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ScienceDirect
Vaccine 25 (2007) 8395–8404
Vaccine
www.elsevier.com/locate/vaccine

Use of a genetic cholera toxin B subunit/allergen fusion molecule as mucosal delivery system with immunosuppressive activity against Th2 immune responses

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Received 7 August 2007; received in revised form 3 October 2007; accepted 3 October 2007
Available online 22 October 2007

Abstract

Induction of peripheral tolerance can be facilitated when the antigen is linked to the B subunit of cholera toxin (CTB), an efficient mucosal carrier. In the present study, a genetic fusion molecule of Bet v 1 and CTB was produced to test whether mucosal application of this construct would lead to suppression of Th2 responses. Intranasal pretreatment of BALB/c mice with rCTB-Bet v 1 prior to allergic sensitisation with the allergen significantly decreased IgE but markedly increased allergen-specific IgG2a levels in sera as well as IFN-γ production of splenocytes. This Th1 shift was supported by an increased T-bet/GATA3 mRNA ratio. IL-5 production within the airways was suppressed after the pretreatment with rCTB-Bet v 1, while local allergen-specific IgA antibodies were markedly enhanced by pretreatment with the construct. Upregulation of Foxp3, IL-10 and TGF-β mRNA expression was detected in splenocytes after pretreatment with unconjugated allergen but not with the fusion molecule, indicating that antigen conjugation to a mucosal carrier modifies the immunomodulating properties of an antigen/allergen.

Keywords: Bet v 1; Conjugated allergen; CTB

1. Introduction

Mucosally induced immunological tolerance has become an attractive strategy for prevention and treatment of autoimmune and allergic diseases [1,2]. Several studies using animal models demonstrated that nasal or oral administration of allergens could be used for prevention and treatment of allergic immune responses [3–6]. Meta-analysis of 22 trials including 979 patients suffering from seasonal or perennial allergic rhinitis showed that sublingual administration of allergen extracts resulted in a significant reduction of both symptoms and medication requirements [7]. Although oral/sublingual or nasal administration offers an effective and safe method for induction of immunological tolerance, the doses of allergen used in these trials and the frequency of allergen administration have been rather high presumably due to failure of absorption of the allergens and their degradation before entering a mucosal tissue [1,8].

The non-toxic B subunit of cholera toxin (CTB) and the closely related *E. coli* heat-labile enterotoxin B subunit (LTB) were used to overcome such limitations by serving as mucosal

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doi:10.1016/j.vaccine.2007.10.003

carrier molecules of chemically or genetically conjugated antigens for the induction of oral tolerance [9–13]. When used as a carrier and given by various mucosal routes in the absence of cholera toxin (CT) as an adjuvant, CTB induced a strong mucosal IgA immune response and antigen-specific tolerance to itself and to the conjugated antigen [14,15]. Conjugation with CTB may greatly facilitate antigen delivery and presentation to the mucosa-associated lymphoid tissues due to its affinity for the cell surface receptor GM1-gangloside which is located on most mammalian cells including dendritic cells, B cells and macrophages [16].

It has been shown that a single oral or nasal administration of microgram amounts of ovalbumin (OVA) chemically coupled to CTB or LTB could suppress an allergen-specific IgE response in naive [12] as well as in sensitised mice [11]. However, in previous studies we demonstrated that the suppressive activity depended on the allergen used when chemically coupled to CTB [13]. Intranasal administration of CTB-OVA prior to sensitisation led to suppression of Th2 immune responses to OVA. In contrast, intranasal administration of chemically coupled CTB-rBet v 1 prior to sensitisation was shown to enhance the production of Th2-promoting cytokines such as IL-4, IL-5, as well as IL-10 and Bet v 1-specific antibodies of all isotypes. It was suggested that, apart from the nature of the antigen, the method of conjugation might influence the effects of CTB/CTB-like molecules [12,17]. The present study was performed to investigate whether recombinant production of a fusion molecule, rather than chemically coupled products, could improve the immunomodulatory capacity of the mucosal delivery system. Therefore, a fusion molecule of CTB and the major birch pollen allergen Bet v 1a was genetically engineered to test whether mucosal application of this rCTB-Bet v 1a could suppress the Th2 response. Using a mouse model of birch pollen allergy we demonstrated that, in contrast to Bet v 1 chemically coupled to CTB, Bet v 1 genetically fused to CTB promoted suppression of systemic and local allergic immune responses.

2. Methods

2.1. Construction of the CTB-Bet v 1a expression vector

The mature CTB coding sequence was amplified by PCR from the CVD 103-HgR live oral cholera vaccine (Orochol, Berna, Switzerland). Prior to construction of the CTB-Bet v 1a fusion gene, the oligonucleotide sequence encoding a flexible tetrapeptide GPGP (hinge) [18] was fused to the 3′ end of the CTB gene. At the 5′ end of CTB the restriction site NcoI was added to the nucleotide sequence. The Bet v 1a encoding cDNA was amplified by PCR from its original cloning vector with DNA primers containing the oligonucleotide sequence encoding a hinge tetrapeptide at the 5′ end and SalI at 3′ end. Both PCR products were gel purified and fused by PCR to obtain a DNA coding for the CTB-Bet v 1a fusion protein. Following cloning into *E. coli* XL-1 Blue cells and confirmation of the DNA, the CTB-Bet v 1a fusion construct was inserted into the expression vector pelBpHis-Parallel 2 [19].

2.2. Expression, purification and refolding of recombinant CTB-Bet v 1 fusion protein

Competent BL21 [DE3] *E. coli* cells were transformed with the CTB-Bet v 1a expression vector and grown in LB medium supplemented with 1 mM ampicillin at 37 °C until an OD_{600} of 0.6–0.8 was reached. Protein expression was then induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM. Cells were harvested 12 h post-induction. 6×His-tagged rCTB-Bet v 1a protein was produced in inclusion bodies and therefore, purified under denaturing conditions. The cells were solubilised in buffer A containing 10 mM Na-phosphate, pH 8, 8 M urea and 20 mM β-mercaptoethanol. The solubilised rCTB-Bet v 1a was purified using a Ni^{2+}-NTA affinity column (Qiagen, Valencia, CA, USA) and subjected to a refolding procedure. Gradual removal of the denaturant agent (8 M urea) was performed by stepwise dialysis. The urea concentration was reduced from 8 to 1 M in refolding buffer containing 20 mM Tris/HCl, pH 7.4, 0.5 M NaCl, 20% glycerol and reduced and oxidized glutathione at a molar ratio of 3:1. After refolding, the sample was dialyzed against the refolding buffer in the absence of the redox pair components and glycerol. The protein was stored at 4 °C or lyophilised and stored at −20 °C. Concentration of bacterial endotoxins was determined using the LAL Endochrome test (Charles River Endosafe, Charleston, MA, USA) according to the user manual. Removal of endotoxins from the sample was achieved using the EndoTrap Blue affinity matrix (Profos, Regensburg, Germany) according to the manufacture's instructions. Protein concentration was determined according to the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA).

2.3. SDS-PAGE and immunoblot analysis

Samples containing 5–10 μg of protein were subjected to 8% SDS-PAGE. Samples were either boiled for 5 min prior to electrophoresis or loaded directly onto the gel without heat treatment. Immunoblot analysis of rCTB-Bet v 1a was performed using a monoclonal mouse anti-Bet v 1 IgG antibody (BIP1) or a serum pool of birch pollen allergic patients containing Bet v 1-specific IgE [4].

2.4. N-terminal sequencing

The protein monomer was blotted onto a polyvinylidene difluoride membrane, Coomassie stained, excised and sequenced using a Procise 491 sequencer (Applied Biosystems, Foster City, CA, USA).