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Mucosal delivery of antigens using adsorption to bacterial spores

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ABSTRACT

The development of new-generation vaccines has followed a number of strategic avenues including the use of live recombinant bacteria. Of these, the use of genetically engineered bacterial spores has been shown to offer promise as both a mucosal as well as a heat-stable vaccine delivery system. Spores of the genus *Bacillus* are currently in widespread use as probiotics enabling a case to be made for their safety. In this work we have discovered that the negatively charged and hydrophobic surface layer of spores provides a suitable platform for adsorption of protein antigens. Binding can be promoted under conditions of low pH and requires a potent combination of electrostatic and hydrophobic interactions between spore and immunogen. Using appropriately adsorbed spores we have shown that mice immunised mucosally can be protected against challenge with tetanus toxin, *Clostridium perfringens* alpha toxin and could survive challenge with anthrax toxin. In some cases protection is actually greater than using a recombinant vaccine. Remarkably, killed or inactivated spores appear equally effective as live spores. The spore appears to present a bound antigen in its native conformation promoting a cellular (T_h1-biased) response coupled with a strong antibody response. Spores then, should be considered as mucosal adjuvants, most similar to particulate adjuvants, by enhancing responses against soluble antigens. The broad spectrum of immune responses elicited coupled with the attendant benefits of safety suggest that spore adsorption could be appropriate for improving the immunogenicity of some vaccines as well as the delivery of biotherapeutic molecules.

1. Introduction

The use of live bacteria as vaccine delivery systems has provided one arm in the push to develop new and more effective vaccines. Live bacterial vaccines include a number of species including those of *Salmonella*, *Shigella*, *Escherichia coli*, *Lactobacillus* and *Bacillus* [1]. In some cases the strategy used is to exploit the life cycle of a pathogen, for example, one oral dose of recombinant, attenuated, *Salmonella enterica* serovar Typhi Ty21a is sufficient to generate a potent immune response because the bacterium efficiently targets the gut-associated lymphoid tissue (GALT) [2]. In the case of *Bacillus* which is the focus of this paper, *Bacillus subtilis* engineered to express heterologous antigens on the surface of the spore or within the germinating spore can be used for oral or nasal delivery of antigens and confer protective immunity. With *B. subtilis* the spore, as a dormant life form, has further advantages of being heat-stable, non-pathogenic and in current use in humans and animals as a probiotic [3]. Recombinant spores expressing protective antigens from *Clostridium tetani* [4], *Clostridium perfringens* [5], *Bacillus anthracis* [6], as well as the parasite *Clonorchis sinensis* [7] have all been shown to confer protection using animal models. With the exception of *B. anthracis*, a mucosal route of delivery has been used in every case demonstrating that recombinant spores could be utilized in a simplified vaccination strategy. On the other hand the use of recombinant bacteria also raises concerns over the use of genetically modified microorganisms and clearance of the bacterium from the host following delivery [1].

A second goal in the development of better vaccines is in identifying new adjuvants that can boost immunogenicity of otherwise, weakly immunogenic antigens (e.g., recombinant protein subunits and synthetic peptides). Until recently only one adjuvant, alum, has been licensed for human use and a number of potential vaccine adjuvants are under development including immunostimulatory adjuvants, mucosal adjuvants, lipid particles and particulate adjuvants [8,9]. Each of these have their strengths but also weaknesses. One of these new classes of adjuvants, referred to as particulate adjuvants (e.g., liposomes, virosomes, virus-like particles,

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poly-lactide-co-glycolide (PLG) microspheres and immune stimulating complexes (ISCOMS)), are particularly encouraging because they mimic the pathogens the immune system has evolved to destroy. Particulate adjuvants efficiently target antigen presenting cells (APCs) and once internalised within the cell are processed by the class I and class II MHC (major histocompatibility complex) pathways leading to antigen presentation on the surface of the APC. Biodegradable PLGs have been shown to induce CTL responses a prerequisite for combating intracellular pathogens [10]. Studies on copolymer adjuvants have shown that for induction of a broad range of immune responses (antibody and cell-mediated) it is necessary that the antigen remains in its native form which can be achieved if bound to the surface of a suitable surface [11]. Many antigens prepared in water-in-oil emulsions are rapidly engulfed by APCs, degraded, and then enter the class II pathway leading to antibody production but in a challenge model, fail to protect. This is thought to be due to the failure to produce the IgG2a isotype that is important for recognition of conformational epitopes [11]. Stabilisation of antigens on inert surfaces in their native form coupled with the ability to induce potent immune responses remain one of the challenges in vaccine and adjuvant formulations.

Previous studies have shown that *Bacillus* spores possess adjuvant properties [12,13] and in this work we show that this is brought about by binding of the antigen to the spore surface. Spores then, serve as antigen carriers, closely resembling the properties of microparticulate adjuvants. Using this approach, we demonstrate the utility of antigen-adsorbed spores in conferring protective immunity to three bacterial pathogens following delivery by a mucosal route.

2. Materials and methods

2.1. Strains

B. subtilis strain PY79, is a standard prototrophic laboratory strain and isogenic to the 168 type strain [14]. HU58 is a non-domesticated isolate of *B. subtilis* [15]. HT251 (*amyE::cotB-GST-Cpa*$_{247–370}$) is isogenic to strain PY79 and carries a recombinant gene on its genome that expresses a modified spore coat protein, CotB, that has been fused to GST-Cpa$_{247–370}$ [5]. RH103 (*amyE::cotB-tetC*) expresses the immunogen, TTFC (tetanus toxin fragment C) from *C. tetani* on the spore surface as a chimera fused to the CotB protein [16].

2.2. Preparation of spores and general methods

Spores used in all experiments were prepared by growth and sporulation in Difco Sporulation Medium (DSM) as described elsewhere [17]. Each batch of spores was heat-treated (68 °C, 45 min) to ensure killing of all vegetative cells. Spores were suspended in sterile PBS (pH 7.4) and stored in aliquots (1×10^{11} spores/ml) at −70 °C until use. Spore counts were determined by (i) direct counting using a haemocytometer and phase-contrast microscopy, and (ii) by serial dilution and plate-counting. Extraction and analysis of spore coat proteins using SDS-PAGE was as described [17].

2.3. Zeta potential measurements

Zeta potentials of the PY79 spore isolates were measured at 24 °C with a 3000HS Malvern Zeta-sizer (Malvern Instruments Ltd., UK). Aliquots of 30 μl of spores suspended in Milli-Q water at a density of 5×10^9 spores/ml were added to 3 ml solutions of defined pH and ionic strength, as described in the text. The pH was adjusted using HCl or NaOH. The mean of two separate measurements from the same sample was determined. The zeta potential was calculated from the electrophoretic mobility using the Smoluchowski equation [18].

2.4. Spore adhesion to hydrocarbon (SATH) assay

The surface hydrophobicity of the PY79 spore isolates was determined using the SATH assay using *n*-hexadecane as hydrocarbon [19]. Purified spores were washed in either Milli-Q water or 1 M NaCl in Milli-Q water by centrifugation at 16,000 × *g* for 10 min and resuspended in 0.1 M NaCl at a density of 1×10^8 spores/ml. Spore suspensions (2 ml) were added to 1 ml *n*-hexadecane (Aldrich) and vortexed for 1 min, incubated at 37 °C for 10 min, and vortexed again for 30 s. The absorbance of the aqueous phase was measured at 600 nm. The mean of two measurements was determined. The percent hydrophobicity (*%H*) was determined from the absorbance of the original spore suspension (A_i) and the absorbance of the aqueous phase after incubation with hexadecane (A_f) according to the following equation: $\%H = [(A_i - A_f)/A_i] \times 100$.

2.5. Recombinant proteins

Proteins were expressed from recombinant plasmids in the *E. coli* strain BL21. With the exception of GST-Cpa$_{247–370}$, the expressed protein carried a poly-histidine tag at its 3′-end and following expression was purified using an AKTA chromatography system (Pharmacia).

(i) TTFC: pET-28b-TTFC expressed *C. tetani* TTFC as a 52.6 kDa polypeptide and has been described elsewhere [4].
(ii) PA: pET-28b-PA expressed the 83.5 kDa protective antigen (PA) from *B. anthracis*. As described elsewhere, in this cassette the secretory signal sequence permitting membrane secretion was deleted [20].
(iii) GST-Cpa$_{247–370}$: The 41 kDa hybrid protein of Sj26GST fused to the carboxy-terminal domain of *C. perfringens* alpha toxin (Cpa$_{247–370}$) has been described elsewhere [21] and was purified using a GST-binding column.
(iv) GST: pET-28b-GST expressed the 26.3 kDa glutathione S-transferase (Sj26GST) from *Shistosomas japonica* [22]. Henceforth Sj26GST is referred to as GST. The *sj26GST* gene was PCR-amplified and cloned from the vector pGEX3X13 (Pharmacia).

2.6. Antibodies

Polyclonal antibodies were raised in mice immunised by the intra-peritoneal (i.p.) route with 2 μg of purified protein on days 1, 14 and 28. Dilutions used were 1:1000 for anti-PA and 1:2000 for anti-TTFC and anti-GST-Cpa$_{247–370}$.

2.7. Binding assays

Unless indicated otherwise the general method for adsorbing proteins to spores was as follows. Suspensions containing 2×10^9 spores were centrifuged and resuspended in 0.2 ml of PBS at pH 4, pH 7 or pH 10. Unless indicated otherwise all reactions were performed in PBS at 0.15 M. Purified recombinant proteins were added to the spore suspension and the binding mixture incubated at room temperature (RT). Spores were centrifuged (1 min, RT) and the pellet washed two times with PBS buffer of the same pH as that used in the binding mixture. The washed pellet was resuspended in 100 μl of spore coat extraction buffer [17], incubated at 68 °C for 1 h to solubilise spore coat proteins and one tenth loaded onto a 12% SDS-PAGE gel.