A DNA Vaccine-Encoded Nucleoprotein of Influenza Virus Fails To Induce Cellular Immune Responses in a Diabetic Mouse Model

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Influenza virus infections cause yearly epidemics and are a major cause of lower respiratory tract illnesses in humans worldwide. Influenza virus has long been recognized to be associated with higher morbidity and mortality in diabetic patients. Vaccination is an effective tool to prevent influenza virus infection in this group of patients. Vaccines employing recombinant-DNA technologies are an alternative to inactivated virus and live attenuated virus vaccines. Internal highly conserved viral nucleoprotein (NP) can be delivered as a DNA vaccine to provide heterosubtypic immunity, offering resistance against various influenza virus strains. In this study, we investigated the efficacy of an NP DNA vaccine for induction of cell-mediated immune responses and protection against influenza virus infection in a mouse model of diabetes. Healthy and diabetic BALB/c mice were immunized on days 0, 14, and 28 by injection of NP DNA vaccine. Two weeks after the last immunization, the cellular immune response was evaluated by gamma interferon (IFN-γ), lymphocyte proliferation, and cytotoxicity assays. The mice were challenged with influenza virus, and the viral titers in the lungs were measured on day 4. Diabetic mice showed significantly smaller amounts of IFN-γ production, lymphocyte proliferation, and cytotoxicity responses than nondiabetic mice. Furthermore, higher titers of the influenza virus were detected after challenge in the lungs of the diabetic mice. The present data suggest that the NP DNA vaccine with the protocol of immunization described here is not able to induce efficient cellular immune responses against influenza virus infection in diabetic mice.

Materials and Methods

Construction of pcDNA3-NP. To construct the DNA vaccine, the NP gene of influenza A/New Caledonia/20/99 H1N1 virus (Obtained from the National Institute for Biological Standards and Control from United Kingdom) was amplified from total RNA of infected Madin-Darby canine kidney (MDCK) cells by reverse transcription-PCR (RT-PCR) with specific primers. The NP gene was confirmed by transient transfection into BHK-21 cells and then inserted into the pcDNA3 plasmid to generate pcDNA3-NP. Expression of the NP protein was confirmed by immunofluorescence staining. BHK-21 cells were transfected with 1 μg of the pcDNA3-NP or pcDNA3 vector using Lipofectamine 2000 (Invitrogen). At 2 days posttransfection, the cells were fixed with 4% formaldehyde-phosphate-buffered saline (PBS). Next, the cells were treated with Triton X-100 for 20 min and then anti-NP monoclonal antibody (Ab) (Serotec), followed by incubation with anti-mouse IgG-rhodamine conjugate (Invitrogen). pcDNA3-NP was amplified in Escherichia coli DH5α and purified using an endotoxin-free plasmid purification kit (Qiagen).

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Establishment of animal model of diabetes. Six- to eight-weeks-old male BALB/c mice were obtained from the animal facilities of the Pasteur Institute (Karaj, Iran). Mice were housed for 1 week before the experiment, given free access to food and water, and maintained in a light/dark cycle with lights on from 6:00 to 18:00 h. All experiments were done according to the Animal Care and Use Protocol of the Pasteur institute of Iran. An animal model of diabetes was successfully established as described previously (30). Briefly, foods were forbidden to mice 12 h before the test. Streptozocin (STZ) was injected intraperitoneally (i.p.) into mice at a dose of 150 mg/kg body weight. After injection with STZ, the plasma samples were collected by tail bleeding for determination of plasma glucose with a digital blood sugar apparatus. The nonfasting plasma glucose concentrations in diabetic mice at 1 week after injection were more than 20 mmol/liter, while nonfasting glucose levels were kept at less than 10.2 mmol/liter in control group of mice.

Immunization. On day 14 of STZ administration, BALB/c mice were injected intradermally with 50 μg of pcDNA3-NP or with PBS (the groups are called pNP-Dia and PBS-Dia, respectively). Healthy control groups were injected with the same protocol (pNP and PBS groups) as well. The vaccination was repeated twice at intervals of 2 weeks in all groups.

Cytotoxicity assay. Two weeks after the last immunization, single-cell splenocyte suspensions were prepared as effector cells without in vitro stimulation (8). The P815 target cells were infected with influenza A/New Caledonia/20/99 H1N1 virus at a multiplicity of infection (MOI) of 5 overnight and washed three times with assay medium. The cytotoxicity activity was measured with the lactate dehydrogenase (LDH) release assay. Supernatants (50 μl/well) were transferred to 96-well flat-bottom plates, and lysis of target cells was determined by the LDH assay kit (Takara, Japan) according to the manufacturer’s instructions. Blank PBS buffer and a solution of 0.1% Triton X-100 in PBS were used as controls. The LDH-mediated conversion of the tetrazolium salt into red formazan product was measured at 490 nm after incubation at room temperature for 30 min. The percentage of specific cytolyis was determined by the following formula.

Specific cytosis (%) = (optical density [OD] of experimental LDH release – OD of spontaneous LDH release of effector cells – OD of spontaneous LDH release from target cells)/(maximum LDH release of target cells – OD of spontaneous LDH release of target cells) × 100%. All experiments were performed in triplicate.

Lymphocyte proliferation assay. The lymphocyte proliferation rate was measured by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, thiazolyl blue (MTT) dye assay. Under sterile conditions, spleens were removed and a single-cell suspension was prepared in phenol red-free RPMI 1640 (Gibco, United Kingdom). Red blood cells (RBCs) were lysed by using 0.75% NH4Cl in Tris buffer (0.02%; pH 7.2). The concentration was adjusted to 1 × 106 cells/ml in phenol red-free RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM l-glutamine, and 25 mM HEPES. One hundred microliters of diluted cell suspensions were dispensed into 96-well flat-bottom culture plates. The mitogen phytohemagglutinin A (PHA) at 5 μg/ml (final concentration) (positive control) or UV-inactivated influenza A/New Caledonia/20/99 H1N1 virus at an MOI of 5 was added to each well, and the volume was adjusted to 0.2 ml. After incubation for 72 h at 37°C in a 5% CO2 humid incubator, cell proliferation was determined by MTT assay (16). Briefly, 20 μl MTT was added in each well, and plates were further incubated at 37°C for 4 h. Following incubation, the supernatant from each well was aspirated carefully, and formazan crystals were solubilized by adding 100 μl dimethyl sulfoxide (DMSO) to each well. The absorbance of each well was then determined at a wavelength of 540 nm. The stimulation index (SI) was calculated as the ratio of the average OD value of wells containing antigen-stimulated cells to the average OD value of wells containing only cells with medium.

IFN-γ assay. Two weeks after the last immunization, spleens of individual mice were removed aseptically and homogenized in RPMI 1640 medium (Gibco-BRL, Germany) supplemented with 10% FCS and antibiotics. Red blood cells were osmotically lysed using ammonium chloride buffer (NH4Cl [0.16 M], Tris [0.17 M]). Tris [0.17 M]. Cells were washed twice with RPMI 1640 and counted, and viability was determined by trypan blue (0.4% [wt/vol]) exclusion. A nominal total of 1 × 106 spleen cells was plated on each well of 24-well plates using RPMI 1640 supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5 × 10−5 M 2-mercaptoethanol. The experiment was performed in duplicate. The cells were restimulated in vitro with UV-inactivated influenza A/New Caledonia/20/99 H1N1 virus at an MOI of 5. Plates were incubated at 37°C in 5% CO2, and 48 h after stimulation, supernatants were removed and kept at −70°C for evaluation of secreted gamma interferon (IFN-γ) levels. The concentration of IFN-γ in the supernatants was estimated using a commercial enzyme-linked immunosorbsent assay (ELISA) kit (R&D systems).

RESULTS

Expression of NP gene in vitro. To confirm the expression of NP, BHK-21 cells were transfected with the pcDNA3-NP plasmid, and expression of NP was confirmed by immunofluorescence (Fig. 1).

Effect of diabetes on cytotoxicity responses induced by pNP immunization. The cytotoxicity response in immunized mice was examined using the LDH release assay. As shown in Fig. 2, the cytotoxicity response was significantly lower in the pNP-Dia immunized mouse group than in pNP-immunized healthy mice (P < 0.001).

Effect of diabetes on lymphocyte proliferation responses induced by pNP immunization. Splenocytes from all four groups of mice were tested for their lymphocyte proliferative response. The results (Fig. 3) indicate a significant decrease in the proliferation index of the lymphocytes in the pNP-Dia group compared to that of the pNP group (P < 0.001).

Effect of diabetes on IFN-γ production induced by pNP immunization. Spleen cells were collected 10 days after the last immunization. The cultured cells were restimulated with UV-inactivated influenza A/New Caledonia/20/99 H1N1 at an MOI of 5, and supernatants were harvested after 48 h. As shown in Fig. 4, IFN-γ production was significantly lower in the pNP-Dia immunized mouse group than in pNP-immunized healthy mice (P < 0.001).
Effect of immunization of diabetic mice on viral clearance from the lungs. To assess the effect of pNP DNA vaccination on the virus clearance rate in diabetic mice, they were challenged with 100 MID\textsubscript{50} of influenza A/New Caledonia\textsubscript{2009} H1N1. As shown in Fig. 5, the influenza virus titer was significantly lower in the pNP-immunized healthy mouse group than in the PBS-immunized healthy group ($P < 0.006$), but there were no significant differences between virus titers in the pNP-Dia and PBS-Dia groups. Furthermore, the influenza virus titer was significantly higher in pNP-Dia immunized mouse group than in pNP-immunized healthy mice ($P < 0.001$).

FIG. 2. Cytotoxicity responses induced by pNP immunization in diabetic mice. Healthy and diabetic mice were immunized three times intradermally with pcDNA3-NP (pNP and pNP-Dia groups, respectively) or PBS (PBS and PBS-Dia groups, respectively) on days 0, 14, and 28. Two weeks after final immunization, splenocytes from immunized and mock-immunized mice were prepared as described in Materials and Methods. LDH release assays were performed in triplicate with splenocytes as effector cells and A/New Caledonia\textsubscript{2009} H1N1 p815 cells as target cells. All experiments were performed more than three times, and each group consisted of five mice. The cytotoxicity activity of the pNP group was significantly higher than that of the pNP-Dia group ($***$, $P < 0.001$). There was no significant difference between the cytotoxicity responses of the pNP-Dia and PBS-Dia groups. E/T, effector-to-target-cell ratio.

FIG. 3. Lymphocyte proliferation responses induced by pNP immunization in diabetic mice. Healthy and diabetic mice were immunized three times intradermally with pcDNA3-NP (pNP and pNP-Dia groups, respectively) or PBS (PBS and PBS-Dia groups, respectively) on days 0, 14, and 28. Two weeks after final immunization, spleens of individual mice (five per group) were removed, and lymphocyte proliferation was evaluated using the MTT method. Values are the means ± standard errors of the means for five experiments. The lymphocyte proliferation of the pNP group was significantly higher than that of the pNP-Dia group ($***$, $P < 0.001$). There was no significant difference between the lymphocyte proliferation of the pNP-Dia and PBS-Dia groups.

FIG. 4. IFN-\textgamma\ production induced by pNP immunization in diabetic mice. Healthy and diabetic mice were immunized three times intradermally with pcDNA3-NP (pNP and pNP-Dia groups, respectively) or PBS (PBS and PBS-Dia groups, respectively) on days 0, 14, and 28. Two weeks after final immunization, spleens of individual mice (five per group) were removed, and IFN-\textgamma\ production was measured with an ELISA kit. IFN-\textgamma\ production of the pNP group was significantly higher than that of the pNP-Dia group ($***$, $P < 0.001$). There was no significant difference between the IFN-\textgamma\ production of the pNP-Dia and PBS-Dia groups.

FIG. 5. Replication of influenza A viruses in the lungs of diabetic and healthy immunized mice. Healthy and diabetic mice were immunized three times intradermally with pcDNA3-NP (pNP and pNP-Dia groups, respectively) or PBS (PBS and PBS-Dia groups, respectively) on days 0, 14, and 28. Two weeks after final immunization, virus titers in the lungs of diabetic and healthy mice (five per group) were measured 4 days after intranasal inoculation with influenza virus. Values are the means ± standard errors of the means for three experiments. The horizontal line shows the minimum inoculation of decimal dilutions (10\textsuperscript{-2}) of lung in MDCK cells. $***$, lung virus titers for pNP were significantly lower than those for the pNP-Dia group ($P < 0.001$), PBS group ($P = 0.006$), and PBS-Dia group ($P < 0.001$). $**$, lung virus titers of PBS group were significantly lower than those of the PBS-Dia group ($P = 0.009$). There was no significant difference between the lung virus titers of the pNP-Dia and PBS-Dia groups.
DISCUSSION

The present study is the first to examine the effects of diabetes on the cell-mediated immune response induced by genetic immunization against influenza virus infection. Patients with diabetes belong to a high-risk population of influenza virus infection (1, 28). However, the current immunization schedule of influenza vaccination for patients with diabetes is the same as that for healthy persons. Diepersloot et al. showed that the patients with diabetes had a poor immune response after immunization with inactivated influenza virus vaccine (3). Furthermore, due to a major concern with the emergence of lethal influenza pandemics, such as that of avian-flu A/H5N1 virus, it is evident that new vaccines are needed to ensure optimal protection against all strains of influenza viruses (18, 25). Cell-mediated immunity has a very important role in inhibiting viral replication and clearance of influenza A virus infections (8, 29). Vaccines designed to induce cellular immune responses are being investigated to create a universal vaccine (13, 18). DNA vaccination with influenza virus NP has been studied in animal models and induced cell-mediated immune responses (7, 12). Data presented in this article show that diabetes reduces the capacity of the pNP DNA vaccine to induce cytotoxicity responses, lymphocyte proliferation, and IFN-γ production. In previous studies, it was demonstrated that to be effective, DNA vaccines need to induce optimal functional activities of many components of the immune system, including T CD4+, T CD8+, and B cells (11, 17). On the other hand, several studies have shown that diabetes causes an abnormality in many immunologic parameters, including cytokine production, such as decrease of interleukin-2, interleukin-12, and IFN-γ production, aberrancies in the maturation of dendritic cells (DCs), and impaired function of CD4+ T cells (6, 9, 14, 15, 20, 24, 30). Clinical studies have suggested the importance of higher vaccination coverage in diabetic patients to reduce the impact of influenza virus infection on morbidity and mortality of infected patients (3, 28). Furthermore, Zhu et al. showed that antibody responses in diabetic mice were reduced and only immunization with higher doses of inactivated influenza virus vaccine provided protection against lethal influenza virus challenge in diabetic mice (31). Another study showed that influenza replication increased in lungs of diabetic mice (21).

Cellular responses are very important in clearing influenza A virus infections in mice (7, 27). In our experiment, reduced viral clearance from lungs of pNP-immunized diabetic mice in comparison to healthy mice may be due to suppression of the cytotoxic responses.

In summary, the present data suggest that the pNP DNA vaccine with the protocol of immunization described here is not able to induce optimal cell-mediated immunity in diabetic mice. These results provide new insights into the field of DNA immunization for infectious diseases in diabetes. Inclusion of Th1 cytokine genes in DNA constructs and employment of other DNA immunization protocols may improve vaccine potency and will be investigated in future studies. Furthermore, follow-up studies are needed to test the effects of diabetes on the potency of genetic immunization in induction of humoral immune responses.

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