**Title**
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Combined insulin B:9-23 self-peptide and polyinosinic-polycytidylic acid accelerate insulitis but inhibit development of diabetes by increasing the proportion of CD4^+Foxp3^+ regulatory T cells in the islets in nonobese diabetic mice

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Abstract

Insulin peptide B:9-23 is a major autoantigen in type 1 diabetes. Combined treatment with B:9-23 peptide and polyinosinic-polycytidylic acid (poly I:C), but neither alone, induce insulitis in normal BALB/c mice. In contrast, the combined treatment accelerated insulitis, but prevented diabetes in NOD mice. Our immuno-fluorescence study with anti-CD4/anti-Foxp3 revealed that the proportion of Foxp3 positive CD4⁺CD25⁺ regulatory T cells (Tregs) was elevated in the islets of NOD mice treated with B:9-23 peptide and poly I:C, as compared to non-treated mice. Depletion of Tregs by anti-CD25 antibody hastened spontaneous development of diabetes in non-treated NOD mice, and abolished the protective effect of the combined treatment and conversely accelerated the onset of diabetes in the treated mice. These results indicate that poly I:C combined with B:9-23 peptide promotes infiltration of both pathogenic T cells and predominantly Tregs into the islets, thereby inhibiting progression from insulitis to overt diabetes in NOD mice.

Key words: insulin, type 1 diabetes, insulitis, nonobese diabetic mouse, CD4⁺ T cell, peptide, polyinosinic-polycytidylic acid, regulatory T cell
Introduction

Type 1 diabetes mellitus is an autoimmune disease that develops when tolerance mechanism(s) fail to control immune responses to islet-specific autoantigens, including insulin, glutamic acid decarboxylase and heat-shock protein [1]. Insulin is an important islet autoantigen in nonobese diabetic (NOD) mice and patients with type 1 diabetes [1]. In NOD mice, insulin-autoreactive CD4$^+$ and CD8$^+$ T cells infiltrate islets of mice, and clones of these T cells can transfer diabetes to young recipients [2, 3]. The B chain peptide, B:9-23, has been suggested to be a primary autoantigenic epitope in the pathogenesis of type 1 diabetes in NOD mice [4, 5]. However, of interest, immunization of NOD mice with exogenous B:9-23 peptide prevents diabetes [6].

In contrast to NOD mice, we have recently found that immunization of non-diabetes prone BALB/c mice with B:9-23 peptide readily induces insulin autoantibodies, but not insulitis or diabetes [7], and that simultaneous administration of B:9-23 peptide and polyinosinic-polycytidylic acid (poly I:C, a Toll-like receptor 3 ligand), but not poly I:C alone, induces insulitis. In addition, we have also developed an experimental autoimmune diabetes model in which B:9-23 peptide induces diabetes in transgenic BALB/c mice expressing the costimulatory molecule B7.1 in their islets [8]. Disease induction is accelerated with simultaneous administration of poly I:C in this model. These data indicate that poly I:C appears to promote anti-insulin autoimmunity in BALB/c mice immunized with B:9-23 peptide.

Since the previous report has demonstrated that poly I:C treatment confers significant protection against diabetes in NOD mice [9], we are left with the question of whether combination treatment of NOD mice with B:9-23 peptide and poly I:C would promote or prevent diabetes. We here first showed that treatment with B:9-23 peptide and poly I:C unexpectedly (or surprisingly) accelerated insulitis, but prevented diabetes in young NOD
mice. We first thought that TGF-β might mediate diabetes protection despite acceleration of insulitis, because it has been reported that the TGF-β producing T cell clone which was found to react to insulin B:9-23 peptide protects NOD mice from disease induced by adoptive transfer of diabetogenic spleen cells [10]. We therefore injected TGF-β monoclonal antibody (2g7, a generous gift from Dr Sylvaine You, INSERM U580 Hopital NECKER, Paris) to NOD mice after B:9-23 peptide immunization [11]. These studies however showed that TGF-β neutralization did not influence the disease inhibition by B:9-23 peptide and poly I:C in NOD mice (our unpublished data).

Another possibility is naturally arising CD4^+CD25^+ regulatory T cells (Tregs). They develop in the thymus and in the periphery, and actively maintain immunological self-tolerance. Tregs have been shown to be essential for regulation of several autoimmune diseases, including type 1 diabetes [12]. The transcriptional factor Foxp3 has been identified to be essential for development and function of Tregs [13]. Insulin-specific Tregs induced by \textit{in vivo} immunization and \textit{in vitro} restimulation with B:9-23 peptide efficiently prevent diabetes induced by adoptive transfer of diabetogenic T cells [14]. In patients with type 1 diabetes, it has been reported that regulatory T cell markers such as Foxp3 mRNA is upregulated in peripheral blood mononuclear cells stimulated with insulin \textit{in vitro} [15]. In this study, we evaluated whether Tregs play a role in the aforementioned action of combined B9-23 peptide and poly I:C.
Materials and methods

Mice

Female NOD mice, 3-4 weeks of age, were purchased from Clea Japan (Tokyo, Japan). All mice were kept under specific pathogen-free conditions at the Laboratory Animal Center for Biomedical Research of Nagasaki University, and were housed in an air conditioned room with a 12-h light-darkness cycle. Animal care and all experimental procedures were performed in accordance with the Guideline for Animal Experimentation of Nagasaki University with approval of the Institutional Animal Care and Use Committee.

Injection of B:9-23 peptide, poly I:C or anti-CD25 antibody

Mouse proinsulin II B chain-derived peptide B:9-23 (SHLVEALYLVCGERG), synthesized and HPLC-purified to greater than 95% homogeneity, was purchased from SIGMA Genosys (Hokkaido, Japan). Polyinosinic-polycytidylc acid sodium salt (poly I:C) was from Sigma-Aldrich KK (Tokyo, Japan). Anti-CD25 antibody was purified from ascites of mice intraperitoneally (i.p.) injected with hybridoma PC61 (a generous gift from Dr.K.Yui at Nagasaki University) using a HiTrap™ protein G HP column (Amersham, Piscataway, NJ).

B:9-23 peptide (100 µg/mouse) in incomplete Freund’s adjuvant (IFA) was subcutaneously injected into the scruff of NOD mice at 4 weeks of age (day 1). Poly I:C (7.5 µg/g body weight) was i.p. administered on days 1-5 and 8-12. PC61 was i.p. injected at the indicated time point.

Monitoring diabetes by blood glucose levels

The blood glucose levels of mice were monitored every other week with a Glutest-Ace meter (Sanwa Kagaku, Nagoya, Japan) starting at 12 weeks of age to determine the development of diabetes following B:9-23 peptide and/or poly I:C injection without PC61. In
the study with PC61, the blood glucose levels were monitored twice a week starting at 4.5 weeks of age. Mice with blood glucose levels above 250 mg/dl for two consecutive measurements were considered diabetic.

**Histology**

Pancreata were obtained at 8 weeks of age after the administration of B:9-23 peptide and/or poly I:C. Pancreata, thyroid tissues and salivary glands were obtained at 6 weeks of age after the administration of PC61 with or without B:9-23 peptide and poly I:C. Each section of the tissues was histologically analyzed by fixing in 10% formalin and staining with hematoxylin and eosin. A minimum of 20 islets from each mouse were microscopically observed by two different observers for the presence of insulitis, and the levels of insulitis were scored according to the following criteria; 0, no lymphocyte infiltration; 1, islets with lymphocyte infiltration in less than 25% of their area; 2, 25-50% of the islet area infiltrated; 3, 50-75% of the islet area infiltrated; 4, more than 75% infiltrated or small retracted islets.

**Immunohistochemistry**

The primary antibodies used were rabbit anti–mouse Foxp3 Ab [16] (the final concentration of 2.5 ug/ml) and FITC hamster anti-mouse CD4 (H129.19) (BD Biosciences Pharmingen, San Diego, CA) (1:250 dilution). The secondary antibodies were Alexa Fluor 555 goat anti-rabbit IgG (0.5 ug/ml) and Alexa Fluor 488 goat anti-hamster IgG (BD Biosciences Pharmingen) (1:250 dilution).

Pancreata and pancreatic draining lymph nodes (PLNs) (for four mice in each group) were embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN), frozen in liquid nitrogen, and cut by a cryostat into 6- to 7-μm-thick sections. The sections were fixed with cold acetone for 10 min at 4°C and use for immunofluorescence. We used an already established double-
immunofluorescence staining protocol for FoxP3 and CD4 [16]. After blocking nonspecific reactions and endogenous biotin activity using Blocking One (Nacalai Tesque, Kyoto, Japan), the samples were incubated with each primary Abs for 1 h at room temperature and subsequently with secondary Abs for 1 h at room temperature, and then fixed for 10 min at 4°C in PBS containing 4% paraformaldehyde. All sections were analyzed with a confocal laser scan microscope LSM5Pascal (Carl Zeiss, Germany). The number of Foxp3+CD4+ cells was counted in four nonconsecutive microscopic fields within the T cell areas of islets and PLN.

**Statistical analysis**

Group differences were analyzed with the Turkey HSD test, and differences between Kaplan-Meier survival curves were estimated by the long rank test using Dr. SPSS II for Windows software (SPSS Inc., Chicago, IL). $P$ values less than 0.05 were considered statistically significant. Insulitis levels were analyzed by Ridit analysis, and levels of $t$ higher than 1.96 or lower than -1.96 were considered statistically significant.
Results

Administration of B:9-23 peptide in combination with poly I:C significantly accelerated the development of peri-insulitis but prevented development of diabetes

We first evaluated the influence of administration of exogenous B:9-23 peptide and/or poly I:C on development of insulitis and diabetes in NOD mice at 4 weeks of age. As determined by life table analysis, B:9-23 peptide alone or in combination with poly I:C significantly suppressed development of diabetes, as compared to the PBS-treated control group (P < 0.0005 and P < 0.001, respectively). Injection of poly I:C alone at 4 weeks of age slightly inhibited development of diabetes, but this inhibition was insignificant (P = 0.1) (Figure 1A).

The pancreata from NOD mice at 8 weeks of age (4 weeks after the beginning of treatment) were used for histological analysis. Unexpectedly, the levels of insulitis were significantly increased by administration of B:9-23 peptide alone or poly I:C alone, compared with the control group (T = 4.304 or T = 6.183, respectively). Simultaneous administration of B:9-23 peptide and poly I:C further accelerated development of insulitis (T = 10.77) (Figure 1B).

Administration of B:9-23 peptide with poly I:C increased the frequency of CD4\(^+\)Foxp3\(^+\) regulatory T cells in the islets, but not in the PLNs

To further characterize insulitis enhanced by B:9-23 peptide and poly I:C, the frequency of Tregs in the islets and PLNs was determined by CD4 and Foxp3 staining as a marker of Tregs. We found that approximately 8 % of CD4\(^+\) T cells were Foxp3-positive both in the islets and the PLNs in 6 weeks-old control mice (Figure 2A, 3A, 3B). Combined treatment of B:9-23 peptide with poly I:C increased the proportion of CD4\(^+\)Foxp3\(^+\) T cells in the islets up to ~17 % (P < 0.0005) (Figure 2A, 3A), but did not change the percentage in the PLNs.
These results indicate that the combination of B:9-23 peptide and poly I:C enhanced insulitis, but concomitantly increased proportion of Tregs in T cells infiltrated into the islets.

Depletion of Tregs induced extremely rapid onset of insulitis and diabetes in mice treated with B:9-23 peptide and poly I:C

To clarify the role played by infiltrating Foxp3⁺CD4⁺ Tregs, we attempted to deplete Tregs by using PC61, a widely used means to examine the function of Tregs. In our preliminary experiment, 500 μg/ mouse PC61 efficiently depleted CD25⁺ cells (data not shown). A single administration of PC61 into mice at 4 weeks of age accelerated the spontaneous development of diabetes (P = 0.0046 vs. PC61(-) PBS) (Figure 4A).

Surprisingly, pre-treatment with PC61 of mice at 3.5 weeks of age abrogated the disease inhibition induced by B:9-23 peptide and poly I:C, and instead greatly accelerated the onset of diabetes. Thus disease developed in 35 and 45 % mice vs. 0 % in non-depleted mice at age of 6 and 10, respectively, weeks (P < 0.005) (Figure 4B).

Pre-treatment with PC61 also decreased the percentage of Foxp3⁺ T cells among the CD4⁺ T cells infiltrated into the islets in untreated NOD mice (P = 0.002 vs. PC61(-) PBS) and in those treated with B:9-23 peptide and poly I:C (P < 0.0005 vs. PC61(-) B:9-23+poly I:C) (Figures 2B and 3A). In the PLNs, in contrast, the frequency of CD4⁺Foxp3⁺ T cells was not influenced by treatment with PC61 (P = 0.954 vs. PC61(-) PBS) (P = 0.613 vs. PC61(-) B:9-23+poly I:C) (Figures 3B).

Massive infiltration of lymphoid cells were observed in the islets from mice treated with PC61, B:9-23 peptide and poly I:C, but only mild to moderate infiltration was seen in those treated with PC61 alone 2.5 weeks after PC61 injection (6 weeks of age) (Figure 2B).

Neither thyroid or salivary glands were intact in all the mice.
These results indicate that B:9-23 peptide and poly I:C promote infiltration of both autoimmune pathogenic T cells and Foxp3⁺ Tregs into the islets, and that the latter keeps the former in check, preventing development of diabetes, and following depletion of the latter with PC61 antibody diabetogenesis is remarkably accelerated.
Discussion

It has been extremely difficult to precisely define environmental determinants of type 1 diabetes of man despite early evidence that viral infections may contribute. We believe that a part of this difficulty may be dependent upon complex interplay between activation of innate and adaptive immunity, that may result in either protection or disease induction in genetically susceptible hosts. For instance the effects of poly I:C on anti-islet autoimmunity vary in different mouse models of type 1 diabetes. Thus our previous studies showed that poly I:C enhances anti-insulin autoimmune reaction in BALB/c mice immunized with B:9-23 peptide [8], whereas our present study (Fig. 1A) demonstrates that poly I:C does not reverse inhibitory effect of B:9-23 peptide on development of diabetes in NOD mice. Although the exact reason(s) for this difference are at present unclear, a recent study show that the effect of poly I:C is dose-related; high dose poly I:C accelerates but low dose prevents diabetes in BioBreeding rats [17].

It was unexpected that the prevention of diabetes of NOD mice by combined poly I:C and B:9-23 peptide administration was accompanied by enhanced insulitis (Fig. 1B). Previous studies suggest that progression from insulitis to overt diabetes is regulated by Tregs in NOD mice. NOD mice only develop diabetes at 3 or 4 months of age, however, insulitis occurs long before the clinical onset of diabetes [18]. Disease transfer into irradiated recipients by spleen cells from diabetic NOD mice is prevented by CD4+ T cells from young pre-diabetic NOD mice were co-transferred with diabetogenic T cells [19]. Recent data have indicated that this protective effect is confined to a CD4+CD25+CD62L+ Treg subset [20, 21].

Our immunofluorescence study indeed showed an elevated proportion of CD4+Foxp3+ T cells in the islets (but not in the PLN) from mice treated with poly I:C and B:9-23 peptide (Figs. 3). The functional significance of Tregs was verified by our depletion study, in which antibody (PC61)-mediated Treg depletion accelerated development of diabetes in untreated
mice and remarkably in those treated with poly I:C and B:9-23 peptide (Figs. 4). Extremely acute onset of diabetes was observed in Treg-depleted, poly I:C and B:9-23 peptide-treated mice (Fig. 4B). Thus PC61 antibody sensitive cells (presumably Tregs) play a role not only in suppression of spontaneous development of diabetes in untreated NOD mice, but also in mice treated with poly I:C and B:9-23 peptide. Our data indicate that combined treatment with poly I:C and insulin peptide B:9-23, recruits both pathogenic T cells and predominantly Tregs into the islets; the latter keeps the former in check, thereby suppressing progression from insulitis to diabetes. However, Treg-depletion in this situation reveals the enhanced action of the pathogenic T cells, by allowing the pathogenic T cells to infiltrate into the islet rapidly, thereby resulting in massive insulitis and acute onset diabetes.

In NOD mice, administration of insulin or insulin-derived peptides can be used to prevent diabetes [22-24] but similar approaches have failed in a clinical setting [25], possibly because of inadequate induction and accumulation of antigen-specific Tregs which can suppress the autoimmune pathogenic T cells over the long disease course. Many therapeutic interventions with antigen-specific Tregs will be attempted to treat autoimmunity [26, 27]. Our results indicate that treatment of NOD mice with insulin B:9-23 peptide, a potential primary autoantigenic epitope, and poly I:C expands the pathogenic T cells as well as Tregs and recruits them into the islets. There appear to be a fine balance between the pathogenic T cells and Tregs resulting in enhancing insulitis but suppressing progression from insulitis to overt diabetes in NOD mice. Thus to obtain disease inhibition by an antigenic peptide vaccination, an efficient therapy which could both induce antigen-specific regulatory cell populations and purge pathogenic T cells should be considered.
Figure legends

Figure 1.

(A) Life table analysis for development of diabetes following administration of B:9-23 peptide and/or poly I:C in NOD mice. Open triangles, B:9-23 peptide alone (n = 10); x, poly I:C alone (n = 16); open squares, B:9-23 peptide + poly I:C (n = 16); open circles, PBS (n = 19). * P < 0.001; ** P < 0.0005. (B) Levels of insulitis at 8 weeks of age determined by Ridit analysis. A level of T > 1.96 was regarded as a significant increase. A level of T < -1.96 was regarded as a significant suppression. * T = 4.304, ** T = 6.183, *** T = 10.77

Figure 2.

(A) Immunofluorescence of the sections of pancreata from NOD mice at 6 weeks of age treated with PBS or B:9-23 + poly I:C without PC61 pretreatment stained with anti-Foxp3 Ab and anti-CD4 mAbs, followed by goat anti-rabbit IgG Alexa555 (red) and goat anti-rat IgG Alexa488 (green). Original magnification: x100. (B) Immunofluorescence of the sections stained and H&E stained paraffin sections of the pancreas from NOD mice at 6 weeks of age treated with PC61(+) PBS or PC61(+) B:9-23+poly I:C 2 weeks before. Original magnification: x200.

Figure 3.

The numbers of Foxp3+ T cells and CD4+ T cells were counted in several nonconsecutive microscopic fields within the T cell area of the pancreas or pancreatic lymph nodes (PLN). The frequency of Foxp3+ T cells in whole CD4+ cells in the T cell area of the pancreas (A) or PLN (B) were compared within each group of treated mice, respectively. The results are presented as the mean ± S.E. * P < 0.005, ** P < 0.0005.
Figure 4.

(A) Life table analysis for the development of diabetes following administration of PC61 alone. Closed circles, PC61(+)PBS (n = 9); open circles, PC61(-)PBS (n = 24). * P < 0.005

(B) Life table analysis for the development of diabetes following pre-treatment of PC61 before the combination therapy with B:9-23 peptide + poly I:C. Closed squares, PC61(+)B:9-23 + poly I:C (n = 8); open squares, PC61(-)B:9-23 + poly I:C (n = 16). * P < 0.005
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References


Figure 3

A

B

PC61 (-)  PC61 (+)  PC61 (-)  PC61 (+)
Figure 4

(A) PC61(-), PBS (n=24) vs PC61(+), PBS (n=9)

(B) PC61(-), B:9-23+poly I:C (n=16) vs PC61(+), B:9-23+poly I:C (n=8)