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Protection of inactivated influenza virus vaccine against lethal influenza virus infection in diabetic mice

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Abstract

Influenza virus infection frequently causes complications and some excess mortality in the patients with diabetes. Vaccination is an effective measure to prevent influenza virus infection. In this paper, antibody response and protection against influenza virus infection induced by vaccination were studied in mouse model of diabetes. Healthy and diabetic BALB/c mice were immunized once or twice with inactivated influenza virus vaccine at various dosages. Four weeks after the first immunization or 1 week after the second immunization, the mice were challenged with influenza virus at a lethal dose. The result showed that the antibody responses in diabetic mice were inhibited. Immunization once with high dose or twice with low dose of vaccine provided full protection against lethal influenza virus challenge in diabetic mice, however, in healthy mice, immunization only once with low dose provided a full protection.

Keywords: Diabetes mellitus; Influenza virus; Vaccine

As a kind of fulminating infectious disease, influenza usually causes high morbidity and mortality in high risk population such as the patients with diabetes [1]. Influenza virus infection and its complications may cause loss of metabolic control in diabetic patients leading to an increase in glycosylated serum protein, ketoacidosis, and thus may increase the hospitalization rate, long-term complications, and mortality rate [2]. Vaccination is an important public health intervention for reducing morbidity and mortality from influenza and pneumonia among persons with diabetes [3,4]. However, some clinical studies showed that, compared with healthy person, the patients with diabetes after immunization once with inactivated influenza virus vaccine had a poor immune response [5,6]. On the contrary, other studies showed no significant difference between the antibody (Ab) response levels in the patients and in healthy person [7–9]. Because of the complexity of influencing factors in clinic, definitive proof of the efficacy of influenza vaccination specifically in people with diabetes is lacking [10]. How great is the influence of simple diabetes on the immunogenicity of influenza vaccine in patients? And, can a reasonable immunization schedule be determined to provide same protection for patients as that for healthy person? Studies were conducted in an animal model of diabetes to explore these problems. Adult BALB/c mice, both healthy and diabetic, were immunized once or twice with monovalent inactivated

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influenza virus vaccine, and their serum-specific IgG antibodies were detected by enzyme-linked immunosorbent assay (ELISA). The mice were challenged with influenza virus at a lethal dose, and the protective effect of vaccine was observed to provide reference for clinical studies.

Materials and methods

Establishment of animal model of diabetes. Specific pathogen free (SPF) BALB/c mice were purchased from Laboratory Animal Center, Hubei Academy of Medical Sciences, and raised in SPF living quarter of laboratory animals of College of Life Science, Hunan Normal University. Foods were forbidden to mice 12 h before test. Streptozotocin (STZ, mixed anomers, Sigma) was dissolved in sterile 0.1 mol/L sodium citrate buffer, pH 4.5, and injected intraperitoneally (i.p.) into mice within 5 min at a dose of 200 mg/kg body weight. The mice after injection with STZ were bled periodically from paraorbital venous plexus for determination of plasma glucose with blood sugar apparatus (Glucotrend 2, Roche). An animal model of diabetes is successfully established if the non-fasting plasma glucose concentrations of the mice 1 and 2 weeks after injection are more than 16.7 mmol/L.

Immunization. Two weeks after injection with STZ, the inactivated split-product influenza virus A/PR/8/34 (PR8, H1N1) vaccine was diluted to a volume of 200 μl with phosphate-buffered saline (PBS). The diabetic and healthy mice were immunized intraperitoneally once with the diluted vaccine at doses of 0.2, 2, and 6 μg, or twice, 3 weeks apart, at doses of 0.02, 0.2, and 2 μg, separately. The mice as control were injected i.p. with 200 μl PBS. Inactivated influenza virus PR8 (H1N1) vaccine was prepared by Shanghai Institute of Biological Products and detected for concentration by BCA kit (Pierce).

Virus infection. Each mouse was anesthetized and challenged with 20 μl PR8 virus suspension (40× 50% lethal dose (LD_{50}) of healthy mice) by nasal drip 4 weeks after the first immunization or 1 week after the second immunization. This infection usually causes rapid, widespread viral replication in the lungs and death of the unimmunized mice within 7 days [11,12].

Specimens. Mice were anesthetized with chloroform and then bled from the heart with a syringe. The serum was separated from the blood and used for IgG Ab assays. After bleeding, the mice were incised ventrally along the midline from the xiphoid process to the point of the chin. The trachea and lung were excised and washed three times with a total of 2 ml PBS containing 0.1% bovine serum saline (BSA). The bronchoalveolar wash was used for virus titration after removal of the cellular debris by centrifugation.

Ab assays. The concentration of IgG Ab against the inactivated influenza virus PR8 vaccine was measured by ELISA. ELISA was performed sequentially from the solid phase using a series of reagents consisting of: first, inactivated influenza virus PR8 vaccine; second, serial 2-fold dilutions of sera from each group of immunized or preimmunized mice; third, goat anti-mouse IgG Ab (γ-chain specific) (Southern Biotechnology Associates) conjugated with biotin; fourth, streptavidin conjugated with alkaline phosphatase (Southern Biotechnology Associates); and finally, *p*-nitrophenyl-phosphate. The amount of chromogen produced was measured based on absorbance at 414–405 nm in an ELISA reader (Labsystems Multiskan Ascent). Ab-positive cut-off values were set as means + 2× SD of preimmunized sera. An ELISA Ab titer was expressed as the highest serum dilution giving a positive reaction.

Virus titration. The bronchoalveolar wash was diluted 10-fold serially. Madin–Darby canine kidney (MDCK) cells were inoculated onto a 24-well microplate, infected with each dilution, and incubated at 37 °C in a carbon dioxide incubator for 48 h, and then the cytopathic effect was observed. The virus titer of each specimen, expressed as the fifty percent tissue culture infection dose ($TCID_{50}$), was calculated by Reed–Muench method. The virus titer in each experimental group is represented by means ± SD of the virus titer per ml of the specimen from all mice in the group.

Statistical analysis. Comparisons of experimental groups were evaluated by Student's *t* test; $P < 0.05$ was considered significant. For survival, the probability was calculated by using Fisher's exact test.

Results

Establishment of mouse model of diabetes

Both female and male BALB/c mice were injected with STZ at a series of doses, and their plasma glucose was determined 1 week after the injection. The results are shown in Table 1. We found that neither female nor male mice injected once with STZ at a dose of 100 mg/kg body weight showed significant elevation of plasma glucose. However, when the dose of STZ for a single injection increased to 150, 180, and 200 mg/kg body weight, the plasma glucose concentration of male mice was elevated significantly 1 week after injection, but that of female mice showed no significant elevation. As shown in Fig. 1, the plasma glucose concentration of male mice was elevated significantly from 3 days after injection once with a high dose (200 mg/kg body weight) of STZ, and the typical symptoms of diabetes, such as thirst, excessive urination, hunger, and weight loss, appeared subsequently. The plasma glucose concentration maintained constantly at a high level (>25 mmol/L) within 70 days after injection. It proved that a stable and persist model of type I diabetes could be established by a single injection with STZ at a high dose in male BALB/c mice. The pathological states of the diabetic mice maintained during the whole study. However, female BALB/c mice were insensitive to STZ.

Susceptibility to influenza virus infection

Diabetic mice and healthy mice were inoculated intranasally with serial 10-fold dilutions of influenza PR8

Table 1
Hyperglycemic response in male and female mice after a single intraperitoneal injection of various doses of STZ

Dose of STZ (mg/kg)	Plasma glucose (mmol/L)	
	Male	Female
Control	6.5 ± 0.9	6.1 ± 0.5
100	10.1 ± 3.2	6.8 ± 0.6
150	20.6 ± 1.6*	5.9 ± 2.6
180	27.1 ± 6.1*	6.3 ± 1.4
200	28.7 ± 3.9*	10.8 ± 9.6

Male and female mice received an intraperitoneal injection with STZ at doses of 100, 150, 180 or 200 mg/kg body weight, respectively. Non-fasting plasma glucose levels were determined 7 days after the injection.

* Plasma glucose concentration was significantly enhanced compared with the control group, $P < 0.01$.

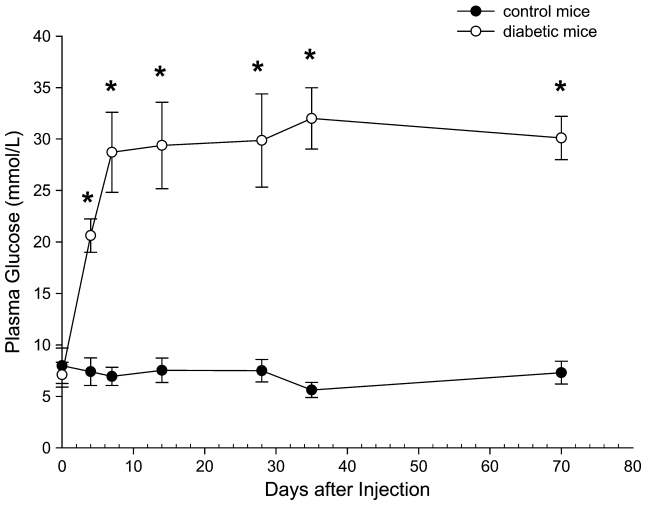

Fig. 1. Plasma glucose concentration in the diabetic mice and the healthy control mice. Male mice were injected with a single high dose of STZ (200 mg/kg body weight) on day 0, and changes in non-fasting glucose levels were measured for 70 days. Data are expressed as means ± SD. *Different from control subjects, $P < 0.01$.

Table 2
Replication of influenza A viruses in the lungs of diabetic and healthy mice

Virus inoculum	Virus titer ($TCID_{50}$)	
	Healthy mice	Diabetic mice
10^{-4}	5.6 ± 0.9	7.0 ± 1.0
10^{-5}	5.3 ± 0.6	6.7 ± 0.6*
10^{-6}	3.9 ± 0.7	5.7 ± 0.6*

Virus titers in the lungs of diabetic and healthy mice were measured 3 days after intranasal inoculation with different doses of PR8 influenza virus. Results are expressed as means ± SD.

* Lung virus titers of diabetic mice were significantly different from those of the corresponding healthy mice, $P < 0.05$.

virus. When the inoculum dose of the virus was increased, a dose-dependent ascent of the mortality was observed. The LD_{50} of influenza virus was calculated as $10^{-5.57}$ for diabetic mice and $10^{-4.81}$ for healthy mice, respectively, indicating that diabetic mice were more susceptible to influenza virus infection than healthy mice.

Virus growth in the lungs of diabetic and healthy mice was measured 3 days after intranasal inoculation with different doses of influenza PR8 virus. As shown in Table 2, virus titers in diabetic group were higher than those in healthy group.

Antibody responses in mice immunized with inactivated influenza virus vaccine

The test consisted of two parts. In part 1, diabetic and healthy mice were immunized once with 0.2, 2, and 6 μg of inactivated influenza virus vaccine separately, and challenged with a lethal dose of homologous influenza virus by nasal drip 4 weeks after the immunization. The sera were collected 3 days after challenge and detected for virus-specific IgG antibody by ELISA. The result showed that the virus-specific IgG antibody levels of healthy mice immunized with 0.2, 2, and 6 μg vaccine were higher than those of diabetic mice (Table 3).

Table 3
Virus-specific IgG titers in the sera of mice vaccinated once with the inactivated influenza virus vaccine

Dose of inactivated vaccine (μg/mouse)	Serum IgG titers; ELISA (2^n)	
Primary	Healthy mice	Diabetic mice
0.2	11.5 ± 1.5*	8.3 ± 2.2
2	13.5 ± 0.6*	9.8 ± 2.9
6	14.5 ± 1.0	13.3 ± 2.1

Healthy and diabetic mice were injected once with the inactivated influenza virus vaccine at a dose of 0.2, 2 or 6 μg separately, and challenged with a lethal dose of PR8 virus 4 weeks after the first immunization. Serum samples from the immunized mice were obtained 3 days after the challenge. Results are expressed as means ± SD.

* Ab titer induced was significantly higher in healthy mice than in corresponding diabetic mice, $P < 0.05$.

In part 2, diabetic and healthy mice were immunized twice, 3 weeks apart, with 0.02, 0.2, and 2 μg of the vaccine separately, and challenged with a lethal dose of homologous influenza virus by nasal drip 1 week after the second immunization. The sera were collected 3 days after challenge and detected for IgG antibody by ELISA. The result showed that, regardless of the dose of vaccine, the IgG antibody levels of healthy mice were significantly higher than those of diabetic mice (Table 4). It proved that, along with the increasing doses of vaccine and times of immunization, the antibody levels of both diabetic and healthy mice increased. The vaccine could induce antibody response in diabetic mice. However, the antibody response level was lower than that in healthy mice.

Table 4
Virus-specific IgG titers in the sera of mice vaccinated twice with the inactivated influenza virus vaccine

Dose of inactivated vaccine (μg/mouse)		Serum IgG titers; ELISA(2^n)	
Primary	Secondary	Healthy mice	Diabetic mice
0.02	0.02	14.5 ± 1.3*	8.8 ± 3.4
0.2	0.2	15.2 ± 0.8*	12.3 ± 1.7
2	2	17.2 ± 0.8*	15.8 ± 0.4

Healthy and diabetic mice were injected twice, 3 weeks apart, with the inactivated influenza virus vaccine at a dose of 0.02, 0.2 or 2 μg separately, and challenged with a lethal dose of PR8 virus 1 week after the second immunization. Serum samples from the immunized mice were obtained 3 days after the challenge. Results are expressed as means ± SD.

* Ab titer induced was significantly higher in healthy mice than in corresponding diabetic mice, $P < 0.05$.

Induction of protection in mice immunized with inactivated influenza virus vaccine

After immunization with influenza vaccine, both diabetic and healthy mice were challenged with a lethal dose (40× LD_{50} of healthy mice) of influenza virus. As shown in Fig. 2, both the percentages of body weight loss of healthy and diabetic mice in immunization group were significantly lower than those in control (unimmunized) group. It indicated the protection provided by the vaccine. However, the periodical observation after challenge revealed that, the diabetic mice immunized once with 0.2 or 2 μg, or twice with 0.02 μg vaccine showed symptoms of severe influenza virus infection, such as ruffled fur and shiver, as compared with those of healthy immunized mice, the symptoms of diabetic mice lasted for a long time, and their physical signs recovered to healthy more slowly.

The survival rate of mice was observed for 21 days to evaluate the protection of induced antibody against virus infection, and their lungs were isolated 3 days after challenge for detection of virus titer. As shown in Table 5, the survival rates of diabetic mice immunized once with 0.2, 2, and 6 μg vaccine were 46.7%, 63.6%, and 100%, respectively. However, all the survival rates of healthy mice immunized once with vaccine at the three doses were 100%. The survival rates of diabetic and healthy mice immunized twice with 0.02 μg vaccine were

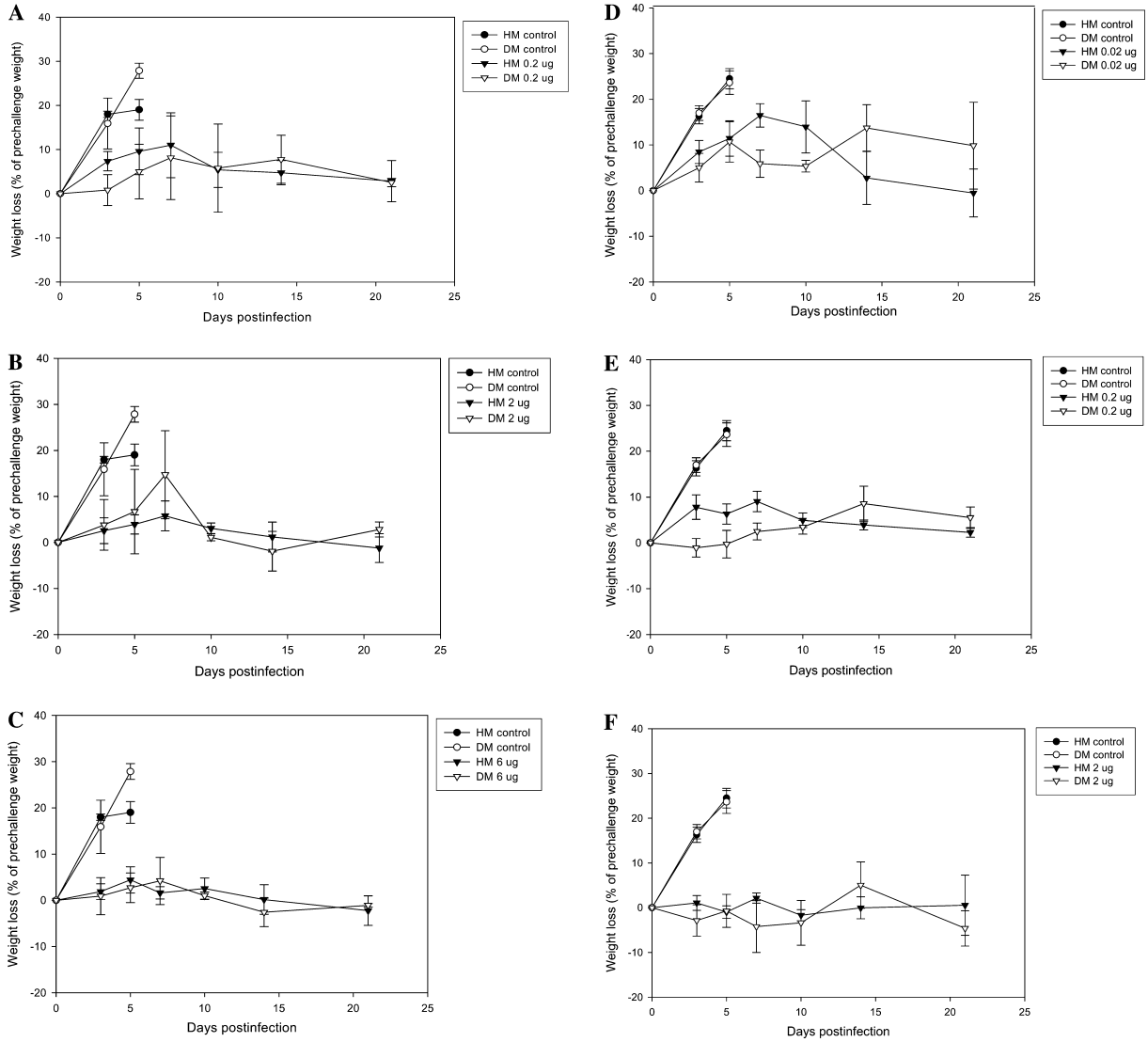

Fig. 2. Body weight changes after the challenge with the PR8 viruses (40× LD_{50}) in the diabetic mice immunized with the inactivated influenza virus vaccine. Mice were immunized once with 0.2 μg (A), 2 μg (B), 6 μg (C) or twice, 3 weeks apart, with 0.02 μg (D), 0.2 μg (E), 2 μg (F) inactivated influenza virus vaccine, respectively. Mice were challenged 4 weeks after the first immunization or 1 week after the second immunization and the body weight was measured from the time of the challenge to 3 weeks after the challenge. Data points represent means ± SD (HM, healthy mice; DM, diabetic mice).

Table 5
Protection against a lethal PR8 virus challenge in the diabetic mice immunized once with inactivated influenza virus vaccine

Dose of inactivated vaccine (μg/mouse)	Mouse	Protection against PR8 virus challenge	
		Lung virus titers ($TCID_{50}$)[a]	Survival mice/total mice (3 weeks)
Control	Healthy	5.2 ± 0.3	0/10
	Diabetic	5.7 ± 0.6	0/10
0.2	Healthy	2.3 ± 0.3[b,c]	10/10[b,c]
	Diabetic	3.7 ± 0.2[b]	7/15[b]
2	Healthy	0.5 ± 0.1[b,c]	10/10[b]
	Diabetic	2.7 ± 0.9[b]	7/11[b]
6	Healthy	0.1 ± 0.1[b]	9/9[b]
	Diabetic	1.2 ± 0.9[b]	9/9[b]

Healthy and diabetic mice were immunized once with 0.2, 2 or 6 μg inactivated influenza virus vaccine and challenged with a lethal dose of PR8 virus. Lung virus titers 3 days later and survival rate of mice 3 weeks after the challenge were measured.

[a] Values represent means ± SD of each group.

[b] Significant difference, compared with corresponding control subjects, $P < 0.05$.

[c] Significant difference, compared with corresponding diabetic subjects, $P < 0.05$.

Table 6
Protection against a lethal PR8 virus challenge in the diabetic mice immunized twice with inactivated influenza virus vaccine

Dose of inactivated vaccine (μg/mouse)	Mouse	Protection against PR8 virus challenge	
		Lung virus titers ($TCID_{50}$)[a]	Survival mice/total mice (3 weeks)
Control	Healthy	5.2 ± 0.3	0/10
	Diabetic	5.7 ± 0.6	0/10
0.02	Healthy	2.7 ± 0.8[b,c]	10/10[b,c]
	Diabetic	4.7 ± 0.6[b]	5/11[b]
0.2	Healthy	0.9 ± 0.7[b,c]	10/10[b]
	Diabetic	2.2 ± 0.3[b]	10/10[b]
2	Healthy	0.1 ± 0.1[b]	10/10[b]
	Diabetic	0.6 ± 0.8[b]	10/10[b]

Healthy and diabetic mice were immunized twice, 3 weeks apart, with 0.02, 0.2 or 2 μg inactivated influenza virus vaccine and challenged with a lethal dose of PR8 virus. Lung virus titers 3 days later and survival of mice 3 weeks after the challenge were measured.

[a] Values represent means ± SD of each group.

[b] Significant difference, compared with corresponding control subjects, $P < 0.05$.

[c] Significant difference, compared with corresponding diabetic subjects, $P < 0.05$.

45.5% and 100%, respectively (Table 6). However, after immunization twice with 0.2 or 2 μg vaccine, both the survival rates of diabetic and healthy mice were 100%. Meanwhile, the lung virus titers of immunized mice were significantly lower than those of unimmunized mice. In each dose group, the lung virus titers of healthy mice were lower than those of diabetic mice. The protection corresponded to the antibody titer induced. It demonstrated that the immunization once with low dose or twice with minimum dose of influenza vaccine provided a full protection for healthy mice, however, only the immunization once with high dose or twice with low dose of vaccine may protect diabetic mice from lethal influenza virus challenge.

Discussion

The patients with diabetes, whether during epidemic or non-epidemic period, belong to high risk population of influenza virus infection [13,14]. It is estimated that the risk of death due to influenza or pneumonia in the adults with diabetes is 70–80% higher than those without the disease [15]. This percentage further increases in the aged patients with diabetes. Since diabetes is a metabolic disease that may cause abnormality of immunologic function including the deficiency of cell-mediated immunity, such as decreases of ratio of CD4 to CD8 lymphocytes, interleukin-2 production, and phagocytic function of monocytes, resulting in the increased risk of viral and bacterial infections [16–19]. Thus, an agreement has been reached for protecting the patients with diabetes from influenza virus infection by vaccination [20]. However, many differences have still existed in the studies on immune response of patients with diabetes after vaccination. It has been reported that some patients may have impaired capacity to produce circulating B cells, and specific IgM and IgG antibodies [16–19,21,22]. The limited data revealed that, in a part of patients with diabetes, antibody responses were not induced following the first vaccination but only induced following the second immunization [22]. The delayed response of patients to vaccination has also been reported [23]. These differences may be due to various factors, such as the type and stages of diabetes, the treatment and composition of vaccine as well as patients' ages, vaccination history, and prevaccination antibody titers. The current immunization schedule (including route, dose, and number of vaccination) of influenza vaccine for the patients with diabetes is the same as that for healthy person. Although it has been reported that the vaccination significantly decreased the hospitalization rate of patients with diabetes during epidemic period of influenza [24], and might induce sufficient immune response in more than 70% of patients [25], there is still lacking definitive proof of the efficacy of influenza vaccination specifically in people with diabetes [10], since discussion of flu vaccine efficacy is more complex than it might seem at first. Many factors, including how well the vaccine strains and the circulating strains match, presence of other cocirculating viruses, and individual difference between the specimens collected, may influence the accuracy of evaluation. So whether vaccination by the current schedule may provide the same protection for the patients with diabetes as that for healthy person is to be further studied.

Unlike most of clinical tests, we established an animal model of diabetes by injecting once with a high dose of STZ into BALB/c mice in this study. Healthy and diabetic mice were immunized once or twice with inactivated influenza virus vaccine at different doses separately, then the mice were challenged with a lethal dose of influenza virus. The immunogenicity of influenza vaccine and the protection of vaccination against lethal virus infection were evaluated by measuring their serum-specific IgG antibody titers, determining the lung virus titers, and observing the survival rate. During the establishment of animal model, we found that a persistently stable animal model of type I diabetes was successfully established with a low mortality by a single injection with STZ at a dose of 150–200 mg/kg body weight into male BALB/c mice. However, using the same procedure, the female mortality was high, and their plasma glucose level showed no significant change. It proved that male BALB/c mice are more sensitive to STZ in comparison with females. Similar results were also observed in other studies [26–29]. Sex hormone level may play an important role in the difference during establishment of animal model [30].

The serum-specific IgG antibody level of diabetic mice 4 weeks after a single immunization with influenza vaccine was significantly lower than that of healthy mice. Kaneshige [6] found that, 4 weeks after initial immunization with influenza vaccine, the hemagglutination inhibition (HI) antibody level of patients with diabetes was significantly lower than that of healthy control. However, the immunization showed no significant influence on hemoglobin A1 (HbA1) level. Diepersloot et al. [5] reported that, after immunization with influenza vaccine, the HI antibody response level of a part of patients with insulin-dependent diabetes mellitus was comparable to that of healthy person, however, the number of patients without antibody response was also significantly larger than that of healthy person. The difference between the antibody responses of patients with diabetes and healthy person was also reported in the studies involving other vaccines [31,32]. On the other hand, some studies also showed that the antibody response level of patients with diabetes was identical to that of healthy person [7,8,33]. The difference among the results of studies on the antibody response of patients with diabetes to influenza vaccine may be related to various factors such as the plasma glucose control states, prevaccination antibody titers, and genetic background [25] of the patients with diabetes. el-Madhun et al. [33] inoculated influenza vaccine to juvenile patients with diabetes and determined the specific antibody-secreting cells (ASC) and relevant antibodies in their peripheral blood. The result showed that, within 17 days after immunization, the B-cell proliferation response of patients was normal, while the magnitude and kinetic profile of serum antibody response of diabetes were similar to those of healthy person. However, only 4–5 patients were included in this study, and their plasma glucose concentrations were under good control. If plasma glucose is poorly controlled, the function of serum-specific antibody may be damaged because of non-enzymatic glycosylation [6]. Besides this, T-cell depletion of patients with type I diabetes has been reported [34]. This may explain the increased incidence of non-responders to influenza antigen.

The result of virus challenge in mice after vaccination was linearly related to the result of ELISA. All the healthy mice immunized once with 0.2, 2, and 6 μg vaccine were fully protected whereas the protective rates of diabetic mice immunized with vaccine at the 3 doses were 46.7%, 63.6%, and 100%, respectively. The IgG antibody levels of diabetic mice immunized twice with 0.02, 0.2, and 2 μg vaccine were significantly different from those of healthy mice. However, the diabetic mice immunized twice with 0.2 and 2 μg vaccine were fully protected, and those with 0.02 μg were partially (44.4%) protected, whereas the healthy mice immunized twice with vaccine at the three doses were fully protected. At present, like healthy person, the patients with diabetes only receive a single dose of trivalent vaccine, which contains 15 μg of each hemagglutinin, annually. Keitel et al.'s [35] study showed that immunization once with a high dose of influenza vaccine provided a better protection for the aged person whose antibody response to the inactivated vaccine was weak, and the corresponding adverse reactions were acceptable. Our study showed that the protective rate of diabetic mice immunized once with a high dose (6 μg) of vaccine was 100%. Taking account of the severe danger of influenza virus infection to the patients with diabetes, the immunization with a higher dose of vaccine is feasible because of its better protective effect and limited adverse reaction.

Kaneshige [6] noticed that, although the antibody level of patients with diabetes 4 weeks after initial immunization was low, it increased significantly 4 weeks after booster immunization and reached a level comparable to that of normal control. Although in this study, the antibody level of diabetic mice immunized twice was lower than that of healthy mice, but the vaccine at a relatively low dose (0.2 μg) provided proper a protection for the diabetic mice. Thus, a booster immunization should be recommended for those patients who show a low antibody response against influenza vaccine. In addition, single administration vaccine (SAV) may simplify the immunization schedule, decrease the cost, and increase the popularization rate of vaccination. This is important to developing countries. However, seeing that the patients with diabetes belong to the high risk population of influenza virus infection, increasing the dose and number of vaccination can provide better protection, thereby decreasing the infection and mortality rates of influenza. Further studies are needed to evaluate the

real effect of vaccine in the patients with diabetes and improve the immunization schedule.

Acknowledgments

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