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In reply

We read with interest the comments made
 by Goossens *et al.* (2007) concerning our
 recent publication (Enkhtuya *et al.*, 2006).
 In our paper we demonstrated the
 specificity of rabbit anti-*Bacillus anthracis*
 (pXO1[−] and pXO2[−]) Pasteur II spore IgG
 and its protective effect in mice against
 lethal challenge by pXO1⁺ and pXO2⁺ *B.
 anthracis* spores. The following are our
 comments addressing their concerns.

1. We are well aware that *Bacillus cereus* and
Bacillus thuringiensis are closely related to *B.
 anthracis*, as they are classified into one
 group based on genomic analysis; thus, we
 examined the cross-reactivity of our rabbit
 anti-anthrax spore IgG to these three
 species plus *Bacillus subtilis* by indirect
 immunofluorescence staining. Fluorescence
 images were all taken with the same exposure
 time and conditions in order to compare the
 relative fluorescence intensity. As clearly
 stated in the text, we obtained remarkably
 strong fluorescence from *B. anthracis* spores,
 while in contrast there was no detectable
 fluorescence from images of other tested
Bacillus spores, including *B. cereus*, *B. thuringiensis*
 and *B. subtilis*, in addition to a negative control.
 Due to space constraints, these data were
 not shown in the original paper (Enkhtuya
et al., 2006). However, we are happy to
 include these findings here (Fig. 1). We are
 certain that anti-spore IgG obtained by
 immunization using formalin-fixed spores
 of plasmidless Pasteur II strain reacts
 specifically with *B. anthracis* spores.

Frankly, the results were unexpected to us at
 first, because we had thought considerable
 levels of cross-reactivity would be observed
 from *B. cereus* and *B. thuringiensis* spores,
 as has been reported by others (Phillips *et al.*,

1983; Stopa, 2000). The works cited by the
 authors as evidence against our work
 demonstrated cross-reactivity of
 fluorescein-conjugated anti-*B. anthracis*
 spore IgG among *B. anthracis*, *B. cereus* or
B. thuringiensis by immunostaining (Phillips
et al., 1983; Stopa, 2000). However, there
 are several differences between the methods
 used. For example, we used formalin-fixed
 spores of *B. anthracis* Pasteur II lacking
 pXO1 and pXO2 as antigens to immunize
 rabbits, whereas Phillips *et al.* immunized
 rabbits, with formalin-fixed spores of *B.
 anthracis* Vollum strain (Phillips *et al.*,
 1983), while mixtures of irradiated anthrax
 spores (Ames, Vollum and Sterne) were
 used in Stopa's work for immunization of
 goats (Stopa, 2000). Furthermore in order
 to determine fluorescence intensity, a
 quantitative immunofluorescence assay
 based on fibre optic microscopy was used in
 the work by Phillips *et al.* (1983), a flow
 cytometer was employed by Stopa (2000),
 while we used fluorescence microscopy to
 determine not only the intensity but also the
 location of fluorescence. Thus, the strain of
 spores, purity of immunogens and methods
 used in each study were different, and
 subsequently it is not surprising that the
 reactivity of antibodies is different in each
 study. In fact, as stated in their paper
 (Phillips *et al.*, 1983), rabbit anti-Vollum
 IgG reacted well with both vegetative cells
 and spores of *B. anthracis*. However, our
 purified anti-spore IgG did react with
 endospores without cross-reacting with the
 vegetative form of *B. anthracis* as shown in
 Fig. 1 of our paper (Enkhtuya *et al.*, 2006).
 Thus, the nature of the spore samples is
 likely to affect the reactivity of antibodies
 obtained. Previous studies have shown that
 high variation in fluorescence intensity can
 be obtained from spores of different *Bacillus*
 strains; although the antibody reacts with
 the three strains of *B. anthracis* (Ames,
 Vollum and Sterne), there are significant
 differences in the reactivity of antibody
 among them (Stopa, 2000). Similar findings
 have reported that various degrees of cross-
 reactivity are detected from various strains
 of *B. cereus* (Phillips *et al.*, 1983). These
 findings suggest that molecular profiles
 expressed on the spore surface are variable,
 even among the same species. We have no
 doubt about the accuracy of the results
 published by other authors, but we are also
 confident about the results and conclusions
 demonstrated in our work.

Fig. 1. Immunostaining of *Bacillus* spp. spores with the affinity-purified rabbit anti-*B. anthracis* (pXO1⁻ and pXO2⁻) spore IgG. The *B. anthracis* (pXO1⁺ and pXO2⁺), *B. cereus* and *B. thuringiensis* spores were inactivated and immobilized by overnight fixation with 1 % paraformaldehyde for immunostaining. The images were taken with the same exposure time, were visualized using Olympus DP70-BSW software. The merged images are enlarged. Bar, 5 μ m for the light and fluorescence microscopic images and 2 μ m for the enlarged image.

2. (i) As clearly stated in our paper (Methods), the strain used in our study for anthrax challenge was Pasteur II strain carrying two virulent plasmids pXO1 and pXO2, which is atypical Pasteur II and produces toxins and capsule. The definition of 'fully virulent' is a contentious issue; however, one widely used definition involves *B. anthracis* being toxigenic and encapsulated (Koehler, 2000). Both plasmids are required for full pathogenicity, and this criterion is fulfilled by the strain used in our study. Indeed, all mice died as a result of infection with the Pasteur II containing pXO1 and pXO2 (5×10^3 spores per mouse intraperitoneally). Pasteur II is also recommended by the OIE (Office International des Epizooties, 2004) as a challenge strain to evaluate the efficacy and immunogenicity of Sterne 34F2 vaccines. Furthermore the 'Carbosap' strain mentioned by the authors (Goosens *et al.*, 2007) as used as an attenuated vaccine strain in Italy is different from Pasteur II 74.12 strain as characterized by using a ninth

genotype marker called pXO2-A (Muscillo *et al.*, 2005). Carbosap vaccine strain also harbours pXO1 and pXO2, and is shown to be pathogenic in mice and guinea pigs (Fasanella *et al.*, 2001). Pasteur-type vaccines, the first anthrax vaccine, varied greatly in their degree of attenuation. Some cultures were sufficiently virulent to kill the vaccinated animals, while others were too attenuated to give immunity (Ivins *et al.*, 1986). Thus, it is no longer used as vaccine strain because of the heterogeneity. There is likely to be diversity within clones derived from original Pasteur strain.

(ii) The authors questioned the *in vivo* relevance of our protective humoral immune response experiments. We are working on active immunization experiments that support our published data, and hope to publish the data in the near future. We think anti-spore IgG performs its function at a very early stage of *B. anthracis* infection, as anthrax spores germinate rapidly into vegetative cells, and therefore pre-incubation

of spores with IgG was performed based on this premise. Consistent with this, reduced protection was observed when anti-spore IgG was administered 30–40 min after spore inoculation, while no protection was observed by 24 h post-infection treatment (Fig. 6 in our paper, Enkhtuya *et al.*, 2006). Thus, as stated in our paper, other virulence factors, such as protective antigen, are likely to be required in order to achieve full protection against the complex virulence of *B. anthracis*.

We acknowledge that intraperitoneal challenge of anthrax spores is not the natural route of infection, and that intranasal or intratracheal inoculation may better reflect human inhalation of anthrax. However, the infection model used in our study (intraperitoneal route of infection) has been widely employed by many investigators to study the pathogenesis of anthrax (Beedham *et al.*, 2001; Perkins *et al.*, 2005; Popov *et al.*, 2004; Cote *et al.*, 2006; Joyce *et al.*, 2006). It is widely accepted that macrophages play a critical role in host–pathogen interactions of anthrax, irrespective of the mode of spore delivery. We agree with the opinion mentioned by Povov *et al.* (2004) that spores inoculated into the peritoneal cavity are transported to lymph nodes by resident macrophages by (or through) a mechanism similar to inhalation anthrax. Therefore, intraperitoneal delivery of *B. anthracis* spores appears to be an acceptable method for studying the pathogenicity of anthrax. No less important, due to consideration for the accuracy of spore delivery, safety concerns posed by the creation of spore-containing aerosols, and the requirement for expensive facilities and equipment, we chose intraperitoneal challenge for this study. Each mode of delivery has advantages and disadvantages; however, we are eager to examine various routes of infection in future studies.

3. (i, ii) As mentioned in a published report (Welkos *et al.*, 2004), the classically used spore germination method of monitoring the decline of optical density is insensitive and inconsistent. Macrophages are required for *B. anthracis* spore germination and therefore we chose to examine the inhibitory effect of anti-spore IgG on germination in an *in vitro* macrophage system. We feel this system reflects the physiological requirements of spore

germination. In our paper (Methods) it is clearly stated how the experiments were performed (in triplicate), including the total number of spores added. We do, however, apologize for not including numbers of total interacting spores, which were very similar between all samples. To calculate the number of total interacting spores, we enumerated c.f.u. of supernatants and washing fractions after 30 min incubation with spores (as non-interacting spores), and subtracted these values from the number added. Then, in order to differentiate from germinated spores, replicated vegetative cells and ungerminated spores, lysates were heat-treated and c.f.u. numbers were determined. Again we think the results adequately support our conclusions.

I hope we have addressed all your concerns surrounding our study and would like to point out that our study holds the promise of potentially useful antibodies. Certainly the editor and qualified reviewers of our manuscript thought the same and we hope that readers take into consideration that studies based on this antibody and plasmidless spores remain ongoing.

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