Co-immunisation with a plasmid DNA cocktail primes mice against anthrax and plague

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Abstract

The protective antigen (PA) of Bacillus anthracis and the V antigen of Yersinia pestis are potent immunogens and candidate vaccine sub-units. When plasmid DNA encoding either PA or V antigen was used to immunise the Balb/c mouse, a low serum IgG titre was detected (log10 1.0 or less) which was slightly increased by boosting with plasmid DNA. However, when mice immunised with plasmid DNA were later boosted with the respective recombinant protein, a significant increase in titre (up to 100-fold) was observed. Mice primed with a combination of each plasmid and boosted with a combination of the recombinant proteins, were fully protected (6/6) against challenge with Y. pestis. This compared favourably with mice primed only with plasmid DNA encoding the V antigen which were poorly protected (1/6). Combined immunisation with the two plasmid DNA constructs followed by boosting with a combination of the encoded recombinant proteins enhanced the protective immune response to Y. pestis compared with priming only with plasmid DNA encoding the V antigen and boosting with rV. This enhancement may be due to the effect of CpG motifs known to be present in the plasmid DNA construct encoding PA.

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Keywords: DNA vaccines; Plague; Anthrax; Protective efficacy

1. Introduction

The use of plasmid DNA has been demonstrated to be an effective means of generating protective immune responses to a number of viral and bacterial pathogens [1–4]. The possibility of combining a number of plasmids in a cocktail to create a multivalent vaccine could be very attractive. As well as conceptual and logistical advantages, there could be practical advantages in that one plasmid may act as an adjuvant for a co-administered plasmid. The compatibility of co-administered plasmid DNA encoding up to four different viral antigens has been studied previously and the responses to the expressed antigens have been shown to be modulated by the immune response to a co-administered protein [5]. To date, DNA immunisation to protect against bacterial infection has generally received less attention than protection against viral infection.

Immunisation with plasmid DNA has been demonstrated to be an effective means of priming the immune system, and can result in pronounced secondary immune responses when the encoded protein is introduced at a later date either from a different vector or as a free protein [6]. This may have benefits in terms of amplifying the immune response and reducing the time required to achieve protective immunity. This DNA prime with protein boost strategy has been exploited by us previously to amplify the immune response to the protective antigen (PA) of the organism causative of anthrax, Bacillus anthracis [7]. In that study, protein boosting was observed to be effective in amplifying the response for up to 1 year after the DNA priming.

We have also previously demonstrated a DNA construct encoding the V antigen of the plague-causing organism Yersinia pestis, to be effective in inducing an immune response to this protein in the mouse [8] following either manual delivery by the intra-muscular route or automated gene gun delivery intra-dermally.

In the current study, we set out to explore some of these concepts using, as a model system, the plasmid DNA constructs encoding these key protective antigens, PA and V antigen, of the organisms which are causative of anthrax and of plague, respectively. The diseases of anthrax and plague are naturally-occurring in areas of the world and are
life-threatening in their severity. Anthrax is caused by the gram-positive bacterium, *B. anthracis*, which produces an array of virulence factors. Key amongst these is PA. PA, one of the three plasmid-encoded major toxins produced by *B. anthracis*, provides a binding site for either of the other two, lethal factor (LF) or edema factor (EF) and in an A–B toxic mechanism, promotes internalisation into the host cell of the resultant lethal toxin complex [9]. Thus, it is important to target PA in any prophylaxis of infection with *B. anthracis*. The organism can infect via the skin through contamination of an open wound or by the inhalational route to establish pulmonary anthrax.

The causative organism of plague is the gram-negative bacterium *Y. pestis*, which can be transmitted to man by the bite of an infected flea, that flea having fed typically on an infected rodent, for example a rat or a ground-squirrel. This means of transmission sets up bubonic plague, which can progress to septicemic plague and secondary pneumonic plague, which is fatal. Alternatively, direct inhalation of the infective organism from an infected animal or from man, as an aerosol, can cause primary pneumonic plague (reviewed in [10]). *Y. pestis* also produces an array of virulence factors which are encoded for by one of three plasmids and which promote the organism’s transmission by the flea and entry into and infection of the warm-blooded host [10]. The *V* antigen is a protein secreted by the organism under the influence of the 70k low calcium response (ler) plasmid [11]. It has a key regulatory role in *Y. pestis*. As virulence factors, both PA and *V* antigen function extracellularly, at least for a part of the pathogenic process. Following binding to the surface of the target cell, PA is internalised as part of the lethal toxin complex [12]. *V* antigen is an intracellular protein in *Y. pestis*, which under the appropriate conditions, is secreted from the bacterium and has been observed on the bacterial cell surface [13]. Here, it is thought to participate in the Type III secretion system by facilitating, in a way not yet fully elucidated, the translocation of a number of *Yersinia* outer proteins (Yop’s) with anti-host properties into the host eucaryotic cell [14–16], to achieve host cell death possibly through apoptosis. This, in turn, allows the extracellular proliferation of the organism and contributes to its severe pathogenic effects. Both proteins therefore exist extracellularly for a sufficient length of time to be targeted by specific antibody. From the current understanding of each of the infectious processes, the pre-existence of a specific antibody titre to PA or to *V* antigen in the infected host would be expected to be protective and for each protein this has been demonstrated in an animal model of the individual infections [7,17–20,32].

As recombinant proteins, both PA and *V* antigen are potent immunogens, inducing high titres of circulating IgG in the mouse. Each of these proteins represents a sub-unit vaccine candidate for anthrax and plague, respectively. The protective efficacy observed for *V* either on its own [17] or in combination with the F1 sub-unit of *Y. pestis* in recombinant form, has been demonstrated to correlate significantly with the magnitude of the IgG1 titre it induces in the mouse [20]. High titres of circulating IgG to PA were detected in the rhesus macaque immunised with rPA, although in this model no significant correlation with protective efficacy against aerosolised *B. anthracis* could be achieved [21]. A DNA construct encoding PA has been reported to protect mice against lethal toxin challenge [22].

The objective of the present study was to determine whether a DNA vaccine approach could be protective against plague. A further objective was to determine whether there was any advantage in combining plasmid DNA constructs encoding, respectively, rPA and r*V* into a single immunisation regimen incorporating a combined protein boost, in terms of conferring protection against infection with virulent plague bacilli whilst also priming mice against anthrax infection.

2. Materials and methods

2.1. Plasmid DNA constructs and recombinant proteins

The plasmid DNA constructs were prepared by isolating DNA fragments encoding the PA from *B. anthracis* or the *V* antigen from *Y. pestis* and cloning them into the mammalian expression vector pCMVβ (Cambridge Bioscience, Cambridge, UK). For the *V* antigen, a DNA fragment encoding a fusion protein of glutathione *S*-transferase with *Y. pestis* V antigen [GST-*V*] was isolated from the plasmid pVG100 [17] and cloned into pCMVβ. The pCMVβ contains the immediate early gene promoter/enhancer from CMV, an intron (splice donor/splice acceptor) and polyadenylation signal from SV40, and the *E. coli* β-galactosidase gene with eucaryotic translation initiation signals. The Not I fragment containing the β-galactosidase gene was replaced with either an Ssp I fragment containing the PA coding sequence to construct pABV1. The control DNA for these constructs was pStu2 which included the β-galactosidase gene of pCMVβ had been replaced with an oligonucleotide containing multiple restriction enzyme cleavage sites.

For preparation of stocks of plasmid DNA, an Endofree Plasmid Preparation Kit was used (Qiagen Ltd., Dorking, UK). The kit uses a modified alkaline lysis procedure, followed by isolation and purification of plasmid DNA on a column of ion-exchange resin. Plasmid stocks were resuspended in distilled water (AnalR grade, BDH, Poole, UK) and stored at −20°C.

Recombinant PA was produced as previously described [32]. Briefly, rPA was expressed in *B. subtilis* and purified by ammonium sulphate precipitation with anion exchange chromatography followed by gel filtration chromatography. The V antigen was produced as a fusion protein with glutathione *S*-transferase in *E. coli*, cleaved with PreScission Protease (Pharmacia) and purified by affinity absorption [33].
Concentrated stocks of either protein were maintained in phosphate buffered saline (PBS) at ~20 °C until required.

2.2. Animals

Age-matched female Balb/c (Charles River Laboratories, Margate, UK) which were free of mouse pathogens, were used throughout this study. Immunisation was started at 6 weeks of age. All animal experimentation strictly adhered to the 1986 Scientific Procedures Act and to the Guidance on the Operation of the Animals (Scientific Procedures) Act, as promulgated by the Home Office in the UK and adopted by the Ethics Committee on Animal Procedures within this research establishment.

2.3. Immunisation

2.3.1. Preliminary dose-response trial

Mice were primed with the V-encoding plasmid (pABV1) intra-muscularly (i.m.) in 0.5% (v/v) bupivicaine hydrochloride (Antigen Pharmaceuticals Ltd., Roscara, Ireland) in PBS. Groups of six mice were primed with doses of pABV1 in the range 10–65 μg. The total volume administered was 0.1 ml divided equally between a site in each hind-limb and mice were blood-sampled for antibody titration 25 days after the priming dose.

2.3.2. Plasmid DNA and recombinant protein combination trial

In a subsequent study, Balb/c mice were primed i.m. with 50μg plasmid DNA encoding either PA or V antigen, administered in a 0.1 ml volume comprising 0.25% (v/v) bupivicaine hydrochloride. In this trial, the concentration of bupivicaine used in the formulation was reduced compared with the preliminary dose-response trial, because central side-effects (initial excitation followed by prolonged sedation) had been observed when animals were dosed with 0.5% (v/v) bupivicaine. Booster doses of plasmid DNA or of the respective recombinant protein were given on day 21.

One group of mice was primed i.m. with a combination of the plasmid DNA encoding each antigen in a total volume of 0.1 ml comprising 0.25% (v/v) bupivicaine hydrochloride in PBS. This group of mice was subsequently boosted on day 21 with a combination of 2.5 μg of each protein adsorbed to 25% (v/v) alhydrogel in a final volume of 0.1 ml PBS. Control animals received vehicle alone and were primed with rPA in alhydrogel concomitantly with the rPA boosting of the cohort primed with plasmid DNA.

In an alternative schedule, mice were immunised in groups of six with rPA or rV in an i.m. schedule in which 2.5 μg of either protein was administered adsorbed to 25% (v/v) alhydrogel (Superfos Biosector, Denmark) in a total volume of 0.1 ml divided between two sites. On day 21, mice were boosted with either the respective recombinant protein as for the primary immunisation, or with 50μg plasmid DNA encoding either PA or V antigen. The plasmid DNA was administered i.m. to mice in 0.25% (v/v) bupivicaine hydrochloride (Antigen Pharmaceuticals Ltd., Roscara, Ireland) in PBS. The total volume administered was 0.1 ml divided equally between a site in each hind-limb.

Additional Balb/c female mice were included in the trial to provide naive controls for the immune response analysis and to control the live organism challenge.

2.4. Immune response analysis

2.4.1. Antibody titre development with time

Mice were blood-sampled 21 days after the priming dose and also 92 days after boosting and the serum antibody response determined by titration of specific IgG against rPA or rV in a modified ELISA [34]. The group mean IgG titre is presented as log10 of the reciprocal of that dilution of immune serum giving an O.D. at 0.1 units over normal mouse serum.

2.4.2. Recognition of protective regions within the V protein in vitro

The recognition of protective regions [23] within the V protein by antibody induced to the pABV1 construct was determined by coating onto the solid phase of a microtitre plate GST fusions with the individual truncated proteins [GST-V1–135], [GST-V135–275] at 5 μg ml−1 protein in PBS and probing with the polyclonal mouse serum induced by pABV1. Recognition of the truncated proteins was detected by use of an horseradish peroxidase-labelled anti-mouse IgG reagent (Sera-Lab) and was positively controlled by means of reference mouse sera raised in-house to the truncated proteins. The degree of cross-reactivity with the truncated proteins was compared with that to the full-length V protein, coated onto microtitre plates as a fusion with GST [GST-V]. Equal aliquots of sera from individuals in group 7 were pooled for this test of cross-reactivity and compared with a positive control reference sample of pooled sera raised in-house in Balb/c mice immunised with [GST-V].

2.4.3. Assay of the in vitro lymphocyte recall response for rPA induced by immunisation with pStuPA

The in vitro recall response for rPA by mixed splenic lymphocytes isolated as previously described [17] from mice immunised either with the pStuPA construct or with rPA, was determined by co-culture of a minimum of 5×104 cells ml−1 with rPA in a concentration range 25–0.2 μg ml−1. Briefly, mixed lymphocytes purified by density gradient separation, were cultured in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 4% 20 mM L-glutamine, 100 IU penicillin ml−1, 0.1 mg streptomycin ml−1 and 10% foetal calf serum, to which was added rPA, for 72 h at 37 °C with 5% CO2, prior to pulsing with 5 μCi 3H methyl-thymidine per ml of culture for 24 h. The uptake of 3H methyl-thymidine into proliferating cells was measured.
by lysing the cells in water and harvesting the cellular fragments onto a synthetic membrane prior to counting the incorporated \( ^3 \)H thymidine in a liquid scintillation medium. The results are presented as the stimulation index derived as shown below from triplicate culture of stimulated or control cells:

- mean counts per minute (cpm) of stimulated cells;
- mean cpm of untreated control cells.

2.5. Organism challenge in vivo

To determine whether the immunisation regimens used above were able to induce protective immunity against live organism challenge with \( Y. pestis \), animals were challenged with 10 colony-forming units (cfu) \( Y. pestis \) strain GB, equivalent to 10 lethal doses (LDs) in the mouse [35]. This dose level has been used previously in our laboratory, and although low, can be prepared very consistently. The \( Y. pestis \) challenge inoculum was prepared at 28°C as previously described [35] and administered sub-cutaneously (s.c.) to mice in 0.1 ml volumes. Animals were carefully observed for 14 days post-challenge for symptoms of disease. No animal was allowed to become distressed and was humanely culled if displaying clinical signs known to lead to death.

2.6. Statistical analysis

The significance of the difference in treatment group means was determined by Student’s \( t\)-test.

3. Results

In a preliminary dose-response trial, the effect of priming and boosting with escalating doses of pABV1 in 0.5% (v/v) bupivacaine as a facilitator, was studied in groups of Balb/c mice. The group mean titres of IgG to V antigen assessed at day 21 and at each dose level, are shown in Fig. 1. Immunoglobulin G titres specific for rV antigen were detected in all groups and these showed an upward trend with dose. From this trial, the 50 μg dose of plasmid DNA was selected for subsequent use as either a priming or boosting immunisation dose, since this dose induced a consistently high antibody response. Subsequently, the concentration of

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**Fig. 1.** Dose-response effect of i.m. immunisation with pABV1 on IgG titre to rV. In a preliminary dose-response trial, groups of six female Balb/c mice were immunised with pABV1 on a single occasion. Animals were immunised in the dose-range 10–65 μg pABV1 in 0.5% (v/v) bupivacaine hydrochloride in 0.1 ml PBS per mouse. The antibody response was determined by blood-sampling at day 21. Antibody titre to V antigen is presented as a group mean log_{10} IgG titre ± standard error of the mean (S.E.M.).
bupivicaine used was reduced to 0.25% (v/v), because of unacceptable side-effects with the 0.5% (v/v) concentration.

The effect of reducing the concentration of bupivicaine was a reduction in total IgG titres observed in subsequent trials.

In a subsequent trial, mice were primed with plasmid DNA encoding either V antigen or PA. Alternatively, mice were primed with rPA protein. The IgG titre to rPA or to rV in each of the eight treatment groups at days 21 and 93 is shown in Fig. 2a and b and Table 1. Modest titres were detected in most groups primed with either of the DNA constructs. The response to priming with pStuPA was consistent across groups, whereas the response to priming with pABV1 was more variable, with group 7 responding much less well than group 2. DNA-primed groups were boosted with protein or with DNA at day 21, and sera was taken at day 93 for antibody titration. All groups boosted with protein responded with an enhanced antibody titre representing a 100-fold or greater increase. The maximum increase (approximately 300-fold) was seen in the PABV1-primed group after boosting with rV protein. A booster dose of pABV1 in pABV1-primed mice had the effect of increasing titre, approximately 10-fold. The titre to rV achieved in pABV1-primed mice boosted with rV was significantly higher than in pABV1-primed mice boosted with pABV1 (P < 0.001). However, the IgG titre to rV in group 1 primed with a combination of the DNA constructs and

| Table 1
<p>| The combinations of treatments |</p>
<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Primary immunisation on day 1</th>
<th>Booster immunisation on day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 μg pStuPA + 50 μg pABV1 in 0.25% (v/v) bupivicaine hydrochloride i.m.</td>
<td>2.5 μg rPA + 2.5 μg rV in 25% (v/v) alhydrogel i.m.</td>
</tr>
<tr>
<td>2</td>
<td>50 μg pABV1 in 0.25% (v/v) bupivicaine hydrochloride i.m.</td>
<td>2.5 μg rV in 25% (v/v) alhydrogel i.m.</td>
</tr>
<tr>
<td>3</td>
<td>50 μg pStuPA in 0.25% (v/v) bupivicaine hydrochloride i.m.</td>
<td>2.5 μg rPA in 25% (v/v) alhydrogel i.m.</td>
</tr>
<tr>
<td>4</td>
<td>2.5 μg rPA in 25% (v/v) alhydrogel i.m.</td>
<td>50 μg pStuPA in 0.25% (v/v) bupivicaine hydrochloride i.m.</td>
</tr>
<tr>
<td>5</td>
<td>2.5 μg rPA in 25% (v/v) alhydrogel i.m.</td>
<td>2.5 μg rPA in 25% (v/v) alhydrogel i.m.</td>
</tr>
<tr>
<td>6</td>
<td>50 μg pStuPA in 0.25% (v/v) bupivicaine hydrochloride i.m.</td>
<td>50 μg pStuPA in 0.25% (v/v) bupivicaine hydrochloride i.m.</td>
</tr>
<tr>
<td>7</td>
<td>50 μg pABV1 in 0.25% (v/v) bupivicaine hydrochloride i.m.</td>
<td>50 μg pABV1 in 0.25% (v/v) bupivicaine hydrochloride i.m.</td>
</tr>
<tr>
<td>8</td>
<td>PBS</td>
<td>2.5 μg rPA in 25% (v/v) alhydrogel i.m.</td>
</tr>
</tbody>
</table>
boostered with a combination of rV and rPA, it was not significantly different from that of group 2 which was primed with pABV1 and boosted with rV. This suggests that there was no significant impairment of the response to V antigen by co-immunisation with the DNA construct encoding PA and of co-boostering with rPA. This effect did not appear mutual, in that co-immunisation with pABV1 and co-boostering with rV (group 1) did not enhance the anti-PA response over and above that achieved by priming with pStuPA alone and boosting with rPA (group 3) and the difference in titres developed was not statistically significant.

The maximum antibody response to rPA was achieved by priming with rPA and this response was boosted with either pStuPA (group 4) or rPA (group 5). The titre developed in group 4 was significantly higher than the titre developed in group 1 (\(P < 0.01\)); but the titre developed in group 5 was maximum and significantly higher than the titre for group 4 (\(P < 0.01\)).

### 3.1. Recognition of protective regions within the V protein

In order to predict whether immunisation with the pABV1 construct is able to induce neutralising antibody specific for the V protein and thus to predict whether protection against organism challenge is likely, sera from the group of animals primed and boosted on two occasions with pABV1 only, were tested in a modified ELISA for cross-reactivity with full-length V, or with fragments of V known to contain protective epitopes (Table 2). Both full-length rV and the fragments of rV, were prepared and used as N-terminal fusions with the carrier protein GST. The maximum antibody titre was raised to the GST fusion with full-length V, or with fragments of V known to contain protective epitopes (Table 2). The determination of a specific recall response (SI) of lymphocytes isolated from immunised animals was investigated (Table 3). The determination of a specific recall response with a stimulation index of 5.77, compared with lymphocytes from PBS-primed controls, indicated that T-cells in immunised animals had been exposed to sufficient rPA by in vivo expression from the pStuPA construct to establish a memory response for the protein. By comparison, lymphocytes isolated from animals primed with rPA had an in vitro stimulation index of 5, when compared with the background stimulation seen in the PBS-primed controls. Thus, the pStuPA construct was as efficient as rPA in inducing a memory T-cell response for rPA in vaccines.

### 3.2. The in vitro lymphocyte recall response for rPA induced by immunisation with the pStuPA construct

In the absence of a high titre of specific antibody induced by pStuPA to rPA, the specific recall response for rPA of lymphocytes isolated from immunised animals was investigated (Table 3). The determination of a specific recall response with a stimulation index of 5.77, compared with lymphocytes from PBS-primed controls, indicated that T-cells in immunised animals had been exposed to sufficient rPA by in vivo expression from the pStuPA construct to establish a memory response for the protein. By comparison, lymphocytes isolated from animals primed with rPA had an in vitro stimulation index of 5, when compared with the background stimulation seen in the PBS-primed controls. Thus, the pStuPA construct was as efficient as rPA in inducing a memory T-cell response for rPA in vaccines.

### 3.3. Protective efficacy

It has been shown previously that the magnitude of the antibody titre raised in the mouse to the V antigen, together with another sub-unit antigen termed F1, correlates with protection against challenge with virulent plague organisms [17,20] and the V antigen is known to contain a major neutralising epitope between amino acids 135 and 275 [23], a region which we have shown to be recognised by antibody induced by the pABV1 construct. Thus, there was a reasonable expectation that sufficient protective immunity would be achieved in this study to protect against live

<table>
<thead>
<tr>
<th>Splenocyte source</th>
<th>Stimulation index</th>
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<tbody>
<tr>
<td>pStuPA immunised mice</td>
<td>5.77</td>
</tr>
<tr>
<td>rPA immunised mice</td>
<td>5.0</td>
</tr>
<tr>
<td>Untreated control mice</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Mixed splenic cells were isolated from immunised animals and cultured \((5 \times 10^7 \text{cells ml}^{-1})\) with rPA (25-0.2 \(\mu\text{g ml}^{-1}\)) for 72 h prior to pulsing with \(^{3}H\) thymidine. The uptake of \(^{3}H\) thymidine into proliferating cells was measured and compared with that for splenocytes derived from untreated control mice, in order to derive the stimulation index. The SI presented here was derived with an optimum concentration of rPA of 1.67 \(\mu\text{g ml}^{-1}\).
Fig. 3. Protection mediated by DNA vaccine priming plus protein or DNA boosting against 10 median lethal doses (MLD) of *Y. pestis* administered s.c. Animals in treatment group 1 (primed with pStuPA + pABV1; boosted with rPA + rV), treatment group 2 (primed with pABV1; boosted with rV), treatment group 7 (primed and boosted with pABV1), together with six untreated controls, were challenged and the number of survivors 14 days post-challenge was determined. All immunised survivors had cleared the challenge infection by day 14.

Organism challenge. Therefore, the immunisation groups achieving the highest antibody titres after two immunising doses (treatment groups 1 and 2) were challenged with 10 LDs of *Y. pestis* strain GB by the s.c. route. Treatment group 7, which had been primed and boosted with pABV1, was included as a control group. The degree of protection was assessed 14 days post-challenge (Fig. 3) when all six animals from group 1, which had been co-immunised with both DNA constructs and boosted with the combined proteins, were fully protected. By comparison, only three of the six animals primed with pABV1 and boosted with rV survived the challenge and only 1/6 primed and boosted with pABV1 survived. Untreated control animals all succumbed to challenge with a mean time to death (ttd) of 5.7 ± 0.3 days. The three animals in the pABV1 + rV group which did not survive, had a mean ttd of 5.3 ± 0.7 days. Bacteriological analysis of spleens removed after death of survivors on day 14 post-challenge demonstrated that all were negative for *Y. pestis* and therefore had cleared the challenge infection.

4. Discussion

This is the first report of combined immunisation with plasmid DNA’s encoding key protective antigens of the causative organisms of two severe infectious diseases: plague and anthrax. Each of the plasmid DNA constructs, used in a mammalian expression vector, has been demonstrated to be able to induce a specific IgG titre in mice which recognises either the PA or V proteins in an in vitro immunoassay. The antibody titre induced to the V antigen has been shown to be protective in immunised mice challenged with *Y. pestis*. Furthermore, co-immunisation of mice with the plasmid DNA constructs encoding PA and V and boosting with the combined recombinant antigens, has been shown to enhance the development of protective immunity against *Y. pestis* challenge, so that all the co-immunised animals (6/6) withstood 10 mouse lethal doses of virulent plague organisms, whereas mice immunised with plasmid DNA encoding V antigen and boosted with rV, were only partially protected (3/6) against the same challenge dose. Although this level of protection against plague is orders of magnitude below that achieved with rV immunisation in three intra-peritoneal doses in mice in Incomplete Freund’s Adjuvant [17] or in two s.c. doses in alhydrogel [36], it indicates that DNA immunisation can be an effective strategy against plague.

At 50 μg, the dose of plasmid DNA selected from the preliminary dose-response study with pABV1 as optimum for immunisation of mice, was the median of the range (0.3–100 μg) used in other studies [24–26]. Nevertheless, only a modest titre IgG response was observed to priming with either plasmid DNA, from the enhanced titres observed following boosting with either rV or rPA or a combination of both. Furthermore, lymphocytes isolated from mice primed with the pStuPA construct proliferated in response to in vitro exposure to rPA, indicating that sufficient rPA had been expressed in vivo from the DNA construct to achieve T-cell memory. Although the co-immunised group received double the doses of DNA and protein during the priming and booster immunisations compared with any of the other immunisation groups, it is apparent from comparison of the end-point titres achieved by them and by the group primed with pABV1 only and boosted with rV, that increasing the immunising doses did not confer any significant additional advantage on this group in terms of higher antibody titres. Indeed, the highest fold increase in titre induced by protein boosting, was seen in the pABV1-primed, rV-boosted group. Clearly, however,
the co-immunised group had a significant advantage over the pABV1rV group in terms of increased protection against challenge and since this could not be explained by a significant difference in antibody response, it may be due to an enhancement of the cellular immune response. A combination schedule comprising priming with plasmid DNA and boosting with free protein would appear to have advantages in terms of achieving a rapidly enhanced secondary immune response in vivo. Any positive influence of the plSmPa construct on the enhancement of cellular immune responses which enhanced protection against Y. pestis challenge, is likely to be a non-specific effect of the construct. It may be due to the presence of a CpG motif contained in the PA gene sequence between bases 3671 and 3676 (AACGTT) which has been recognised as immunostimulatory [28,29]. Recently, synthetic oligonucleotides containing CpG motifs have been co-administered with a peptide derived from the nucleoprotein of influenza virus and found to promote CD8+ T-cell priming for the peptide, which is thought to be mediated by the activation and presentation of antigen to dendritic cells [30]. CpG oligonucleotides have also been shown to act as adjuvants for co-administered polysaccharide-protein conjugate vaccines, enhancing the antibody response across a range of isotypes, an effect which may be mediated by the direct and indirect (through T-cell activation or alteration of the local cytokine milieu) activation of B-cells [31]. In this study, it has been demonstrated that multivalent priming with DNA encoding antigens from different micro-organisms, followed by combined boosting with the recombinant proteins encoded by the plasmid DNA’s, can be achieved with the same degree of success as single specificity DNA priming and protein boosting, with no interference in the authentic expression simultaneously of two recombinant proteins. Co-immunisation with the combined plasmid DNA’s resulted in enhanced protective efficacy against plague compared with immunisation with pABV1 only.

References