Of spore opsonization and passive protection against anthrax

Passive protection mediated by antibodies directed against *Bacillus anthracis* inactivated spores has been suggested by Enkhtuya *et al.* (2006) in a recent paper in this journal. In our view, the main conclusions drawn in this article are overstatements in regard to the data presented and to the currently published studies, and thus, are misleading.

1. The authors report as 'data not shown' that rabbit IgG directed against anti-formaldehyde inactivated (FIS) *B. anthracis* spores do not cross-react with spores of *Bacillus cereus* and *Bacillus thuringiensis*. This is surprising as spore surface components of *B. anthracis*, *B. cereus* and *B. thuringiensis* share common epitopes. In the course of studies on spore surface structures, we and others have repeatedly encountered difficulties in obtaining *B. anthracis* spore-specific antibodies; a high level of cross-reactivity is consistently observed with most strains of *B. cereus* (for example Philips *et al.*, 1983; Stopa, 2000; Sylvestre, 2003). This point is well known to those working on specific detection of spores of *B. anthracis* relative to *B. cereus* and *B. thuringiensis*. Indeed this is why the design of a means for rapid and specific detection of *B. anthracis* spores is still one of the aims of current bioterrorism countermeasure programmes.

2. The experimental conditions reported in the study for observing passive protection against *B. anthracis* infection with rabbit anti-FIS IgG have two biases. (i) The *B. anthracis* Pasteur II strain, described in 1985 as 'attenuated' by Uchida *et al.* (1985), is not 'fully virulent' as stated in the article by Enkhtuya *et al.* (2006). Moreover it has been used until recently as a live veterinary vaccine in Italy (World Health Organization, 1998). It is known that some attenuated strains still possess both virulence plasmids and are not considered fully virulent strains (Cataldi *et al.*, 2000;

Muscillo *et al.*, 2005). Mislabelling of the challenge strain used thus compromises the significance of the protection data reported.

(ii) The experimental conditions used in this study are very far from the natural *in vivo* conditions in which a protective humoral immune response functions. Ideally, passively transferred humoral immunity should mimic what would be observed in *in vivo* immunized hosts in which circulating antibodies could be recruited and exert their protective function in the infected sites. Protection should thus be observed even if the *B. anthracis* spore challenge is performed in a different location than the administered antibodies (for example challenge by subcutaneous route and antibody injection by intraperitoneal or intravenous route). The experimental procedures reported by Enkhtuya *et al.* (2006) artificially favour spore-antibody interaction: spores are either coated and opsonized with antibodies before infection, giving an 80 % survival, or injected in the same body compartment as the antibodies (the peritoneum) with a short delay of 30–40 min between each injection, leading to only 40 % protection. It would be informative about the *in vivo* relevance of this passive transfer of protection (a) to follow the level of protection achieved when more time is allowed to elapse between spore challenge and antibody inoculation, and (b) to ascertain the level of protection achieved when the challenge is performed by the classical subcutaneous route. Indeed, in our hands, in experiments of homologous passive transfer of FIS-immune serum (mouse FIS-immune serum intraperitoneally transferred in recipient

mice challenged subcutaneously) we did not observe any protective effects (Glomski *et al.*, 2007).

3. The cell infection experiments, from which the inhibition of germination by the anti-FIS IgG is concluded, lack essential information. (i) The informative experiment in this regard would have been to follow the optical density decrease of a pure spore suspension as classically used by many colleagues studying germination kinetics and as already described for *B. anthracis* (Titball & Manchee, 1987; Moir, 1990; Welkos *et al.*, 2001). (ii) In a similar cell infection experimental system, Welkos *et al.* (2002) have shown that phagocytosis by macrophages, and subsequent germination and killing of the spores, was enhanced by antibody coating of the spores. Since, in the study by Enkhtuya *et al.* (2006), all germinated spores are heat-killed before c.f.u. counting is performed, essential information about total and germinated spore c.f.u. counts is lacking. This hampers evaluation of the actual effect of antibody coating on the extent of germination, and thus does not adequately support the conclusions drawn.

In summary, the results reported by Enkhtuya *et al.* (2006) do not appear relevant, as such, to an *in vivo* protective role of anti-spore antibodies in an actively immunized animal. However, the study, which relies on an experimentally forced system, confirms the ability of rabbit antibodies reactive with *B. anthracis* antigens to stimulate mouse macrophages to efficiently kill the bacteria and thus help control infection (Welkos *et al.*, 2001, 2002).

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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.