Pathogenesis of the Influenza Virus in Diabetes Model Mice

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Authors’ contributions

This work was carried out in collaboration between all authors. Author MI designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Authors HK, TT, SQ, MT, HT, AM, MN, YI managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

ABSTRACT

When diabetes model mice and control mice were intranasally infected with the influenza virus (1 LD₅₀), a significant higher mortality rate was observed in the diabetes model. The mortality rate became 100% at 10 days post infection. Furthermore, histopathological examination of lung tissue revealed a more enhanced inflammatory response in the diabetes model mice infected with the
influenza virus. On the contrary, viral clearance from the lungs was suppressed in the diabetes model mice. The recruitment of macrophages was observed in the case of virus-infected control mice at 1 and 3 days post infection. Intriguingly, the number of macrophage in the lungs was not changed through the period of observation, thus the recruitment of macrophages in the lungs was not found in the virus-infected diabetes model mice.

Keywords: Influenza virus; diabetes model mice; pathogenesis.

1. INTRODUCTION

As stated the International Diabetes Federation “IDF” [1], diabetes prevalence (including type 1 and type 2) in the world reached 366.2 million in November 2011, showing a rapid increase of about 20% compared to 284.6 million in the year 2010. The IDF also predicts and warns that diabetes prevalence in the world will reach 551.8 million by the year 2030. The finding means 10% of the adult population will become diabetes prevalent. In addition, type 2 diabetes accounts for about 90% of total diabetics [2].

It is well known that diabetic patients are prone to infectiousness, thus infection is considered as an important complication. Furthermore, this characteristic of not only being more susceptible to infection, but also more resistant to medical treatment. A cohort study in the United States [3] showed that infection prevalence of a diabetic is higher compared to that of a non-diabetic. In some studies, it has been reported that the mortality rate due to infectious disease in diabetics is about two to three times compared to that of non-diabetic patients [3].

Influenza is widespread every year. Especially in 2009, the first flu pandemic of the 21st century occurred. Empirically diabetes is considered as a risk factor for influenza onset conditions. Furthermore, it is reported that there is a high pneumonia merger rate in diabetic patients with an influenza virus infection [4]. However, the scientific evidence about interrelationships between diabetes and the influenza virus infection is insufficient.

Through this study, we have aimed to make clear the susceptibility of diabetic mice to the influenza virus infection, and the pathogenesis of influenza virus infection in diabetic mice by using diabetes model mice. In this research, a significant higher mortality rate was observed and viral clearance from the lung was decreased in the diabetic mice infected with influenza virus.

The aim of our animal study is that the obtained data apply to human cases. A lot of disease-

2. MATERIALS AND METHODS

2.1 Experimental Animals

Female BKS.Cg+Lepr-db/+Lepr-db/Jcl (7 weeks old) mice for the diabetic model group (DM+) and female BKS.Cg-m +/+Lepr-db/Jcl (7 weeks old) mice for the control (DM-) group were purchased from Charles River Laboratories. To establish the infection of influenza virus, 2.3 x10^7 PFU/ml (1 LD_{50}) of A/Puerto Rico/8/34 strain was infected intranasally with PIPEMAN (inf(+)) while the control group was infected with PBS (inf(-)). Lung tissue was collected at 0, 0.5, 1, 3, 5 and 8 days post infection. Virus titer (LD_{50} Dose) was calculated using the method of Read & Muench [6]. All experiments were approved by the Institutional Animal Care and Use Committee of Chubu University (No. 2710037).

2.2 Cell and Virus

Madin-Darby canine kidney (MDCK) cell monolayers were maintained in Eagle’s minimum essential medium (MEM) supplemented with 5% fetal bovine serum.

The influenza virus, A/Puerto Rico/8/34 (H1N1) strain was used. Stock was prepared by allantoic inoculation of 10-day-old embryonated eggs with 0.1 ml of a 10^-2 dilution of infected fluid. After incubation for 2 days at 35˚C, the allantoic fluid was harvested, then the fluid was centrifuged for 10 minutes at 1500 rpm under 4˚C for semipurification of the virus, and stored at -80˚C. The infectious titer of the virus was determined by plaque assay method with MDCK cells.
2.3 Plaque Assay

Infectivity titrations of the influenza virus were performed by plaque assay in monolayer cultures of MDCK cells cultured in 12 well plates. The virus solution was 10 fold-diluted, and the cells were inoculated with 0.1 ml of each diluted solution. After adsorption of the virus at 37°C in 5% CO₂ incubator for 1 hour, the cells were overlaid with 0.5% agar solution containing 20 µ g/ml acetylated trypsin. After two days of incubation at 37°C the cells were stained with neutral red.

2.4 Histopathological Analysis of the Lung

Animals were deeply anesthetized and a blood sample was taken directly from the heart for the death with the de-blood. Immediately after the mice were euthanized, the lungs were obtained and fixed in buffered 4% paraformaldehyde. The fixed lungs were cut at 10 µm serial sections. Sections were stained with hematoxylin and eosin, and subsequently the degree of inflammation was semi-quantitatively scored. The sections were scored in an unbiased fashion from 0 to 3. A score of 0 indicated the absence of inflammation; 1, minimal or mild perivascular and peribronchiolar infiltrates of lymphocytes mixed with fewer neutrophils; 2, moderate perivascular and peribronchiolar infiltrates of predominantly neutrophils mixed with fewer lymphocytes, and infiltrates of neutrophils within the alveolar sacs; and 3, severe perivascular and peribronchiolar infiltrates of predominantly neutrophils mixed with lymphocytes, alveolar sac accumulations of neutrophils and fewer lymphocytes.

2.5 Immunohistochemical Staining

Immunohistochemical staining using a specific antibody against macrophages (F4/80; Abcam, ab6640), or integrin α2 (Abcam, ab64692) was carried out. The tissue slides were incubated briefly with 10% normal goat serum with 0.3% Triton X-100 in phosphate buffer (PBS, pH7.2) for 30 minutes, followed by incubation with a primary antibody in 5% normal goat serum with 0.1% Triton X-100 overnight at 4°C. After being washed three times with PBS, secondary staining was performed with Alexa 568 rat-IgG (Santa Cruz, A11077) or Alexa 488 hamster-IgG (molecular probes, A21110) for 1 hour at 37°C. After washing three times with PBS, the slides were counterstained with DAPI (DOJINDO, 340-07971), and were observed at x20 magnification with a microscope (BX51, Olympus, Tokyo).

2.6 Statistics

The data was analyzed with the two-tailed Student’s t test using SPSS software (Ver.17).

3. RESULTS

3.1 Virus Infection and Survival Rate

The diabetes model mice and control mice were intranasally infected with the influenza virus (1 LD₅₀), and then body weights were measured chronologically. LD₅₀ was previously determined by intranasally inoculation of tenfold dilution of virus containing fluids to the diabetes model and control mice (Table 1). As demonstrated in Fig. 1, no weight loss was observed in the non-infected group of BKS.Cg +/+Lepdb/Jcl mice (DM-), while weight gain was found in the non-infected group of BKS.Cg+Lepdb/+Lepdb/Jcl (DM+). On the contrary, weight loss was observed in the infected group of mice (Fig. 1). Although weight loss of the infected DM-mice appeared to be more severe than that of the infected DM+ mouse, there was no difference in real weight loss between the DM+mouse and DM-mice after allowing for weight gain of the non-infected DM+ mice.

The survival rate of virus-infected mice is shown in Fig. 2. A significant high mortality rate was observed in the diabetes model mouse from 5 days post infection, and the mortality became 100% at 10 days post infection. Conversely, we firstly observed a death in the case of a control mouse at 7 days post infection, then the death rate increased to 60% at 6 days post infection, and from then on, no deaths were observed.

3.2 Histopathological Analysis of the Lung

DM+ mice and DM-mice were infected with the 1 LD₅₀ influenza virus. At varying periods after virus infection, the lung tissues of mice were isolated, and then sections were stained with hematoxylin and eosin (Fig. 3). Furthermore, the degree of inflammation was semi-quantitatively scored according to the materials and methods. As shown in Fig. 4, the histopathological examination of lung tissues revealed profound differences between the two groups of animals at 3 and 5 days post infection, that is, a more enhanced inflammatory response in the virus infected DM(+) group mice. At 8 days post infection, both groups turned toward a recovery, but were still left with some regions of inflammation.
infection, the body weight

We specified in quantity the load of virus in the lungs by using the plaque method. Both lungs of infected mice were isolated at appropriate periods post infection, and then the tissues were ground. Subsequently, titrations of influenza virus in the supernatant were performed by plaque assay in MDCK cells. As shown in Fig. 5, the virus titers in the lungs of diabetes model mice show an increasing trend from day 0.5 to day 5 post infection, and even after the fifth day, the decreasing trend was very minimal. On the other hand, an increasing trend was also observed in the case of control mice from day 0.5 to day 3 post infection, and the peak of virus titer in the lung was observed at day 3 post infection. Subsequently, a significant decline in viral titer was observed through days 5 to days 8 post infection in the control mice. These findings indicate that viral clearance in the lung of diabetes model mice was worse than that of the control mice.

3.4 Detection and Evaluation of Macrophages in the Lung

We observed 2 fields of views per one leaf of lung tissue on a slide glass under a microscope, and counted the number of macrophages and nuclei of total cells per field of view. We, then calculated the percentage by dividing the number of macrophages by the number of total cells seen in one field of view. The exudation rate of macrophages in the lung was evaluated by the averaged value (Fig. 6). In the case of the control mice, the recruitment of macrophages was observed at day 1 and day 3 post infection. Intriguingly, in the case of the diabetes model mice, the number of macrophages in the lung remained unchanged through the period of observation. Thus the recruitment of macrophages in the lung was not found on any post infection date, indicating that there were

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**Table 1. Estimation of LD<sub>50</sub> at the diabetes model and control mice**

| Dose  | Mortality | Survival | Cumulative mortality | LD<sub>50</sub> (Dose) |
|-------|-----------|----------|----------------------|------------------------|
| DM+   | 10<sup>2</sup> | 6        | 0                    | 17/17                  | -5.25                  |
|       | 10<sup>3</sup> | 4        | 0                    | 11/11                  |                        |
|       | 10<sup>4</sup> | 5        | 0                    | 7/7                    |                        |
|       | 10<sup>5</sup> | 2        | 1                    | 2/3                    |                        |
|       | 10<sup>6</sup> | 0        | 8                    | 0/9                    |                        |
| DM-   | 10<sup>1</sup> | 5        | 0                    | 15/15                  | -3.21                  |
|       | 10<sup>2</sup> | 5        | 1                    | 10/11                  |                        |
|       | 10<sup>3</sup> | 2        | 2                    | 5/8                    |                        |
|       | 10<sup>4</sup> | 3        | 2                    | 3/8                    |                        |
|       | 10<sup>5</sup> | 0        | 6                    | 0/11                   |                        |

The diabetes model and control mice were intranasally inoculated by tenfold dilution of virus containing fluids. At 15 days post infection, mortality was finally determined, and LD<sub>50</sub> was calculated by using the method of Read & Muench.

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**Fig. 1. Weight loss of mice infected with influenza virus A/Puerto Rico/8/34 (H1N1)**

Six female BKS.Cg-+/Lep<sup>db</sup>-/+ and seven female BKS.Cg-+/-/Lep<sup>db</sup>-/+cl mice (DM+) were inoculated with 10<sup>3</sup> influenza virus A/Puerto Rico/8/34 (H1N1). At varying intervals after virus infection, the body weights of the mice were estimated. The body weights of mice preinfectected with the virus is indicated as 0. Bars represent the standard deviations of each mean. DM-mice infected with virus; DM+mice infected without virus; DM+mice infected without virus.

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3.3 Viral Clearance in the Lung

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significant differences in the exudation rate of macrophages in the lung between diabetes model mice and control mice at day 1 (P <0.01) and day 3 (P <0.001) post infection. However, on day 5 and day 8 post infection, there was no significant difference in the number of macrophages between diabetes and control mice.

Intriguingly, the recruitment of macrophages in the lungs was not found at any post infection date in virus-infected diabetic mice. Low viral clearance may be related to the higher susceptibility of diabetes model mice. Although it has been already well known clinically that diabetic patients are compromised hosts for infection, only a few accounts of animal experiments about the problem have been reported.

In connection with the hypersusceptibility of diabetics to microorganisms, it has been reported that hyperglycemia itself attenuates the migration of neutrophil, adhesive action to the vascular endothelium, phagocytosis, and the bactericidal action opsonization [7,8,9]. This study shows that no recruitment of macrophages in the lungs was found in virus infected diabetic mice. Relationships between low viral clearance and no recruitment of macrophages in the lung of the diabetic mice are worth further investigating.

Although viral load in the upper respiratory tract has been said as not necessarily being proportional to the severity, the viral load in the lung was higher in the case of diabetes model mice in our study, suggesting that the higher viral load in the lung is one of the factors for higher sensitivity in diabetics.

The influenza virus has a high affinity to the respiratory system. Since viral pneumonia due to the influenza virus itself and the secondary infection bacterial pneumonia after being infected becomeare serious complications involved in serious complications, particularly in mortality, the vulnerability with regard to pneumonia or respiratory infection in diabetic patients.

4. DISCUSSION

In this study, susceptibility to the influenza virus was found to be significantly higher in the case of diabetes model mice, in comparison to control mouse. A higher mortality was observed in the diabetes model mice. Furthermore, the virus-infected DM(+) group mice showed a more enhanced inflammatory response. However, the viral clearance in the lungs of the diabetes model mice was worse than that of the control mice.

In Fig. 3, the lung histology of influenza virus infected mice at day 0-8 p.i. is shown. DM+ mice and DM- mice were infected with 1 LD50 influenza virus. At varying periods after virus infection, the lung tissues of mice were isolated, and then sections were stained with hematoxylin and eosin.
has significant meaning as a risk [10]. In a study using diabetes model mice [11], it has also been shown that the presence of hyperglycemia inhibits influenza virus neutralizing effect of SP-D, a collectin surfactant protein that is an important non-specific immune mechanism in the lungs.

**Fig. 4. Semiquantitatively scored for the degree of inflammation**
Sections shown in Fig. 3 were scored on the degree of inflammation in an unbiased fashion from 0 to 3 according to the materials and methods. A score of 0 indicated the absence of inflammation; 1, minimal or mild perivascular and peribronchiolar infiltrates of lymphocytes mixed with fewer neutrophils; 2, moderate perivascular and peribronchiolar infiltrates of predominantly neutrophils mixed with fewer lymphocytes, and infiltrates of neutrophils within the alveolar sacs; and 3, severe perivascular and peribronchiolar infiltrates of predominantly neutrophils mixed with lymphocytes, alveolar sac accumulations of neutrophils and fewer lymphocytes.

**Fig. 5. Viral titres in the lung of mice infected with influenza virus**
DM+mice and DM-mice were infected with 1 LD50 influenza virus. At varying periods after virus infection, both lungs of infected mice were isolated at various periods post infection, and then the tissues were ground. Subsequently, titrations of influenza virus in the supernatant were performed by plaque assay in MDCK cells. Data showed a mean titer of 5 mice + SD.

For diabetes, there is a report showing that various macrophage functions [12] such as chemotaxis ability, adhesion ability, phagocytic activity, and sterilization ability of phagocytosing pathogens, are widely impaired [13]. In this study, the decrease of macrophage mobilization was also found in diabetic mice.

As mentioned above, this experiment is a study focused on the effect of the influenza virus on diabetes model mice, and it clarified that the high susceptibility to influenza virus is revealed in diabetic mice. From much research, the reduction of macrophage functions is considered as one of the important factors in the higher susceptibility of diabetes to influenza infection. However, the molecular mechanism by which macrophage dysfunction is caused in diabetic hosts remains unclarified [14,15]. Given that the infection is closely related to the prognosis of diabetes, the elucidation of the mechanism is highly expected.

**5. CONCLUSION**
In conclusion, when the diabetes model mice and control mice were intranasally infected with the
influenza virus, a significant higher mortality rate was observed in the diabetes model, with the mortality rate becoming 100% at 10 days post infection. A more enhanced inflammatory response was found in the diabetes model mice, while viral clearance from the lungs was suppressed in such mice. The recruitment of macrophages in the lungs was not found in the virus-infected diabetes model mice.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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