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Intranasal vaccination with p277 tandem repeat sequences carried by Hsp65 prevented type 1 diabetes in NOD mice

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ABSTRACT **AIM**: To improve the prevent efficacy of peptide p277 in autoimmune diabetes. **METHODS**: The recombinant expression plasmid pET28-Hsp65-6× p277 was constructed by inserting 6× p277 which were amplified by PCR into the vector pET28-Hsp65. The plasmid pET28-Hsp65-6× p277 was transformed into *E. coli* BL21 (DE3) and the fusion protein (Hsp65-6× p277) was expressed effectively as soluble protein after inducing by lactose. The fusion protein was purified and then used to immunize 4-week old female NOD mice with three times of i.n. inoculations in the absence of adjuvants. Serum samples from the immunized mice were collected at monthly interval. The concentrations of blood glucose and antibodies were measured by automatic analyzer. **RESULTS**: Administration with the Hsp65-6× p277 to NOD mice could prevent the development of diabetes. **CONCLUSION**: The fusion protein Hsp65-6× p277 might be further developed to a vaccine against insulin-dependent diabetes mellitus.

KEY WORDS heat shock protein 65; p277; insulin-dependent diabetes mellitus; immune

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It is now generally agreed upon that type 1 diabetes mellitus, also called IDDM (insulin-dependent diabetes mellitus), is caused by an autoimmune process in which T cells invade the pancreatic islets and destroy the insulin-producing β cells[1]. The 60-kD heat shock protein (Hsp 60) has gained notoriety for its apparent involvement in the disease[2]. A peptide, p277, from the sequence of the mammalian Hsp 60 molecule was identified as containing a target epitope for diabetogenic T cells[3]. Prediabetic nonobese diabetic (NOD) mice manifest spontaneous antiboby formation and T cell responses to Hsp 60 and to peptide p277 prior to the onset of diabetes[4,5]. Vaccination with the p277 peptide by itself can induce resistance to spontaneous diabetes in NOD mice[6].

The immune response has an important role in the pathogenesis of type 1 diabetes mellitus. Inflammation and autoimmunity to heat shock proteins are part of the progression of the disease[7]. In this regard, immune-based therapies can mitigate the disease process. More and more evidence supports that mucosal administration of autoantigens can decrease organ-specific inflammation and disease in several models of autoimmunity, such as atherogenesis[8]. In this work, in order to improve the efficacy of the peptide p277 against diabetes, a fusion protein of Hsp65-6× p277 was constructed by genetic engineering.

1 MATERIALS AND METHODS

1.1 Materials 4-week-old female NOD/Lt mice were purchased from Shanghai Slaccas Experiment Animal Limited Company (SCXK 2003-0003). The incidence of diabetes in the colony is 70%-80% at 7 months of age. The protein rhVEGF-p277 and HSP65 were gifts from ZHU Ai-hua (PhD) in our laboratory. All other chemicals and

reagents were obtained from chemical or biological companies (Takara, AMRESCO, Sigma, *et al*). All the chemical reagents used in the experiment were analytical grade.

1.2 Construction of the vector Hsp65-6× p277

Bacterial genomic DNA containing the Hsp65 gene was extracted from Mycobacterium tuberculosis var. bovis as described[9]. The Hsp65 gene was amplified by PCR from the bacterial genomic DNA template using H1 (5'-TTCGCCATGGCCAAGACAATTGCGTACG-3') and H2 (5'-AAAAAGATCTGCGAAATCCATGCCACCCATGT-3') as forward and reverse primers. Amplified Hsp65 DNA fragments (1.6 kb) were analyzed in 1.0% (*w*/*v*) agarose gel (Fig 1). After purification, the amplified fragments were digested by endonuclease NcoI and BglII and then inserted into the pET28a previously digested by NcoI and BamHI to form a new expression vector pET28-Hsp65. The p277 gene was amplified by PCR using A1 (5'-ATGGGCTAGCGTTCTGGGTGGTGGTGTT-GCTCT-GCTGCGCGTTATCC-CGGCTCTG-3') and A2 (5'-TAT-GTCTAGAATCTTCGTTAGCTGGGGTCAGGGAGTCCAA-AG-CGGGGATAAC GC G-3') as forward and reverse primers, respectively. Amplified p277 DNA fragments were analyzed in 1.0% (*w*/*v*) agarose gel (Fig 2). After purification, the amplified fragments were digested by endonuclease NheI and XbaI and then inserted into the pET28-Hsp65 previously digested by NheI to form a new expression vector pET28-Hsp65-p277. The plasmid pET28-Hsp65-p277 still has NheI site. The amplified fragments p277 still can be inserted into pET28-Hsp65-p277. The same process was repeated six times. Thus a vector expressing the fusion protein Hsp65-6× p277 was successfully constructed. It contained Hsp65 and six times 25-amino-acid residue of p277 (PVLGGGVALL-RVIPALDSLTPANED). The plasmid pET28-Hsp65-6× p277 was transformed into *E. coli* BL21 (DE3) and the resultant colonies were screened by kanamycin resistance, and then sequenced to confirm correct insertion. A schematic presentation of Hsp65-6× p277 is shown in Fig 3.

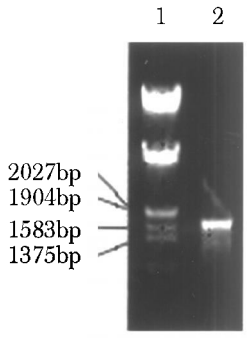

Fig 1 PCR products analyzed by 1.0% agarose electrophoresis

Lane 1: λDNA/EcoRI/HindIII; Lane 2: PCR products (BCG as template)

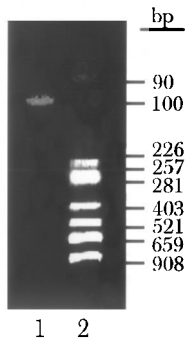

Fig 2 PCR products analyzed by 1.7% agarose gel electrophoresis

1: DNA marker (pBR322/AluI); 2: p277 DNA fragment amplified by PCR with A_1, A_2 as up- and down-stream primers

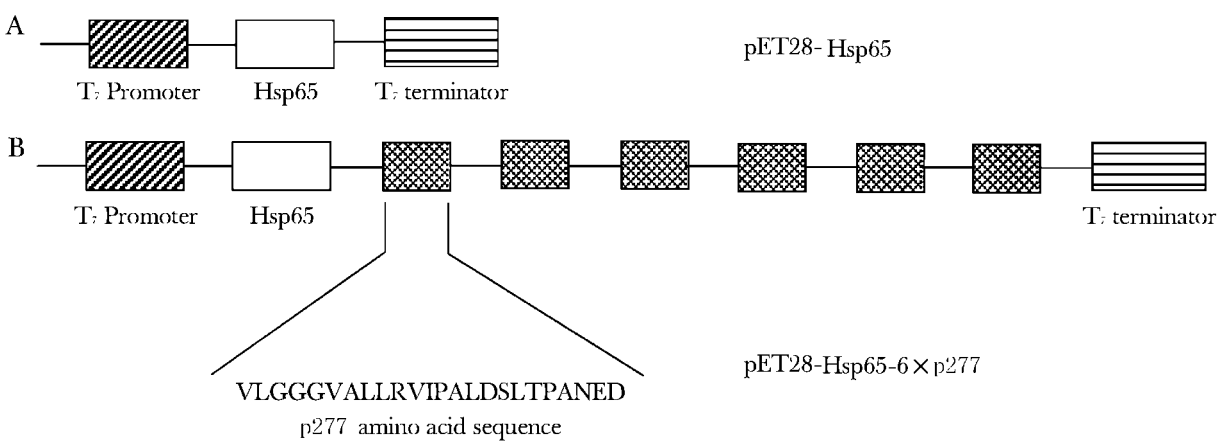

Fig 3 Schematic diagram of the plasmid of Hsp65 and Hsp65-6× p277

Hsp65 (A) and Hsp65-6× p277 (B) fusion protein were expressed in *E. coli* using the pET28 vector under the direction of the T7 promoter. The T7 promoters are represented in hatched boxes. The Hsp65 gene is represented by open box. In addition, the p277 are represented by cross boxes. The T7 terminator is represented by linear boxes.

1.3 Expression and purification of Hsp65-6× p277 fusion protein In our laboratory, several fusion proteins had been prepared successfully[10]. The same strategy was used in expression and purification of the fusion protein Hsp65-6× p277. The resultant bacteria were grown in Luria-Bertani (LB) medium supplemented with kanamycin (50 $\mu g \cdot ml^{-1}$) and incubated for 4 h after induction with lactose when the optical density value at 600 nm (OD_{600}) of the bacterial suspension reached 0.50. Cultures were harvested analyzed by 12% SDS-polyacrylamide gel electrophoresis (PAGE).

After harvest, cells (wet weight of 10.0 g) were suspended in 100 ml of 10 $mmol \cdot L^{-1}$ PB supplemented with 40 μl lysozyme (10 $\mu g \cdot ml^{-1}$) and 10 μl DNaseI (1 $mg \cdot ml^{-1}$) at 37 ℃ for 30 min with shaking, then centrifuged at 12 000× g for 15 min. The supernatant was precipitated using ammonium sulfate at 20%—40% saturation. Approximately 80% of Hsp65-6× p277 was precipitated. The precipitate was solubilized in 100 ml PBE (10 $mmol \cdot L^{-1}$ PB [pH 7.4] and 5 $mmol \cdot L^{-1}$ EDTA). The solution was dialyzed to double-distilled H_2O for 24 h, and then loaded in a DEAE52-cellulose (Whatman, USA) column pre-equilibrated with PBE. The column was washed with the equilibration buffer until the absorbance value at 280 nm was below 0.2 Hsp65-6× p277 was eluted using a linear gradient of 50—500 $mmol \cdot L^{-1}$ NaCl in PBE protein-containing fractions were collected and then analyzed by 12% SDS-PAGE.

1.5 Vaccination 4-week-old female NOD mice were divided into three groups of 10 animals each (n=10 per group). Two groups, respectively, received three i.n. inoculations of 100 μg of purified Hsp65-6× p277, HSP65 solubilized in sterilized phosphate-buffered saline at 4, 7 and 10 weeks of age; the control mice received three i.n. inoculations of phosphate-buffered saline.

1.6 Hyperglycemia After the final administration, serum samples were collected at monthly interval. The concentration of blood glucose was measured by Hitachi automatic analyzer (model-7150, Tokyo, Japan). A mouse was considered to be diabetic if the blood glucose level was > 11 $mmol \cdot L^{-1}$ on two consecutive examinations. The data were expressed as ($\bar{x} \pm s$). Paired Student's t-test was used.

1.7 ELISA analyses of specific antibodies against p277 or Hsp65 Purified rhVEGF-p277 or Hsp65 was diluted with 0.1 $mmol \cdot L^{-1}$ carbonate-bicarbonate buffer (pH 9.5) and applied to flat-bottom ELISA plates (Costar, USA) at 100 μg per well. ELISA plates were incubated overnight at 4 ℃. Wells were blocked with 10 $mmol \cdot L^{-1}$ phosphate buffer (pH 7.4) containing 5% (w/v) bovine serum albumin (Sigma, USA) for 1 h. Then the wells were incubated with 1∶100 two-fold dilutions of serum samples collected from immunized animals overnight at 4 ℃. The wells were washed three times with 10 $mmol \cdot L^{-1}$ phosphate buffer (pH 7.4) containing 0.1% (v/v) Tween-20. Each well was added 100 μl goat anti-mouse IgG horseradish peroxidase (Promega, USA) which was diluted 1∶20 000 with 10 $mmol \cdot L^{-1}$ phosphate buffer (pH 7.4) containing 1% (w/v) BSA and then incubated at 37 ℃ for 1 h. The wells were washed intensively six times with 10 $mmol \cdot L^{-1}$ phosphate buffer (pH 7.4) containing 0.1% (v/v) Tween-20. To each well, 50 μl 0.01% (w/v) 3, 3', 5, 5'-tetramethylbenzidine and 50 μl 0.2 $mol \cdot L^{-1}$ Na_2HPO_4 — 0.1 $mol \cdot L^{-1}$ citrate buffer (pH 5.5) containing 0.24% (w/v) H_2O_2-urea was added and incubated at 37 ℃ for 15 min. The reaction was stopped by adding 50 μl 2 $mol \cdot L^{-1}$ H_2SO_4. OD_{450} value of the reaction solution was measured by multiskan spectrum microplate spectrophotometer (Thermo, USA).

2 RESULTS

2.1 Construction of the vector Hsp65-6× p277 Hsp65 gene (1.6 kb) was successfully amplified using BCG genomic DNA as the template and two primers designed on the basis of Hsp65 DNA sequence, and inserted into pET28a to form the pET28a-Hsp65 plasmid. The p277 gene was successfully amplified using the two oligonucleotides and inserted into pET28a-Hsp65 to form the pET28a-Hsp65-6× p277 plasmid. The amount of Hsp65-6× p277 in engineered *E. coli* strains increased to the highest level when cells were further cultured in LB medium for 5 h after lactose induction. The fusion protein Hsp65-6× p277 was expressed as soluble proteins in engineered stains (shown in Fig 4).

2.2 Expression and purification of Hsp65-6× p277 fusion protein After impurity was partially removed by precipitation in 25% saturated ammonium sulfate, about 80% of Hsp65-6× p277 was precipitated with 45% saturated ammonium sulfate. The precipitations was solubilized and dialyzed to 10 $mmol \cdot L^{-1}$ PB to remove most of ammonium sulfate in solutions. Then it was further purified by DEAE52-cellulose. The fusion protein was eluted at 100—130 $mmol \cdot L^{-1}$ NaCl in PB. It was purified to

approximate homogeneity using the above procedure (Fig 5).

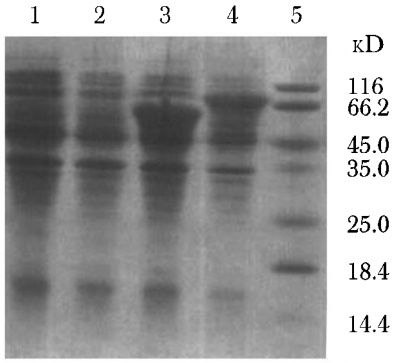

Fig 4 Expression of Hsp65-6× p277

Hsp65-6× p277 fusion protein was analyzed in 12% SDS-PAGE gel stained with Coomassie blue. Lane 1: total proteins of *E. coli* BL21 (DE3); lane 2: Total proteins of *E. coli* BL21 (DE3) containing pET28a induced by lactose; lane 3: total proteins of *E. coli* BL21 (DE3) containing pET28-Hsp65 induced by lactose; lane 4: total proteins of *E. coli* BL21 (DE3) containing pET28-Hsp65-6× p277 induced by lactose; lane 5: protein molecular weight markers

2.3 Administration of Hsp65-6× p277 could prevent diabetes in the absence of adjuvants We immunized NOD female mice three times with Hsp65-6× p277 in PBS at 4, 7 and 10 weeks of age respectively. Control mice treated with PBS for spontaneous IDDM appearing. The results indicated that the fusion protein Hsp65-6× p277 could reduce significantly the severity of spontaneous diabetes developing (Tab 1).

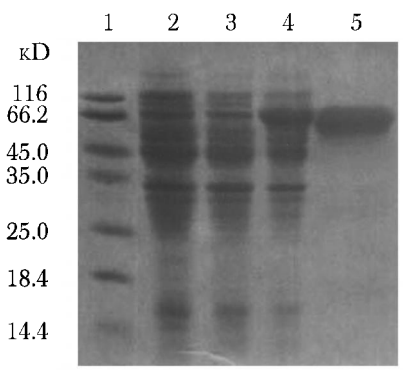

Fig 5 Purification of Hsp65-6× p277

Hsp65-6× p277 fusion protein was analyzed in 12% SDS-PAGE gel stained with Coomassie blue; Lane 1: protein molecular weight markers; lane 2: total proteins of *E. coli* BL21 (DE3); lane 3: total proteins of *E. coli* BL21 (DE3) containing pET28a induced by lactose; lane 4: total proteins of *E. coli* BL21 (DE3) containing pET28-Hsp65—6× p277 induced by lactose; lane 5: purified Hsp65-6× p277

2.4 Specific anti-p277 antibody formed in NOD mice ELISA assay showed that the serum samples collected from mice immunized with Hsp65—6× p277 contained anti-p277 antibodies. The anti-p277 antibodies could be detected as early as the age of 7-week-old post the first immunization (Fig 6). Specific antibodies against p277 continued to be made at high levels and last for more than 12 weeks. ELISA assay showed that the serum samples collected from mice immunized with Hsp65-6× p277 by intranasal administration contained anti-Hsp65 antibodies also (Fig 7).

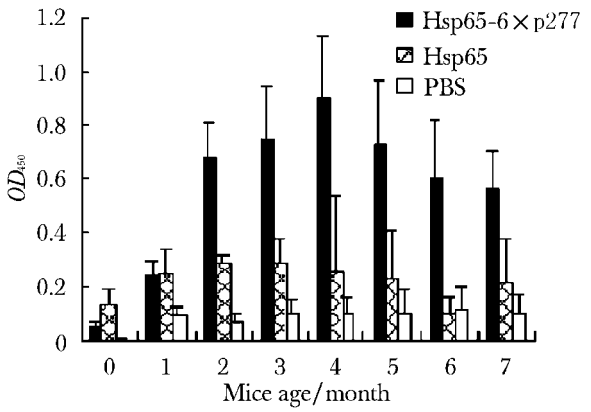

Fig 6 Anti-p277 antibodies in immunized mice

Anti-p277 antibodies were detected by ELISA at several time points. OD_{450} value of the reaction solution was indicated with ordinate, and the ages of mice were indicated with abscissa. Serum samples from mice immunized with the fusion protein Hsp65-6× p277; Serum samples from mice immunized with the fusion protein Hsp65; Serum samples from mice in control group. High level anti-p277 antibodies have been induced in Hsp65-6× p277-treated mice compared with PBS-treated mice ($P < 0.01$).

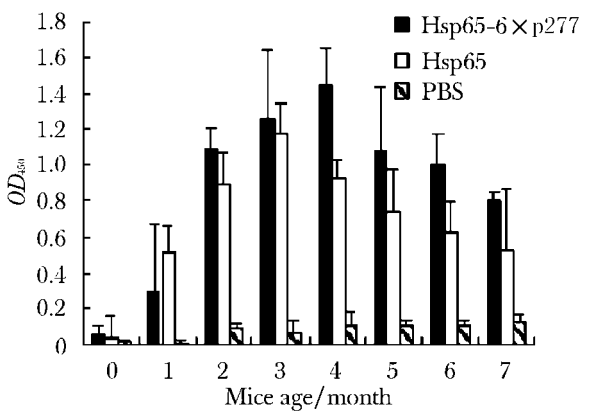

Fig 7 Anti-Hsp65 antibodies in immunized mice

Anti-Hsp65 antibodies were detected by ELISA at several time points. A_{450} value of the reaction solution was indicated with ordinate, and the ages of mice were indicated with abscissa.

Tab 1 Effect of administering antigens on development of diabetes($\bar{x}\pm s$, $n=10$, mmol·L^{-1})

Group	Treatment Antigens	Spontaneous IDDM					
		6 months		7 months		8 months	
		Incidence	Blood glucose	Incidence	Blood glucose	Incidence	Blood glucose
A	PBS	2/10	8.4±14.5	6/10	18.2±4.8	8/10	20.9±8.5
B	Hsp65	2/10	12.8±16.3	3/10	13.6±14.8	5/10	15.3±14.9
C	Hsp65-6× p277	0/10	3.71±2.90	0/10	4.28±3.50	0/10	5.09±2.30

4-week-old NOD female mice were treated by i.n. inoculation with PBS or with 100 μg of the indicated antigens. The mice were inoculated other twice at 7 weeks and 10 weeks of age respectively. Spontaneous IDDM was assayed at ages 6, 7 and 8 months. A mouse was considered to be diabetic if the blood glucose level was >11 mmol·L^{-1} on two consecutive examinations

3 DISCUSSION

Studies showed that the peptide p277, an immunomodulatory peptide from Hsp60, could arreste β-cell destruction and maintain insulin production in newly diabetic NOD mice and in patients with newly diagnosed (< 6 months) type 1 diabetes[11,12]. The p277 peptide can be exploited to regulate the spontaneous autoimmune process of β-cell destruction in NOD mice[13]. But the peptide, p277, only contained 25-amino-acid residue (PVLGGGVALLRVIPALDSLTPANED), which apparently is very weak antigen on its own. The weak antigenicity of the self-antigen could be overcome by several methods such as linking the self-antigen with strong T-helper epitopes[14], carrying the self-antigen by carrier molecules[15] or other methods. In this paper we use the fusion protein strategy to enhance the antigenicity of p277. Mycobacterium heat shock protein-65 was chosen as carrier and six copies p277 as strong T-helper epitopes. Special immune response against p277 was successfully triggered in body after immunizing mice with the Hsp65-6× p277 without any adjuvants.

In recent years more and more self-antigens have been selected for vaccine development, such as anti-luteinizing hormone releasing hormone (LHRH), anti-human human chorionic gonadotrophin (hCG)[16] and other vaccines, which have been tested for immunocontraception in primates or other animals. Administration of the p277 peptide by itself can induce resistance to spontaneous diabetes in NOD mice[15], but the weak immunogenicity limits its efficiency. Although the 65 kD heat shock protein (Hsp65) of mycobacterium tuberculosis could be used to vaccinate against autoimmune diabetes in NOD mice[4] also, it lied on the approach of administration and it needed adjuvants. Now we reported that the fusion protein Hsp65-6× p277 could reduce significantly the severity of spontaneous diabetes developing in NOD mice even in the absence of adjuvants, and the efficiency outgo administration of the p277 peptide by itself only.

Mucosal administration of autoantigens not only has proven effective as a treatment for a large number of animal models of autoimmune disease[17] but also has provided numerous advantages over conventional subcutaneous injection. These include convenience, safety and acceptability by more people. However, despite these attractive features, in practice, it is rather difficult to stimulate strong immune responses by mucosal administration of antigens. Our study showed that using Hsp65 as a carrier for presenting repeat p277 epitopes could successfully stimulate long-lasting immune response against p277 in mucosal administered mice even in the absence of adjuvants. It provides convenience for human to develop vaccine in the future.

Special immune response against p277 and Hsp65 was successfully triggered in body after immunizing mice with the Hsp65-6× p277 without any adjuvants. The titer of anit-p277 antibodies could be last long term. NOD mice vaccinated with the fusion protein Hsp65-6× p277 could decrease significantly the incidence of spontaneous IDDM. It was verified that the strategy was effective for developing vaccines. The fusion protein Hsp65-6× p277 has the potential to be developed as a suitable vaccine against insulin-dependent diabetes mellitus.

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鼻粘膜免疫融合蛋白 Hsp65-6× p277 预防 NOD 小鼠 1 型糖尿病的发生

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摘要　目的: 提高多肽 p277 的免疫原性, 从而提高其对自身免疫性糖尿病的预防作用。方法: 将 p277 6 次重复与 Hsp 65 融合置于 pET28a 中构建重组 Hsp 65-6× p277 表达质粒。该重组质粒在大肠杆菌 BL21 中以高效可溶形式表达。依次通过细胞裂解、硫酸铵沉淀、双蒸水透析、DEAE 纤维素 52 柱层析纯化获得目的蛋白。用纯化后的融合蛋白 Hsp 65-6× p277 通过鼻腔给药方式, 在不添加任何佐剂的情况下 3 次免疫 4 周龄雌性 NOD 小鼠。每月眼角取血, 检测抗体和血糖浓度。结果: 初步药效学实验表明融合蛋白 Hsp 65-6× p277 可抑制 NOD 小鼠中 1 型糖尿病的发生。结论: 融合蛋白 Hsp 65-6× p277 有可能发展成为一种具有防治胰岛素依赖性糖尿病作用的疫苗。

关键词　热休克蛋白 65; p277; 胰岛素依赖性糖尿病; 免疫