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 overstated 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 (Tibball & 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).
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-\textit{Bacillus anthracis} (pXO1$^+$ and pXO2$^-$) Pasteur II spore IgG and its protective effect in mice against lethal challenge by pXO1$^+$ and pXO2$^+ \textit{B. anthracis}$ spores. The following are our comments addressing their concerns.

1. We are well aware that \textit{Bacillus cereus} and \textit{Bacillus thuringiensis} are closely related to \textit{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 \textit{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 \textit{B. anthracis} spores, while in contrast there was no detectable fluorescence from images of other tested \textit{Bacillus} spores, including \textit{B. cereus}, \textit{B. thuringiensis} and \textit{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-spor IgG obtained by immunization using formalin-fixed spores of plasmidless Pasteur II strain reacts specifically with \textit{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 \textit{B. cereus} and \textit{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-\textit{B. anthracis} spore IgG among \textit{B. anthracis}, \textit{B. cereus} or \textit{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 \textit{B. anthracis} Pasteur II lacking pXO1 and pXO2 as antigens to immunize rabbits, whereas Phillips et al. immunized rabbits, with formalin-fixed spores of \textit{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 \textit{B. anthracis}. However, our purified anti-spor IgG did react with endospores without cross-reacting with the vegetative form of \textit{B. anthracis} as shown in Fig. 1 of our paper (Enkhtuya et al., 2006). Thus, the nature of the spor 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 \textit{Bacillus} strains; although the antibody reacts with the three strains of \textit{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 \textit{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.