HOST RESPONSE TO INFECTION

In-vitro characterisation of the phagocytosis and fate of anthrax spores in macrophages and the effects of anti-PA antibody

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Antibodies (Abs) to the protective antigen (PA) component of the anthrax toxins have anti-spore as well as anti-toxin activities. Anti-PA antisera and purified anti-PA Abs enhance the phagocytosis by murine-derived macrophages (MQs) of spores of the Ames and Sterne strains and retard the germination of extracellular spores in vitro. The fate after phagocytosis of untreated and anti-PA-treated spores was further studied in culture medium that supported phagocytosis without stimulating spore germination (Dulbecco’s minimal essential medium with horse serum 10%). The spores germinated within cells of primary peritoneal murine MQs (C3H/HeN) and MQs of the RAW264.7 MQ-like cell line; germination was associated with a rapid decline in spore viability. Exposure of MQs to inhibitors of phago-endosomal acidification (bafilomycin A and chloroquine) reduced the efficiency of MQ killing and allowed outgrowth and replication of the organisms. Treatment of spores with anti-PA Abs stimulated their phagocytosis and was associated with enhanced MQ killing of the spores. The enhanced killing of spores correlated with the greater extent of germination of anti-PA-treated spores after phagocytosis. A PA null mutant of the Ames strain exhibited none of the effects associated with anti-PA Ab treatment of the parental strain. Thus, the anti-PA Ab-specific immunity induced by vaccines has anti-spore activities and its role in impeding the early stages of infection with Bacillus anthracis needs to be assessed.

Introduction

The effective treatment and prophylaxis of disease caused by Bacillus anthracis require knowledge of the pathogenesis of infection in non-immune and immune hosts. Protection of experimental animals can be induced by vaccination with live vaccines, the purified protective antigen (PA) component of the anthrax toxins, or partially purified preparations containing PA such as Anthrax Vaccine Adsorbed (AVA), the licensed human anthrax vaccine [1–3]. Animal studies have shown that the primary immunogen in AVA is PA and recent studies suggest that the titre of anti-PA antibodies (Abs) in immune animals correlates with protection [4, 5]. Toxin-neutralising antibodies are induced but the exact mechanism of protection against infection afforded by AVA and other PA-based vaccines and the role of the antitoxin immune response are not fully elucidated.

Immune sera from animals vaccinated with a live non-capsulate, toxin-producing strain of B. anthracis have, in addition to toxin-neutralising activity, anti-spore activities. The latter are manifested as immune serum-mediated stimulation of phagocytosis of spores by rabbit peritoneal macrophages (MQs) and the inhibition of spore germination in vitro, in the absence of MQs [6]. It was shown recently that antisera and purified Abs to PA similarly inhibited the germination of spores in vitro and enhanced their phagocytosis by murine peritoneal MQs [7]. The presence of spore-associated proteins recognised by anti-PA Abs was detected by electron microscopy and confirmed by immunoblot analysis of spore coat extracts that revealed antigens staining specifically with anti-PA Abs. In the present work, spores were treated with anti-PA Abs and the rate of phagocytosis and of intracellular germination, and killing by macrophages were examined to determine
whether antitoxin Abs in vaccinated individuals have anti-spore effects that might be protective early in the infection before outgrowth and toxin secretion by the bacilli.

Materials and methods

Bacterial strains and spore preparations

Strains of *B. anthracis* used included the virulent capsulate toxigenic Ames strain and the non-capsulate toxigenic Sterne vaccine strain. Two double-mutant derivatives of Sterne strain 7702 were also provided by M. Mock (Pasteur Institute, Paris, France). Strain RP4 has deletions in the loci encoding PA and oedema factor (EF) and produces only the lethal factor (LF) component of anthrax lethal toxin; RP42 has deletions of both the EF and LF genes and produces only PA [8, 9]. All growth media for strains RP4 and RP42 contained erythromycin 10 mg/L and kanamycin 20 mg/L. A PA null mutant of the Ames strain was created by a directed signature-tagged mutagenesis procedure in which the *pagA* locus encoding PA was interrupted by integration of a vector harbouring an erythromycin-resistance cassette (L. Mendelson, unpublished data). Spores were prepared and purified from broth cultures of the strains, as described previously [7, 10, 11]. The spores were activated by heating at 65°C for 30 min [10] and were evaluated by phase microscopy; they were used only if ≥95% were refractile (ungerminated).

Preparation of macrophages and *in vitro* phagocytosis of spores

MOs used included primary peritoneal cells obtained from C3H/HeN mice (Charles River, Frederick, MD, USA) 4 days after intraperitoneal (i.p.) inoculation of soluble starch 2% as described previously [11] and cell lines RAW264.7 (American Type Culture Collection, ATCC, Rockville, MD, USA) and MH-S (ATCC), originating from transformed murine peritoneal or alveolar MOs, respectively. The cell lines retain MO functions and characteristics such as phagocytosis, bactericidal activity and the presence of Fc receptors. The primary MOs and the MQ cell lines were cultured in Dulbecco’s Minimal Essential Medium (DMEM) with fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA) 10% or horse serum (HS, Gibco BRL) 10%. Seed cultures were subcultured into 24-well plates, with or without coverslips, and incubated at 37°C in air with CO₂ 5% for 1–2 days. The primary peritoneal exudate MOs were cultured for 3–5 days at 37°C in CO₂ 5% on coverslips in 24-well plates, as described previously [11]. The wells, which contained (1–3) × 10⁶ MOs, were washed and the cells were infected with spores of *B. anthracis* – (1–2) × 10⁹ cfu/well, with a usual multiplicity of infection (m.o.i.) of 3–10 – suspended in DMEM with FBS or HS 10%. In some experiments, the spores were opsonised on ice for 30 min by immune serum or IgG, pre-immune serum or IgG, or medium alone. They were then added to the MOs, and incubated at 37°C in CO₂ 5% for 45 min (C3H/HeN) or 1 h (cell lines), except as described in the text, to allow phagocytosis. Unphagocytosed spores were then removed by washing the wells 10 times in Hank’s Balanced Salts Solution (HBSS) containing 10 mM Hepes, pH 7.0, or 20 mM PBS without Ca²⁺ or Mg²⁺. The coverslips were removed from the wells and further washed by rinsing sequentially in three 150-mm disposable sterile beakers (Falcon). Some of the washed coverslips were either stained or used to determine the number of viable bacteria as described below (t₀ samples), and others were re-incubated at 37°C in CO₂ 5% after adding fresh medium.

Processing and staining of spore-infected macrophages

Coverslips to be evaluated for phagocytosis and germination of spores by light microscopy were fixed in methanol and then stained twice, first with malachite green spore stain and then with Wright-Giemsa stain [11]. Phagocytosis was measured by direct microscopic counts of the double-stained samples, and the data were expressed as the phagocytic index (PI), where PI = the mean number of spores/infected MQ × 100. The number of spores present in ≥10 fields, and in a total of at least 100 MQs (to include both infected and uninfected MQs), were counted on each of the duplicate coverslips per sample [7, 11]. In some experiments, germinated and ungerminated spores were distinguished by fixing replicate coverslips overnight in formaldehyde 4% in 20 mM PBS (Ca²⁺/Mg²⁺-free). Fixed cells were then washed, incubated for 15 min with Triton X-100 0.1% in PBS and then incubated for 15 min with PBS containing 5 μg Sytox Green™ (Molecular Probes, Eugene, OR, USA). The dye stains DNA of permeabilised MQs as well as that of germinated spores or bacilli. All the fluorescent spores and MQs in a field were counted under epifluorescence microscopy. The dye does not stain ungerminated spores, which were detected by phase contrast microscopy.

Assays for spore and macrophage viability

The viability of phagocytosed spores was determined. Infected and washed MOs were lysed by adding 0.5 ml of a solution of bovine serum albumin (BSA) 0.01% in distilled water. The samples were incubated for 5 min; the coverslips were scraped with a sterile plastic transfer pipette; and the lysates were diluted and spread on trypticase soy agar plates. In some experiments, samples of the lysates were heated at 65°C for 30 min to kill germinated spores and bacilli before dilution and plating. MQ viability was assessed by trypan blue dye uptake and by differential nuclear staining with Syto-13™ and propidium iodide (Molecular Probes), as described previously [12]. It was also
assessed by direct microscopic observation of the macrophage morphology and changes in total counts of adherent cells during the experiments as described previously [13]. The effects on spore and MQ viability of culture in the presence of inhibitors of phagolysosome function were also determined. The inhibitors tested (all from Sigma-Aldrich, St Louis, MO, USA) included bafilomycin A1 (0.05–2 μM), chloroquine (100 μM) and cytochalasin B or D (CB or CD, 2 mg/L). They were present in the medium during both the initial period of phagocytosis and the subsequent incubation of the washed MQs.

*Sera, toxin and antibody preparations*

Rabbit antisera, designated anti-rPA antiserum, were from animals hyperimmune to PA purified from ΔSterne-1 (pPA102) CR4, a pX01-cured, non-spore-forming derivative of the Sterne strain that carries the PA gene cloned into the plasmid vector pUB110 [14,15]. Affinity-purified rabbit anti-PA IgG was obtained by chromatography of rPA antiserum over a PA antigen column followed by a protein A column. IgG from sera collected from non-immune normal rabbits (NRS) was also prepared by protein A column chromatography. These reagents were described previously [7]. Polyclonal rabbit anti-spore antiserum, provided by Covance Inc. (PA, USA), were prepared from rabbits inoculated two to three times with germinated or ungerminated spores killed by gamma-irradiation and mixed with Ribi Trimix adjuvant (Ribi Immunocchem Research, Hamilton, MT, USA) (P. Fellows and B. Ivins, USAMRIID). Purified recombinant PA was described previously [7].

**Statistical analysis**

The data were analysed by standard statistical methods (mean, SEM, analysis of variance and unpaired Student’s t test). In comparing groups, a p value <0.05 was considered to indicate a significant difference.

**Results**

*Influence on spore germination of the serum used in the macrophage culture medium*

To characterise the phagocytosis and fate of spores within MQs, a cell culture medium that supported MQ growth but did not significantly stimulate the germination and outgrowth of spores before their phagocytosis was needed. Although spores did not germinate in the unsupplemented culture medium (DMEM) used in the phagocytosis experiments, phagocytosis by RAW264.7 cells was poor in medium without serum (data not shown). Adding serum to the medium improved phagocytosis; however, as observed by others (J. Ireland, personal communication), the sera from some species promoted germination before phagocytosis. For example, when incubated for 1 h in medium with FBS 10%, spores of both the Ames and Sterne strains of *B. anthracis* germinated; Sterne to a greater extent than Ames (95.8 SEM 0.05% versus 44.0 SEM 3.6%); but they germinated much more slowly in medium with HS (8.6 SEM 0.52% versus 2.0 SEM 0.80%). When incubated with RAW264.7 MQs for 1 h in DMEM with HS10 (DH10), c. 20% of the spores of both the Sterne and Ames strains had germinated. The decreased spore germination in DH10 did not appear to be secondary to an effect on viability of the organisms. Spores incubated in DH10 for 24 h were able to germinate, albeit slowly, and multiply, increasing two-fold over 24 h (data not shown). Therefore, DH10 was selected for use in the spore phagocytosis assays unless otherwise indicated.

Phagocytosis of *B. anthracis* strain Ames spores and germination within murine macrophages

RAW264.7 and MS-H cells and primary peritoneal MQs from mice were exposed to preparations of heat-activated, ungerminated spores (at m.o.i. values of 3–20 cfu/MQ) and incubated to allow phagocytosis for various periods up to 1 h. As shown in Fig. 1 for RAW264.7 MQs, significant numbers of phagocytosed spores began to germinate after the 1-h incubation period. In Fig. 1a, ungerminated phagocytosed spores were stained with malachite green whereas germinated organisms appeared blue (Wright-Giemsa stain). Germinated organisms could also be visualised by the fluorescent stain, Sytox Green; ungerminated spores were unstained but could be visualised by phase-contrast microscopy or could be stained with rabbit anti-spore antiserum and red fluorescent dye-conjugated anti-rabbit IgG (data not shown). The number of phagocytosed spores, detected microscopically and by plate counts of MQ lysates, increased in samples collected after incubation periods up to 1 h, at which time both parameters reached their maximal value (data not shown).

*Viability of *B. anthracis* strain Ames after phagocytosis*

After the initial 1-h period to allow maximal phagocytosis, the number of intracellular organisms decreased over 24 h, both as detected microscopically in stained preparations of infected RAW264.7 MQs and by quantitative viable counts of MQ lysates (Fig. 1 and Table 1). A rapid decline in the viability of Ames spores from t0 (at the end of the 1-h phagocytosis period) to 4 h post-phagocytosis was observed in MQs cultured in both DF10 and DH10 (Fig. 2 and Table 1). Gentamicin (2.5–5 mg/L) was added after removal of unphagocytosed spores at t0 only in experiments with DF10. The kinetics of killing did not correlate with differences in the m.o.i. of 3–100 cfu/MQ or with the total number of MQ/well (data not shown). Similar results were obtained with either RAW264.7 cells or primary peritoneal MQs elicited from C3H/HeN mice.
In some experiments, lysates were divided into aliquots that were heated or kept on ice for 30 min before plating. For both heated and unheated aliquots, most of the MQ-associated decline in spore viability (3–170-fold) again occurred between t₀ and 4 h and was observed in cultures grown in DF10 or DH10 (Fig. 2 and data not shown). The decreased cfu/MQ in the heated compared with the unheated aliquots of the samples plated at t₀ revealed that significant germination occurred during the period of MQ uptake of the spores. The decline in spore viability and in the total number of organisms from t₀ to 4 h appeared to be due to MQ killing of spores and not to a decrease in MQ numbers. Trypan blue and fluorescent dye staining of the monolayers indicated that nearly all of the cells remained viable for the duration of the experiment; 8% of the MQs infected with Ames spores and incubated for 24 h were positive for nuclear staining by propidium iodide (which crosses the nuclear membrane of non-viable cells). Also, the total number of MQs/well often increased by 2–3-fold over the 24-h course of the experiment. A decline in the number of phagocytosed spores after extended incubation was also observed microscopically. This apparent decrease appeared to be due to poor staining of killed, partially digested spores (Fig. 1c). By 24 h post-phagocytosis, few spores could be detected intracellularly and the infected MQs often appeared to be returning to a more quiescent, pre-exposure-like state (data not shown). Residual viable bacteria present in the 4-h and 24-h samples appeared to be mostly viable.

Table 1. Phagocytosis and germination of spores of *B. anthracis* strain Ames in RAW264.7 macrophages

<table>
<thead>
<tr>
<th>Phagocytosis medium*</th>
<th>Time (h)</th>
<th>Mean (SEM) number of spores/MQ</th>
<th>Mean (SEM) percentage of spores germinated</th>
</tr>
</thead>
<tbody>
<tr>
<td>DF10</td>
<td>t₀</td>
<td>1.20 (0.3)</td>
<td>80.6 (7.0)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.34 (0.09)</td>
<td>83.0 (6.6)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.13 (0.05)</td>
<td>82.4 (9.6)</td>
</tr>
<tr>
<td>DH10</td>
<td>t₀</td>
<td>7.60 (1.4)</td>
<td>14.8 (2.6)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5.40 (0.7)</td>
<td>27.4 (1.1)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1.96 (0.7)</td>
<td>12.7 (4.1)</td>
</tr>
</tbody>
</table>

*DMEM with glucose, glutamine, 25 mM Hepes and FBS 10% (DF10) or DH10.

†Means (SEM) of triplicate values from six experiments. The values were derived by counting all phagocytosed organisms and macrophages in at least 10 fields and include ungerminated spores (malachite green-positive) and germinated *B. anthracis*. Killed organisms were not stainable and were not detected microscopically.

§A higher percentage of spores had germinated and were stainable at 4 h than the corresponding values at t₀ and 24 h (p = 0.026).

Significantly fewer spores/MQ detected in comparison with the corresponding values at t₀ and 24 h (p = 0.004).
unphagocytosed spores that had not been removed from the cultures by washing and that germinated upon plating (discussed below).

Spores of the Sterne strain were phagocytosed and germinated intracellularly to an extent similar to that of Ames spores. As observed with the Ames strain, there was no evidence microscopically or by viable count for intracellular replication of germinated Sterne spores. However, Sterne spores were much more adherent to the MQs than Ames strain spores and were more difficult to remove by washing. The addition of CD to the medium to inhibit phagocytosis had little effect (<2-fold) on the number of Sterne spores MQ as detected both microscopically and by viable count of RAW 264.7 cell lysates in comparison to the number of spores MQ in untreated cultures. These small differences were obtained in samples processed at both t₀ (the end of the 1-h phagocytosis period) and after incubation for a further 4 h. In contrast, CD-treated MQs infected with Ames spores exhibited a 15-fold decrease in the number of phagocytosed spores compared with the number in untreated MQs. As observed in bafilomycin A1-treated cultures, MQs

Effect of exposure of macrophages to inhibitors of endosomal function on the efficiency of macrophage killing

To begin identifying activities of MQs potentially involved in impeding spore outgrowth, the effects of inhibitors of endosomal acidification on the uptake and viability of Ames spores were studied. Compounds tested included bafilomycin A1 and the lysosomotropic amine chloroquine [16–20]. MQs treated with bafilomycin A1 exhibited larger numbers of germinated spores microscopically after the 1-h phagocytosis period than untreated cultures and the treated MQs permitted the outgrowth and replication of the organisms (Figs 1b and 3a). The lower PI of the untreated MQs compared with the bafilomycin A1-treated ones at t₀, and the decline from t₀ to 4 h in the number of viable organisms recovered from lysates of the untreated, but not bafilomycin A1-treated, MQs (Figs. 1c, 1d and 3b) suggests that killing of B. anthracis by untreated MQs began during or shortly after the uptake period and continued during the subsequent incubation period. Control experiments verified that the addition of bafilomycin A1 (or chloroquine – see below) alone to spores in DH10 medium did not cause spores to germinate.

As observed in bafilomycin A1-treated cultures, MQs
The number of viable cfu spores in lysates from treated and untreated MQs were determined. The number of viable cfu/well in the bafilomycin-treated samples was greater than the corresponding values at t₀ and at 4 h of the untreated samples (p < 0.01, for both). The viable counts of phagocytosed spores in lysates from treated and untreated MQs were determined. The number of viable cfu/well in the bafilomycin-treated samples was greater than the corresponding values at t₀ of the CB-treated MQs (p = 0.002) and at 4 h of the untreated (p = 0.031) and CB-treated (p = 0.018) MQs; the bar symbols are the same as described in (a). The data are the means and SEM of triplicate samples from each group and are from one experiment representing four.

![Graph](image)

**Fig. 3.** The effects of bafilomycin A1 and CB or CD treatment on the viability of phagocytosed Ames spores. Bafilomycin A1 (2 μM) was added to the medium of treated RAW264.7 MQs during both the phagocytosis and the 4-h incubation periods. (a) The PI values of the bafilomycin-treated samples (■) were greater than the corresponding values at t₀ and at 4 h of the untreated samples (■), p = 0.01, for both, and the CB-treated (□), p < 0.01 for both. (b) The viable counts of phagocytosed spores in lysates from treated and untreated MQs were determined. The number of viable cfu/well for the bafilomycin-treated samples was greater than the corresponding values at t₀ of the CB-treated MQs (p = 0.002) and at 4 h of the untreated (p = 0.031) and CB-treated (p = 0.018) MQs; the bar symbols are the same as described in (a). The data are the means and SEM of triplicate samples from each group and are from one experiment representing four.

treated with chloroquine (100 μM) exhibited larger numbers of germinated spores microscopically after the phagocytosis period than untreated cultures (data not shown). However, in contrast to the effects of bafilomycin A1, chloroquine-treated samples exhibited a decline of almost 10-fold in viable counts of *B. anthracis* from MQ lysates between the t₀ and 4-h samples compared with a 2–3-fold decrease in the untreated samples. The decline in lysate viable counts in the chloroquine-treated MQs was associated with the presence of nine times more organisms in the culture medium (3.8 × 10⁵ cfu) than in MQ lysates (4.2 × 10⁵ cfu). The inclusion of gentamicin 2.5 mg/L in the cultures greatly reduced the concentration of *B. anthracis* recovered from the medium in samples collected after incubation for 4 h; i.e., the antibiotic caused a 273-fold reduction in viable counts from the medium of chloroquine-treated MQs, in comparison with chloroquine-treated MQs not incubated with gentamicin (data not shown). Thus, as observed with bafilomycin A1, chloroquine treatment reduced the killing of spores by MQs. Adding inhibitors of phagocytosis CB or CD (2 mg/L) to the MQ cultures at the time of infection resulted in a 10-fold reduction in lysate and medium viable counts compared with the counts from untreated cultures. Despite efforts to remove unphagocytosed spores after the uptake period, some remained as suggested by the recovery of viable organisms from CB-treated cultures (Fig. 3 and data not shown).

**Stimulation of phagocytosis and enhancement of MQ killing of Ames spores by anti-PA Abs**

Pretreatment of spores with anti-PA Abs has been shown to enhance their phagocytosis by murine primary peritoneal MQs [7]. In this study, pretreating spores with anti-rPA Abs was often associated with an increased rate of phagocytosis by the RAW264.7 cell line as detected microscopically by counting stained cells at the end of the 1-h phagocytosis period (Fig. 4a); these data were obtained by using cultures stained with the malachite green spore stain and Wright-Giemsa. The anti-rPA Ab-treated spores also germinated more readily and to a greater extent by the end of the phagocytosis period (Fig. 4a, inset) than did spores pretreated with NRS or medium alone. Similar results were obtained when either the whole anti-rPA antiserum or the affinity-purified anti-rPA IgG (and NRS IgG) were used.

Pretreatment of spores with anti-rPA Abs resulted in enhanced killing by MQs. As shown in Fig. 4b, at the end of the 1-h phagocytosis period, there were significantly fewer cfu/MQ in the anti-rPA antiserum-treated samples than in the untreated samples (p = 0.015); in contrast, the concentration of organisms in lysates of the NRS-treated cells was not significantly less than in the untreated lysates. This enhanced killing of the anti-rPA-treated spores occurred despite the increased number of organisms phagocytosed after anti-rPA IgG treatment (p = 0.04) compared with the number taken up by MQs exposed to untreated spores (Fig. 4a and data not shown) [7]. Reduced concentrations of viable organisms were cultured from both the anti-rPA IgG- and NRS IgG-treated samples after incubation for 4 h. However, whereas the viable counts present at 4 h in NRS-treated cultures declined by three-fold versus the counts at t₀, the comparable reduction from t₀ to 4 h in the anti-rPA-treated cultures...
was 38-fold. The cfu/MQ recovered from anti-rPA-treated MQs at 4 h was significantly less than that from NRS-treated cells (p = 0.006). Residual adherent, unphagocytosed spores appeared to account for most of the viable counts obtained at 4 h in these cultures; the latter was suggested by the recovery of spores from washed MQs that had been incubated in medium with CB (Fig. 3b). It should be noted that the enhanced rate of killing of the anti-rPA IgG-treated spores (Fig. 4b) led to the loss of stainability and, hence, to the underestimation of the number of phagocytosed spores (Fig. 4a). The difference in phagocytosis by MQs of anti-rPA antiserum- and NRS-pretreated spores (Fig. 4a) did not reach statistical significance (p = 0.06), a finding in agreement with this observation. Anti-rPA antibodies enhanced the uptake and killing of spores by MQs regardless of whether the cultures were incubated in DH10 (without antibiotics) or DF10 (with gentami-
Two approaches were used to confirm a specific role and intracellular fate of B. anthracis spores

Role of anti-PA antibodies in the phagocytosis of spores

To verify that the increased extent of germination of anti-PA Ab-treated spores was associated with enhanced germination intracellularly and not with an increase in germination of extracellular spores during the phagocytosis period, organisms present in MQ culture supernates after phagocytosis were stained by fluorescent stain-labelled anti-spore antiseraum and with Sytox Green, which stains only germinated spores. The extent of germination of organisms remaining in the medium at the end of the phagocytosis period was not influenced by pre-incubation in the presence of anti-PA Abs; 12.6% of the organisms present in culture medium supplemented with DH10 alone had germinated. Comparable levels of germinated spores were detected in samples from medium supplemented with anti-PA IgG or NRS IgG (17.5% and 16.2%, respectively).

To compare the opsonising activity for spores of anti-PA Ab to that of other anti-spore opsonins, and to examine the specificity of the stimulatory effect of anti-PA Ab on germination, the phagocytosis of Ames spores pretreated with polyclonal rabbit antisera to either germinated or ungerminated spores was characterised. The kinetics and extent of phagocytosis of the anti-spore and anti-PA antiserum-treated spores were not significantly different. All three preparations stimulated significantly more phagocytosis than pre-immune serum-treated or untreated spores after incubation for 15 and 30 min, although not after incubation for 1 h (data not shown). Thus, the anti-PA antiserum opsonised the spores as well as did whole anti-spore antiserum. However, there were significant differences between the samples in the extent of germination of intracellular spores; after incubation for 30 min, only 30.1% SEM 4.2% of the spores pretreated with the antispore antiserum had germinated, compared with 70.4% SEM 3.3% of the anti-PA treated spores (p = 10^-6).

Role of anti-PA antibodies in the phagocytosis and intracellular fate of B. anthracis spores

Two approaches were used to confirm a specific role for anti-PA Abs in the augmented phagocytosis, germination and killing of B. anthracis spores. In the first approach, mutants derived from the Ames and Sterne parent strains with inactivated PA genes were tested in the MQ assays. They were unaffected by pretreatment with anti-PA Abs in their uptake, germination and killing by MQs. Although treatment with anti-PA Abs enhanced the phagocytosis of Ames spores, it had no effect on the uptake of strain IM78, a PA null mutant of the Ames strain (Fig. 5). Moreover, there were no significant differences in the number of cfu recovered at either t0 and 4 h after phagocytosis from MQs infected with IM78 spores treated with anti-PA IgG, NRS IgG, or medium. In contrast, anti-PA Ab-treated parental Ames spores were recovered from MQs in significantly lower numbers than NRS-treated spores (three-fold less) after incubation for 4 h (data not shown). Similar results were observed with Sterne mutant RF4 (LF^+, PA^-, EF^+) in contrast to mutant RP42 (PA^+, LF^-, EF^-). Treating Sterne RP42 with anti-PA Abs was associated with the increased phagocytosis and killing of spores compared with NRS treatment, whereas no differences were associated with treatment of the RP4 mutant.

In the second approach, the effect of adding excess rPA on the phagocytosis of anti-rPA-treated spores by RAW264.7 cells was examined. The addition of rPA 10 mg/L to suspensions of spores and anti-rPA Abs was associated with a nearly two-fold decrease in the number of phagocytosed spores at the end of the 1-h phagocytosis period (0.58 versus 1.06 spores/MQ in the cultures with or without added PA, respectively; p = 0.04). No effects attributed to pretreatment with rPA were observed in the uptake of NRS IgG-treated spores.

Discussion

The pathogenesis of inhalational anthrax has not been completely characterised. However, several steps in the process have been delineated or hypothesised based on observations in animals and in vitro model systems [21–28]. Upon inhalation of a lethal dose of B. anthracis spores by a susceptible host, such as a guinea-pig, the spores appear to be taken up by cells lining the alveoli and by free phagocytes [26,28]. Some of the phagocytosed spores appear to be cleared locally, whereas others are transported within MQs to the regional lymphatics where they germinate and develop into toxin-producing bacilli [21,26,28,29].

As reported recently in experiments with attenuated Sterne-like pX01^+, pX02^- strains [22–25], spores of the fully virulent pX01^+, pX02^+ B. anthracis Ames strain were readily phagocytosed by and germinated within the RAW 264.7 MQ-derived cell line (Fig. 1, Table 1, data not shown). Furthermore, phagocytosis was followed by a decline in the numbers of viable organisms that could be recovered from the MQs. This
decline was observed in MQs infected with spores and incubated in either antibiotic-free DH10 or DF10 with gentamicin. The data indicated that the apparent decline in the total number of viable spores observed by the end of the phagocytosis period as well as at later time points was due to killing by MQs (Figs 1 and 2, Table 1); it was not due to a decrease in the viability or number of MQs. As suggested by the recovery of c. (1 – 2) × 10^3 cfu of viable organisms from the wells of cytochalasin-treated cultures after extensive washing (Fig. 3), bacteria present in the 4-h and 24-h samples appeared to a large extent to be unphagocytosed spores that had not been removed from the washed cultures, and had germinated after plating. These results confirmed those reported previously in studies with spores of the pX01^þ pX02^− Sterne strain and primary peritoneal MQs [11]. They are also consistent with an in-vivo study by Guidi-Rontani et al. [24] who reported a decline in spore counts recovered from alveolar MQs present in bronchial alveolar lavage fluids collected from mice 3 h and 24 h after infection with B. anthracis. The results of the present study are also consistent with older studies showing a decrease in spores detectable in the lungs early after exposure without an increase in spores in local lymph nodes [21, 28]. They are in contrast to those reported by Hanna and co-workers who observed that germination of B. anthracis strain Sterne within RAW264.7 cells was accompanied by outgrowth and replication of the germinated spores, lysis of the infected MQ, and release of the vegetative bacilli from the MQs [22, 23, 25]. Guidi-Rontani and co-workers reported that Sterne strain 7702 germinated but failed to multiply within MQ; however, the organisms survived and appeared to be cytotoxic for MQs [12]. The differences reported in the fate of B. anthracis phagocytosed by RAW264.7 cells and in the effects of the spore infection on MQs in vitro remain unexplained. They might be due to differences in the MQs, the strain of B. anthracis used (e.g., virulent or attenuated), the m.o.i. or other parameters of in-vitro phagocytosis, or the growth conditions used to prepare the spores. Interestingly, microscopic observations on the fate of spores in infected guinea-pigs suggest that both situations – the killing of spores by MQs and the outgrowth and lysis of MQs by the invading organism – are present in vivo. In a guinea-pig model, Ross [28] showed that inhaled spores were phagocytosed by alveolar cells. Some of the phagocytosed spores appeared to be digested and killed, whereas others were transported within MQs to the regional lymphatics where they germinated and outgrew into vegetative bacilli. The data thus suggest that there is an apparent balance between the clearance of infection by MQs in the host and intracellular survival and release of the germinated organisms from lysed macrophages. The

Fig. 5. The effect of anti-rPA Abs on the phagocytosis of a PA null mutant of the Ames strain. The parental Ames strain and a PA-negative mutant derivative, IM78, were treated with anti-rPA IgG or NRS IgG. ■ anti-PA-treated IM78; □ anti-PA-treated Ames; □ NRS-treated IM78 spores; and □ NRS-treated Ames. RAW 264.7 MQs were infected with the spores and samples were collected as described in Figs. 1 and 4. The PI values of the anti-PA-treated parental strain at 15 min and 30 min were greater than those of the corresponding NRS-treated spores (p = 0.05 and 0.005, respectively) and were greater than the PIs of the anti-PA-treated IM78 spores (p = 0.043 and 0.025). The extent of phagocytosis of anti-PA-treated IM78 spores did not differ from that of the NRS-treated IM78 spores for all three time points.
sequence of events occurring after an individual exposure probably depends on the resistance of the host, the route, virulence and size of the spore challenge and the source of the responding MQs. Attempts are being made to characterise the fate of the spore within MQs obtained from both naive and immune hosts.

Phagocytosed spores were observed within phagosomes, structures that, upon fusion with lysosomes, possess an acidic environment containing hydrolytic enzymes and other antimicrobial substances. To investigate a possible role of this environment in the sporiclidal activity of MQs, the effect of inhibitors of phagolysosomal acidification on viability of the phagocytosed spore was studied. Bafilomycins are specific inhibitors of the vacuolar ATPase proton pump, which is responsible for the acidic pH of endosomes and lysosomes [20]; the lysosomotropic amine chloroquine also dissipates intracellular proton gradients and raises the pH of intracellular vesicles [16]. MQs treated with either bafilomycin A1 or chloroquine were compromised in their sporiclidal activity and the phagocytosed spores were able to germinate, outgrow and escape from the MQ (Fig. 3 and data not shown). The data suggest that the loss in spore viability after phagocytosis may be associated with the antimicrobial environment of the phagosome; further studies are needed to define the sporiclidal attributes of this compartment.

Antispore activities have recently been attributed to antitoxin Abs [6, 7]. As shown in Fig. 4 and previously [6, 7], pretreatment of spores with antitoxin Abs enhances spore phagocytosis. With culture medium (DH10) that supported phagocytosis without stimulating spore germination or requiring the inclusion of an antibiotic, this finding was extended by showing that anti-PA Abs also enhanced the rate of germination of phagocytosed spores (Fig. 4a, inset) and the more rapid germination of anti-PA Ab-treated spores was associated with the increased sporiclidal activity of RAW264.7 MQs (Fig. 4b).

Three approaches were used to further demonstrate that anti-PA antibody had specific modulating effects on the pH of intracellular vesicles [16]. MQs treated with either bafilomycin A1 or chloroquine were compromised in their sporiclidal activity and the phagocytosed spores were able to germinate, outgrow and escape from the MQ (Fig. 3 and data not shown). The data suggest that the loss in spore viability after phagocytosis may be associated with the antimicrobial environment of the phagosome; further studies are needed to define the sporiclidal attributes of this compartment.

In conclusion, the observations suggest that treating spores of the Ames strain with anti-PA Abs is associated with enhanced phagocytosis and an increased subsequent rate of germination. We hypothesise that the germinated Ames spores are more readily killed than ungerminated ones, possibly due to a greater susceptibility of the former to MQ phagolysosomal antimicrobial activities. Further studies are being done to investigate this hypothesis.

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References
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