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Expression of Cholera Toxin B Subunit and Assembly as Functional Oligomers in Silkworm

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The nontoxic B subunit of cholera toxin (CTB) can significantly increase the ability of proteins to induce immunological tolerance after oral administration, when it was conjugated to various proteins. Recombinant CTB offers great potential for treatment of autoimmune disease. Here we firstly investigated the feasibility of silkworm baculovirus expression vector system for the cost-effective production of CTB under the control of a strong polyhedrin promoter. Higher expression was achieved via introducing the partial non-coding and coding sequences (ATAAAT and ATGCCGAAT) of polyhedrin to the 5' end of the native CTB gene, with the maximal accumulation being approximately 54.4 mg/L of hemolymph. The silkworm bioreactor produced this protein vaccine as the glycoslated pentameric form, which retained the GM1-ganglioside binding affinity and the native antigenicity of CTB. Further studies revealed that mixing with silkworm-derived CTB increases the tolerogenic potential of insulin. In the nonconjugated form, an insulin : CTB ratio of 100 : 1 was optimal for the prominent reduction in pancreatic islet inflammation. The data presented here demonstrate that the silkworm bioreactor is an ideal production and delivery system for an oral protein vaccine designed to develop immunological tolerance against autoimmune diabetes and CTB functions as an effective mucosal adjuvant for oral tolerance induction.

Keywords: Autoimmune diabetes, Baculovirus, Cholera toxin B subunit, Edible vaccine, Oral tolerance, Silkworm

Introduction

Cholera toxin B subunit (CTB) is the pentameric non-toxic portion of cholera toxin (CT), responsible for the holotoxin binding to the GM1 ganglioside receptor present on most nucleated cells (Cuatrecasas, 1973). When conjugated to autoantigens, the CTB dramatically increases their tolerogenic potential after oral administration (Pierre *et al.*, 1992; Sun *et al.*, 1994, 1996 and 2000; Bergerot *et al.*, 1997; McSorley *et al.*, 1998; Rask *et al.*, 2000). This effect is probably mediated by the ability of CTB to act as a mucosal carrier system (Sun *et al.*, 1994), although CTB might also have direct effects on the immune system (Li and Fox, 1996; Burkart *et al.*, 1999). Recent studies have showed that CTB is an effective mucosal adjuvant in potentiating immune responses or increasing immunological tolerance to corresponding antigens (Tochikubo *et al.*, 1998; Wu *et al.*, 1998; Yasuda *et al.*, 1998; Sun *et al.*, 2000; Maeyama *et al.*, 2001; Anjuere *et al.*, 2003; Bregenholt *et al.*, 2003; Holmgren *et al.*, 2005; Zhang *et al.*, 2005). These investigations indicate that CTB is a powerful edible vaccine if expressed in large-scale production in an edible tissues or organism.

Up to now, the CTB gene has been expressed in *Escherichia coli* (Areas *et al.*, 2002), Swiss 3T3 cells (Hashimoto *et al.*, 1996), transgenic tobacco (Hein *et al.*, 1996; Wang *et al.*, 2001; Daniell *et al.*, 2001), potato (Arakawa *et al.*, 1997), and tomato (Jani *et al.*, 2002). However, the recombinant CTB expressed in *Escherichia coli* was in an insoluble form, which required extensive purification. In addition, bacteria-derived CTB is inappropriate as an edible vaccine because of the contamination of endotoxin. Despite the suitability of plant expression systems for the production of CTB as functional oligomers, the expresssion levels in transgenic plants are not satisfied with the therapeutic applications in humans.

The development of recombinant protein production in the silkworm baculovirus expression vector system (BEVS) may overcome this limitation and thus may facilitate the use of genetically modified edible proteins for the production and

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delivery of vaccine antigens (Miller, 1988; Wang *et al.*, 1991). There is the potential for not only low-cost but also high-capacity production with the capability to scale-up to agricultural levels. Using the *Bombyx mori* nuclear polyhedrosis virus (BmNPV) of the baculovirus expression system also eliminates concerns regarding pathogens that could potentially be transmitted to humans. The baculovirus is non-infectious to vertebral animals, and the system itself is safe (Carbonell and Miller, 1987; Herrington *et al.*, 1992). Collectively, these features make the silkworm system an ideal expression and delivery package for producing oral vaccines.

Generally, silkworm is a high-level expression system (Miller, 1988). The production levels of foreign genes are very different in the silkworm baculovirus system. Several extra techniques are used to improve the production level in this system, such as selecting appropriate codon, introducing 5'-untranslated sequences and coexpressing chaperones (Kost *et al.*, 2005).

In this paper, the partial non-coding and coding sequences of polyhedrin are specially fused to the 5'-end of the native CTB gene and this modified CTB gene were expressed in BmN cells and silkworm larvae with BEVS. We found that the production level in silkworm larvae was encouraging, reaching 54.4 mg/L of hemolymph. Furthermore, the expressed recombinant protein was released into the hemolymph in a pentameric form, retaining the GM1-ganglioside binding affinity and the native antigenicity of CTB. The oral administration of this silkworm-derived CTB and insulin admixtures suppressed pancreatic islet inflammation in non-obese diabetic (NOD) mice. This investigation demonstrates the potential of other oral protein vaccines to be expressed and assembled properly in the silkworm bioreactor.

Materials and Methods

Reagents, *B. mori* larvae, cell line and mice DNA manipulation and PCR amplification kit were purchased from *TaKaRa Biomedicals* (Japan). The pBacPAK8 transfer vector was obtained from *Clontech* (Palo Alto, USA). DOSPER liposomal transfection reagent, DIG DNA labeling and detection kit and chromogenic Western blotting kit were supplied by *Roche Diagnostics* (Germany). The recombinant plasmid pBlue-CTB was provided by Dr. Shengwu Ma. Fifth-instar silkworm *B. mori* larvae (Jingsong × Haoyue, Showa) were fed fresh mulberry leaves and reared under a photoperiod schedule of 12 h light and 12 h darkness at 25 ± 1°C. BmN cells were cultured in TC-100 medium (Gibco-BRL, Gaithersburg, USA) containing 10% fetal calf serum (Gibco-BRL) and 50 μg/ml gentamycin at 27°C. Female non-obese diabetic (NOD) mice were purchased from Shanghai Laboratory Animal Center (SLAC, CAS, China) and housed at the central animal facility, where they were screened for bacterial and viral pathogens.

Construction of the recombinant transfer vector pBacPAK-CTB In order to introduce the polyhedrin non-coding and coding sequences (in bold type) up-/down-stream of the start codon of the coding sequence of the native CTB gene, the primers for PCR amplication from pBlue-CTB were designed as follows: forward primer 5' GG*GGATCC***ATAAATATGCCGAAT**ATTAAATTAAA ATTTGGTGT 3' (*Bam*HI) and reverse primer 5' GG*GAATTC*TTA ATTTGCCATACTAATTG 3' (*Eco*RI). Then the CTB gene was inserted into the transfer vector pBacPAK8 downstream of the polyhedrin promoter. Following confirmation of the DNA sequences, the recombinant transfer vector pBacPAK-CTB was constructed and verified.

Transfection and isolation of the recombinant baculovirus Co-transfection was performed as described in our previous work (Gong *et al.*, 2005). Briefly, the purifed recombinant transfer vector pBacPAK-CTB and linearized viral DNA of modified baculovirus Bm-BacPAK6 digested with *Eco*81I were used for co-infection in cultured BmN cells. After purification by three rounds of plaque isolation, the recombinant virus was selected and then identified further by PCR amplification and genomic DNA hybridization. Finally, the recombinant virus stock was prepared and the dilution of the virus was calculated using the Reed-Muench method.

Expression of recombinant CTB in *B. mori* cells and larvae BmN cells (2×10^6 cells/flask) were infected with the recombinant virus at a medium dose (MOI = 10). The infected cells were cultured at 27°C. Then the cultured cells were harvested at 2-7 d post-infection respectively. The BmN cells were resuspended in 0.2 ml of PBS, and then the cells were lysed by repeated freezing and thawing or gentle sonication. After centrifugation to remove the insoluble debris, the supernatant was collected. The fifth instar *B. mori* larvae were needle inoculated with the recombinant viral solution (1×10^7 pfu/ml) into the body cavity, using wild-type virus injection as control. The infected larvae were reared at 25°C. Hemolymph was then collected at 2-7 d post-inoculation and centrifuged to remove the insoluble impurities. For further analysis, the hemolymph samples were stored at −20°C.

ELISA quantification of CTB The recombinant CTB protein levels in BmN cells and silkworm larvae were determined by quantitative ELISA assay. A 96-well microtiter plate (JET, Canada) was loaded with serial dilutions of the cell-lysed supernatant or hemolymph in bicarbonate buffer, pH 9.6 (15 mmol/l $NaCO_3$, 35 mmol/l $NaHCO_3$) and incubated overnight at 4°C. The plate was washed three times in PBST (phosphate buffered saline (PBS) containing 0.05% Tween-20). The background was blocked by incubation in 1% bovine serum albumin (BSA) in PBS (100 μl/well) for 2 h at 37°C, followed by washing three times with PBST. The plate was incubated in a 1 : 8000 dilution of rabbit anti-cholera toxin primary antibody (Sigma) (100 μl/well) at 37°C for 2 h, followed by washing the wells three times with PBST. Then the plate was incubated with a 1 : 2000 dilution of anti-rabbit IgG conjugated with horseradish peroxidase (SABC, China) (100 μl/well) for 2 h at 37°C and washed three times with PBST. Finally the chromogenic substrate O-phenylenediamine (SCRC, China) (100 ìl/well) was added to the wells and the plate was incubated for 20 min at 37°C to develop color, followed by adding 2 mol/l H_2SO_4 (50 μl/well) to stop the reaction. The plate was cooled to room temperature before the absorbencies were measured in a Labsystems Multiskan MS ELISA plate reader (Labsystems, Finland) at 492 nm. Comparison of the absorbance at 492 nm of a known amount of bacterial CTB-

antibody complex (linear standard curve) and that of a known concentration of transformed plant total soluble protein was used to estimate CTB expression levels.

Western blot analysis For detection of the presence of monomeric or pentameric recombinant CTB, the cell-lysed supernatant or hemolymph samples were electrophoresed in a 12% SDS-PAGE for 45-60 min at 20 mA in Tris-glycine buffer (25 mM Tris, 250 mM glycine, pH 8.3, 0.1% SDS). Prior to electrophoresis, samples of the hemolymph were either boiled for 5 min prior to electrophoresis or loaded directly on the gel without heat treatment. The separated proteins were transferred from the gel to Hybond-P membrane (Amersham Biosciences) by electroblotting on a wet blotter (Bio-Rad, Richmond, USA) at 60 v for 2 h. The following immunoreaction and detection was performed by using a chromogenic western blotting kit according to manufacture's protocol. In this procedure, a rabbit anti-cholera toxin antiserum (Sigma, St. Louis, USA) and rabbit anti-insulin primary antibody were used for the immunoreaction respectively.

Enzymatic deglycosylation of silkworm-expressed CTB The cell-lysed supernatant or hemolymph containing CTB protein were digested with the deglycosylating enzyme peptide N-glycosidase F and O-glycosidase respectively (Roche) according to the manufacturer's instructions with a minor modification. In brief, 10 mg of silkworm-expressed CTB was heated at 100°C for 3 min in 20 mM sodium phosphate buffer, pH 7.5, containing 20 mM EDTA, 0.5% SDS and 1% 2-mercaptoethanol (only in the N-deglycosylation reaction). Nonidet P-40 was added to give the final concentration of 1% and the extract was digested overnight with 50 mU/ml peptide N-glycosidase F (or 0.2 mU/ml O-Glycosidase) at 37°C. After different enzymatic deglycosylation, the samples were subjected to SDS-PAGE and Western blot analysis with the anti-CTB antibody.

GM1-ganglioside binding assay A GM1-ELISA was performed to detect the affinity of silkworm-derived CTB protein for GM1-ganglioside. The microtiter plate was coated with monosialoganglioside-GM1 (Sigma) by incubating the plate with 50 μl/well of GM1 (10 μg/ml) in methanol at 4°C overnight. Alternatively, the wells were coated with 100 μl/well of 1% BSA, and the same dilution of either hemolymph-infected wild-type baculovirus or bacterial CTB (Sigma) were used as negative and positive controls respectively. The remainder of the procedure was identical to the ELISA assay described above.

Oral tolerance induction by admixtures of Silkworm-drived CTB and insulin Four-week-old female NOD mice were divided into five groups for different treatments: Group 1, fed 100 μg of insulin (Novo Nordisk); Group 2, fed 100 μg of insulin + 1 μg of CTB; Group 3, fed 100 μg of insulin + 10 μg of CTB; Group 4, fed 10 μg of CTB and Group 5, fed equal volume of wild-type hemolymph used as a control. The silkworm-derived CTB protein consisted in the hemolymph. At 5 weeks of age, each mouse was treated every other day until 10 weeks of age (3-4 times per week). Oral antigen was administrated via a blunt-ended curved feeding tube inserted into the esophagus/stomach. The animals were killed at 10 weeks of age for histopathological analysis of pancreatic islets.

Histopathological analysis of pancreatic islets For evaluation of insulitis levels in mice that had been treated by different methods, the extent of lymphocyte infiltration of islets was measured. Each group consisted of five mice. At 10 weeks of age, the mice were killed and the pancreata were removed. Each pancreas was fixed in 10% buffered formalin and embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin. The degree of insulitis was evaluated using a standardized scoring system by an observer that was blind to the group designations, as described previously (Charlton and Mandel, 1988). At least 15 islets were scored for each animal.

Results

Construction of recombinant baculovirus and expression of CTB in *B. mori* cells and larvae In our previous work, the expression level of the native CTB gene in silkworm is extremely low (data not published). To improve the production level of CTB, the partial non-coding and coding sequences (ATAAAT and ATGCCGAAT) of polyhedrin gene were introduced to the 5'-end of the native CTB gene. The 15 base-pair (bp) of corresponding homologous region of polyhedrin enhanced the expression level of foreign genes when this modified CTB gene was driven by the strong AcMNPV polyhedrin promoter (Fig. 1). The presence of the CTB gene in recombinant virus was confirmed by direct PCR of viral genomic DNA and further analyzed by genomic DNA hybridization. As expected, the PCR amplification of recombinant viral genomic DNA showed a specific band of 381 bp. No nonspecific amplification was observed in any of the samples (Fig. 2A). Then we used the fragment of the full-length CTB gene as a probe, and the recombinant plasmid as a positive control. The Southern blot revealed the presence of a fusion gene identical to the positive control, while the wild-type baculovirus did not show the gene-specific band (Fig. 2B). These evidences indicated that the CTB gene was inserted into the baculovirus genome immediately downstream of the polyhedrin promoter. The recombinant baculovirus was thus successfully constructed. According to the Reed-Muench formula, the dilution of the recombinant virus was 6.69×10^8 pfu/ml. After infection by this recombinant virus, BmN cells and silkworm larvae started to present the typical symptoms of NPV infection and produced CTB protein in the cytoplasm and body cavity, respectively.

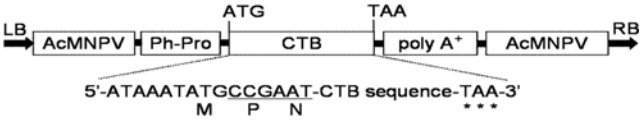

Fig. 1. Schematic structure of the transfer vector pBacPAK-CTB. AcMNPV, *Autographa Californica* Nuclear Polyhedrosis Virus; Ph-Pro, AcMNPV polyhedrin promoter; ATAAAT ATGCCGAAT, partial 5'-non-coding and coding sequences of polyhedrin; poly A^+, polyadenylation signal; LB, left border; RB, right border.

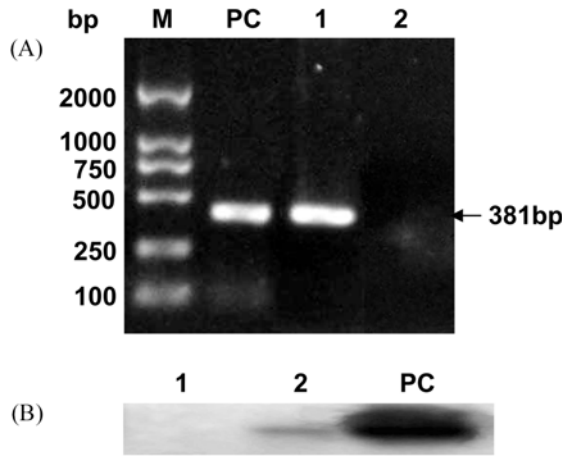

Fig. 2. Identification of the recombinant baculovius by PCR and southern blot. (A) PCR amplification of the recombinant baculovirus. M, DNA ladder; PC, positive control, the recombinant plasmid pBacPAK-CTB; lane 1, genomic DNA of the recombinant virus; lane 2, wild-type genomic viral DNA. The arrow indicates the size of CTB gene. (B) Southern blot of genomic viral DNA (digested with *Bam*HI and *Eco*RI). Lane 1, wild-type genomic viral DNA; lane 2, genomic DNA of recombinant virus; PC, positive control, recombinant plasmid pBacPAK-CTB.

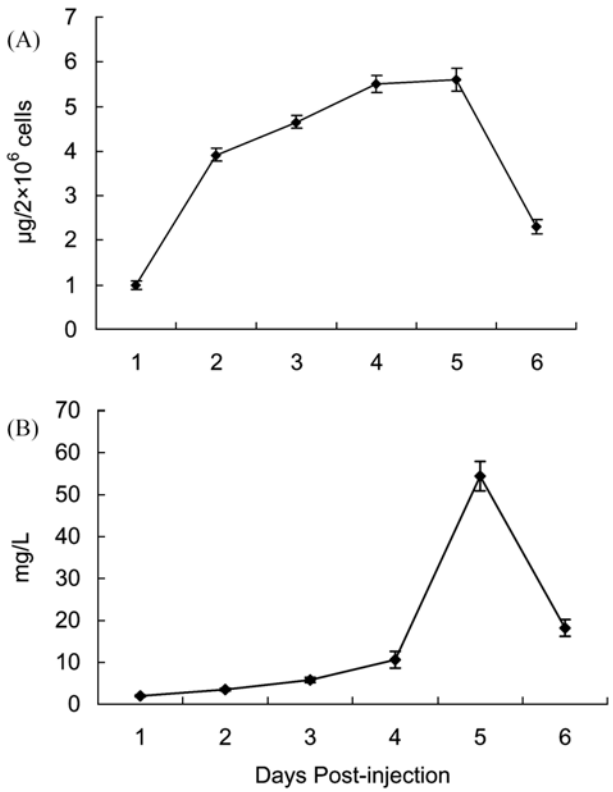

Fig. 3. Quantitative analysis of protein production levels in BmN cells (A) and silkworm larvae (B). Data are presented as the mean concentration ± SD on each day.

ELISA quantification of CTB expression To confirm the level of CTB expression in BmN cells and silkworm larvae, the infected cells and hemolymph of larvae were collected at 1-6d post-infection and detected by quantitative ELISA. The ELISA results showed that the highest detectable level of CTB protein in cells yielded up to 5.6 μg/2 × 10^6 cells at the fifth day post-infection (Fig. 3A). At the late phase of infection, the infected cells started to lyse. In the infected silkworm larvae, the recombinant fusion protein was efficiently released into the larval hemolymph. On average, 0.4 ml of hemolymph was obtained per larva. At the sixth day post-infection, the maximum amount of the recombinant CTB in the hemolymph reached 54.4 mg/L (Fig. 3B).

Silkworms produce CTB protein in a pentameric form In unboiled samples, silkworm-expressed CTB appeared as 70 kDa-pentamers (Fig. 4A, lanes 4 and 5). However, a nonspecific band with an apparent molecular weight of 50 kDa was found in all samples. This is due to nonspecific cross-reaction between anti-cholera toxin antibodies and silkworm protein. We found that the oligomeric fusion protein was dissociated into monomers by boiling for 5 min and subsequently migrated as two or three specific bands in both boiled silkworm cell and hemolymph samples (Fig 4A, lanes 3 and 6). This is not due to inadequate boiling or low concentrations of dithiothreitol or β-mercaptoethanol. Increasing these agents to reduce disulfide bridges or increasing duration of boiling did not alter polypeptide profiles significantly. In addition, the size of these bands is not identical to dimers, trimers or multimers of CTB. We presumed that these polypeptide profiles might result from the various degrees of glycosylation of silkworm-expressed CTB. The arrows in Fig 4A indicated the presence of various glycosylated proteins of CTB in BmN cells and hemolymph. The subsequent experiments of enzymetic deglycosylation supported this scientific hypothesis.

Silkworm-expressed CTB is partially glycosylated To verify that the silkworm-expressed CTB is glycosylated, the expressed products were treated with PNGase F and O-glycosidase respectively. No evident change of CTB proteins was found after O-glycosidase treatment (Fig. 4B, lanes 1c and 2c). However, after PNGase F treatment, a new band (indicated by arrow) was detected in silkworm cells and hemolymph respectively (Fig. 4B, lanes 1b and 2b). Owing to the incomplete deglycosylation, the apparent molecular weight of this new band was larger than the nonglycosylated CTB. These results indicate the recombinant CTB proteins in silkworm were partially glycosylated at various degrees.

Silkworm-derived CTB demonstrates a strong affinity for GM1-ganglioside For confirmation of the specific affinity of the pentameric protein for GM1-ganglioside, we used a GM1-ELISA method with GM1-ganglioside as the capture molecule and the bacterial pentameric CTB to produce a

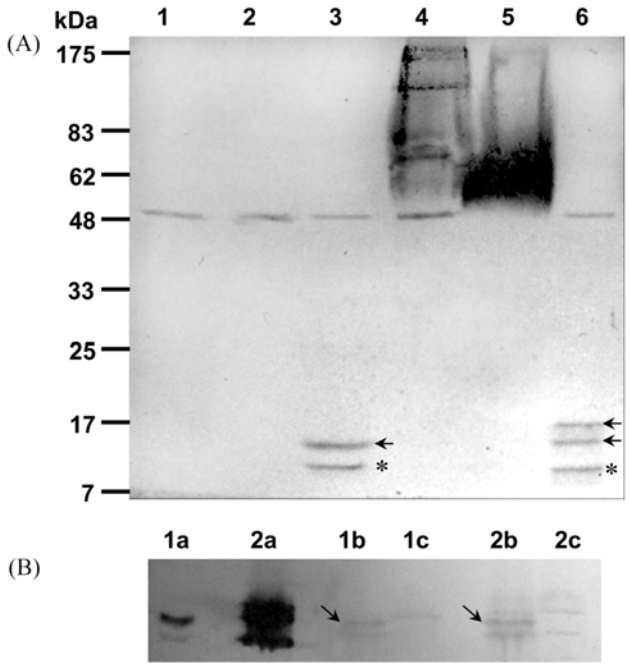

Fig. 4. (A) Western blot analysis of CTB expression in silkworm cell and larvae. Lane 1, boiled wild-type hemolymph; lane 2, unboiled wild-type hemolymph; lane 3, boiled cell extracts containing CTB protein; lane 4, unboiled cell extracts containing the same protein; lane 5, unboiled hemolymph containing the same protein; lane 6, boiled hemolymph containing the same protein. Numbers on the left indicate the positions of protein size markers. Asterisks indicate the nonglycosylated form of CTB. Various glycosylated form of CTB is indicated by arrows. (B) Deglycosylation of the recombinant CTB produced in BmN cells and silkworm larvae. The silkworm-expressed CTB were treated with PNGase F and O-glycosidase. Lane 1a, untreated cell extracts; lane 1b, PNGase F-treated cell extracts; lane 1c, O-glycosidase-treated cell extracts; lane 2a, untreated hemolymph; lane 2b, PNGase F-treated hemolymph; lane 2c, O-glycosidase-treated hemolymph. The deglycosylated form of recombinant CTB is indicated by arrows.

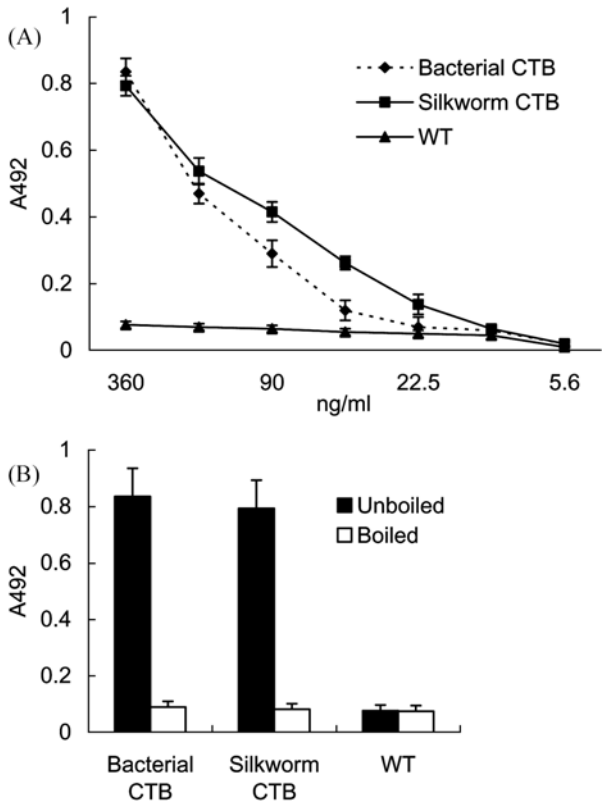

Fig. 5. Analysis of the binding affinity for the GM1-ganglioside pentameric CTB by GM1-ELISA. (A) Reactivity of CTB with the GM1-ganglioside and the antibody to CTB in GM1-ELISA in comparison with a native CTB control. WT indicate the hemolymph infected by wild-type virus. All samples were serially diluted two-fold and incubated for 1 h prior to the addition of the CTB-specific antibody. (B) Boiling induced pentamer dissociation into monomers. Approximately equal amounts of the three different samples indicated were used to measure A492 signal levels. Data represent the mean A492 values ± SD of each sample.

standard curve. An increase in the concentration-specific absorption signal was observed, indicating that the recombinant CTB protein existed as a pentamer, because only pentameric CTB can bind to GM1-ganglioside (Fig. 5A). However, the heat-treated protein completely lost its affinity for GM1-ganglioside (Fig. 5B). The silkworm-derived CTB protein exhibited the biochemical and antigenic properties necessary for the purposes of this study. The GM1 binding ability also suggests proper folding of CTB molecules resulting in the functional pentameric structure.

Silkworm-synthesized CTB is a mucosal adjuvant for oral tolerance induction in NOD mice To investigate whether silkworm-synthesized CTB would be effective mucosal adjuvant in the reduction of insulitis, female NOD mice were treated with insulin and silkworm-derived CTB admixtures, beginning at 5 weeks of age. After 5 weeks of supplementation, the mice were killed, their pancreatic tissues were harvested, and the islets of each animal were scored. A representative islet from an animal fed insulin and CTB admixtures showing focal peri-islet lymphocyte infiltration was given an insulitis score of 1 (Fig. 6A). Pancreatic islets from an animal fed CTB had an insulitis score of 2 (Fig. 6B), whereas animals fed insulin or wild-type hemolymph had insulitis scores of 3 and 4 (Fig. 6C and 6D). Student's *t*-test revealed a significant reduction of insulitis in mice fed 100 μg of insulin and 1 μg of CTB admixtures compared with that in mice fed insulin alone (Fig. 7; 1.97 ± 0.56 vs. 2.5 ± 0.4, $p < 0.05$). In addition, this suppressive effect of insulin was increased further when the CTB concentration was lowered. This result indicated that the adjuvant effect of CTB was presented. No significant differences in the insulitis scores were found in two groups of mice fed insulin and CTB admixtures. Interestingly, the recombinant CTB alone had the direct effect on the immune system, as there was a marked reduction of insulitis in animals fed 10 μg

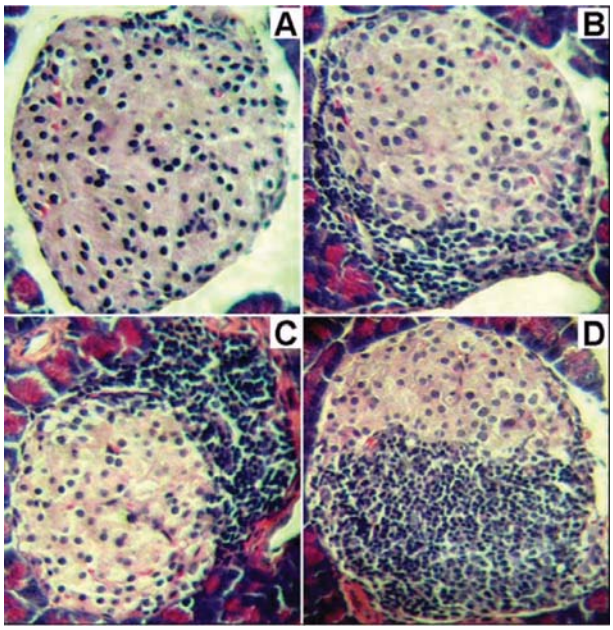

Fig. 6. Oral CTB-insulin admixtures treatment suppresses lymphocyte infiltration into the NOD pancreatic islets. (A) Representative islet from an animal fed insulin and CTB admixtures with minimal lymphocyte infiltration (histopathological score = 1), (B) Representative islet from an animal fed 10 μg of CTB with more extensive infiltration (histopathological score = 2), (C) Representative islet an animal fed 100 μg of insulin with intraislet infiltration in one-third to one-half of the islet area (histopathological score = 3), (D) Representative islet an animal fed wild-type hemolymph with extensive intraislet infiltration involving more than half of the islet area (histopathological score = 4).

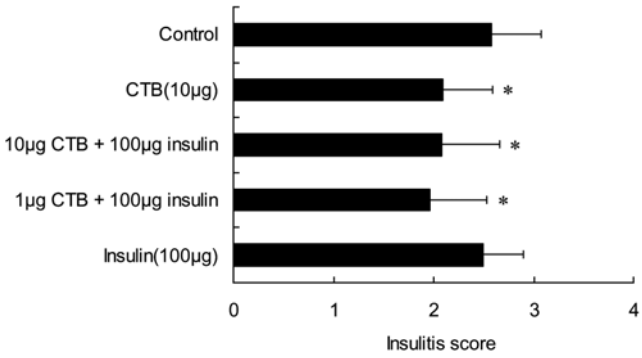

Fig. 7. Effect of feeding CTB or CTB-insulin admixtures on insulitis in NOD mice. Semiquantitative insulitis score. Data are expressed as the mean score of each group ± SEM (*$p < 0.05$).

of CTB vs. those fed wild-type hemolymph (Fig. 7; 2.09 ± 0.56 vs. 2.58 ± 0.5, $p < 0.05$). These findings demonstrate that the silkworm-synthesized CTB protein can function as a mucosal adjuvant, even in its nonconjugated form, increasing the specific tolerogenic effect of oral insulin. Meanwhile, when CTB is at a higher concentration, it can directly induce oral tolerance in NOD mice, providing an effective suppression of insulitis.

Discussion

Our investigation demonstrates the use of the polyhedrin coding and non-coding sequences can enhance the expression levels of the CTB gene with BEVS. Furthermore, these short sequences have no effect on the biological activity and immunogenicity of the fusion protein. Taken together, the method for fusing the partial non-coding and coding sequences is a feasible approach for overcoming the extremely low-level expression in silkworm. We found that recombinant CTB expressed in silkworm retains the significant immunological properties and it is an effective mucosal adjuvant, with the ability to increase the tolerogenic potential of orally administered insulin. This suggests that silkworm can be used to express CTB to develop a subunit edible vaccine against cholera. In addition to its relevance for the treatment of type 1 diabetes, the use of CTB as an adjuvant might prove to be an important tool in the development of new immune-regulatory treatment regimens in other autoimmune disorders.

The potential use of silkworm as an expression system or "bioreactor" for production of antigenic proteins for clinical use offers several advantages. It can be used for (i) large-scale production of foreign proteins, (ii) as an edible vaccine if expressed in an edible silkworm pupa or (iii) as a delivery system for oral protein drugs, to either enhance mucosal immunity or induce oral tolerance to the products of these peptides. In contrast to the conventional prokaryotic expression system, large-scale production of purified CTB in bacteria involves the use of expensive fermentation techniques and stringent purification protocols (Lebens *et al.*, 1993), making this a prohibitively expensive technology for developing countries. As compared with other eukaryotic expression systems, the expression of heterologous proteins in the silkworm bioreactor is under the control of the strong polyhedrin promoter, allowing levels of expression of up to 20% of total cell protein (Massotte, 2003). It is obvious that the protein production capacity of silkworms predominates over that of any other industrial system in use today. In addition, the cost of producing 1 kg of recombinant protein in silkworm is much lower than the cost of producing the same amount by *E. coli* fermentation or transgenic plants. The expression levels of CTB in silkworm in the present study are extremely encouraging, but there is still plenty of room to improve the current expression level. Further selection of strong promoters, regulatory elements (i.e. signal sequences, 5'-untranslated leader sequence and 3'-untranslated region) and appropriate codon choice should make it possible to produce very high levels of CTB (Ma *et al.*, 2005).

The recombinant CTB expressed in silkworm system can form various degrees of N-glycosylation. This is due to the glycosylated modification in baculovirus expression system at post-translational level (Ishida *et al.*, 1994; Kulakosky *et al.*,

1998; Joosten *et al.*, 2003). The glycosylated form could be a more stable conformation, which may contribute to enhancing cell recoganition and regulating immune response (Bolmstedt *et al.*, 2001; Rudd *et al.*, 2001; Lowe, 2001). Whether CTB function as a mucosal adjuvant or a direct oral tolerance antigen, it appears correlated with dose. At a lower dose, the CTB demonstrated the effect of adjuvant. In addition, 1 μg was more effective than 10 μg. At the same time, the direct oral tolerance was induced by CTB alone. A probable explanation could be that the glycosylated CTB induce immunoregulatory cells and suppress anti-islet effector cells, providing protective effect at the T cell level (Li and Fox, 1996; Sobel *et al.*, 1998; Burkart *et al.*, 1999).

In summary, we have demonstrated the feasibility of using silkworm for the production of CTB in a pentameric form with immunological properties as a mucosal adjuvant. To our knowledge, this is the first report on the production of functional CTB using silkworm. It is hoped that silkworm will not only become a valuable commercial source of CTB, but will also provide a convenient, feasible and effective means of treating clinical diseases, such as type 1 diabetes, by the oral delivery of insulin-CTB admixtures. Future development of edible vaccines for human consumption would be more appealing if the vaccines were expressed in silkworm. Therefore, we anticipate that elevated edible vaccine expression in silkworm is both feasible and forthcoming.

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References

Anjuere, F., George-Chandy, A., Audant, F., Rousseau, D., Holmgren, J. and Czerkinsky, C. (2003) Transcutaneous immunization with cholera toxin B subunit adjuvant suppresses IgE antibody responses via selective induction of Th1 immune responses. *J. Immunol.* **170**, 1586-1592.

Arakawa, T., Chong, D. K., Merritt, J. L. and Langridge, W. H (1997) Expression of cholera toxin B subunit oligomers in transgenic potato plants. *Transgenic Res.* **6**, 403-413.

Areas, A. P., Oliveira, M. L., Ramos, C. R., Sbrogio-Almeida, M. E., Raw, I. and Ho, P. L. (2002) Synthesis of cholera toxin B subunit gene: cloning and expression of a functional 6XHis-tagged protein in *Escherichia coli*. *Protein Expr. Purif.* **25**, 481-487.

Bergerot, I., Ploix, C., Petersen, J., Moulin, V., Rask, C., Fabien, N., Lindblad, M., Mayer, A., Czerkinsky, C., Holmgren, J. and Thivolet, C. (1997) A cholera toxoid-insulin conjugate as an oral vaccine against spontaneous autoimmune diabetes. *Proc. Natl. Acad. Sci. USA* **94,** 4610-4614.

Bolmstedt, A., Hinkula, J., Rowcliffe, E., Biller, M., Wahren, B. and Olofsson, S. (2001) Enhanced immunogenicity of a human immunodeficiency virus type 1 env DNA vaccine by manipulating N-glycosylation signals. Effects of elimination of the V3 N306 glycan. *Vaccine* **20**, 397-405.

Bregenholt, S., Wang, M., Wolfe, T., Hughes, A., Baerentzen, L., Dyrberg, T., von Herrath, M. G. and Petersen, J. S. (2003) The cholera toxin B subunit is a mucosal adjuvant for oral tolerance induction in type 1 diabetes. *Scand. J. Immunol.* **57**, 432-438.

Burkart, V., Kim, Y., Kauer, M. and Kolb, H. (1999) Induction of tolerance in macrophages by cholera toxin B chain. *Pathobiology* **67**, 314-317.

Carbonell, L. F. and Miller, L. K. (1987) Baculovirus interaction with nontarget organisms: a virus-borne reporter gene is not expressed in two mammalian cell lines. *Appl. Environ. Microbiol.* **53**, 1412-1417.

Charlton, B. and Mandel, T. E. (1988) Progression from insulitis to beta-cell destruction in NOD mouse requires L3T4+ T-lymphocytes. *Diabetes* **37**, 1108-1112

Cuatrecasas, P. (1973) Gangliosides and membrane receptors for cholera toxin. *Biochemistry* **12**, 3558-3566.

Daniell, H., Lee, S. B., Panchal, T. and Wiebe, P. O. (2001) Expression of the native cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. *J. Mol. Biol.* **311**, 1001-1009.

Gong, Z., Jin, Y. and Zhang, Y. (2005) Oral administration of a cholera toxin B subunit-insulin fusion protein produced in silkworm protects against autoimmune diabetes. *J. Biotechnol.* **119**, 93-105.

Hashimoto, Y., Oshima, A., Narimatsu, H. and Suzuki, A. (1996) Expression of the cholera toxin B subunit in the Golgi apparatus of Swiss 3T3 cells inhibits DNA synthesis induced by basic fibroblast growth factor. *J. Biochem. Tokyo* **119**, 985-990.

Hein, M. B., Yeo, T., Wang, F. and Sturtevant, A. (1996) Expression of cholera toxin subunits in plants. *Ann. NY. Acad. Sci.* **792**, 50-56.

Herrington, D. A., Losonsky, G. A., Smith, G., Volvovitz, F., Cochran, M., Jackson, K., Hoffman, S. L., Gordon, D. M., Levine, M. M. and Edelman, R. (1992) Safety and immunogenicity in volunteers of a recombinant Plasmodium falciparum circumsporozoite protein malaria vaccine produced in Lepidopteran cells. *Vaccine* **10**, 841-846.

Holmgren, J., Adamsson, J., Anjuere, F., Clemens, J., Czerkinsky, C., Eriksson, K., Flach, C. F., George-Chandy, A., Harandi, A. M., Lebens, M., Lehner, T., Lindblad, M., Nygren, E., Raghavan, S., Sanchez, J., Stanford, M., Sun, J. B., Svennerholm, A. M. and Tengvall, S. (2005) Mucosal adjuvants and anti-infection and anti-immunopathology vaccines based on cholera toxin, cholera toxin B subunit and CpG DNA. *Immunol. Lett.* **97**, 181-188.

Ishida, N., Tsujimoto, M., Kanaya, T., Shimamura, A., Tsuruoka, N., Kodama, S., Katayama, T., Oikawa, S., Matsui, M.,

Nakanishi, T., Kobayashi, J. and Nakazato, H. (1994) Expression and characterization of human bone morphogenetic protein-2 in silkworm larvae infected with recombinant Bombyx mori nuclear polyhedrosis virus. *J. Biochem (Tokyo)*. **115**, 279-285.

Jani, D., Meena, L. S., Rizwan-ul-Haq, Q. M., Singh, Y., Sharma, A. K. and Tyagi, A. K. (2002) Expression of cholera toxin B subunit in transgenic tomato plants. *Transgenic Res.* **11**, 447-454.

Joosten, C. E., Park, T. H. and Shuler, M. L. (2003) Effect of silkworm hemolymph on N-linked glycosylation in two Trichoplusia ni insect cell lines. *Biotechnol. Bioeng.* **83**, 695-705.

Kost, T. A., Condreay, J. P. and Jarvis, D. L. (2005) Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nat. Biotechnol.* **23**, 567-575

Kulakosky, P. C., Hughes, P. R. and Wood, H. A. (1998) N-Linked glycosylation of a baculovirus-expressed recombinant glycoprotein in insect larvae and tissue culture cells. *Glycobiology* **8**, 741-745.

Lebens, M., Johansson, S., Osek, J., Lindblad, M. and Holmgren, J. (1993) Large-scale production of Vibrio cholerae toxin B subunit for use in oral vaccines. *Biotechnology (NY)* **11**, 1574-1578.

Li, T. K. and Fox, B. S. (1996) Cholera toxin B subunit binding to an antigen presenting cell directly co-stimulates cytokine production from a T cell clone. *Int. Immunol.* **8**, 1849-1856.

Lowe, J. B. (2001) Glycosylation, immunity, and autoimmunity. *Cell* **104**, 809-812.

Ma, S. W., Huang, Y., Davis, A., Yin, Z. Q., Mi, Q. S., Menassa, R., Brandle, J. E. and Jevnikar, A. M. (2005) Production of biologically active human interleukin-4 in transgenic tobacco and potato. *Plant Biotechnol. J.* **3**, 309-318.

Maeyama, J., Isaka, M., Yasuda, Y., Matano, K., Kozuka, S., Taniguchi, T., Ohkuma, K., Tochikubo, K. and Goto, N. (2001) Cytokine responses to recombinant cholera toxin B subunit produced by Bacillus brevis as a mucosal adjuvant. *Microbiol. Immunol.* **45**, 111-117.

Massotte, D. (2003) G protein-coupled receptor overexpression with the baculovirus-insect cell system: a tool for structural and functional studies. *Biochim. Biophys. Acta.* **1610**, 77-89.

McSorley, S. J., Rask, C., Pichot, R., Julia, V., Czerkinsky, C. and Glaichenhaus, N. (1998) Selective tolerization of Th1-like cells after nasal administartion of cholera toxoid-LACK conjugate. *Eur. J. Immunol.* **28**, 424-432.

Miller, L. K. (1988) Baculoviruses as gene expression vectors. *Annu. Rev. Microbiol.* **42**, 177-199.

Pierre, P., Denis, O., Bazin, H., Mbongolo, E. M. and Vaerman, J. P. (1992) Modulation of oral tolerance to ovalbumin by cholera toxin and its B subunit. *Eur. J. Immunol.* **22**, 3179-3182.

Rask, C., Holmgren, J., Frederiksson, M., Lindblad, M., Nordstrom, I., Sun, J. B. and Czerkinsky, C. (2000) Prolonged oral treatment with low doses of allergen conjugated to cholera toxin B subunit suppresses immunoglobulin E antibody responses in sensitized mice. *Clin. Exp. Allergy* **30**, 1024-1032.

Rudd, P. M., Elliott, T., Cresswell, P., Wilson, I. A. and Dwek, R. A. (2001) Glycosylation and the immune system. *Science* **291**, 2370-2376.

Sobel, D. O., Yankelevich, B., Goyal, D., Nelson, D. and Mazumder, A. (1998) The B-subunit of cholera toxin induces immunoregulatory cells and prevents diabetes in the NOD mouse. *Diabetes* **47**, 186-191.

Sun, J. B., Holmgren, J. and Czerkinsky, C. (1994) Cholera toxin B subunit: An efficient transmucosal carrier-delivery system for induction of peripheral tolerance. *Proc. Natl. Acad. Sci. USA* **91**, 10795-10799.

Sun, J. B., Li, B. L., Czerkinsky, C. and Holmgren, J. (2000) Enhanced immunological tolerance against allograft rejection by oral administration of allogenic antigen linked to cholera toxin B subunit. *Clin. Immunol.* **97**, 130-139.

Sun, J. B., Rask, C., Olson, T., Holmgren, J. and Czerkinsky, C. (1996) Treatment of experimental autoimmune enchephalomyelitis by feeding myelin basic protein conjugated to cholera toxin B subunit. *Proc. Natl. Acad. Sci. USA* **93**, 7196-7201.

Tochikubo, K., Isaka, M., Yasuda, Y., Kozuka, S., Matano, K., Miura, Y. and Taniguchi, T. (1998) Recombinant cholera toxin B subunit acts as an adjuvant for the mucosal and systemic responses of mice to mucosally co-administered bovine serum albumin. *Vaccine* **16**, 150-155.

Wang, X. G., Zhang, G. H., Liu, C. X., Zhang, Y. H., Xiao, C. Z. and Fang, R. X. (2001) Purified cholera toxin B subunit from transgenic tobacco plants possesses authentic antigenicity. *Biotechnol. Bioeng.* **72**, 490-494.

Wang, X. Z., Ooi, B. G. and Miller, L. K. (1991) Baculovirus vectors for multiple gene expression and for occluded virus production. *Gene* **100**, 131-137.

Wu, H. Y. and Russell, M. W. (1998) Induction of mucosal and systemic immune responses by intranasal immunization using recombinant cholera toxin B subunit as an adjuvant. *Vaccine* **16**, 286-292.

Yasuda, Y., Matano, K., Asai, T. and Tochikubo, K. (1998) Affinity purification of recombinant cholera toxin B subunit oligomer expressed in Bacillus brevis for potential human use as a mucosal adjuvant. *FEMS Immunol. Med. Microbiol.* **20**, 311-318.

Zhang, P., Yang, Q. B., Balkovetz, D. F., Lewis, J. P., Clements, J. D., Michalek, S. M. and Katz, J. (2005) Effectiveness of the B subunit of cholera toxin in potentiating immune responses to the recombinant hemagglutinin/adhesin domain of the gingipain Kgp from Porphyromonas gingivalis. *Vaccine* **23**, 4734-4744.