Oral Administration of *Bacillus subtilis* Endospores Displaying Influenza Virus Matrix Protein 1 Elicit Cellular Immune Response in Mice

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Abstract

Bacterium *Bacillus subtilis* is a gram-positive bacilli which produce endospores. Being metabolically dormant, spores are resistant to many environmental stressors such as UV radiation, desiccation, heat or freezing. There are evidence that oral or intranasal administration of spores presenting antigens induces a specific, both cellular and humoral immune response which can protect animals from infection. In our study, using a genetic approach we constructed *Bacillus subtilis* strains producing spores presenting influenza A virus matrix protein 1 (M1) on their surface. M1 protein was fused to spore coat CotZ protein and was stably exposed on the spore surface as demonstrated by the immunostaining of intact, recombinant spores. The immunogenicity of recombinant spores was tested by oral administration in mice. As proved by an IFN- γ ELISPOT assay, constructed spores elicited significant cell-mediated immune response.

Keywords: Bacterial spores; *Bacillus subtilis*; Spore display; Influenza virus; Flu

Introduction

Development of an effective vaccine against infection influenza virus is a real challenge. A key problem for the development of effective vaccines against influenza is a high degree of antigenic variability of the virus strains circulating during the year. The constantly ongoing antigenic drifts and shifts of influenza virus cause the need for annual vaccination with a formulation against a specific strains circulating in a given season. Most existing vaccines are effective due to the ability to induce production of neutralizing antibodies directed against the capsular antigen-hemagglutinin [1]. Although the immunity which is based on antibodies is effective it is not universal and provides protection only against selected strains as a result of the antigenic variability of the virus. In contrast cellular-mediated

immune response targets conserved antigens and can provide cross-immunity against different subtypes [2]. Influenza virus-specific Cytotoxic T Lymphocytes (CTLs) have been shown in animal studies to limit influenza A virus replication and to protect against lethal influenza A virus challenge [3]. In last year's a new live antigen carrier system emerged [4]. Recent studies show that recombinant endospores of *Bacillus subtilis* can serve as antigen carriers by fusing peptide of interest to spore coat proteins [5]. In our study we constructed *Bacillus subtilis* strain producing spores presenting influenza M1 protein on their surface. M1 is most abundant protein in influenza virions and it is conserved among various strains. Moreover it has been shown that vaccine based on M1 protein can elicit significant cellular response in vaccinated individuals [6]. Recombinant spores were orally administrated to mice to evaluate the immunogenic properties of constructs. This work indicates that spores can serve as influenza antigen carriers and induce cell-mediated immune response. Moreover co-administration of constructed spores with spores presenting interleukin 2 significantly boosted observed cell-mediated response.

Materials and Methods

Bacterial Strains and Transformation

All strains used in this study are listed in Table 1. All cloning experiments were done using *Escherichia coli* DH5 α [7]. Bacteria were transformed according to the previously described methods: CaCl₂-induced competence of *E. coli* cells [8] and transformation of *B. subtilis* [9].

Construction of Recombinant *Bacillus subtilis* Strain

To generate genetic fusion, gene *cotZ* coding for the coat protein was PCR-amplified together with its natural promoter using the *B. subtilis* 168 chromosomal DNA as a template and

oligonucleotide pair cotZ-F/ cotZ-R listed in Table 2. Amplification product was digested with *EcoRI* and *BamHI* and cloned into the pDL vector [10] yielding plasmid pDL-CotZ.

DNA sequence coding for influenza A virus matrix protein 1),

(M S L L T E V E T Y V L S I I P S G P L
K A E I A Q R L E S V F A G K N T D L E A L M E W L K T R P
ILGFVFTLTVPSEGLQRRRFVQNALNGNDPNMMDRAVKLYKKLK
REITFHGAKEVSLSYSTGALASCMGLIYNRMGTVTTEAAGLVLCATC
EQIADSQHRSHRQMATTNPLIRHENRMVLASTTAKAMEQMAGSS
E Q A A E A M E V A N Q T R Q M V H A M R T I G T H P S S A G L K
DDLLENLQAYQKRMGMVQMQRFK)

was synthesized (Life Technologies) with codon optimization for *Bacillus subtilis* and restriction site *BamHI* was added at the 5' and *SacI* at the 3' end of the ORF. Synthesized fragment was digested with *BamHI* and *SacI* and cloned at the 3' end of the *cotZ* gene carried by pDL-CotZ obtaining plasmid pNC02.

Plasmid pNC02 was linearized by digestion with a *BsmBI* restriction enzyme and used to transform *B. subtilis* 168. Transformation resulted in the integration of the plasmid into bacterial chromosome at the *amyE* locus in a double homologous recombination manner. Obtained Chloramphenicol-Resistant (Cm^R) clones were PCR-tested for the incorporation of the fusion gene at the *amyE* locus in *B. subtilis* 168 chromosome using oligonucleotide pair AmyS/AmyA Table 2. Selected clone was called BNC02 [Table 1].

Production of Spores

Sporulation was induced using the previously described

Table 1: Strain list		
Strain	Relevant genotype	Source or reference
Bacillus subtilis		
168	trpC2	[17]
BNC02	amyE::cotZ-M1	This work
BKH121	amyE::cotB- GGGEAAAKGGG-IL-2	[18]
Escherichia coli		
BL21(DE3)	fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHI ΔEcoRI-B int::lacI::PlacUV5::T7 gene1) i21 Δnin5	NEB Inc., USA
DH5α	fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	[7]

Table 2: List of the PCR Oligonucleotides		
Name	Sequence	Restriction site
cotZ-F	GCTTAGGATCCATGATGATGTGTACGATTG	<i>BamHI</i>
cotZ-R	CGTAGCGAATTACGTTATCACTCTTGT CCTC	<i>EcoRI</i>
AmyS	CCAATGAGGTAAAGAGTATTCC	-
AmyA	CGAGAAGCTATCACCGCCAGC	-

nutrient depletion method in Difco Sporulation Medium (DSM) [11]. Briefly, 48-hour bacterial cultures were harvested and washed in 1 M KCl, treated with lysozyme followed by washing steps in 1 M NaCl, 0.05% (w/ v) Sodium Dodecyl Sulfate (SDS) and water. The purified spores were titrated using Thoma chamber and stored at -20°C.

Western Blotting Analyses

To extract spore coat proteins a buffer containing 0.1 M NaCl, 0.1 M NaOH, 0.1 M Dithiothreitol (DTT) and 1% (w/ v) SDS was used. 5×10^8 spores were resuspended in 50 μL of decoating buffer and incubated for 30 min at 70°C with shaking (1000 rpm). Suspension was centrifuged (10 000 RCF, 10 min, RT) and supernatant kept for further analysis. Extracted spore coat proteins were separated in NuPAGE® Novex® 4-12% Bis-Tris pre-cast polyacrylamide gels (Life Technologies), electrotransferred on a nitrocellulose using iBlot® 2 Dry Blotting System (Life Technologies). Membranes were incubated overnight at 4°C with GA2B anti-influenza A matrix protein 1 Mab (Pierce). Western blots were visualized developing with BCIP/ NBT according to the manufacturer's instructions (Thermo Scientific).

Immunofluorescence Microscopy

Samples were fixed directly in the medium as described by Negri A, et al. [12]. Briefly, intact spores were incubated overnight at 4°C with GA2B anti-influenza A matrix protein 1 mAb (Pierce), followed by incubation with anti-mouse Cy3 (Jackson Immuno Research) overnight at 4°C. Samples were loaded on microscope slides and viewed using a Zeiss Axioplan fluorescence microscope.

OBALB/c mice were purchased from the breeding facilities at the Medical University of Gdańsk. The animals were kept in polycarbonate cage, housed in well aerated rooms with a 12-h light/12-h dark cycle at $25 \pm 2^\circ\text{C}$, fed with normal pellet diet and water ad libitum. The physical condition of the animals was monitored daily. None of the animals exhibited clinical signs indicative of severe illness during experiment and 100% of mice reached humane endpoint euthanasia which was performed by carbon dioxide inhalation followed by cervical dislocation.

Three groups of five mice (female, 8 weeks) were immunized using intragastric gavage with water suspension of recombinant spores expressing CotZ-M1 (BNC02), mixture of CotZ-M1 (BNC02) CotB-IL-2 (BKH121) fusions or control, non-recombinant spores (strain 168). Oral immunizations contained 1.0×10^{10} spores in a volume of 0.2 ml in case of spores BNC02 and 168 or contained 1:1 ratio of 0.5×10^{10} spores in a volume of 0.2 ml in case of mixture of BNC02 and BKH121 and were administered by oral gavages on days 1, 3, 5, 22, 24, 26, 43, 45, 47. Spleens were collected from all of the animals on day 61.

IFN-γ ELISpot Assay

Spleens were isolated and single cell suspension was prepared as described elsewhere [13].

The number of IFN-γ-secreting cells was determined by using mouse IFN-γ ELISpot kit according to manufacturer's instructions

(BD ELISpot). Splenocytes (1×10^5) were cultured for 48 h in presence of recombinant M1 antigen previously described [14]. The spots were counted using automated ELISpot plate reader (CTL-ImmunoSpot S6 Micro Analyzer, USA).

Statistics

ELISpot tests have been performed for each animal in three technical repeats. Statistical significance of the data was determined by one-way analysis of variance followed by the Bonferroni posttest.

Results and Discussion

Spore Coat Expression and Surface Display of M1 Antigen

The presence of fusion protein in the recombinant endospore coat was tested by western blotting with mouse monoclonal anti-influenza A M1 antibodies (Pierce). As demonstrated by western blotting [Figure 1A], CotZ-M1 fusion protein was expressed and located in the spore coat. However, observed molecular weight (35 kDa) of the fusion protein differ from the calculated (45 kDa). This could be due to the reported previously instability of the M1 protein in the bacterial expression systems [15,16]. In our work M1 protein was fused to the C-terminus of spore coat CotZ protein. It has been shown that M1 C-terminal domain (residues 165 to 252) is quite susceptible to degradation by endogenous bacterial proteases. This falls with our observation as the residues 165 to 252 is 10 kDa. The surface exposition of fusion protein was analyzed by immunofluorescence microscopy of dormant spores of wild type and recombinant strains using mouse monoclonal (GA2B) anti-influenza A M1 antibodies (Pierce) as primary antibodies and anti-mouse IgG-Cy3 (Jackson Immuno Research) as secondary antibodies. We observed a specific, fluorescent signal only from recombinant spores [Figure 1B].

Immune Response to Recombinant Spores

To assess immunogenic properties of constructs and whether spores presenting IL-2 will act as an adjuvant we immunized

Figure 1: (A) Western blot analysis of expression of fusion gene. Spore coat proteins were extracted and analyzed by western blotting with anti-influenza M1 mAb. Numbers of lanes refer to the strain used in experiment: 1-*B. subtilis* 168; 2-BNC02(CotZ-M1, ~35kDa). **(B)** Immunofluorescence staining of recombinant spores. Purified spores were incubated with anti-influenza M1 mAb, followed by anti-mouse Cy3 conjugates. Spores were visualized by phase contrast (PC) and immunofluorescence (IF) microscopy. The same exposure time was used for all IF images. Scale bar 10µm.

Figure 2: IFN- γ response of sensitized mouse splenocytes to M1 as assessed by the ELISpot. The splenocytes were isolated from mice orally immunized with 168 spores (black closed bar), BNC02 (CotZ-M1) (grey closed bar) or 1:1 mixture of BNC02 and BKH121 (CotB-IL-2) (light-gray closed bar). Cells were incubated with purified M1 protein for 48 h and then the IFN- γ -secreting cells were enumerated by ELISpot procedure. Error bars represent standard deviation. * p -value < 0.05, ** p -value < 0.01 against 168(wt).

orally mice either with spores presenting M1 antigen (BNC02), mixture of spores presenting M1 antigen and interleukin 2 (BNC02+BKH121) or non-recombinant spores (168). To test induction of cellular immune response we conducted IFN- γ ELISpot using splenocytes from immunized mice. A significant increase in the number of IFN- γ -secreting cells stimulated with recombinant M1 was observed in case of mice immunized with BNC02 spores [Figure 2]. These results suggest the induction of specific cell-mediated immune response. Moreover, when co-administered with BKH121, spores presenting M1 protein increased the strength of observed immune response. The key question is whether observed response would provide a robust therapeutic or protective immunity? In our opinion spores constructed in this research rather have the potential to serve as a specific immunostimulant adjunctive the standard immunization procedures than as a standalone oral vaccine.

In summary, the system presented in this work seems to be a promising candidate as a formulation stimulating the immune system to fight the infection with influenza virus. Although co-administration of IL-2 presenting spores as an adjuvant increases the efficiency of immunization, further research is required to assess protective and therapeutic potentials of such formulation.

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Conflict of Interests

The authors declare that they have no conflict of interests.

Ethical Approval

The animal procedures protocol was approved by the Committee on the Ethics of Animal Experiments of the Medical University of Gdańsk (Permit Number: 3/2014).

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