México). All viruses used for the experiments came from a single viral preparation batch prepared in Madin-Darby Canine Cells (MDCK) as previously described[3].

The titer of the virus stock was evaluated by viral hemagglutination[8]. One hemagglutinating unit (1 HA) corresponded to $2x10^5$ infective viral particles. The human virus stock were stored at -80°C. MDCK cells were maintained in Minimal Essential Medium (MEM, Invitrogen, USA).

Adaptation of pandemic A/H1N1 influenza viruses in mice: Mouse-adapted variants of pandemic A/H1N1 influenza virus were derived by three sequential mouse lung-to-lung passages[9]. Briefly, we used female mice of the Balb/C strain from 6 to 8 weeks of age with a body mass of 18 to 23 g. Animals had food and water free access (Purina chow standard diet, Purina, USA) and subject to 12 h light-dark cycles, receiving human care based on the criteria of the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publ. 86-23, rev., 1985), also according to the guidelines and Official Mexican Standard NOM_062 -ZOO- 1999. Animals were infected intranasally, instilling 60 µL the viral solution with a dose of 1 HA, corresponding to the first pass (n=7).

Four days later, animals were sacrificed and lungs were collected, homogenized and centrifuged. Then, 60 µl of the supernatants per mouse were used for the next passage (second pass, n = 13). After three passages (third pass, n = 21), we obtained pandemic influenza A/H1N1 viruses adapted in mice.

Determination of viral infectivity: The infectivity of third passage of mouse-adapted of pandemic A/H1N1 influenza viruses was determined in MDCK cells by viral [8, 9.] briefly, confluent MDCK cells were inoculated with 100 µL of virus at 37°C for 1 h. The cells were then washed and cultured in MEM/antibiotics medium (Sigma, USA) at 37°C for 48°C.

Pathogenicity of virus in BALB/C mice: Titrated virus were used to prepare infective doses of 8 HA, 16 HA, 32 HA, 64 HA, 128 HA and 150 HA in a 50 µL DMEM medium. Then, animals were infected intranasally instilling 50 µL of the viral solution, weighted and observed daily and lung tissues were obtained, weighted and fixed in 10% formaldehyde, 7 days post-infection.

RESULTS AND DISCUSSION

Influenza A is an acute infectious disease that causes inflammation in the airways in humans and whose symptoms are fever, headache and fatigue. Under certain conditions exacerbated immune responses deteriorate the patient's health causing the death[10-12].

To understand the processes occurring during pulmonary A/H1N1 virus infection, here we established a mouse model of infection. The adaptation of the 2009 pandemic A/H1N1 virus strain in Balb/C mice were made making three serial animal passages, using the methodology previously reported[9,13,14]. Animals subjected to viral adaptation, had a gradual deterioration in health status.

After carrying out the serial passages, we proceeded to determine the viral titer by hemagglutination in the MDCK cell line. The pandemic H1N1/A mouse-adapted virus showed high cytopathic effect, having morphological changes in the cell line MDCK, such as rounding of cells suggesting the induction of apoptosis (Figure 1).

Also we observed an increased in the viral titer from the third pass in comparison to that from the first pass (from 2 x 10^5 p.v.i. to 9.2 x 10^6 p.v.i.), confirming viral replication and increase of virulence.

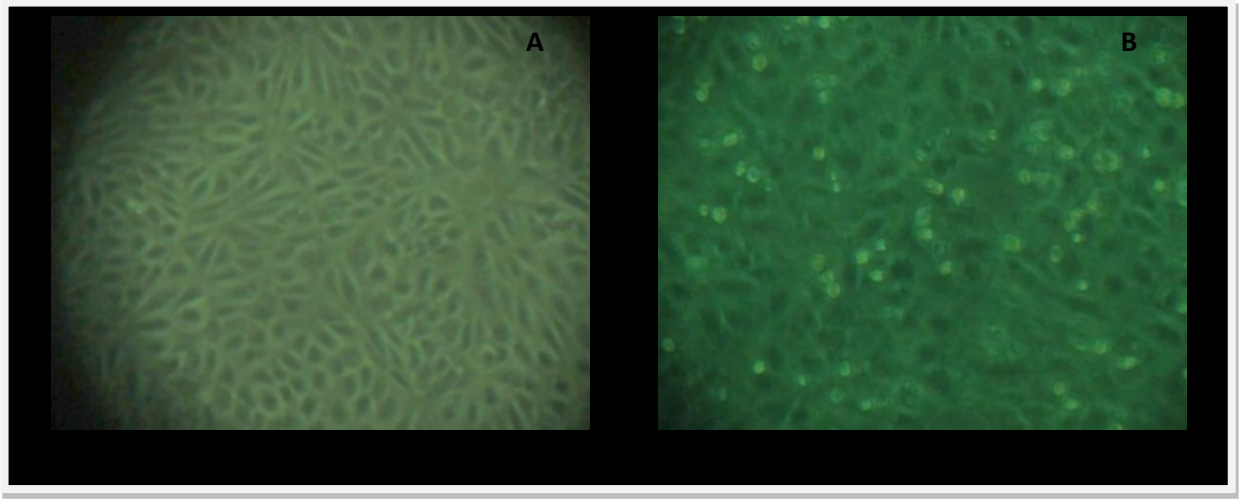

Figure 1: Cytopathic effect caused by the 2009 pandemic A/H1N1 virus adapted to Balb/C mice. A) MDCK cells before virus infection, B) MDCK cells post-infection.

Pathogenicity of mouse-adapted H1N1 viruses *in vivo*

Clinical and histopathology analysis of groups infected with different doses of the H1N1 mouse-adapted virus : The clinical analysis using the scale of Menéndez and collaborators, showed that non-infected animals had a score of 1 (Table 1), with no signs of disease with a normal lung architecture, thin interalveolar walls, alveoli preserved with thin walls in bronchi and vessels (Figure 2A).

However, using the infective doses from 8 HA to 150 HA, animals had a score of 5, related to loss of mobility, labored breathing and cyanosis of tail and ear. The clinical signs were more severe and evident in those animals infected with higher viral doses (128 HA and 150 HA), exhibiting 66.6% and 33.3% mortality, respectively (Table 1).

Histopathology from animals infected with higher doses (128 HA and 150 HA), showed the destruction of the alveolar parenchyma, edema, large lymphocytic infiltrate, intra-bronchial and peri-bronchial hemorrhage, septal congestion, perivascular and pleural inflammation, severe interstitial pneumonitis, and the formation of the surfactant membrane, which resembles a Diffuse Alveolar Damage (DAD) (showed in figure 2B-C). Similar results have been observed in murine models of infection[15] with influenza A strain A/H5N1.

Table 1: Identification of the infective dose 2009 pandemic A/H1N1 virus in mice of the Balb/C.

EXPERIMENTAL GROUP	SCORE AND GRADE OF CLINICAL SIGNS	MORTALITY RATE
Control 	1.Healthy, no sign of disease	0 %
8 HA 	5. Step or abnormal gait, decreased mobility, respiratory distress, cyanosis, tail and ears.	0 %
16 HA 	5. Step or abnormal gait, decreased mobility, respiratory distress, cyanosis, tail and ears. Moderate.	0 %
32 HA 	5. Step or abnormal gait, decreased mobility, respiratory distress, cyanosis, tail and ears.	0 %
64 HA 	5.Step or abnormal gait, decreased mobility, respiratory distress, cyanosis, tail and ears.	0 %
128 HA 	5. Step or abnormal gait, decreased mobility, respiratory distress, cyanosis, tail and ears. Severe signs.	66.6 %
150 HA 	5. Step or abnormal gait, decreased mobility, respiratory distress, cyanosis, tail and ears. Severe signs.	33.3 %

The establishment of the immunopathogenic process was evident clinically and histopathologically from 8 HA, which corresponds to 1.6 x 10^6 p.v.i.; presenting edema, lymphocytic infiltrates, acute interstitial pneumonitis (Figure 2D) and pulmonary fibrosis (Figure 3B). In Figure 2E, the histopathological changes of the group infected with 16 HA, presented an acute interstitial pneumonitis, light bleeding, deformation of cells, lymphocytic infiltrate and dense fibrosis (Figure 3C); at 32 HA the pathological process increased

having a greater loss of alveolar morphology, intrabronchial hemorrhage and alveolitis (Figure 2F). A more severe damage was presented at a dose of 64 HA, where the alveoli is lost, due to alveolar collapse, increased lymphocytic infiltrates, peribronchial and septal congestion and intrabronchial hemorrhage, triggering a process of severe pneumonia (Figure 2G).

The pathological changes or diffuse interstitial pneumonitis are comparable with severe clinical cases reported in human patients infected with 2009 pandemic A/H1N1 viruses and caused the Distress Acute Respiratory Syndrome (ARDS) and the DAD, defined by the formation hyaline membrane lining the alveoli and alveolar ducts, accumulation of inflammatory cells in the lung edema and even death in some human[4, 16, 17].

Our results also showed pulmonary fibrosis by Masson trichromic staining in four infective doses, showing collagen deposition in alveolar walls and spaces (Figure 3B-E), probably due to the migration of fibroblasts and inflammatory cells producing pro-inflammatory cytokines that exacerbate viral pathogenic process.

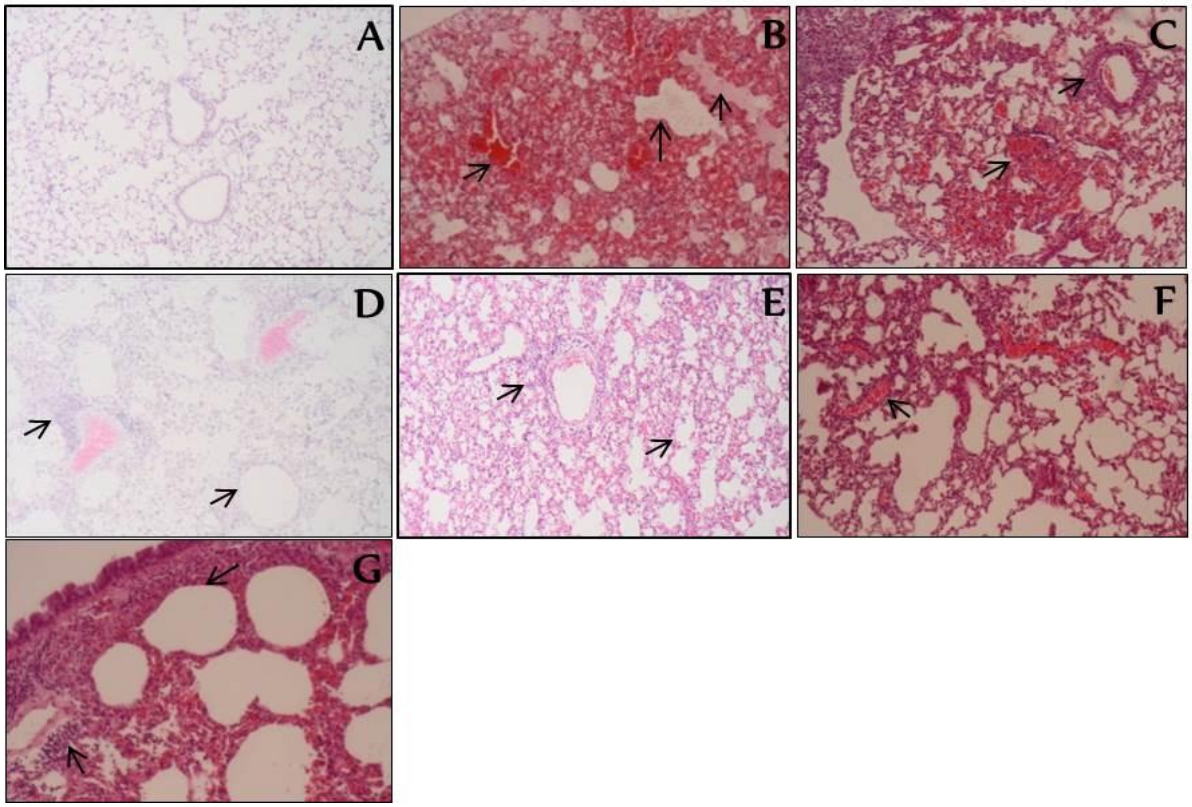

Figure 2: Histopathological changes of lungs infected with 2009 pandemic A/H1N1 virus adapted to Balb/C mice. A) Non-infected mice lungs. B) Infected mice with 128 HA, C) Infected mice with 150 HA, D) Infected mice with 8 HA, E) Infected mice with 16 HA, magnification 20 X, F) Infected mice with 32 HA, G) Infected mice with 64 HA. Arrows indicate the morphological changes of lung caused by influenza virus, such as lymphocytic infiltration and septal congestion, intrabronchial and peribronchial hemorrhage, interstitial pneumonitis, hyaline membrane formation and alveolar collapse. Magnifications 10X.

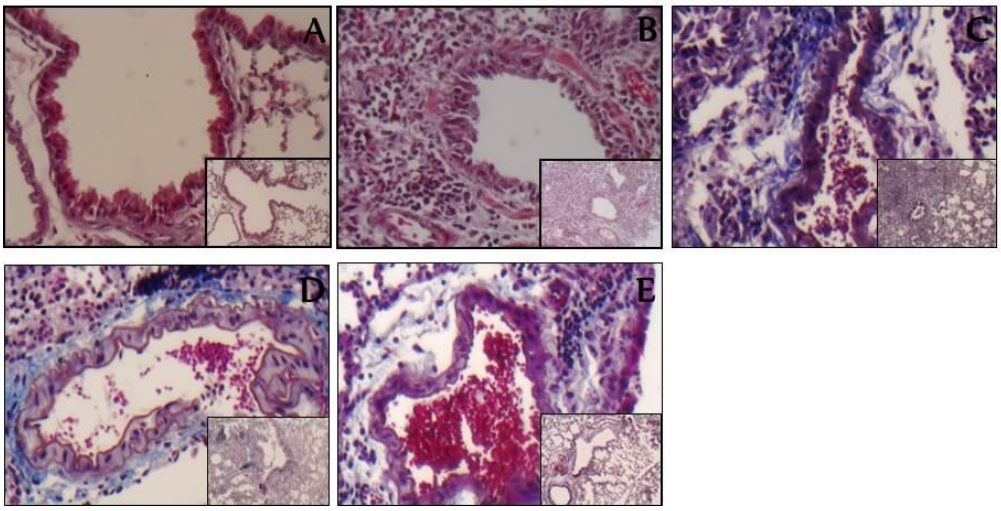

Figure 3: Masson trichromic staining of lungs infected with 2009 pandemic A/H1N1 virus adapted to Balb/C mice by. A) Non-infected mice or control group. B) Infected mice with 8 HA, C) Infected mice with 16 HA, D) Infected mice with 32 HA, E) Infected mice with 64 HA. Magnification 10X and 40X.

CONCLUSIONS

The present study demonstrated the relationship between clinical signs and histopathological changes in the different experimental groups. Low viral dose (8 HA) tested in this study, produce acute interstitial pneumonitis and pulmonary fibrosis. Infection with 16 HA, produce severe interstitial pneumonitis, alveolar collapse, hemorrhage, septal congestion due to lymphocytic infiltrate and dense fibrosis simulating diffuse alveolar damage as reported in humans. The infection with 16 HA is an ideal, fast and efficient model to evaluate immunomodulatory drugs that diminished the acute respiratory distress syndrome (ARDS) and the diffused alveolar damage (DAD).

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