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**References**

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A Th1-Recognized Peptide P277, When Tandemly Repeated, Enhances a Th2 Immune Response toward Effective Vaccines against Autoimmune Diabetes in Nonobese Diabetic Mice

Liang Jin,* Aihua Zhu,† Yu Wang,* Qingmei Chen,* Qiyan Xiong,* Jianping Li,* Yunxia Sun,* Taiming Li,* Rongyue Cao,* Jie Wu,* and Jingjing Liu2*

Subunit immunogens containing tandemly repeated copies of T and B cell epitopes have been shown to be more immunogenic than the respective immunogen containing only a single copy of the sequence. To investigate whether the increased copies of the Th2-activated peptide sequence will enhance the Th2-like immune response, we compared the cytokine secreted in mice that inoculated with two immunogens containing one or six tandemly repeated copies of a Th2-activated peptide sequence P277. Immunization of mice with a 6×P277 fusion protein elicited much higher levels of Th2-type cytokines and lower Th1-type cytokines than with a fusion protein with one P277 peptide. The data of tandemly repeated peptide P277 potentiate the anti-inflammatory in NOD mice, most likely associated with a Th1 to Th2 cytokine shift specific for the autoimmune T cells, which suggested that application of multiple tandem repeats of a Th2-activated epitope is an efficient method to enhance the anti-inflammatory immune response by shifting the immune response from Th1-like to Th2-like. The subunit immunogens containing tandemly repeated copies of peptide P277 might be effective vaccines against autoimmune diabetes in NOD mice. The Journal of Immunology, 2008, 180: 58–63.

Recent studies of the factors important in the pathogenesis of the autoimmune diabetes have implicated certain islet proteins as early targets of the autoreactive T cells (11), one of which is the 60-kDa heat shock protein (HSP60)3 (12). Peptide P277 is a 24-aa fragment of the HSP60 molecule, first discovered to be an Ag for diabetogenic T cell clones in NOD mice (13). Maryam et al. (14) showed that the peptide P277 recognized by Th1 cell clone. Therapeutic vaccination with P277 can arrest the spontaneous diabetogenic process associated with a Th1 to Th2 cytokine shift specific for the autoimmune T cells (15, 16). The results suggest that the peptide P277 leads to activation of T cells specific for Th2 clone.

In recent years, it has been established that immunogens containing tandemly repeated B or T cell epitopes increased immunogenicity associated with increased copies of the target sequence (17, 18). We have been interested in whether the increased Th2 immune response is also attributable to copies of the Th2-activated peptide sequence. Therefore, two immunogens were constructed containing either a single or six tandemly repeated copies of the Th2-activated peptide P277 (19). The hypotheses have been examined by comparing the cytokine secreted in the mice that inoculated with the two immunogens. It is shown that the effectiveness of anti-autoimmune diabetes of the tandem repeat construct is superior to that of the single copy construct. This effect appears to result from an enhanced recognition of the tandemly repeated P277 sequence by the Th1 cells, leading secondarily to more efficient inhibition of the Th1 cells and the consequent increased recruitment of Th2 cells. These results demonstrated an important role of the multiple tandem repeats of a Th2-activated peptide in enhancing the Th2 immune response.

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Materials and Methods

Mice

Four-week-old female NOD/Lt mice, with average weight of 18.8 g, were purchased from Slaccas Experimental Animal (SCXK 2003-0003) and maintained under pathogen-free conditions at the Animal Center of our institute. The onset of clinical insulitis begins at 3 mo of age and reaches a cumulative incidence of 80% or greater by 8 mo of age in this colony for females.

Antigens

The fusion proteins HSP65-6P277, HSP65-P277, and HSP65 were prepared as described (19). Briefly, a single or six tandemly repeated copies of the peptide were fused to mycobacterial HSP65 to construct the fusion protein HSP65-6P277 and HSP65-P277. The resulted recombinant proteins were expressed in Escherichia coli and subsequently purified as immunogens in the studies. All proteins were dissolved in sterilized PBS (pH 7.4). The sequence of P277 residues (437–460 in the H-HSP65 sequence) is VLGGGCALLRCIPALDSLTPANED. Purified human rVEGF-P277 was supplied by Z. Aihua (Shanghai, China) in our laboratory. OVA, Con A, and BSA were purchased from Sigma-Aldrich.

Vaccination

Four-week-old female NOD/Lt mice were divided into 4 groups of 10 animals each (n = 10 per group). Three groups of mice received three in trans inoculations of immunogen containing 100 μg of purified HSP65-6P277, HSP65-P277, and HSP65, respectively, at 4, 7, and 10 wk of age; the control mice received three in trans inoculations of PBS (pH 7.4). The serum samples were collected before every inoculation. After the third administration, serum samples were collected at monthly interval for 5 mo. All collected samples were stored at −20°C until use.

Hyperglycemia

The mice blood glucose level was measured monthly by Hitachi automatic analyzer (model 7150). A mouse was considered to be diabetic if the blood glucose level was ≥11 mM on two consecutive examinations.

Pancreas histopathology

Mice from each treatment group were analyzed at the age of 8 mo, when almost all the control NOD mice receiving phosphate buffer were sick. The pancreata were fixed with 10% formalin solution. Formalin-fixed paraffin blocks of pancreas tissue were sectioned with a microtome and stained with H&E (Sangon). The degree of insulitis was evaluated by a pathologist in a blinded fashion. The average degree of insulitis was assessed over 20 islets scored per pancreas. Each islet was classified as follows: clear, if no infiltrate was detected; mildly infiltrated, if peri-insulitis or an intraislet infiltrate occupied <25% of the islet; infiltrated or heavily infiltrated, if 25–50% or >50% of the islet was occupied by inflammatory cells.

FIGURE 1. Effect of HSP65-6P277 treatment on type 1 diabetes in NOD mice. To test whether inoculation with HSP65-6P277 might indeed protect these mice from developing diabetes and whether the level of protection is higher than in mice inoculated with HSP65-P277, we immunized 4-wk-old female NOD mice with HSP65-6P277, HSP65-P277, HSP65, and PBS three times at 4, 7, and 10 wk of age, respectively, and followed their glucose levels. A, The actual progression of hyperglycemia recorded in real time. B, The mortality of the mice immunized with HSP65-6P277 (represented by beeline, n = 10) in each month comparing with PBS (arrow). C, The mortality of the mice immunized with HSP65-P277 (beeline) in each month comparing with PBS (arrow). D, The mortality of the mice immunized with HSP65 (beeline) in each month comparing with PBS (arrow). E, The concentration of blood glucose and cumulative incidence of diabetes at the age of 8 mo. In control group, eight of the mice eventually developed type 1 diabetes and only two escaped the disease, and the highest blood glucose level arrived at 24.28 mmol/L; in the HSP65-P277 group, five of the mice eventually developed type 1 diabetes. However, none the mice of group HSP65-6P277 eventually developed type 1 diabetes, and all escaped the disease. The level of protection was higher than in mice inoculated with HSP65-P277 (p < 0.05).
reaction was stopped by 1 drop of 2M H2SO4, and the samples were read.

The plates were washed, and alkaline phosphatase biotinylated Abs was added for 1 hour at 37°C, then extensively washed and incubated with streptavidin conjugated with alkaline phosphatase for 1 hour at 37°C. The detection limit for the experiments described in this study was 15 pg/ml for IL-10 and IFN-γ.

Statistical significance

Data generated from animals immunized with HSP65-6 × P277 were compared with animals that received HSP65-P277 and PBS. Student’s t test was conducted to assay significant differences between the different experimental groups.

Results

Effect of HSP65-6 × P277 treatment on type 1 diabetes in NOD mice

Fig. 1A shows the progression of hyperglycemia development recorded in real time. At the beginning of treatment, all the 4-week-old female NOD/Ltj mice were healthy and had normal blood glucose. Approximately 80% of the mice either developed hyperglycemia or were dead in control group within 6–8 mo period. Of the total of 10 mice that received HSP65-P277, 4 were dead from severe diabetes by 8 mo. By contrast, none of the 10 mice treated with HSP65-6 × P277 at 8 mo of age died (Fig. 1A). Fig. 1E shows the concentration of each mouse and the cumulative incidence of each group at the age of 8 mo. In control group, 8 of the 10 mice eventually developed type 1 diabetes and only 2 escaped the disease; the incidence of HSP65-P277 group was 50%; but none of the mice in group HSP65-6 × P277 developed type 1 diabetes disease (p < 0.05).

Effect of HSP65-6 × P277 treatment on insulitis

At the end of the observation period, when the mice were 8 mo old, the pancreata from all mice were obtained for histological examination. The predication of the pancreas in mice treated at 20 wk showed a difference between the HSP65-6 × P277-treated and the HSP65-P277-treated mice as follows: ~80% of islets in HSP65-6 × P277-treated mice, but 30% of those in HSP65-P277-treated mice were free of insulitis (p < 0.05) (Fig. 2A). Fig. 2B demonstrated the results obtained on histological examination of the pancreas showed that there was a significant increase in the number of islets free of insulitis, fewer necrosis areas formed in the pancreas tissue, and a few lymphocytes filtrated around the islets of pancreas (original magnification, ×200). C, From HSP65-P277-vaccinated mice: a few necrosis areas formed in the pancreas tissue and a few lymphocytes filtrated around the islets of pancreas (original magnification, ×200). D, From control mice: much necrosis and marked atrophy of pancreas islets showed and many lymphocytes filtrated around the islets (original magnification, ×200).

Effect of HSP65-6 × P277 treatment on T cell proliferation

Increasing spontaneous T cell reactivity to HSP60 has been previously related to the progression of diabetes (20), and modulation of the HSP60-specific immune response was associated with the control of the diabetogenic process (21). We therefore analyzed the splenocytes isolated from HSP65-6 × P277-treated and HSP65-P277-treated animals to check their proliferative response to HSP65-6 × P277 and HSP65-P277. As shown in Fig. 3, PBS-treated NOD mice manifested spontaneous reactivities to HSP65-6 × P277 and HSP65-P277. In contrast, splenocytes from the mice showed a significant increase in the number of islets free of insulitis, fewer necrosis areas formed in the pancreas tissue, and a few lymphocytes filtrated around the islets of pancreas (original magnification, ×200). From HSP65-P277-vaccinated mice: a few necrosis areas formed in the pancreas tissue and a few lymphocytes filtrated around the islets of pancreas (original magnification, ×200). D, From control mice: much necrosis and marked atrophy of pancreas islets showed and many lymphocytes filtrated around the islets (original magnification, ×200).
vaccinated with HSP65-6 × P277 showed diminished reactivates to HSP65-6 × P277, and the ability of down-regulation of spontaneous proliferative T cell responses much stronger than HSP65-6 × P277 (p < 0.05). However, T cells from both treated and untreated mice showed similar reactivities to Con A and indicated that there was no general inhibition of T cell reactivity induced by HSP65-6 × P277 vaccination. This result suggested that prevention of diabetes was associated with down-regulation of spontaneous proliferative T cell responses to the fusion protein HSP65-6 × P277. No responses were detected by the same preparation of E. coli harboring no HSP65-6 × P277 gene; this result excluded the possibility that the above observed phenomenon was due to E. coli-derived artifacts.

**Effect of HSP65-6 × P277 treatment on cytokines**

To test whether the increased copies of the Th2-activated peptide P277 would enhance the Th2-like immune response, we compared the levels of cytokine secreted in mice that inoculated with two immunogens, HSP65-6 × P277 and HSP65-P277. The amount of IL-10 and IFN-γ secreted by spleen cells after HSP65-6 × P277 and HSP65-P277 stimulation in vitro was analyzed. Because different cytokines are secreted in different physiological amounts, we incubated the control mice of spleen cells with a prototypic T cell mitogen Con A. As shown in Fig. 4, the different experimental groups did not differ in their response to Con A, and immunization of mice with the fusion protein HSP65-6 × P277 elicited much higher levels of Th2-type cytokines (Fig. 4A) and lower Th1-type cytokines (Fig. 4B) than with HSP65-P277 (p < 0.05). However, compared with the amount of cytokine released in response to Con A stimulation, IFN-γ secretion on stimulation with HSP65-6 × P277 was lower in the HSP65-6 × P277-treated animals. This down-regulation of IFN-γ secretion was associated with an increase in IL-10 secretion in response to stimulation with HSP65-6 × P277.

**Discussion**

The major findings of the present study were the increased effectiveness of prevention of autoimmune diabetes in NOD mice associated with the copies of the peptide P277 in the immunogen. It is possible that the Th2-activated peptide P277, when tandem repeated, enhanced the Th2 immune response. We examined this hypothesis by comparing the cytokine secreted in mice that inoculated with two immunogens containing one or six tandemly repeated copies of a Th2-activated peptide sequence P277. Immunization of mice with the fusion protein HSP65-6 × P277 elicited much higher levels of Th2-type cytokines and lower Th1-type cytokines than with HSP65-P277 (p < 0.05).

More and more evidence supported the idea that the peptide P277 was a bidirectional bomb. Peptide P277 has been found to serve as a functionally important target in mouse type 1 diabetes, but a single s.c. administration of peptide P277 (100 μg in oil), even late in the autoimmune process, can arrest β cell damage in most NOD mice (22). Thus, specific Abs to P277 have been found to mark the arrest of spontaneous type 1 diabetes induced by treatment of NOD mice with peptide P277 itself (21, 23). To overcome the potential poor immunogenicity of the peptide P277, we have constructed the fusion protein HSP65-6 × P277, as described (19). Oscherwitz et al., as well as Richard et al. (17, 18) reported that the immunogens containing tandemly repeated B or T cell epitopes increased immunogenicity associated with increased copies of the target sequence. Our data confirmed their results. The anti-P277 Ab level induced by the tandem repeat construct is superior to that of the single copy construct (p < 0.01). We may not elucidate the mechanisms leading to the results of that tandem repeat epitope can enhance immunogenicity of the target sequence; however, Ab-producing cells, B cells, might be, very possibly, recruited by the immunogens containing tandemly repeated cell epitopes in vivo. B cells are not only central to the production and amplification of humoral immune response, they also play an important role as APCs in the generation of T cell-mediated immune responses (24, 25). In addition, recent studies indicated that human HSP60 induced naive mouse B cells to proliferate and to secrete IL-10 (26) and the activated B cells inhibited spontaneous Th1 autoimmunity (27, 28). But we cannot confirm whether or not the activated B cells play a primary role in enhancing the Th2-type immune response in our studies, because the possibilities have not been tested experimentally in depth.

Peptide P277 was recognized by Th1 cell clone (14) and identified as containing a target epitope for diabetogenic T cells (13). However, administration of P277 can arrest the diabetogenic process (22). Peptide treatment was marked by down-regulation of T cell proliferation to P277 and by up-regulation of anti-P277 Abs of
the IgG1 and IgG2b isotypes. These changes in autoimmune reactivity were accompanied by a shift of the autoimmune process from a proinflammatory Th1-like response to an anti-inflammatory Th2-like response (21). The results suggest that the peptide P277 leads to activation of T cells specific for Th2 clone. It is conceivable that the increased copies of the Th2-activated peptide P277 will enhance the immune response specific for Th2 type. Interestingly, in our studies, we found that the mice inoculated with the fusion protein HSP65-6 × P277 elicited much higher levels of Th2-type cytokines and lower Th1-type cytokines than with HSP65-P277 (p < 0.05). At present, we do not know how the Th2-activated peptide P277, when tandem repeated, inhibited the Th1 cells and recruited Th2, but the results reported in this study and elsewhere suggest the following possibility: 1) mice vaccinated with HSP65-6 × P277 elicited high level Ab formation, which suggested that Ab-producing cells, B cells, must be activated, and the activated B cells secreted IL-10 and inhibited spontaneous Th1-like response (26–28); 2) successful treatment of NOD mice with peptide P277 is associated with the recognition of Th1 clone to P277 (13) along with a decrease in the proliferation of T cells in response to HSP60 and P277 (21). Peptide P277, when tandem repeated, may be facile recognized by diabetogenic T cells, leading secondarily to more efficient inhibition of the Th1 cells and the consequent increased recruitment of Th2 cells. Irrespective of how HSP65-6 × P277 might function as a immunogen in mouse type 1 diabetes, the present results show that a Th1-recognized peptide P277, when tandem repeated, inhibited the proliferation of T cells in response to HSP65-6 × P277 and elicited much higher levels of Th2-type cytokines (such as IL-10) and lower Th1-type cytokines (such as IFN-γ). HSP65-6 × P277 might be used as a preventive agent to arrest the autoimmune process in NOD mice.

It is also worth noting that we inoculated the mice by mucosal administration. Mucosal administration not only has provided numerous advantages over conventional s.c. injection, such as convenience, safety, and acceptability by more people, but also has proven effective as a treatment for a large number of animal models of autoimmune disease (29). Now, mucosal administration has been a novel therapeutic approach for treating processes not classically considered to be autoimmune, but involving an inflammatory component. For example, mucosal administration of HSP in mice lacking the receptor for low density lipoprotein can cause significant decrease in the size of atherosclerotic plaques, and suppress inflammation and atherosclerosis development (30). Weiner et al. (31) showed that nasal administration of amyloid A-β peptide limits decreased amyloid plaque deposition in a transgenic animal model of Alzheimer’s disease. The results of the present study suggest similar consideration for the treatment of type 1 diabetes. On the basis of our results, we postulate that mucosal treatment with HSP65-6 × P277 stimulates the development of adaptive immune cells that secret anti-inflammatory cytokines such as IL-10. However, whether mucosal administration plays a key role in inducing anti-inflammatory response to prevent the β-cell destruction needs to be tested experimentally in depth, because we did not adopt any other approach of inoculation in this study.

Our data, taken together with those cited above, can support the following possible model: an enhanced recognition of the tandemly repeated P277 sequence by the Th1 cells, leading secondarily to more efficient inhibition of the Th1 cells and the consequent increased recruitment of Th2 cells. Certainly, the evidence we have provided in this study that the mechanism contributing to the increased copies of the Th2-activated peptide P277 will enhance the immune response specific for Th2 type is plausible. Nevertheless, the beneficial effects of HSP65-6 × P277 on the type 1 diabetes are clear. We conclude that the behavior of the mouse immune system is susceptible to specific modification by the immunogens harboring tandem repeat peptide P277. These observations support the idea that the immune system is a cognitive system in which behavior can be self-correcting if the system is given suitable information (32, 33).

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Disclosures

The authors have no financial conflict of interest.

References


Corrections


Fig. 1E of the article cited above was published previously as Fig. 10 in Liang Jin, Yu Wang, Qiyan Xiong, Qingmei Chen, Jianping Li, Aihua Zhu, Rongyue Cao, Jie Wu, Jingjing Liu. 2007. Long-lasting specific antibodies against P277 induced by mucosal administration of P277 repeat sequences carried by Hsp65 in the absence of adjuvants. *Vaccine* 25: 2043–2050.

The authors regret the duplicate publication of this figure and apologize to the scientific community for the need to publish this correction.

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