

Oral Administration of Recombinant Live Yeast Producing Altered Peptide Ligand Derived from Insulin B:9-23 Peptide Linked to Cholera Toxin B Subunit Suppresses Development of Autoimmune Diabetes in NOD Mice

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Insulin is a major autoantigen responsible for the pathogenesis of type 1 diabetes, and its B-chain peptide B:9-23 has been suggested to contain critically important epitopes in the NOD mouse. We have previously demonstrated that the altered peptide ligand of B:9-23 peptide with alanine substitutions at positions 16 and 19 (A^{16,19}APL) suppresses insulinitis and reduces the incidence of diabetes when administered intranasally together with cholera toxin (CT). In this study, we extended the previous findings to determine whether mucosal administration of the A^{16,19}APL without the use of toxic CT is efficacious for diabetes prevention. To this end, we linked the A^{16,19}APL to the C-terminus of non-toxic cholera toxin (CTB) B subunit and the chimeric fusion protein was produced in methylotrophic yeast *Pichia pastoris*. Although intranasal administration of the recombinant CTB-A^{16,19}APL fusion protein extracted from the yeast cells failed to prevent the progression to diabetes, oral administration of the live yeast cells producing the chimeric protein resulted in more than 50% reduction of the development of diabetes, albeit the fact that protective efficacy did not reach the significance level by life table analysis ($P=0.08$). Furthermore, the expression of insulin autoantibodies was not altered throughout the course of oral immunization experiment. These results demonstrated that the efficacy of recombinant CTB-A^{16,19}APL for oral immunization has much room for improvement. Nevertheless, oral administration of recombinant yeast or other edible materials such as food plants engineered to produce altered autoantigenic peptide could potentially become a novel non-invasive immunotherapeutic strategy for type 1 diabetes. (250 word)

ACTA MEDICA NAGASAKIENSIA 54: 33 - 38, 2009

Keywords: Type 1 diabetes; Insulin; Oral tolerance; Yeast; Altered peptide ligand; Cholera toxin B subunit

Introduction

Type 1 diabetes is an autoimmune disease and immune modulation with autoantigens has been proposed as a promising strategy for type 1 diabetes management. Insulin and proinsulin are key targets for prevention of type 1 diabetes^{1,2} and the insulin B chain peptide, B:9-23, has been identified to contain a primary autoantigenic epitope responsible for the pathogenesis of type 1 diabetes in NOD mice.³ Immunization of NOD mice with the peptide B:9-23 effectively prevented diabetes,⁴ while a similar approach such as DPT-1

has failed in a clinical trial.⁵ The DPT-1 trial tested whether insulin administered either subcutaneously or orally could prevent the development of diabetes in healthy, islet antibody-positive relatives of patients with type 1 diabetes. Subcutaneous injection or oral administration of insulin did not provide promising results for the prevention of type 1 diabetes.^{5,6} However, a delay in the onset of diabetes was observed for a subgroup of relatives with high levels of insulin autoantibodies when they were treated orally with insulin,⁶ suggesting that mucosal (oral or nasal) insulin or insulin peptide attenuates anti-islet autoimmunity in the subgroup of type 1

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Received June 11, 2009; Accepted July 8, 2009

diabetes patients. In NOD mice, it has been suggested that mucosal administration of autoantigenic peptide is a "double-edged sword" with potential to induce both protective and pathogenic immunity, especially when a CTL epitope is present within the peptide.⁷ We have recently demonstrated that intranasal administration of the altered peptide ligand with alanine substitution at position 16 and 19 (A^{16,19}APL), abolishes the insulin-specific CTL response and prevents diabetes when co-administered with mucosal adjuvant cholera toxin (CT).⁸ The use of CT is unacceptable for clinical application, hence we sought to determine whether the non-toxic B moiety of the CT (CTB) becomes an alternative molecular platform to deliver the APL through mucosal route for the induction of systemic tolerance for prevention of diabetes.

Yeasts, lactic acid bacteria, and edible plants are an attractive production as well as oral delivery system for small peptides when linked to mucosal delivery proteins such as CTB.^{9,10} CTB, unlike its parental holotoxin, is a good immunogen as well as effective delivery molecule for linked materials to breach mucosal surface of various organs of animals.¹¹ Prevention of type 1 diabetes in NOD mice by recombinant potato plant producing CTB-proinsulin fusion protein was the first demonstration of edible vaccine against autoimmune type 1 diabetes.¹²

In this study we demonstrated that recombinant yeast expressing CTB-APL chimeric fusion protein potentially becomes a novel oral vaccine platform for type 1 diabetes prevention.

Materials and methods

Construction of *Pichia pastoris* expression vector containing CTB-A^{16,19}APL fusion gene

Pichia pastoris expression vector containing the full length CTB gene was constructed as described previously.¹³ Briefly, the CTB gene containing leader peptide was PCR amplified with a primer pair (sense primer:

5'-GCGCCAATTGGCCACCATGATTAAATTAATAAATTTGGTGT-3' where the underlined sequence is a *MunI* restriction endonuclease recognition sequence and italic letters represent the initiation codon; anti-sense primer:

5'-GCGCCAATTGTTAGAATCTGACCGGGCCATTGGCCATACTAATTGCGGC-3' where the single and double underlined sequences are *MunI* and *EcoRI* restriction endonuclease recognition sequences, respectively, and italic letters represent the termination codon) and plasmid psB containing the CTB gene as template.¹³ The amplified fragment was digested with *MunI* restriction endonuclease, and then was inserted at the unique *EcoRI* site of pAO815 (Invitrogen Japan KK, Tokyo, Japan). The constructed plasmid pAO815-CTB contains the unique *EcoRI* site immediately downstream of the hinge-encoding sequence (Gly-Pro-Gly-Pro). Next, a complementary 5'-phosphorylated oligonucleotide pair (sense:

5'-PO₄-AATTCTCTCATTGGTTGAAGCTTTGGCTTTGGTTGCTGGTAAAAGAGGTC-3'; anti-sense:

5'-PO₄-AATTGACCTCTTACCAGCAACCAAGCCAAAGCTT

CAACCAAATGAGAG-3') encoding A^{16,19}APL oligopeptide fragment was mixed at equimolar amount, and then heated to 95 degrees Celsius for 5 min, followed by gradual cooling to room temperature for annealing. The ends of the resultant double-stranded oligonucleotide fragment was designed to contain "sticky ends" of *EcoRI* and *MunI* restriction endonuclease cleavage ends, hence it was directly inserted at the unique *EcoRI* site of pAO815-CTB vector (Figure 1). The generated chimeric gene was sequenced (ABI-3130, Applied Bioscience) and the CTB fused to one copy of A^{16,19}APL gene with correct orientation was selected and used for *P. pastoris* GS115 transformation experiment as described before.¹³ The predicted amino acid sequence of the fusion protein is as follows (underlined 15 amino acids is A^{16,19}APL and its flanking amino acids, i.e. EF and QF, are derived from sequence used for cloning):

MIKLKFGVFFTVLLSSAYAHGTPQNITDLCAEYHNTQIHTLNDK
IFS^YTESLAGKREMAIITFKNGATFQVEVPGSQHIDSQKKAIERM
KDTLRIAYLTEAKVEKLCVWNNKTPHAI^AISMANGPGPEFSHL
VEALALVAGERGQF

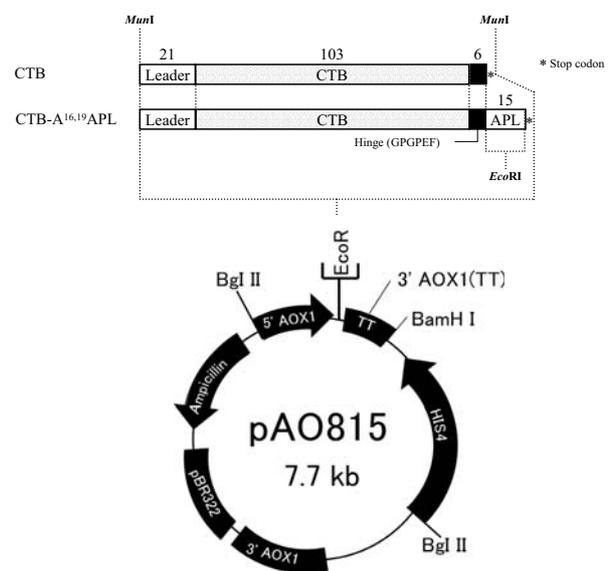


Figure 1. Schematic drawing of CTB and CTB-A^{16,19}APL fusion gene cloned into *EcoRI* site of pAO815 vector

CTB and CTB-A^{16,19}APL fusion genes are drawn at scales proportional to the amino acid lengths for each constitutive moiety. Numbers above each moiety refer to numbers of amino acids. The full-length CTB gene fused to the hinge sequence followed by the termination codon was first inserted into the unique *EcoRI* site within the pAO815 vector. The reconstituted unique *EcoRI* sequence (provided within the anti-sense CTB primer) within the vector immediately before the termination codon was utilized to insert the double-strand A^{16,19}APL-coding deoxyoligonucleotide sequence. See section Materials and methods for detailed construction procedure.

CTB-A^{16,19}APL expression in *P. pastoris* determined by GM1-ELISA and Immunoblot analyses

Approximately 100 transformed yeast colonies were screened for high level producers of recombinant CTB-A^{16,19}APL protein. After methanol induction of 3-ml culture of each clone,¹³

supernatant was applied to a 96-well microtiter plate (50 μ l/well in duplicate, Microlite™ 2, Dynatech Laboratories) which was pre-coated with GM1-ganglioside (5 μ g/ml), followed by blocking with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at 37 °C for 2 hrs. The GM1-bound fusion protein was detected by anti-CT antiserum (1:3000 dilution with 0.5% BSA in PBS) (C3062, Sigma-Aldrich, St. Louis Missouri, USA), followed by anti-rabbit IgG conjugated with alkaline phosphatase (1:5,000 dilution with 0.5% BSA in PBS) (A-2256, Sigma-Aldrich). Wells were washed three times with PBST between each step. To confirm the presence of APL epitope in the fusion complex, GM1-ELISA was conducted by using anti-A^{16,19}APL immune sera developed in NOD mice by subcutaneous immunization with 50 μ g of a synthetic A^{16,19}APL peptide emulsified with the incomplete Freund's adjuvant. To collect sufficient amounts of recombinant protein from cells for animal immunization experiments, cells were lysed by Y-PER yeast protein extraction reagent (Wako, Osaka, Japan) in the presence of 100 mM PMSF and 1 M DTT, and the supernatant was collected by centrifugation at 13,500 rpm for 10 min.

Yeast cells (approximately 500 μ g by wet weight) transformed with pAO815-CTB-A^{16,19}APL, pAO815-CTB or pAO815 alone were extracted by Y-PER yeast protein extraction reagent as described above. An equal amount of each sample was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) immobilon-P membranes (Minipore Corp., Bedford, MA, USA). After blocking with 5% skim milk in TBS-T for 1 hour at room temperature, membranes were sequentially incubated at 4 °C with 1:20,000 dilution of rabbit anti-cholera toxin antiserum (in 5% skim milk) and 1:2,000 dilution of anti-rabbit IgG (HRP-conjugated species-specific whole antibody from donkey) (Amersham Biosciences UK limited, Buckinghamshire, England). The membranes were then washed as before and protein bands were detected with ECL (peroxide buffer and enhancer solution) (Amersham Pharmacia Biotech).

Mice and reagents

Female NOD mice at 3-4 weeks of age were purchased from Clea Japan (Tokyo, Japan). All mice were kept under specific pathogen-free condition at the laboratory of Animal Center for Biomedical Research of Nagasaki University, and were housed in an air-conditioned room with a 12-h light-dark cycle. CT was purchased from List Biological Laboratories. Animal experimental procedures described in this study were approved by the institutional animal experimentation committee and all animal experiments were conducted in accordance with the committee's guidelines for animal experimentation.

Intranasal administration of CTB-A^{16,19}APL fusion protein

Intranasal administration started at 4 weeks of age (days 1-5 and 8 and then weekly until 10 weeks of age) with 20 μ g of yeast cell extract mixed with or without 2 μ g of CT. Mice were anesthetized

with ether, and 20 μ l of vaccine mixtures were introduced into each nostril with a micropipette.

Oral administration of GS115 cells expressing CTB-A^{16,19}APL

Animals were orally inoculated through a syringe filled with a ball-type feeding needle in a volume of 0.5 ml of solution with 50 μ g of yeast. Immunization started at 4 weeks of age with two doses per week until 10 weeks of age.

Monitoring diabetes by blood glucose level

The blood glucose levels were monitored using Glutest-Ace meter (Sanwa Kagaku, Nagoya, Japan) at every other week starting at 12 weeks of age. Mice with blood glucose levels above 250 mg/dl for two consecutive measurements were considered diabetic.

Measurement of insulin autoantibody (IAA)

Mice were bled and sera were stored at -20 °C until antibody assay was conducted. The levels of IAA in serum was evaluated prospectively at 4, 8 and 12 weeks of age by using a 96-well filtration plate micro IAA assay as previously described.¹⁴ The IAA levels were determined based on the differences in counts per minute (delta cpm) between wells with and without cold insulin, and the results were expressed as an index defined as follows: (sample cpm - human negative control cpm)/(human positive control cpm - human negative control cpm). The index value of 0.01 was set as cut-off limit of normal serum level of IAA in non-diabetic mouse strain.

Statistics analysis

Statistical analysis was conducted by using the Turkey HSD test and differences between Kaplan-Meier survival curves were estimated by the log rank test with the use of Dr. SPSS II for windows software (SPSS Inc., Chicago, IL). P values less than 0.05 were considered statistically significant.

Results

Intracellular expression of recombinant CTB-A^{16,19}APL fusion protein.

To confirm the expression of CTB-A^{16,19}APL fusion protein in yeast, GM1-ELISA was performed using anti-CT or anti-A^{16,19}APL antiserum. As expected the cell extract from unfused CTB-gene transformed yeast was reactive with anti-CT, but not with anti-A^{16,19}APL antiserum, and the cell extract from vector-transformed yeast was unreactive with either antibodies. On the other hand, the CTB-A^{16,19}APL-gene transformed yeasts were detected as double positive clones for anti-CT and anti-A^{16,19}APL antiserum. Among the double positive clones, we selected yeast clone #3, which has the highest expression level, for intranasal or oral immunization experiments (Figure 2).

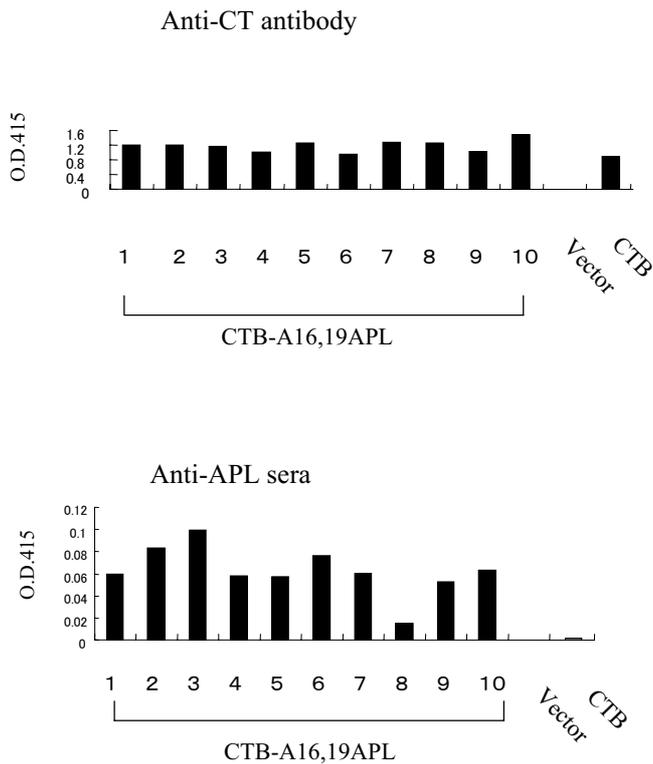


Figure 2. Expression analysis of the CTB-A^{16,19}APL fusion protein from recombinant *Pichia pastoris* by GM1-ELISA

Cell extracts derived from several clones of recombinant yeast transformed with CTB-A^{16,19}APL fusion gene (10 clones were shown in this figure), unfused CTB gene, or vector only were applied to microtiter plate wells pre-coated with GM1-ganglioside, and then captured proteins were detected by anti-CT or anti-APL antiserum. Only the fusion protein was found reactive to both antisera. Extract derived from yeast transformed with unfused CTB gene did not react with anti-APL antiserum, and no reaction with both antisera was detected for cell extract derived from vector-transformed recombinant yeast. The results were expressed as OD₄₁₅.

Immunoblot analysis using anti-CT antibody revealed that the extract from CTB- or CTB-A^{16,19}APL-gene transformed yeasts showed bands with a molecular mass of 12 and 13.5 kDa, respectively. The molecular size of the bands were closely matched to CTB or CTB-A^{16,19}APL fusion monomer (Figure 3).

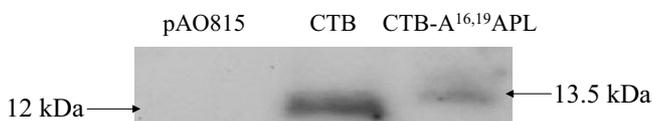


Figure 3. Expression analysis of the CTB-A^{16,19}APL fusion protein from recombinant *Pichia pastoris* by immunoblot

Cell extracts from recombinant yeast transformed with CTB-A^{16,19}APL fusion gene, unfused CTB gene, or vector only were separated by 12% SDS-PAGE and blotted to PVDF membrane. The protein bands were reacted with rabbit anti-CT antiserum and anti-rabbit monoclonal antibody conjugated to horseradish peroxidase (HRP), followed by HRP substrate. The molecular mass of the bands correspond well with the expected molecular mass for the fusion protein and unfused CTB monomer.

Intranasal administration of recombinant CTB-A^{16,19}APL failed to prevent diabetes.

A^{16,19}APL peptide was known to be capable of preventing diabetes in NOD mice when subcutaneously¹⁵ or intranasally administered.⁸ In this study, we established a yeast expression system for production of CTB-A^{16,19}APL fusion protein, and examined whether the extracted recombinant protein could prevent diabetes when intranasally administered. Thirty-six NOD mice were randomly divided into 4 groups (n = 9/group) and were administered with crude lysate of CTB-A^{16,19}APL fusion protein (20 μg/mice) with or without CT. Mice were administered intranasally with PBS or CT alone as a control. Intranasal administration of CTB-A^{16,19}APL fusion protein did not suppress the development of diabetes when compared to the PBS-administered control group ($P = 0.95$) (Figure 4). Furthermore, CT co-administered with CTB-A^{16,19}APL did not alter the course of diabetes development. These results indicate that the amount of autogenic peptide A^{16,19}APL might be too low to modulate the development of diabetes.

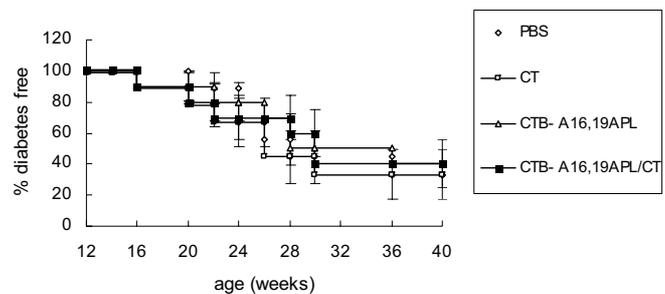


Figure 4. Life table analysis for the development of diabetes following intranasal immunization with recombinant proteins

NOD mice were intranasally immunized with CTB-A^{16,19}APL, a mixture of CTB-A^{16,19}APL with CT, CT only, or PBS as described in section Materials and methods. Intranasal immunization was started at 4-10 weeks of age. There was no statistically significant differences between CTB-A^{16,19}APL (or CTB-A^{16,19}APL + CT) and CT or PBS treated groups ($P = 0.95$). PBS (n = 9); CT (n = 9); CTB-A^{16,19}APL (n = 10); CTB-A^{16,19}APL + CT (n = 10)

Oral administration of live yeasts expressing CTB-A^{16,19}APL was moderately effective in reducing the incidence of diabetes.

In order to increase the amount of antigen uptake in vivo by protecting the antigen from the digestive enzymes in the gastrointestinal tract, live yeast cells were directly administered orally. Twenty-seven NOD mice were randomly separated into 3 groups (n = 9/group) for oral administration with CTB-A^{16,19}APL or unfused CTB in 0.5 ml of PBS, or with PBS alone. Accumulated incidence of disease at 40 weeks of age was 37% in CTB-A^{16,19}APL, 55% in CTB, and 76% in PBS-treated mice. Reduction of the disease incidence by 51% level was observed for CTB-A^{16,19}APL as compared with PBS-treated mice, although the difference analyzed by Kaplan Meier analysis did not reach a statistically significant level ($P = 0.08$) (Figure 5).

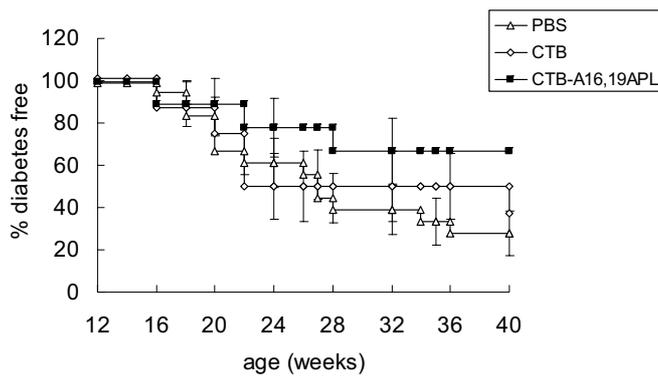


Figure 5. Life table analysis for the development of diabetes following oral administration of live recombinant yeast expressing CTB-A^{16,19}APL in NOD mice

Oral administration started at 4 weeks of age and mice were fed with yeast twice a week until 10 weeks of age. The blood glucose levels higher than 250 mg/dl for two consecutive measurements was regarded hyperglycemic. The CTB-A^{16,19}APL showed 51% reduction in the development of diabetes, but the efficacy did not reach a statistically significant level when analyzed by log Rank test ($P = 0.09$). PBS; CTB; CTB-A^{16,19}APL ($n = 9$).

Insulin autoantibody expression in oral CTB-A^{16,19}APL-treated NOD mice.

We have previously reported that A^{16,19}APL co-administrated with CT eliminated the insulin autoantibody (IAA) expression in NOD mice.⁸ In this study we also examined the effect of oral CTB-A^{16,19}APL treatment on IAA expression. We compared the levels or the prevalence of IAA in CTB-A^{16,19}APL treated mice with those in the control groups at 4, 8 and 12 weeks of age and no significant differences were found between any groups (Figure 6).

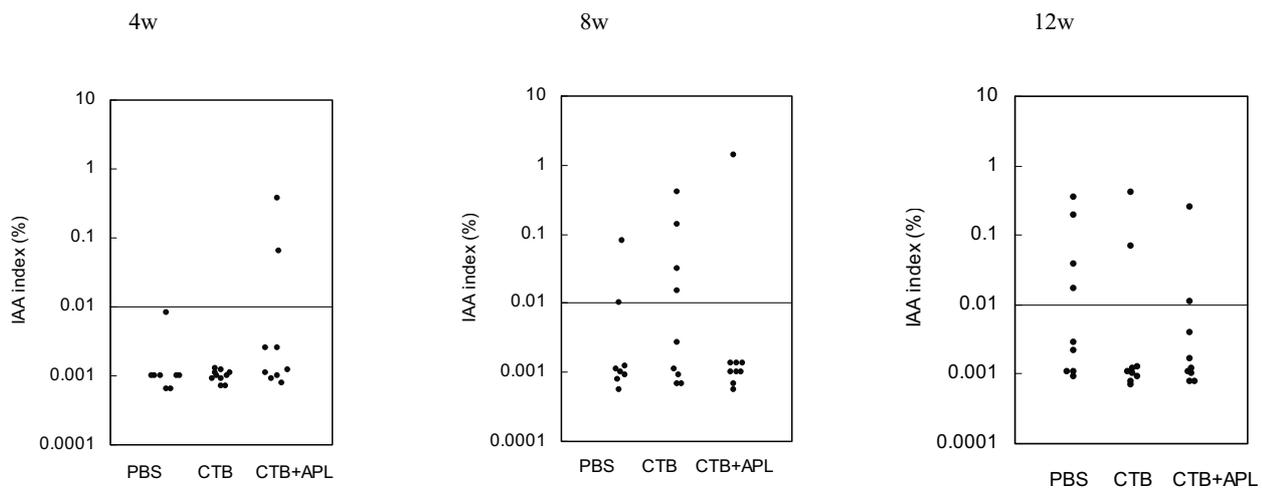


Figure 6. Insulin autoantibody level analyzed in mice orally treated with live recombinant yeasts

IAA level induced by oral administration of CTB-A^{16,19}APL to NOD mice was measured ($n = 9$). There was no significant difference in IAA levels for three groups at 4, 8 or 12 weeks of age. The cut-off level of IAA index was set at 0.01%.

Discussion

We have previously demonstrated that the altered B:9-23 peptide with alanine substitutions at position 16,19 (A^{16,19}APL), but not native B:9-23 peptide, provides a strong suppression of insulinitis and diabetes when co-administered intranasally with cholera toxin adjuvant.⁸ A significant improvement has been achieved by co-administering immunomodulating agents, such as CTB, to enhance the tolerogenic activity of autoantigen given orally as well as intranasally. CTB conjugated with several autoantigens can markedly suppress progression of various autoimmune diseases including autoimmune diabetes.^{9, 16-18} In this study we engineered recombinant yeast expressing CTB-A^{16,19}APL fusion chimeric protein for nasal and oral vaccination for type 1 diabetes prevention.

Intranasal administration of CTB-A^{16,19}APL even with the presence of CT adjuvant failed to prevent diabetes. The amount, purity or protein folding of the fusion protein could be insufficient to induce protective immunity in NOD mice, although the precise mechanism of the failure was not investigated in this study.

We next evaluated oral route and used a yeast live cell system in which the antigen was encapsulated by yeast cells to protect it from degradation by gastric digestive enzymes. This strategy is therefore expected to prolong the recombinant protein's half-life in vivo. This strategy of encapsulating antigens by live cells¹² is expected to deliver more intact recombinant antigens into the small intestine where antigens are efficiently taken up through GM1-gangliosides by CTB's affinity for the receptor. In this study we found that oral delivery of yeast live cells expressing CTB-A^{16,19}APL tended to decrease the onset of diabetes but did not alter the spontaneous expression of IAA, indicating that this immunization method is less effective than the intranasal vaccination using synthetic peptide together with CT. We speculated that possible reasons for insufficient efficacy of our oral strategy could be caused

by less quantity or quality of antigen from yeasts than synthetic one and weaker adjuvant efficacy of CTB compared to that of CT. A recent study suggested that oral-CT administration could efficiently induce the villous M cells on the small intestine, which plays a critical role for the induction of systemic Ag-specific immune responses via the oral route.^{19,20} Since oral-CTB does not induce such systemic responses, our strategy using CTB fusion protein might not affect on the autoantibody production or prevent autoimmune disease. We should consider an additional approach to target important intestinal epithelial cells such as villous M cells to develop more effective oral vaccination.²¹

A clinical trial for the prevention of type 1 diabetes with alum-adsorbed glutamic acid decarboxylase (GAD), which is another islet specific autoantigen, is currently underway²² and its results are promising. Subcutaneous injection of GAD-alum at disease onset induced a GAD-specific immune response with higher levels of GAD antibodies and significantly less decline of the endogenous insulin secretion as compared to the placebo group. However, the GAD-alum treatment does not unnesessitate the insulin therapy requirement since frequent subcutaneous administration of antigen with adjuvant is invasive and not readily acceptable for clinical application, especially for children. To improve the efficacy of oral vaccination with altered insulin peptide (A^{16,19}APL) delivered by food-grade vehicles such as yeast and edible plants, a novel vaccination system should be developed, which may include a combination of systemic priming with strong immunogens like GAD-Alum, followed by a non-invasive thus less stressful mucosal vaccination. This new vaccine platform may potentially become a safe and effective immunotherapeutic strategy to cure type 1 diabetes.

Acknowledgments

We would like to thank Dr. George S Eisenbarth (Barbara Davis Center for childhood diabetes, Denver, CO., USA) for supporting IAA measurements. This work was supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan and by research grants from the Japan Society for the Promotion of Science (#17590940).

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