Phagocytosis, germination and killing of *Bacillus subtilis* spores presenting heterologous antigens in human macrophages

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*Bacillus subtilis* is a Gram-positive spore-bearing bacterium long used as a probiotic product and more recently regarded as an attractive vehicle for delivering heterologous antigens to be used for mucosal vaccination. This report describes the *in vitro* interaction between human macrophages and *B. subtilis* spores displaying the tetanus toxin fragment C or the B subunit of the heat-labile toxin of *Escherichia coli* on their surface in comparison to spores of the parental strain. Recombinant and parental *B. subtilis* spores were similarly internalized by human macrophages, at a frequency lower than 2.5%. Inside macrophages, nearly all spores germinated and were killed within 6 h. Using germination-defective spores and inhibiting spore germination inside macrophages, evidence was produced that only germinated spores were killed by human macrophages and that intracellular spore germination was mediated by an alanine-dependent pathway. The germinated spores were killed by macrophages before any round of cell duplication, as estimated by fluorescence microscopy analysis of macrophages infected with spores carrying the *gfp* gene fused to *abrB*, a *B. subtilis* gene shown here to be expressed at the transition between outgrowth and vegetative growth. Monitoring of macrophage infection never revealed cytotoxic effects being exerted by *B. subtilis* spores. These *in vitro* data support the hypothesis that *B. subtilis* spores may potentially be used as a suitable and safe vehicle for administering heterologous antigens to humans.

INTRODUCTION

*Bacillus subtilis* is a Gram-positive spore-forming microorganism, which has been rated as safe by the Food and Drug Administration of the United States and by the European Food Safety Authority. This bacterium has been long assumed as a probiotic product for human use and licensed to be orally administered as live spores for oral bacteriotherapy. A wide literature supports the use of oral administration of *B. subtilis* spores for both the prevention and the treatment of a broad variety of gastrointestinal disorders (Hong et al., 2005; Fujiya et al., 2007; Williams, 2007). The advantage of using *B. subtilis* spores mainly relies on the particular attributes of bacterial spores, which are extremely robust life forms, capable of withstanding extreme temperatures, desiccation and exposure to solvents and noxious chemicals (Driks, 1999). Moreover, unlike vegetative forms, *B. subtilis* spores are unaffected by the gastric fluid, and can transit across the stomach and reach the small intestine alive (le Duc et al., 2003a).

Based on their stability and safety once ingested, *B. subtilis* spores may be considered as an ideal vehicle for delivering heterologous antigens to the gastrointestinal tract. In the gastrointestinal tract, spores can reach the Peyer’s patches and carry heterologous antigens to phagocytes and B cells, thus priming specific humoral and cell-mediated immune responses (Spinosa et al., 2000; Hoa et al., 2001; le Duc et al., 2003a; Mauriello et al., 2007). In addition, *B. subtilis* spores can be easily engineered to display, on their surface, a large variety of heterologous antigens in association with spore coat proteins. The spore coat proteins CotB and CotC have already been used as fusion partners for various proteins, such as the tetanus toxin fragment C (TTFC) (Isticato et al., 2001), the B subunit of the heat-labile toxin of *Escherichia coli* (LTB) (Mauriello et al., 2004), the

**Abbreviations**: FBS, fetal bovine serum; LTB, B subunit of the heat-labile toxin of *Escherichia coli*; TTFC, tetanus toxin fragment C.
protective antigen (PA) of *Bacillus anthracis* (le Duc et al., 2007), and the *Clonorchis sinensis* 22.3 kDa tegumental protein (CSP22.3) (Zhou et al., 2008). Immunization of mice with *B. subtilis* spores displaying TTFc, PA or CSP22.3 was shown to induce a protective immune response against a challenge with the tetanus toxin, *B. anthracis* spores or *C. sinensis*, respectively (le Duc et al., 2003b, 2007; Zhou et al., 2008). Nevertheless, information on the interaction of *B. subtilis* spores with human macrophages is lacking, while this step has a crucial role in both controlling the initial host colonization/invasion by infectious agents (Aderem & Underhill, 1999) and in instructing the adaptive immune response by presenting pathogen-derived peptides to local T-cells (Underhill et al., 1999). The few available data describing the *in vitro* interaction of *B. subtilis* with phagocytes have been produced with murine macrophages and non-recombinant spores. By these studies, it was demonstrated that *B. subtilis* spores germinated in infected phagocytes and initiated vegetative gene expression, although vegetative cell-growth was not detected (le Duc et al., 2004).

In this study, the *in vitro* interaction of *B. subtilis* spores with human macrophages was analysed by co-culturing THP-1 cells, which represent a good model mimicking blood monocyte-derived macrophages (Stokes & Doxsee, 1999), and isogenic recombinant spores carrying heterologous antigens on their surface, either TTFc or LTb in association with CotB or CotC. The focus was to investigate whether heterologous antigens expressed on the spore surface could modify spore–cell interactions and the intracellular fate of spores ingested by human macrophages. The results obtained clearly show that: (i) spores of all strains are phagocytosed by human macrophages at a frequency lower than 2.5%; (ii) most internalized spores germinate by an alanine-mediated germination pathway; and (iii) only the germinated spores are rapidly killed. By the use of *B. subtilis* strains carrying the *abrB::gfp* gene fusion, evidence is also provided that germinated *B. subtilis* spores never initiate expression of the recombinant protein inside macrophages. Moreover, during the whole time-frame of spore/cell co-cultures, no damage was ever observed in *B. subtilis*-infected macrophages. These data support the idea that recombinant *B. subtilis* spores may be used as safe carriers for heterologous antigen delivery.

**METHODS**

**Bacterial strains and molecular procedures.** *B. subtilis* strains are listed in Table 1. *E. coli* strain DH5α was used for cloning experiments (Sambrook et al., 1989). *B. subtilis* was transformed by the two-step method (Cutting & Vander Horn, 1990). Isolation of plasmids, restriction enzyme digestion and ligation of DNA were carried out by standard methods (Sambrook et al., 1989). Chromosomal DNA from *B. subtilis* was extracted as previously described (Cutting & Vander Horn, 1990). AZ413 was obtained by transforming strain TB1 (Mauriello et al., 2007) with chromosomal DNA of strain IM201 (Mauriello et al., 2004) and selecting for chloramphenicol resistance. In order to obtain the *abrB::gfp* gene fusion, a 814 bp genomic fragment containing the *abrB* promoter region and upstream sequences was PCR-amplified from *B. subtilis* using the primers *abrBs* (5'-gaattcAATGACAACTTTATGCCG-3') and *abrB* (5'-gaattcTCTCGTCCCAAGAGTACT-3'), respectively annealing at positions –814–796 and –19–1 (underlined, lower-case letters indicate restriction sites inserted in the primers). The amplification product was cloned upstream of the *gpmu*3a gene of plasmid pAD123 (BGSC collection) (Dunn & Handelsman, 1999) previously digested with BamHI and EcoRI. The resulting plasmid, pGC92, was used to transform competent cells of *B. subtilis* wild-type strain PY17. Chloramphenicol-resistant clones were the result of a single Campbell-like cross-over event between homologous DNA regions present on the plasmid and on the chromosome. Several clones were analysed by immunofluorescence microscopy and one of the positive clones, GC254, was selected for further studies. Chromosomal DNA of strain GC254 was used to move, by chromosomal DNA-mediated transformation, the *abrB::gfp* fusion into strains RH103 (Istitaco et al., 2001) and IM201 (Mauriello et al., 2004), whose *cat* gene was previously replaced with a spectinomycin-resistance gene cassette (*spc*) by transformation with a linearized form of the pHL62 plasmid (*cat::spc*) (LeDeaux & Grossman, 1995). The recombinant strains derived from RH103 and IM201 were designated GC255 and GC256, respectively.

**Preparation of *B. subtilis* spores.** Sporulation of *B. subtilis* strains was induced by the exhaustion method (Nicholson & Setlow, 1990) in Difco sporulation medium (DSM). Cultures were harvested 24 h after the initiation of sporulation. Spores were purified as described (Nicholson & Setlow, 1990) using lysozyme treatment to break any residual sporangial cells followed by washing in 1 M NaCl, 1 M KCl and twice in water. The spore suspension was heat-treated (80 °C, 30 min) to kill any residual vegetative cells, titrated for colony-forming units (c.f.u. ml⁻¹) and stored at –20 °C. Inocula were prepared by

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<th>Strain</th>
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<td>PY17</td>
<td>trpC2</td>
<td>Youngman et al. (1984)</td>
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<tr>
<td>RH103</td>
<td>trpC2 ameE::cotB-tetC (cat)</td>
<td>Istitaco et al. (2001)</td>
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<td>IM201</td>
<td>trpC2 cotC::lb (cat)</td>
<td>Mauriello et al. (2004)</td>
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<td>TB1</td>
<td>trpC2 gerD-cwD::neo</td>
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<td>AZ413</td>
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diluting frozen spore samples in the medium used for coculturing spores and macrophages. Quantification of the inocula was performed by plating serial dilutions of spore suspensions on Müller–Hinton agar (Oxoid) and counting c.f.u. after 24 h incubation at 37 °C.

Germination assay. The extent of spore germination was evaluated as previously described (Senesi et al., 1975). The germination mixtures used were: (i) sterile phosphate-buffered saline (PBS, EuroClone); (ii) RPMI 1640 (EuroClone) supplemented with 10% heat-inactivated fetal bovine serum (PBS, EuroClone) and 2 mM glutamine (EuroClone) (complete medium); (iii) serum-free complete medium; (iv) complete medium with 3 mM D-alanine or 3 mM L-alanine (Sigma). Spores were suspended to a final OD625 of 0.5–0.7 and germination kinetics recorded at 37 °C using an 8453 UV–visible spectrophotometer (Agilent Technologies). The decrease in OD625 was taken as an index of spore germination, with a 60% decrease corresponding to 100% spore germination.

Macrophages. The human monocytic cell line THP-1 (human monocytic leukaemia) was purchased from the European Collection of Animal Cell Cultures (ECACC). Cells were propagated in complete medium supplemented with 1% Penstrep (EuroClone) and 0.2 µg amphotericin B ml⁻¹ (EuroClone) at 37 °C in air and 5% CO2 at 95% humidity. Two days before infection, monocytes were dispensed into 24-well plates at a density of approximately 3 × 10⁵ cells per well and differentiated into macrophages by the addition of 50 nM phorbol 12-myristate 13-acetate (PMA, Sigma). For fluorescence microscopy experiments, 24-well plates containing 12 mm diameter coverslips were used. Cell viability was monitored by Trypan Blue (Sigma) exclusion.

Phagocytosis and killing of B. subtilis spores. Macrophages were infected with B. subtilis spores (strain GC254, GC255 or GC256) at an m.o.i. of 1:10 (approx. 3 × 10⁵ spores per well) in serum-free complete medium. Phagocytosis was allowed to proceed for 2 h at 37 °C. To evaluate the intracellular killing of spores, infected macrophages were incubated for an additional 2 (t₁), 4 (t₂) and 6 h (t₃) in complete medium. Macrophages were washed with PBS, incubated with 2.5 µg gentamicin ml⁻¹ (Sigma) for 30 min, further washed with PBS, and finally lysed by resuspending in cold 0.1% Triton X-100 (Sigma). The complete removal of macrophages from the wells was verified by microscopic inspection using an inverted microscope (Olympus BH-2). Lysates from multiple wells were pooled, serially diluted and plated onto Müller–Hinton agar to determine the total number of intracellular bacteria. To evaluate the number of heat-resistant forms, lysates were heated at 80 °C for 10 min before being diluted and plated. The number of intracellularized bacteria was expressed both as absolute count and as percentage of the corresponding infectious dose. Each experiment was performed in triplicate on different days. To quantify the amount of spores physically bound to macrophages, cells were pre-incubated with 20 µM cytochalasin B (Sigma), a phagocytosis inhibitor, for 1 h and phagocytosis and killing assays were performed in the presence of 20 µM cytochalasin B.

Inhibition and reversal of spore germination within macrophages. Macrophages were pre-incubated in complete medium plus 3 mM D-alanine for 1 h and maintained in serum-free complete medium with 3 mM D-alanine during infection with B. subtilis spores. At the end of infection, non-phagocytosed spores were removed by PBS washings and gentamicin treatment and the infected macrophages were incubated for 2 h in complete medium containing 3 mM D-alanine. Afterwards, macrophages were washed with PBS and further incubated for 2 h in complete medium charged with either 3 mM D-alanine or 3 mM L-alanine. The number of phagocytosed spores as well as the numbers of total viable bacteria and heat-resistant spores recovered from infected macrophages were determined as described above.

Evaluation of abrB expression by fluorescence analysis. Spores of strains GC254, GC255 and GC256 were induced to germinate as described by Keijser et al. (2007), and samples collected at various time points. Samples were fixed with 2.8% formaldehyde and 0.04% glutaraldehyde for 15 min at room temperature, followed by incubation on ice, and analysed with an Olympus BX51 microscope equipped with a ×100 UPlanFl objective and a U-WIBA filter cube (excitation filter 460–490 nm, barrier filter 515–550 nm). Exposure times were always between 500 and 1000 ms. Images were captured and cropped with analySIS software (SIS).

Evaluation of GFP expression in infected macrophages and immunofluorescence assays. For the evaluation of GFP expression by internalized bacteria, macrophage monolayers on coverslips were infected with spores of strain GC254, GC255 or GC256, as described above. For the immunofluorescence assays, macrophages were infected with the strain IM201 or AZA413 in serum-free complete medium and centrifuged at 2000 g for 10 min. After 1 h incubation, macrophages were washed with PBS and further incubated for 1 or 2 h in complete medium. At each time point, monolayers were washed with PBS supplemented with 1 mM CaCl2 and 1 mM MgCl2 and fixed with 4% paraformaldehyde (Sigma) for 15 min. Free aldehydic groups were blocked with 50 mM NH4Cl in PBS for 10 min and macrophages were permeabilized by incubation for 5 min in 0.2% Triton X-100. Immunofluorescence labelling was performed with a rabbit polyclonal antibody directed against LTB and anti-rabbit IgG (whole molecule) F(ab')2 fragment-FITC (Sigma). Coverslips were immediately mounted onto microscope slides in mounting solution (Advanced Biotechnologies) and observed with an Eclipse 55i fluorescence microscope (Nikon), equipped with a DS-2M digital camera (Nikon). The number of intracellular fluorescent spores per infected macrophage was estimated by counting ten randomly selected microscope fields per slide.

Statistical analysis. All values are expressed as the mean ± SD. Statistical analysis was performed using the two-tailed Student’s t test. A P-value ≤0.05 was considered statistically significant.

RESULTS

Co-cultivation of B. subtilis spores and macrophages

To analyse the interaction of human macrophages with B. subtilis spores, preliminary experiments were performed to exclude the possibility that spores could germinate in the medium used for macrophage/spore co-cultures. Germination of B. subtilis spores was recorded by monitoring the time-dependent decrease in OD625 of spore suspensions, as a lowering in their optical density reflects the extent of their germination (Powell, 1950). When dormant spores of B. subtilis strains GC254, GC255 and GC256 were tested for germination in complete medium, the OD625 of spor suspensions dropped by 30% in 20 min, indicating that a rapid rate of spore germination occurred in this culture condition. No change in OD625 was ever detected, over a 2 h time-frame, when spores were suspended in PBS or in serum-free complete medium (data not shown). Viability of the macrophages, tested by Trypan Blue exclusion, revealed that 10% of macrophages died in
the presence of PBS alone, while almost 100% of the cells remained viable in serum-free complete medium for up to the 2 h incubation period that we chose for spore uptake by macrophages. Therefore serum-free complete medium was used to avoid extracellular spore germination and to ensure macrophage viability during the time-course of spore phagocytosis by macrophages. FBS, which acts as a potent inducer of spore germination, was included in all co-cultures only after spore uptake by macrophages was complete.

Phagocytosis and intracellular killing of spores

The efficiency of phagocytosis and intracellular killing by macrophages was compared for *B. subtilis* spores displaying or not heterologous antigens on their surface. Macrophages were infected with spores of strain GC254, GC255 or GC256 and lysed to recover ingested bacteria at the end of the 2 h infection (*t*0). Total counts of viable bacteria were not significantly different for the three strains used (Fig. 1a), being 1.95 ± 0.66% (GC254), 2.42 ± 0.83% (GC255) and 1.90 ± 0.69% (GC256) of the corresponding infectious spore suspension. This finding suggested that non-recombinant and recombinant *B. subtilis* spores, displaying either the TTFC or the LTB on their surface, were similarly phagocytosed and that the heterologous antigens did not modify the interaction of spores with macrophages. The intracellular burden of viable bacteria was found to rapidly decrease, when expressed either as an absolute number (Fig. 1a) or as a percentage of the ingested spores (Fig. 1b), with no statistical difference being noted among the three strains during the time-course of infection. Interestingly, at each time point counts of total viable bacteria and heat-resistant spores were not significantly different (Fig. 1a), thus indicating that almost all recovered bacteria were heat-resistant spores. This finding demonstrates that modifications of the spore surface, due to heterologous antigens, do not affect spore killing by macrophages and strongly suggests that the forms susceptible to macrophage killing could be germinated spores or vegetative cells.

When co-cultures of spores and macrophages were performed in the presence of cytochalasin B approximately 10^3 spores were recovered from macrophages at each time point of the incubation period (Fig. 1a). In view of this finding and considering that the number of spores surviving killing recovered at *t*0 without the addition of the phagocytosis inhibitor was approximately 10^3, we concluded that almost all ingested spores were killed by macrophages within 6 h.

Macrophages kill germinated spores

To address the question of whether the decrease in the intracellular spore number was due to the killing of dormant or germinated spores, phagocytosis and killing experiments were carried out in the presence of *D*-alanine, an efficient inhibitor of spore germination for many bacilli (Hills, 1949; Moir *et al.*, 2002). The concentration of 3 mM *D*-alanine completely inhibited spore germination of all strains even in complete medium (data not shown); therefore this concentration was used in all spore/macrophage co-cultures. The presence of *D*-alanine did not significantly affect spore internalization, as the extent of phagocytosis recorded for GC254, GC255 and GC256 was respectively 1.99 ± 0.36%, 1.75 ± 0.4% and 2.06 ± 0.5% of the corresponding infectious dose. As already shown for co-cultures set up without *D*-alanine, no difference was found between total viable counts and

![Fig. 1. Phagocytosis and intracellular survival of *B. subtilis* spores.](http://mic.sgmjournals.org)
counts of heat-resistant spores recovered from macrophages at \( t_0 \), \( t_2 \) and \( t_4 \) (data not shown). A high percentage of spores of the three strains was recovered from macrophages at \( t_2 \) (Fig. 2); these values were significantly higher \((P \leq 0.01)\) than those obtained in the absence of D-alanine at the corresponding incubation time (see Fig. 1b). At \( t_4 \), spore numbers recovered from D-alanine-treated macrophages were lower than those recovered at \( t_2 \) (Fig. 2). However, the recovered spores were roughly 40–50% of those internalized at \( t_0 \) and fourfold higher than those recovered from cultures incubated in the absence of D-alanine at the corresponding incubation time (see Fig. 1b). These data indicated that D-alanine inhibition of spore germination inside macrophages was responsible for the decrease in the intracellular killing of ingested spores. Therefore, to restore intracellular spore germination and to confirm that heat-resistant spores are less susceptible to macrophage killing than germinated spores, half of the co-cultures were extensively washed at \( t_2 \) and resuspended in complete medium containing 3 mM l-alanine (replacing D-alanine) (Fig. 2). The addition of l-alanine to the medium restored intracellular killing. In fact, at \( t_4 \), numbers of intracellular spores were significantly lower \((P \leq 0.01)\) than those obtained in the presence of D-alanine (Fig. 2).

To further validate the results of the germination-inhibition assays, macrophages were infected with spores of \textit{B. subtilis} strains IM201 and AZ413. Both these strains display the LTB heterologous antigen in association with CotC, but AZ413 also carries the \textit{gerD-cwlD} mutation that leads to an early block in spore germination and to inability to respond to various germinants (Mauriello \textit{et al.}, 2007).

The fate and persistence of spores within infected macrophages was examined by intracellular immunofluorescence, with a polyclonal serum against LTB. To better detect the internalized spores by immunofluorescence, macrophage/spore co-cultures were centrifuged to enhance spore adhesion, thus facilitating their uptake. Microscopic observation revealed that comparable amounts of spores of both strains were detectable within macrophages at \( t_0 \) (7.2 ± 2.5 spores per infected macrophage for strain IM201 and 6.8 ± 3.1 spores per infected macrophage for strain AZ413) (Fig. 3). However, a rapid reduction in fluorescent spores of IM201 was observed over time, and none could be seen after 2 h of incubation (Fig. 3a). In contrast, fluorescent spores of the germination-defective strain AZ413 were clearly detectable inside macrophages at the same incubation time (2.7 ± 0.8 spores per infected macrophage) (Fig. 3b). Since the germination-defective strain persists longer inside cells, escaping macrophage killing, this finding confirms that human macrophages are able to rapidly kill the germinated spores.

**B. subtilis** spores do not initiate vegetative growth inside macrophages

To evaluate whether germinated spores could initiate vegetative growth within human macrophages, phagocytosis assays were carried out with spores of strain GC254, GC255 or GC256 and cell monolayers were analysed by fluorescence microscopy. GC254, GC255 and GC256 are characterized by the presence of the GFP coding region under the transcriptional control of the \textit{abrbB} promoter. AbrbB is a transition-state regulator that is expressed during the transition between the lag and exponential growth phases (O’Reilly & Devine, 1997). Nevertheless, timing of its expression in germinating cells has never been studied in detail. To analyse \textit{abrbB} expression during germination, spores of strains GC254, GC255 and GC256 were induced to germinate as described by Keijser \textit{et al.} (2007). Aliquots were collected at various time points after the induction of germination and analysed by phase-contrast and fluorescence microscopy. For all aliquots, the OD\textsubscript{600} was also measured. In accordance with previous reports (Keijser \textit{et al.}, 2007), at 10 min into germination the OD\textsubscript{600} of the culture had decreased to less than 50% of the initial value. A minor decrease (to about 40%) was observed at later stages (10–80 min). Thereafter, the optical density started to increase and reached a value similar to the initial one within the following 40 min (data not shown). At this stage, the increase in optical density is due to spore swelling and not to cell division (Keijser \textit{et al.}, 2007). Microscopic analysis of the various time points showed all the germination stages, as previously described (Keijser \textit{et al.}, 2007): phase-bright spores became phase-dark within 10 min after the induction of germination, then the size of phase-dark spores started to increase and after 80 min cells started to burst out of the protective layers. Remnants of the spore coat and cortex were still visible at later times (Fig. 4). Cells started to become fluorescent 120 min after the induction of ger-[Fig. 2. Inhibition and reversal of inhibition of \textit{B. subtilis} spore germination inside macrophages. Macrophages were infected with spores in the presence of D-alanine \((t_0)\). After a 2 h incubation with D-alanine \((t_2)\), infected macrophages were further incubated with either D-alanine or L-alanine \((t_4)\). The percentage of spores recovered from macrophages was calculated at each time point of incubation. Asterisks (*) denote statistical significance \((P<0.01)\).]
mination (Fig. 4). As shown in Fig. 4, a cell still attached to remnants of the spore protective layers is fluorescent, indicating that the abrB::gfp fusion is expressed at that time. Therefore, in our experimental conditions, 120 min after the induction of germination, cells are at the end of the outgrowth phase and are just starting vegetative growth.

In phagocytosis assays performed with spores carrying the abrB::gfp fusion, no fluorescent recombinant protein was ever observed within macrophages (Fig. 5), indicating that internalized B. subtilis spores did not initiate vegetative growth. Fluorescent cells, only visualized 6 h after infection (Fig. 5a), were outside the macrophages and likely derived from a few spores that had remained physically bound to the macrophage surfaces. Indeed, treatment of infected monolayers with low (2.5 μg ml⁻¹) concentrations of gentamicin, allowing killing of extracellular bacteria (Ohya et al., 1998; Drevets et al., 1994), revealed that these vegetative forms were present only outside macrophages (Fig. 5b). Taken together, these results demonstrate that B. subtilis spores, which are able to germinate inside human macrophages, do not initiate vegetative growth within macrophages, as they are intracellularly killed soon after germination.

**Recombinant B. subtilis spores do not damage human macrophages**

To analyse the potential cytotoxic effect of recombinant B. subtilis spores on macrophages, cell monolayers were infected with strain GC254, GC255 or GC256 and macrophage viability was monitored at t₀, t₁, t₄ and t₆ by Trypan Blue exclusion. Microscopic examinations of cell layers revealed that viability of infected and non-infected macrophages was similar and higher than 99% at all the incubation time points (data not shown). This finding indicates that human macrophages survive the infection with spores and that recombinant B. subtilis spores do not exert any cytotoxic effect on phagocytes.

**DISCUSSION**

The first line of immunological defence against a pathogenic insult is the innate immune system, which plays an important role in engulfing and destroying infecting micro-organisms, as well as in activating the acquired immune response with the expansion of antigen-specific T and B cells (Medzhitov & Janeway, 1997).

In this investigation, co-cultures of the human macrophage cell-line THP-1 and recombinant B. subtilis spores have been used to examine the effectiveness of macrophages in controlling cellular infection with spores presenting heterologous antigens on their surface, to further support the development of a B. subtilis-based safe vaccine. Spore uptake by human macrophages, as already shown for murine macrophages (le Duc et al., 2004), was very inefficient, the percentage of phagocytosis being lower than 2.5% regardless of the heterologous antigen that the spores present.
expressed. When the amount of spores internalized by macrophages was increased by centrifugation, the efficiency of phagocytosis was still lower than that reported for pathogenic micro-organisms (Hu et al., 2006; Rajavelu & Das, 2007). The low extent of B. subtilis spore uptake by human macrophages may be considered a further indication of the safety of this micro-organism. Indeed, it has been recently demonstrated that the pathogen B. anthracis survives macrophage killing through a mechanism of autoinhibition of spore germination, also known for B. subtilis (Anmuth et al., 1956), and that autoinhibition is activated at high spore densities (McKevitt et al., 2007). Consequently, high numbers of spores within the macrophage may inhibit their own germination and increase intracellular survival, thus contributing to the virulence exerted.

Inside macrophages, the number of intracellular B. subtilis spores was found to rapidly decrease, independently of the heterologous antigen presented. At 6 h post-infection, the intracellular bacterial counts were similar to the counts obtained with the addition of a phagocytosis inhibitor, thus suggesting that they mostly resulted from residual bacteria adhering to the macrophage surfaces (Fig. 1). B. subtilis was shown to be unable to replicate inside human macrophages, since no increase in the intracellular bacteria was ever detected during infection. Therefore, human macrophages appear to be able to efficiently control the infection by recombinant B. subtilis spores. Killing of internalized B. subtilis spores appears to require intracellular spore germination. Indeed, comparable numbers of total and heat-resistant forms were found intracellularly during infection, suggesting that dormant spores are not susceptible to the mechanisms activated by macrophages to destroy internalized bacteria. In addition, when germination of engulfed spores was blocked by adding D-alanine, a potent germination inhibitor, an increased number of heat-resistant spores remained viable inside macrophages (Fig. 2). This conclusion was also supported by the longer intracellular persistence of spores we observed for a germination-defective B. subtilis strain (Fig. 3). The relationship between spore germination and intracellular killing was confirmed by replacing D-alanine with L-alanine.

Fig. 4. Timing of expression of the abrB::gfp fusion in strain GC255. Samples shown were collected at the induction of germination (t₀) and 2 h later (t₂). The same microscope fields are shown by phase-contrast (PC) and by fluorescence (F) microscopy (taken at ×1000; reproduced at ×600). The arrow indicates remnants of spore coat and cortex still attached to the cell pole. Similar results were obtained for strains GC254 and GC256.

Fig. 5. Germinated spores do not initiate vegetative gene expression inside macrophages. Macrophages were infected with spores of B. subtilis strain GC255 carrying the abrB::gfp fusion and incubated for 6 h. Representative fluorescence microscopy images (taken at ×1000; reproduced at ×850) of infected macrophages before (a) and after (b) treatment with 2.5 μg gentamicin ml⁻¹ to kill extracellular bacteria.
in spore/macrophage co-cultures that had already been treated with D-alanine. L-Alanine induced spore germination inside macrophages and restored intracellular killing, with approximately 60% of the spores being killed within 2 h after the addition of the L-amino acid (Fig. 2). This finding demonstrates that macrophages rapidly kill germinated spores and that an L-alanine-dependent germination pathway is activated inside human macrophages. The slight reduction in the number of intracellular spores observed in the presence of D-alanine (Fig. 2) also suggests that other compounds, different from L-alanine, and/or FBS can act as germinants on spores inside macrophages.

The finding that, at an m.o.i. of 1 macrophage:10 spores, B. subtilis spores may escape intracellular killing only in the presence of D-alanine, given as an exogenous germination inhibitor, again highlights the differences in the intracellular behaviour of B. subtilis and the highly pathogenic B. anthracis. In fact, at the same m.o.i., B. anthracis is internalized to a higher extent, thus allowing the D-alanine-mediated autoinhibition of germination to be active inside macrophages (McKevitt et al., 2007).

Expression of an abrB::gfp fusion was analysed in germinating spores and shown to initiate before cells entered the vegetative growth cycle. Thus, the appearance of fluorescence can be taken as an indication of the initiation of vegetative growth during spore–macrophage infection. In spore/macrophage co-cultures, no fluorescent forms were detectable inside macrophages at each time of incubation, indicating that B. subtilis spores could not initiate vegetative growth inside phagocytes and confirming that, once germinated, spores are extremely susceptible to macrophage killing.

In conclusion, this report provides evidence showing that recombinant B. subtilis spores are phagocytosed by human macrophages and do not persist inside the cells, but they are rapidly killed upon their germination and do not grow inside the infected phagocytes. In addition, B. subtilis spores do not exert any cytotoxic activity on human THP-1 cells, as the viability of infected macrophages is not affected by spore infection. The overall data obtained support the notion that B. subtilis spores are suitable and safe carriers for administering heterologous antigens to humans.

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