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The effect of anti-spore antibody responses on the use of spores for vaccine delivery

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ABSTRACT

Among the different types of bacteria being exploited as live vaccines *Bacillus* endospores have a number of distinct advantages most importantly being their heat stability. Typically antigens are displayed on the spore surface or expressed in the germinating spore, i.e., the vegetative cell. How antigens are delivered by spores can significantly affect the nature of the resulting immune response. When antigens are expressed in the germinating spore, then, after the first dose of recombinant spores, ensuing humoral responses are abruptly arrested. We have investigated this phenomenon by first demonstrating that while immune responses against the encoded antigen are impaired this is not the case for anti-spore responses that progressively increase until hyperimmunity is reached. We reasoned that anti-spore IgG or sIgA could be responsible for inhibiting spore germination, thus preventing expression of the vegetatively expressed antigen. In this work we have demonstrated that antisera from both immunised and naïve animals can bind to spores non-specifically yet only IgG or sIgA from immunised animals can bind specifically. Both immune and naïve antibodies were found to inhibit germination *in vitro* and most probably non-specific binding could account for this. On the other hand only immune antibodies were capable of opsonising spores and enhancing their uptake by macrophages and we reason that specific antibody-spore binding is required. Opsonophagocytosis could be an important tool to reduce spore germination and subsequent outgrowth. With regard to use for heterologous antigen delivery though, this severely curtails the use of a repetitive dosing regime.

1. Introduction

Spores of the Gram-positive bacterium *Bacillus subtilis* have been shown to be effective as live bacterial vaccines in a number of reports. In this approach genetically engineered spores have been constructed that express and display heterologous antigens on the spore coat and thus serve as the vaccine delivery system. In each case the antigen is fused, as a chimera, to one of the principal proteins found on the outermost layer of the spore coat. The 43 kDa CotB and the 15 kDa CotC proteins have been shown most suitable for display of potential protective antigens. This includes the tetanus toxin fragment C (TTFC) from *Clostridium tetani* [1], domains 1b-3 and 4 of the protective antigen (PA) of *Bacillus anthracis* [2], a tegumental protein of *Clonorchis sinensis* [3], the carboxy-terminus of the alpha toxin of *Clostridium perfringens* and subunit B of *Escherichia coli* labile toxin (LTB) [4]. With the exception of *B. anthracis* PA, in every case the recombinant spore has been used for the oral or nasal delivery of heterologous antigens in murine models. Analysis of humoral and mucosal (IgG and sIgA) immune responses has demonstrated seroconversion and in the case of *C. tetani* TTFC, *C. perfringens* alpha toxin and *C. sinensis* tegumental protein, protection has been demonstrated following oral immunisation [3–6]. Accordingly, the inherent heat-stability and robustness of bacterial spores make them attractive as simple vaccine delivery systems. Although the spore is extremely robust and exhibits extraordinary resistance to environmental insults this may not be the case for immunogens expressed on the spore surface. Indeed, TTFC and alpha toxin are both partially degraded when recombinant spores are immersed in fluids mimicking gastric conditions [5,7]. While simulated conditions may not perfectly mimic the stomach it is apparent that proteins exposed on the spore surface are potentially subject to degradation. Using the oral route, in particular, this raises the question of how seroconversion, or indeed protection, can be invoked? Two explanations might account for this, firstly, using a large spore dose it is possible that a shielding effect may be achieved permitting sufficient numbers of spores to transit the gastric barrier unaffected. In a second model spores passing through the stomach germinate in the ileum and proliferate before re-sporulating and entering the colon. It is at the second stage of re-sporulation that the cells would express a second dose of immunogen. Molecular experiments using PCR analysis of sporulation-specific mRNA *in vivo* has now proven that spores do

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indeed germinate, replicate and then re-sporulate confirming the validity of this second hypothesis [7].

Consideration of the potential negative constraints of exposing antigens on the spore surface has lead to an alternative approach for antigen delivery. In this strategy an immunogen is expressed only in the vegetative cell. Spores are administered orally and, following spore germination in the small intestine, the antigen expressed in the vegetative cell [8]. High levels of expression can be achieved by fusing the open reading frame of the candidate gene to a strong, vegetatively expressed, promoter [8,9]. Interestingly, when comparing the humoral immune responses following oral and nasal dosing with spores expressing the antigen on the spore surface with those using the germinating spore approach, clear differences are apparent. Specifically, when using spore coat display, after each successive dose of spores antigen-specific antibody titres subsequently increase. By contrast, when the antigen is expressed in the germinating spore, antibody titres only increase after the first dose. Following the second dose, IgG titres level off and show no apparent increase. In this paper we have investigated the underlying reason for how and why antigens, delivered by the germinating spore, fail to generate humoral responses.

2. Materials and methods

2.1. Preparation of spores and general methods

Spores used in all experiments were prepared by growth and sporulation in Difco Sporulation Medium (DSM) as described by Nicholson and Setlow [10]. Each batch of spores was heat-treated (68 °C, 30 min) to ensure the presence of no viable vegetative cells, suspended in sterile PBS (phosphate buffered saline) and stored in aliquots (1×10^{11} spores/ml) at −70 °C until use. Spore counts were determined by serial dilution and plate counting. Extraction and analysis of spore coat proteins using SDS-PAGE was as described [10]. DNA-mediated transformation of competent cells was made as described [11]. Plasmid amplification for nucleotide sequencing, subcloning experiments and transformation of *E. coli* competent cells were performed in the strain TG1 [12]. Bacterial strains were transformed according to previously described procedures: $CaCl_2$-mediated transformation of *E. coli* competent cells [12] and a two-step transformation of *B. subtilis* competent cells [11].

2.2. Bacterial strains and strain constructions

PY79 is a prototrophic, wild type, strain of *B. subtilis* derived from the 168 type-strain [13]. JH334 (*thrC*::*PrrnO-hylA*^{L461T}) is derived from strain PY79 and expresses a modified form of listeriolysin O (LLO_{L461T}) encoded by the *hylA* gene of *Listeria monocytogenes*. The construction of the *hylA*^{L461T} gene has been described elsewhere [14] and in strain JH334 the gene is contained at the *thrC* locus on the *B. subtilis* genome.

All strains used in this study were isogenic to PY79 and the following additional strains were made in this work:

(i) LH78 (*amyE*::*PrrnO-synmpt64*, *thrC*::*PrrnO-hylA*^{L461T}): LH78 was created by transforming competent cells of JH334 with plasmid pLH65 (see Section 2.3) followed by selection for chloramphenicol-resistance, Cm^R) using plates supplemented with chloramphenicol (5 μg/ml). LH78 would express the *Mycobacterium tuberculosis* MPT64, and in vegetative cells of *B. subtilis* this would produce a 26.3 kDa species (241 amino acids).

(ii) LH106 (*amyE*::*PrrnO-synmpt64*^{sec}, *thrC*::*PrrnO-hylA*^{L461T}): LH106 was created by transforming competent cells of JH334 with plasmid pLH81 (see Section 2.3). Expression of MPT64 from this construction in vegetative cells of *B. subtilis* would produce a 32.8 kDa species (301 amino acids) that after membrane translocation would secrete a cleaved MPT64 of 243 amino acids (26.5 kDa).

2.3. Plasmid constructions

2.3.1. pLH65

pLH65 carries a codon-optimised *mpt64* gene placed under the control of the *PrrnO* promoter of *B. subtilis*. *PrrnO* is a promoter utilised during vegetative cell growth and has been used previously for directing high levels of gene expression in the vegetative cell [8,15]. Initial attempts to express the *mpt64* gene were unsuccessful, resulting in at best, low levels of protein synthesis. Accordingly, in a second approach, a codon-optimised gene *synmpt64*, was synthesised (Shanghai Genery Company, Shanghai, China) with approximately 60% of codons (of a total of 243) changed to those preferred by *B. subtilis* (see Supp. Fig. 1). This synthetic DNA was made without the secretory *M. tuberculosis* signal sequence at its 5′-end. The *synmpt64* DNA carried EcoR1 and Not1 restriction sites at its 5′ and 3′-ends respectively. Following cleavage with EcoR1 and Not1 the *synmpt64* gene was sub-cloned into plasmid pDL205 cleaved with the same enzymes. pDL205 carries an expression cassette comprising the *PrrnO* promoter adjacent to a strong ribosome binding site (RBS) and a multiple cloning site (carrying the EcoR1 and Not1 sites) [15]. Insertion of *synmpt64* would provide a promoter, RBS and also an ATG start codon enabling expression in *B. subtilis* as a 26 kDa species. pDL205 also carries the front and back portions of the *amyE* locus allowing introduction of sub-cloned DNA into the amylase locus with selection for chloramphenicol resistance.

2.3.2. pLH81

In a similar approach, as used for pLH65 (above), a secreted version of *synmpt64* was made, referred to as *synmpt64*^{sec}. In this construction the *synmpt64* gene was fused at its 5′-end to the secretory signal sequence of listeriolysin O (*hylA*). To achieve this, the 5′-end of the *hylA* sequence that encodes the signal sequence (residues 1–180 of the *hylA* ORF) was PCR-amplified from *L. monocytogenes* strain NCTC 19115. The PCR product was designed to carry BamH1 and EcoR1 restriction endonuclease sites at its 5′ and 3′-ends to enable sub-cloning into plasmid pDL205, yielding plasmid pLH66. The *synmpt64* DNA carried EcoR1 and Not1 restriction sites at its 5′ and 3′-ends respectively and was next sub-cloned into plasmid pLH66 following cleavage with EcoR1 and Not1 of both *synmpt64* and plasmid pLH66 to generate pLH81.

2.4. MPT64 protein

Recombinant MPT64 (26 kDa) was produced in *E. coli* BL21 (DE3 pLysS) from a pET28b expression vector (Novagen) that carried the *mpt64* gene fused to an N-terminal polyhistidine tag. High levels of expression were obtained upon induction with isopropyl-β-D-thiogalactoside (IPTG). MPT64 was purified by passage of the cell lysate through a nickel affinity column. Eluted His-MPT64 protein was checked for integrity by SDS-PAGE and eluted fractions were pooled and dialyzed against PBS extensively. The concentration was determined using the Bio-Rad DC Protein Assay kit.

2.5. Animals

Animals used in this work were pathogen-free, C57BL/6 mice (female, age 6–8 weeks; obtained from Harlan, UK). Animals were housed in the Royal Holloway, University of London's animal house and work described in this paper was performed under the Home Office project licence PPL 70/6126.

2.6. Immunisations

For evaluation of immune responses groups of six mice were dosed in two ways, parenterally by intra-peritoneal (i.p.) injection, and mucosally by intra-nasal dosing. For the latter, mice were lightly anaesthetised and inoculated with 2×10^9 spores (PBS) in a volume of 20 μl using a pipette tip on days 0, 2, 21, 23, 42 and 44. We considered this as three separate doses, dose 1 (0, 2), dose 2 (21, 23) and dose 3 (42, 44) and chose to split dosings to allow a reduction in the volume of the nasal dose and a more effective animal recovery following anaesthesia. For i.p. injection, a dose of 1×10^9 spores in a volume of 0.1 ml of PBS on days 0, 21 and 42 was used. For sampling, blood was taken on days −1, 20, 41 and 62 for nasal dosings and −1, 13, 27 and 42 for i.p.

2.7. ELISA for detection of serum IgG

Immuno Maxisorp plates (Nunc) were coated with 50 μl per well of the specific antigen (in 0.5 M carbonate/bicarbonate buffer, pH 9.6) and left at 4 °C overnight. Antigen was either, extracted spore coat protein (5 μg/ml) or purified MPT64 (5 μg/ml). After blocking with 0.5% BSA in PBS for 1 h at 37 °C, serum samples were applied using a 2-fold dilution series starting with a 1/50 dilution in ELISA diluent buffer (0.01 M PBS, pH 7.4; 3% (w/v) NaCl; 0.5% (w/v) BSA (Sigma); 0.05% (v/v) Tween-20). Every plate carried replicate wells of a negative control (a 1/50 diluted pre-immune serum) and a positive control (serum from mice immunised parenterally with MPT64). Plates were incubated for 2 h at 37 °C before addition of the appropriate anti-mouse immunoglobulin HRP conjugates (all obtained from Sigma). Plates were incubated for a further 1 h at 37 °C then developed using the substrate TMB (0.1 mg/ml TMB [Sigma] in 0.1 M sodium acetate buffer, pH 5.5). Reactions were stopped using 2 M H_2SO_4. OD_{450} was measured with a Rosy Anthos HT3 ELISA plate reader using dual wavelength function against 620 nm. Dilution curves were drawn for each sample and end-point titres calculated as the dilution producing the same optical density as the 1/50 dilution of a pooled pre-immune serum. Statistical comparisons between groups were made using the Student's unpaired *T* test. A *P* value of >0.05 was considered non-significant.

2.8. Preparation of serum IgG and lung sIgA

Serum IgG from immunised mice was purified using a Protein A column (Prochem, USA) following the instructions provided by supplier. The perfusion and saponin extraction method was used to obtain lung sIgA as previously described [16,17] with some modification. Briefly, animals were sacrificed 20 days after the final immunization and 0.1 ml of 1% heparin in PBS was injected by the i.p. route before blood was drawn from the heart. Immediately after bleeding, at least 20 ml of 0.1% heparin–PBS was infused into the heart to maximally remove blood from the lungs. The lungs were collected and weighed before being stored at −20 °C in PBS (1 μl/mg tissue) containing 2 mM phenymethylsulfonylfluoride (PMSF), 0.1 mg of trypsin inhibitor from soybean (Sigma) per ml, and 0.05 M EDTA. After thawing the samples, saponin (Sigma) was added to a final concentration of 2% (w/v) to permeabilize the cell membranes, and the samples were stored at 4 °C overnight. The organs were spun down at 16,000 × *g* and the supernatants saved for further analysis.

2.9. Germination experiments

Heat-activated, refractile, spores of *B. subtilis* strain PY79 (2×10^8 spores) were incubated on ice for 30 min with or without serum or lung sIgA diluted in 100 μl of 0.1 M Tris–HCl buffer. The pre-treated spores were then added to 3 ml of 0.1 M Tris buffer (pH 7.4) supplemented with germinant (L-alanine at 10 mM), and incubated with shaking at 37 °C. At defined time intervals, samples were read spectrophotometrically (at OD_{580}) and the extent of germination was assessed by the decline in OD_{580}.

2.10. Opsonohagocytosis experiments

Macrophages (MQs) were cultured according to the method of Welkos et al. [18] with some minor modification. MQs used included (i) primary peritoneal cells obtained from C57BL/6 mice (Harlan, UK) 4 days after intra-peritoneal inoculation of 0.1 ml soluble starch (2%) and (ii) the macrophage-like cell line, RAW264.7 (European Collection of Cell Cultures; E.C.A.C.C.). The primary MQs and the MQ cell line were cultured in Dulbecco's Minimal Essential Medium (DMEM) with horse serum (HS, Sigma) 10%. Previous work has shown that cell culture medium supplemented with fetal bovine serum could stimulate *B. anthracis* spore germination, while the spore would germinate much more slowly in a cell culture medium supplemented with horse serum [18]. We found that *B. subtilis* spores germinated much more slowly in medium (DH10) containing horse serum (<5%) than in medium with fetal bovine serum (DF10; >95%) (Supp. Fig. 2) following 1 h of incubation. Therefore, DH10 was selected for use in all cell culture methodologies. Seed cultures were subcultured into 24-well plates, and each well contained 1×10^5 MQs, and incubated at 37 °C with CO_2 (5%) for 1–2 days. The primary peritoneal exudate MQs were cultured for 3–5 days at 37 °C in CO_2 (5%) on coverslips in 24-well plates. The spores were opsonised on ice for 30 min using, either immune serum, immune lung sIgA, pre-immune serum, naïve lung sIgA, or medium alone. They were then added to the MQs with a usual multiplicity of infection (m.o.i.) of 10 and incubated at 37 °C in CO_2 (5%) for 1 h, to allow phagocytosis. Unphagocytosed spores were then removed by washing the wells three-times in DMEM medium. The coverslips were removed from the wells and further washed by rinsing sequentially with 0.01 M PBS in three 150-mm disposable sterile beakers. Some of the washed coverslips were stained with Malachite Green and Wright-Giemsa solutions, and others were re-incubated a further 3 h at 37 °C in CO_2 after adding fresh medium for staining. A Nikon H55OS microscope was used for phase-contrast examination of slides. The cells in wells without coverslips were lysed at indicated time points with 0.1% Triton X-100 and the c.f.u. determined by plating serial dilutions of cell lysates on DSM agar plates. To determine spore counts lysates were heat-treated (68 °C, 30 min) prior to plating.

2.11. Adhesion experiments

Human, epithelial-like, Caco-2 cells and Hep-2 cells (both obtained from the E.C.A.C.C.) were cultured in Minimal Essential Medium (MEM) with 10% horse serum. Seed cultures were subcultured into 24-well plates and incubated at 37 °C with 5% CO_2 for 2 days. Spores were treated on ice for 30 min with naïve lung sIgA, anti-spore lung sIgA or medium alone. They were then added to the Caco-2 or Hep-2 cells, and incubated at 37 °C with 5% CO_2 for 1 h. Each well contained 1×10^5 cells, and were inoculated with *B. subtilis* PY79 spores (1×10^6), with a m.o.i. of 10. Non-adhering spores were removed by washing the wells with MEM medium three times. Cells were lysed with 0.1% Triton X-100 solution and the numbers of spores determined by heat-treating the lysate (68 °C, 30 min) prior to plating on agar.

2.12. Western blot detection of spore-antibody binding

Western blotting was used to detect the binding of antibody to spores using two different methods. One approach involved extracting spore coat proteins with 200 μl of SDS-DTT extraction buffer

from 4×10^9 PY79 spores as described for isolating spore coat proteins [10]. Extracted spore coat proteins were loaded on SDS-PAGE gels (20 μl/well), fractionated, transferred to nitrocellulose membrane and probed with either mouse anti-spore immune serum or naïve serum diluted 1:2000 in 0.01 M PBS buffer, pH 7.4, supplemented with 5% skimmed milk before addition of anti-mouse immunoglobulin HRP conjugates. A second method was to mix 5 μg of purified anti-spore IgG or naïve serum IgG with 2×10^9 of PY79 spores in 100 μl of 0.01 M PBS buffer (pH 7.0) followed by incubation at room temperature for 30 min. Spores were centrifuged and gently washed with PBS buffer before spore coat proteins were extracted using SDS-DTT extraction buffer. The spore coat extraction was loaded onto SDS-PAGE and transferred to membranes. A HRP-conjugated anti-mouse IgG secondary antibody was used directly to detect the antibody bound to spores.

2.13. Antibodies

The mouse-anti-PA polyclonal was raised in mice in other work [2]. A HRP-conjugated goat anti-mouse polyclonal antibody was obtained from Sigma and used at a 1:5000 dilution (used for Fig. 3C). A polyclonal serum to MPT64 was raised in mice in this work.

3. Results

3.1. Systemic anti-MPT64 IgG responses following parenteral and mucosal dosing of recombinant spores

We first compared the humoral immune responses directed against an antigen expressed in the germinating spore with responses directed against the spore, i.e., the anti-spore response. A recombinant strain, LH78 (*amyE*::*PrrnO-synmpt64, thrC*::*PrrnO-hylA*^{L461T}) was constructed that expressed the *M. tuberculosis* MPT64 antigen. In LH78 we used a codon-optimised synthetic gene, *synmpt64* since attempts to clone and express the native *mpt64* gene under the control of *PrrnO* resulted in little to no detectable expression of MPT64 (data not shown). In our construct *PrrnO* was placed upstream of the *synmpt64* ORF to facilitate expression in the vegetative cell and the signal sequence of *mpt64* was removed to enable intracellular expression of the 26.3 kDa MPT64. One further refinement to LH78 was the co-expression of the pore-forming cytolysin, listeriolysin O (LLO), in a form active at neutral pH and referred to as LLO_{L461T}. As shown previously, expression of LLO_{L461T} in *B. subtilis* vegetative cells enables entry of the bacterium into the cytosol following phagocytosis [14]. In a second construction, LH106 (*amyE*::*PrrnO-synmpt64*^{sec}, *thrC*::*PrrnO-hylA*^{L461T}), we made a similar strain that expressed a modified form of *synmpt64* fused to the LLO secretory signal sequence and therefore enabling MPT64, once synthesised, to be secreted from the bacterial cell as a 26.5 kDa species. In this laboratory we have used the signal sequence of LLO as an efficient tool for protein secretion in *B. subtilis* (unpublished data). In both cases, expression of MPT64 in the vegetative cell (LH78) or in the culture supernatant following membrane secretion (LH106) was confirmed (Supp. Fig. 3). In other experiments expression and delivery of MPT64 into the cytoplasm of phagocytic cells that had taken up spores of LH78 or LH106 was confirmed using intracellular labelling and confocal imaging (data not shown).

Mice were administered intra-nasally, spores of LH78, LH106 and a control strain, JH334 that expressed only LLO_{L461T} in three principal doses. In parallel experiments, mice were given three parenteral (intra-peritoneal) doses of spores. Serum was then examined for anti-MPT64 IgG responses (Fig. 1). Our results showed that for parenteral immunisation (Fig. 1A) MPT64-specific IgG levels increased following the first dose for all three groups receiving spores but after the second dose for LH78 no further increase in antibody titres was observed. For LH106, however, antibody titres, although low, continued to increase until the third dose after which they declined. The levels of IgG found in mice receiving LH106 spores were at levels significantly greater ($P < 0.05$) than the other groups. Nasal immunisations revealed what appeared to be an initial rise followed by an abrupt levelling off of IgG levels (Fig. 1B). sIgA responses were also evaluated in lung extractions and, although low, were significantly ($P < 0.05$) above those found in naïve animals (Supp. Fig. 4).

Anti-spore IgG responses were also measured in mice dosed parenterally (Fig. 1C) and nasally (Fig. 1D). Compared to naïve groups in all groups receiving recombinant spores a steady increase in antibody titre was apparent and with each group responses were significantly greater ($P < 0.05$) than in the naïve groups. These results demonstrate firstly, that using either a parenteral or mucosal route recombinant spores were immunogenic, and secondly, responses against an antigen expressed within, or secreted from, a germinating spore were abruptly terminated after the first or second dose.

3.2. Effect of antibodies on spore germination in vitro

To address the question of if, and how, antibodies might interfere with spore germination we used a simple assay of spore germination. In this assay synchronised spore germination is achieved in the presence of a germinant (10 mM L-alanine) and is monitored over time using the change in absorbance (OD580 nm) to detect germination [10]. A fall in OD580 nm results when phase-bright, refractile, spores change to phase dark spores as a consequence of the rapid uptake of water into the spore following the fracturing of the spore coats. We measured the ability of wild type, PY79, spores to germinate following pre-treatment with IgG (Fig. 2A). IgG was obtained from day 62 bleeds of mice immunised (intra-nasally with LH106 spores) or not immunised from the experiment shown in Fig. 1B. sIgA was obtained from the lung extractions of sacrificed mice taken from the same experiment. Our results demonstrated firstly, that we could detect a clear change in absorbance reading in spores co-incubated with germinant. It is important to realise that even in the absence of germinant spores can still germinate, albeit more slowly, and this event could be attributed to the low levels of contaminating germinants that can carry-through during the spore purification steps. Secondly, in the presence of immune serum (IgG) germination of spores was inhibited in the presence of germinants and this could be correlated with the antibody titer. Finally, serum obtained from unimmunised, naïve mice, was also capable of inhibiting spore germination at levels no different from immune serum. In similar experiments using the same batch of PY79 spores we measured the effect of sIgA antibodies on spore germination (Fig. 2B). Again, compared to spores incubated only with germinant both immune and naïve samples inhibited spore germination but the extent of inhibition was clearly less than when using serum IgG. We attribute this to the lower antibody titres found in lung extractions (see Supp. Fig. 4).

3.3. Binding of antibodies to spores

One explanation for why serum from immunised as well as unimmunised naïve mice was able to inhibit spore germination *in vitro* was that antibodies were able to bind spores and interfere with germination. To address this we first tested the ability of immune and naïve serum (from mice dosed with LH106 by the i.p. route) to bind to spore coat proteins extracted from PY79 using Western blotting (Fig. 3A). These results showed clearly that immune serum contained IgG antibodies that specifically recognised spore coat proteins. At least three principal coat protein species of 65, 50 and 36 kDa were visible as discrete bands; the former most

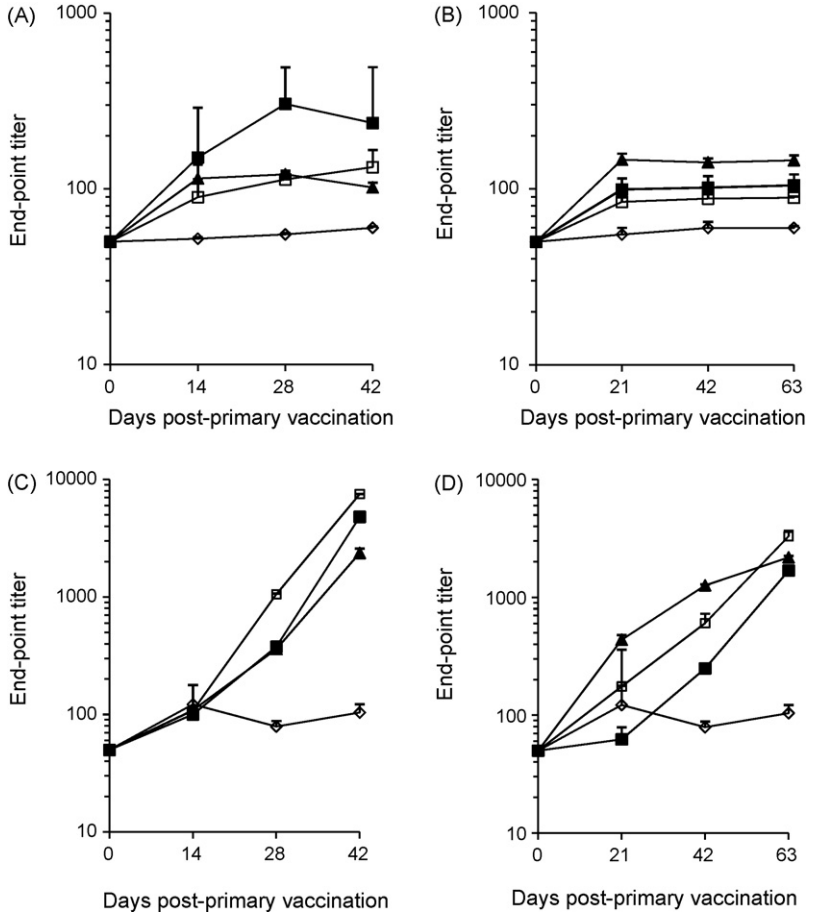

Fig. 1. Systemic immune responses following parenteral or mucosal delivery of spores expressing MPT64 in the germinating spore. Groups of six C57 BL/6 mice were dosed (Panels A and C) by the i.p. route three times at 2 weeks interval with 1×10^9 of different spores or (↓; Panels B and D) intranasally six times at 3 weeks interval with 2×10^9 spores of either LH78 (▲; *amyE*::*PrrnO-synmpt64*, *thrC*::*PrrnO-hylA*^{L461T}) or LH106 (■; *amyE*::*PrrnO-synmpt64*^{sec}, *thrC*::*PrrnO-hylA*^{L461T}). Control groups were, a group immunised with 2×10^9 of JH334 (□; *thrC*::*PrrnO-hylA*^{L461T}) spores and a naïve group of non-immunised animals (◊). Sera were collected and tested by ELISA for anti-MPT64-specific IgG (Panels A and B) and anti-spore-specific IgG (Panels C and D). The end-point IgG titer was calculated as the dilution of serum producing the same optical density as a 1/50 dilution of a pooled pre-immune serum. Error bars are standard error of the means.

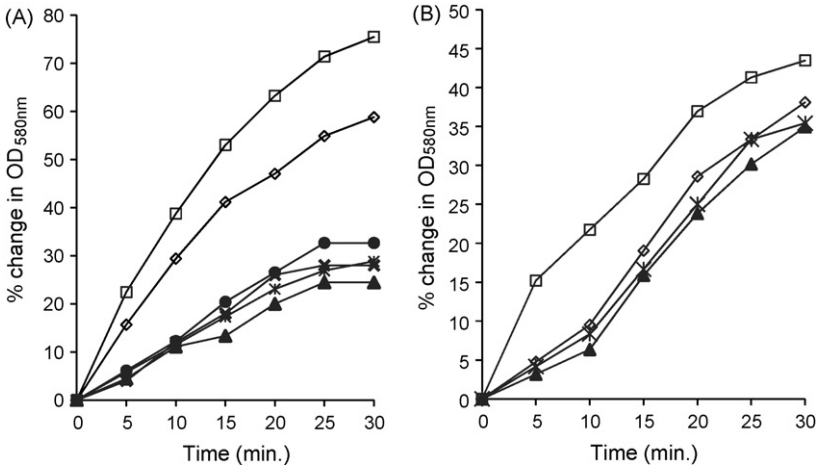

Fig. 2. Effect of immune serum on *in vitro* spore germination. Suspensions of spores of *B. subtilis* strain PY79 (2×10^8 spores) were pre-treated with serum IgG or lung sIgA (from mice immunised with LH106 spores by the i.p. route) and then induced to germinate in the presence of the germinant L-alanine (10 mM). The change in optical density (580 nm) of suspensions incubated at 37 °C was monitored reflecting the rate of germination. Panel A shows the effects of pre-treatment with IgG and in Panel B pre-treatment with sIgA. Symbols; ◊, untreated spores incubated without germinant; □, untreated spores incubated with germinant; ×, pre-treated spores (naïve serum at 1:10 (Panel A) or naïve sIgA undiluted (Panel B)) incubated with germinant; ✳, pre-treated spores (naïve serum at 1:40) incubated with germinant; ▲, pre-treated spores (anti-spore serum at 1:10 or undiluted anti-spore sIgA) incubated with germinant and; ●, pre-treated spores (anti-spore serum at 1:40) incubated with germinant.

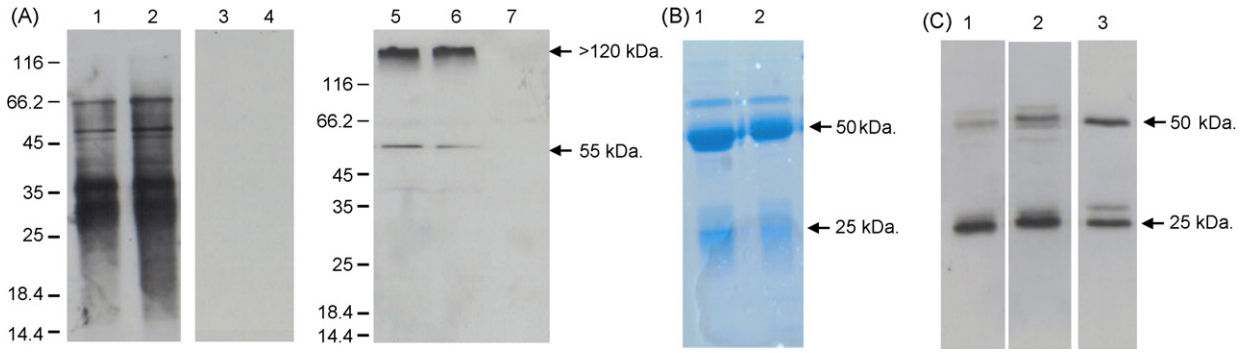

Fig. 3. Binding of IgG to spores. Antibodies present in serum or lung extractions from mice immunised with LH106 spores using the i.p. route were evaluated for their ability to bind to spore coat proteins and to spores. In Panel A coat proteins extracted from PY79 spores were fractionated by SDS-PAGE and then probed with immune serum (lanes 1 and 2) or from unimmunised mice (lanes 3 and 4). In lanes 5–7 sIgA taken from lung extractions has been used to probe immunoblots of spore coat proteins. Lanes 5 and 6 show blots probed with sIgA from immunised animals and lane 7 from naïve mice. Panel B shows the results of SDS-PAGE fractionation of the purified IgG fractions of immune serum (10 μg, lane 1) and naïve serum (10 μg, lane 2). The 50 kDa heavy chain and 25 kDa light chains are indicated. Panel C, Purified IgG (5 μg) was mixed with PY79 spores, gently washed and spore coat proteins extracted and fractionated by SDS-PAGE. Bound IgG (heavy and light chains) was detected using a conjugated secondary anti-mouse IgG. Lane 1, immune IgG; lane 2, naïve IgG; lane 3, a control serum consisting of mouse anti-PA.

likely was CotA and the latter CotG [19]. As expected, naïve serum, carried no antibodies that could specifically recognise spore coat proteins. Using sIgA we could also detect two principal species of coat protein of approximately 55 kDa and another high mwt species of greater than 120 kDa. Interestingly, no spore coat protein is known of this mwt and we cannot rule out the possibility that it is a previously unnoticed dimer of the 55 kDa species. Spore coat proteins are known to often contain multimeric forms even under SDS-PAGE fractionation so the identity of this highly immunogenic species remains elusive. Next, we asked whether IgG present in the serum could bind to spores by first purifying IgG from mouse immune and naïve serum (Fig. 3B). Purified IgG was first mixed with PY79 spores, incubated (30 min) and then spores were washed with PBS. Spore coat proteins were then extracted and fractionated by SDS-PAGE. Using an anti-mouse secondary antibody we were able to detect the heavy and light chains from spores incubated with both immune and naïve serum (Fig. 3C). This demonstrated that both immune and naïve serum IgG was capable of non-specifically binding to spores but only with the immune serum was binding specific. This approach though, could not be used for sIgA, which was present at a concentration too low to be purified. In other work, anti-serum to the *B. anthracis* protective antigen, PA, has been shown to bind to *B. anthracis* spores [18,20,21]. We wondered whether other antisera could bind to *B. subtilis* spores and in parallel studies we co-incubated with a mouse anti-PA serum. As shown in Fig. 3C anti-PA IgG could also bind spores suggesting that antibodies can bind spores non-specifically.

3.4. Pre-treatment of spores with antibodies enhances their phagocytosis

The effect of immune (from mice dosed i.p. with LH106 spores) and naïve serum on phagocytosis of PY79 spores was determined in a RAW264.7 cell line (Fig. 4 and Supp. Fig. 5) as well as in mouse peritoneal macrophages (Fig. 5). Phagocytosis of spores occurred for up to 8 h following addition of spores to RAW264.7 macrophages. Pre-treatment of spores with naïve serum did not alter the rate or uptake of spores (Supp. Fig. 5A). Spores pre-treated with immune serum did show a clear difference in uptake by phagocytes and when treated with the highest concentration of antisera showed an increase in total counts found within macrophages (Supp. Fig. 5A). To determine the proportion of spores that had germinated within

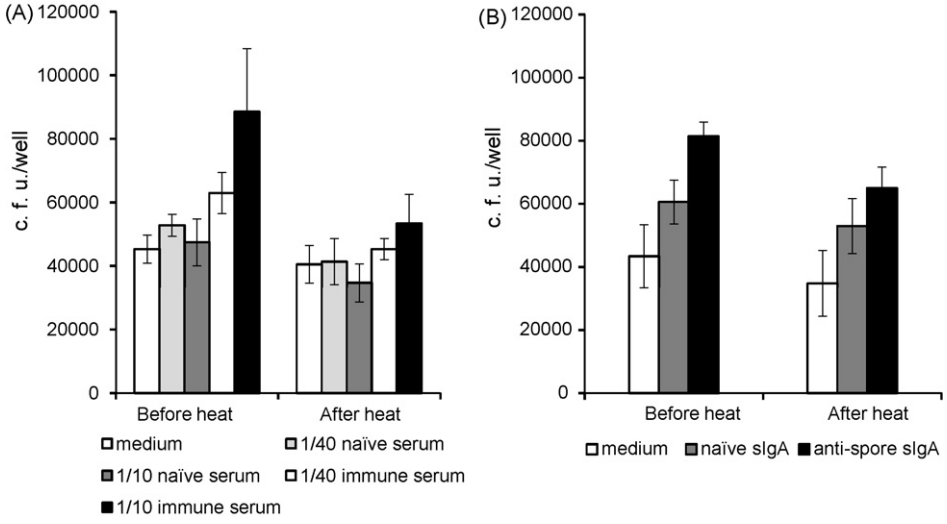

Fig. 4. The effect of anti-spore serum on phagocytosis and germination of spores in macrophages. PY79 spores were pre-treated with different dilutions of naïve serum, immune serum or medium alone. Serum was obtained from mice immunised with LH106 spores by the i.p. route). Spores were then added to a cultured macrophage cell line, RAW264.7. After 1 h incubation cells were washed and the number of spores phagocytosed was determined by plate counting the contents of lysed macrophages. To determine the numbers of ingested spores lysates were heat-treated (68 °C 30 min) prior to plating and determination of c.f.u. Panel A shows uptake of spores pre-treated with serum IgG and Panel B, with lung sIgA.

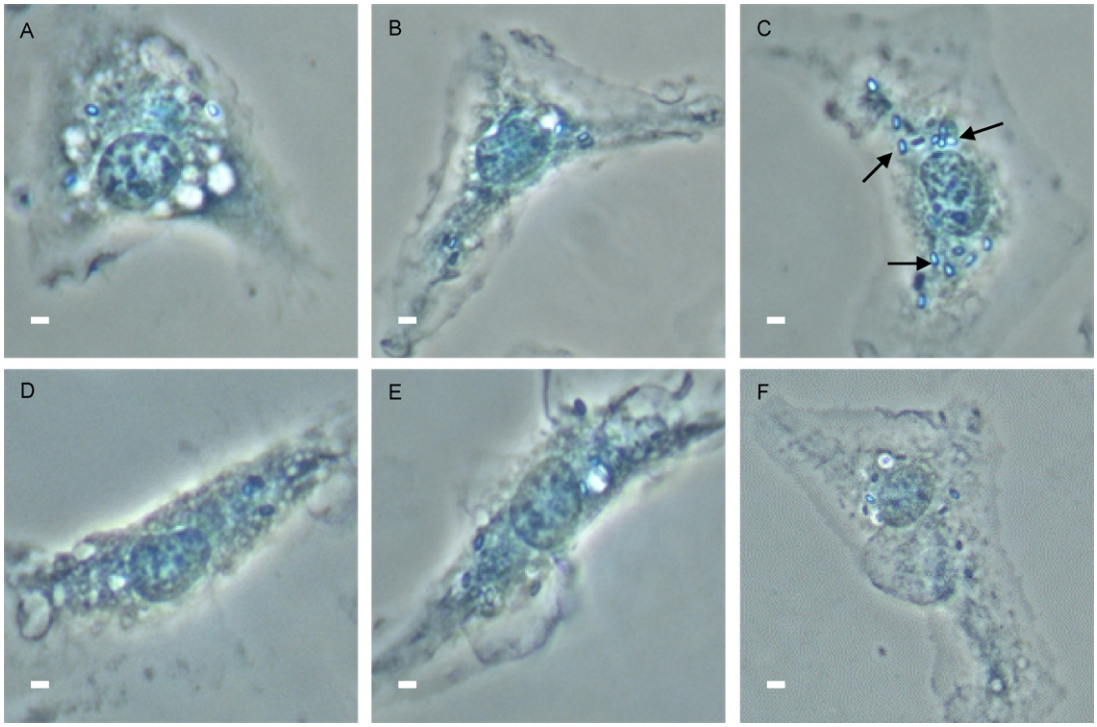

Fig. 5. Phagocytosis in mouse peritoneal macrophages. Murine peritoneal macrophages were infected with spores pre-treated with or without serum in DH10 medium. Infected cells were incubated for 1 h at 37 °C, washed and stained (Wright-Giemsa and malachite green), or re-incubated for 3 h after adding fresh medium. Macrophages infected with untreated spores (A and D), naïve serum-treated spores (B and E) and anti-spore serum-treated spores (C and F) were stained at 1 and 4 h. Arrows mark representative phase-bright spores. The bar marker is 2 μm in every panel.

macrophages we heat-treated phagocytes (to kill vegetative cells) before determination of viable counts. Our results showed that for immune serum-treated spores, following phagocytic uptake, a significant proportion (~50%) had germinated within the phagocyte (Fig. 4A). We performed similar experiments using lung sIgA. Phagocytic uptake of spores was maintained for at least 5 h (Supp. Fig. 5B). Importantly, pre-treatment of spores with lung sIgA from immune animals also showed an increase in uptake of spores at significantly greater levels than with spores pre-treated with sIgA from naïve animals ($P > 0.05$). Heat-treatment revealed that a significant proportion of ingested spores had germinated following uptake (Fig. 4B). Using mouse peritoneal macrophages we performed similar experiments adding pre-treated spores and using direct microscopy to visualise phagocytic uptake (Fig. 5). Our analysis revealed that with spores pre-treated with immune serum we could readily detect greater levels of phase bright spores and vegetative cells within macrophages. After 1 h of phagocytosis the proportion of spores to vegetative cells was approximately the same (50:50) as found in the RAW264.7 studies. These results show then that opsonised spores can be more efficiently phagocytosed leading to spore germination and cell death.

3.5. *Pre-treatment of spores with antibodies enhances their adhesion to epithelial cells*

A further possibility that might explain how anti-spore antibodies inhibit immune responses to administered spores is if these antibodies physically reduced adhesion to the mucosa. We tested this using two epithelial cell lines, Caco-2 and Hep-2. Our approach was to pre-incubate PY79 spores with lung sIgA obtained from the unimmunised mice (naïve sIgA) and from immunised mice (immune sIgA; dosed i.n. with LH106 spores). Antibody-coated spores were then added to Caco-2 or Hep-2 cells and after 1 h of incubation washed and the number of spores associated with cells determined by serial dilution and plating of lysed cells (Fig. 6). Surprisingly, we found that spores incubated with immune serum actually increased the number of spores associated with either Caco-2 cells (Fig. 6A) and Hep-2 (Fig. 6B). Our analysis did not discriminate between spores that adhered to cells or spores that had been endocytosed as has been noted earlier [14]. In any event, we can state that immune serum appears unable to decrease cell adhesion rather, the opposite.

4. Discussion

The aim of this study was to understand and clarify a previous observation that the nature of the humoral immune response directed against a heterologous antigen depends upon how the antigen is presented in a bacterial spore [15]. In these studies, when an antigen (*C. tetani* TTFC) was expressed on the spore surface successive doses of recombinant spores elicited seroconversion and hyperimmunity. By contrast, if expression of the same antigen was expressed in the germinating spore seroconversion appears to abruptly terminate after the first dose of immunogen and hyperimmunity was not reached. In this work we have used the *M. tuberculosis* MPT64 antigen to evaluate anti-MPT64 and anti-spore responses side-by-side using parenteral and mucosal delivery. We have shown that successive doses of spores delivered by a parenteral or local route do in fact, induce seroconversion and hyperimmunity with regard to anti-spore IgG responses yet when MPT64 is expressed in the germinating spore (in strain LH78), with regard to anti-MPT64 IgG (or sIgA), no significant seroconversion occurs beyond the first dose in agreement with earlier observations to the same effect [5,15]. One explanation is that the anti-spore response is capable of inhibiting the germination of recombinant spores. After the initial dose of recombinant spores, anti-spore-specific IgG and, in the case of local delivery, sIgA, would be produced and capable of neutralising spore germi-

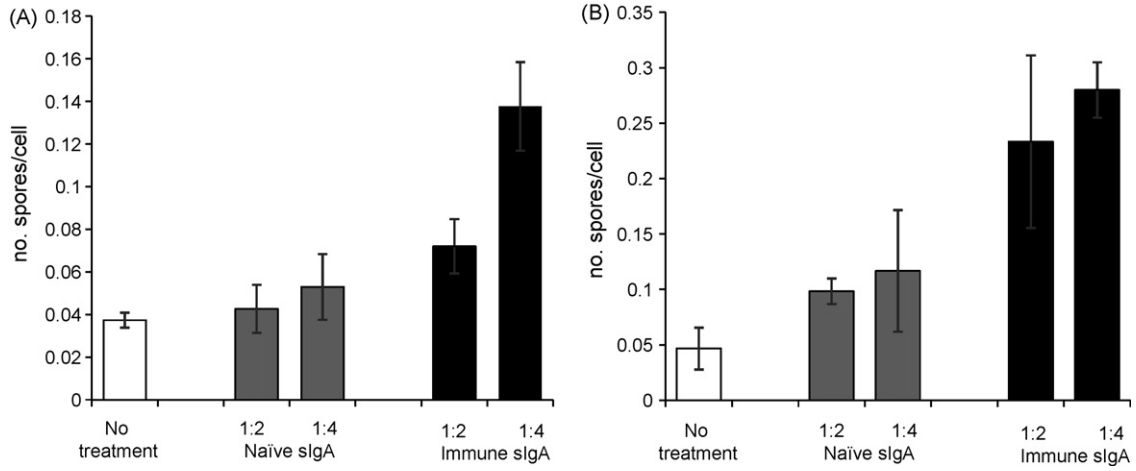

Fig. 6. Influence of antibodies on association of spores to cells. Caco-2 (Panel A) and Hep-2 (Panel B) cells were infected with PY79 spores pre-treated with lung sIgA (from mice immunised with LH106 spores by the i.n. route), naïve lung sIgA or with medium alone. After 1 h incubation, the number of spores adhering to cells was determined by viable counting of the cell lysate. The number of spores/cell is shown and represents the number of adhering + endocytosed spore counts.

nation upon successive doses. Since delivery of the antigen requires spore germination we can imagine a number of ways in which anti-spore antibodies could neutralise seroconversion, (i) by physically inhibiting spore germination, (ii) by enhancing the uptake and destruction of spores (opsonisation) and, (iii) by decreasing the adherence of spores to the mucosa (only relevant when administering spores via the mucosal routes).

Anti-spore antibodies could directly inhibit spore germination by binding or coating spores preventing access to a specific germinant. Our data confirms that antisera from immunised mice did indeed inhibit spore germination *in vitro*. This included serum IgG as well as sIgA obtained from lung extractions. However, we were surprised to find that naïve serum as well as naïve sIgA, that is, serum or lung extractions taken from unimmunised mice, was also a potent inhibitor of spore germination, *in vitro*. A simple interpretation then, is that antibodies, whether IgG or sIgA are coating spores and preventing access of a specific germinant. In the case of *B. subtilis* the germinant, L-alanine, must pass through the spore coat layer to interact with specific germinant receptors in the inner spore membranes [22]. We were able to confirm using immunoblotting that purified IgG, from both immune and naïve animals could indeed bind to the spore coats. We believe that this binding was non-specific though because, using Western blotting of extracted spore coat proteins, only immune serum could bind to specific spore coat proteins. This demonstrates that spores, and specifically the spore coats, are able to adsorb molecules. Interestingly, this offers some parallels to studies showing that the protective antigen (PA) of *B. anthracis* is capable of non-specifically binding to the spore coat of *B. anthracis* spores [20] and understanding the extent and nature of the spore's binding properties could prove interesting.

Opsonisation was also confirmed where only antibodies derived from immunised animals played a direct role in enhancing phagocytosis. Our results showed that opsonised spores were more efficiently phagocytosed and this, in turn, would lead to faster germination and destruction of the spore within the phagocyte. Germination of spores within phagocytes and subsequent vegetative gene expression has been demonstrated previously for *B. subtilis* using *in vitro* studies [14,23]. Studies examining the fate of phagocytosed *B. anthracis* spores has shown that a phagocyte is unable to destroy an intact spore, rather, it requires germination of the spore before cell killing can occur [24]. Accordingly, we must similarly assume that anti-spore antibodies lead to faster uptake and presumably faster germination within phagocytic cells *in vivo*. In this case though, we assume that any role of antibodies in inhibiting spore germination is relinquished once within the phagosome or phagolysosome. It is also interesting that for opsonisation to occur specific binding of antibodies to spore coat proteins must occur and non-specific binding of antibody to spores is not sufficient for opsonisation. What remains unclear is how anti-spore responses are maintained while those to the antigen expressed in the germinating spore are switched off. Presumably, the way in which the antigens is processed is altered. In our constructions we have engineered the germinated spore to enter the cytoplasm by secretion of LLO that permeabilises the phagosomal membrane. Possibly, entry of the bacterium into the cytosol subverts the immune response towards a Th1-bias (leading to a cell-mediated immune response) as has been observed from another study examining the fate of *B. subtilis* in macrophages [14]. Alternatively, the newly germinated spore is simply destroyed too quickly reducing the amount of immunogen that can be synthesised.

A final hypothesis we tested was whether antibodies could decrease adhesion of spores to eukaryotic cells. This is principally an issue that would occur at the mucosa. By its very nature, the studies we applied are artificial and cannot realistically represent the interaction of bacteria with mucus-rich mucosal cells, on the other hand they provide a useful model. We found some evidence that anti-spore sIgA could actually increase adhesion of spores to the cultured cells. Perhaps, making bacteria 'sticky' may not only enhance their adhesion to epithelial cells but could also enhance their endocytosis.

As mentioned at the beginning of this section, germinating spores of a strain, LH106, that expressed the MPT64 antigen in a secretable form did seroconvert and anti-MPT64 responses reached hyperimmunity following parenteral injection. This was not the case when nasal administration was used though, in which case no seroconversion was seen. A similar finding has been observed following parenteral delivery using *B. subtilis* spores that express and secrete *B. anthracis* PA [2]. A number of explanations might account for this. Firstly, is the possibility that a secreted antigen, as an exogenous antigen, is processed differently within a phagocytic cell. Alternatively, a secreted antigen may inadvertently be able to associate with the spore, bound to the spore coat, in which case since spore germination is not required for antigen delivery and an immune response might be produced. Precisely such a possibility has been considered to explain the association of PA with *B. anthracis* spores [20]. Here when *B. anthracis* spores are prepared, high levels of secreted PA fortuitously bind to spores. With *B. anthracis* the presence of anti-PA antibodies would bind to

spore-bound PA and both inhibit spore germination and enhance phagocytosis [18,21]. Using nasal delivery it might be argued that the bound antigen is prematurely degraded. A final, and more likely, possibility is that PA is secreted immediately the spore begins to germinate and while the spore coats are still associated with the hatching cell. Possibly, with LH106 spores the MPT64 antigen is secreted very rapidly and even when spores are opsonised and taken up by macrophages sufficient antigen can be made to generate hyperimmunity. By the nasal route it is less clear that the spore cannot germinate supporting a view that the action of anti-spore sIgA is more potent in preventing spore germination.

In conclusion these studies have demonstrated that the contribution of anti-spore antibodies to the use of *B. subtilis* spores for antigen delivery is important and can dictate the nature of the immune response. Using germinating spores as vaccine vehicles they can only be considered for delivery of a single dose of antigen where anti-spore antibodies prevent subsequent spore germination. On the other hand, secreted antigens, at least with MPT64 studied here, appear able to escape the germinating spore before its intracellular destruction when delivered parenterally. Delivery of antigens on the spore surface though does not interfere with antigen delivery but is constrained only by the susceptibility of exposed antigens.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2009.05.087.

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