

Research Paper

Investigation in a Model System of the Effects of Combinations of Anthrax and Pertussis Vaccines Administered to Service Personnel in the 1991 Gulf War

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KEY WORDS

Gulf War, Anthrax, Pertussis, vaccines, mouse model, toxicity

ABBREVIATIONS

AVP	anthrax vaccine precipitated
EF	oedema factor
FHA	filamentous haemagglutinin
HPA	Health Protection Agency
IL	interleukin
IFN	interferon
LF	lethal factor
LPS	lipopolysaccharide
NO	nitric oxide
PA	protective antigen
PRN	pertactin
PT	pertussis toxin
PWC	plain whole cell pertussis vaccine
PWCA	adsorbed whole cell pertussis vaccine
SHD	standard human dose

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ABSTRACT

The toxicity and immunogenicity of the anthrax and pertussis vaccine combinations used in the 1991 Gulf War was assessed in NIH, A/J and Balb/c mice. Inoculation of pertussis vaccines, vaccine combinations, or aluminium salt caused illness, splenomegaly and significant weight loss. Although some animals recovered eventually, a lethal form of ascites developed in some NIH mice and body weights of A/J and Balb/c mice remained below normal levels. Inoculation of anthrax vaccine produced little effect. Exposure to diluted vaccine combinations produced less serious side effects of shorter duration. Single vaccinations induced specific IgG1 antibodies whereas a mixture of IgG1 and IgG2a was produced after multiple injections. Antigen stimulation of spleen cells from mice exposed to pertussis vaccines induced high levels of NO and IL-6, whereas stimulated spleen cells from mice exposed to anthrax vaccine produced only low levels of IL-6. In mice, pertussis vaccines act as an adjuvant for anthrax vaccine, but these vaccines are also the major cause of toxicity of the vaccine combination. The relatively high vaccine dose used, together with the low sensitivity of mice to anthrax toxin, emphasises that caution should be exercised in applying these results to human recipients of these vaccines.

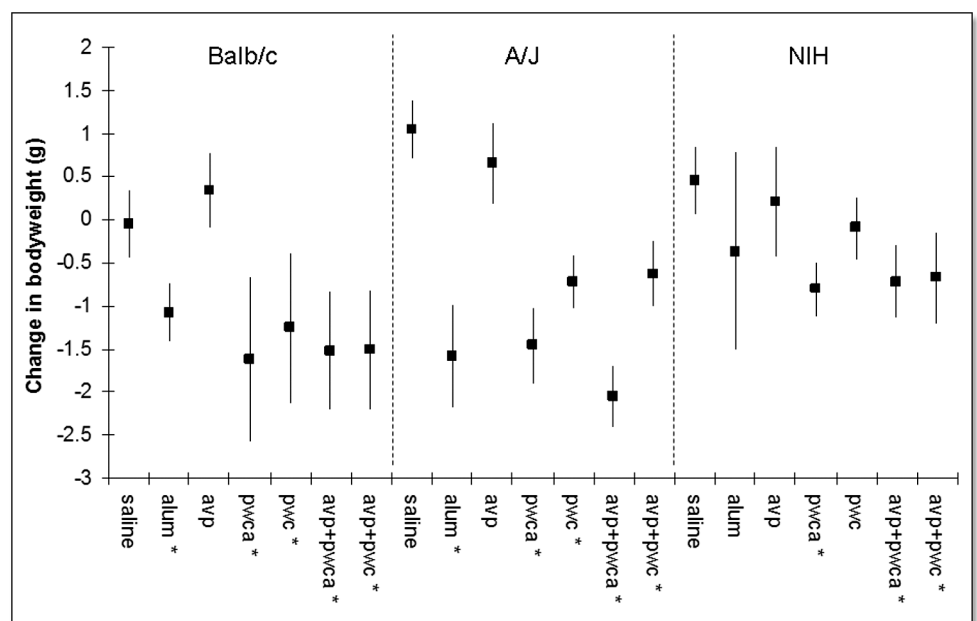
Within months of the end of Operation Desert Storm in February 1991, American veterans began to report various symptoms later collectively referred to as 'Gulf War syndrome'.¹⁻³ Similar reports of illness emerged from UK veterans, whose disease was characterised by an increased frequency of symptoms of ill health including insomnia, headaches, musculoskeletal disorders and fatigue.⁴ The cause of the disease is controversial. In American veterans it has been attributed to a combined exposure to pesticides, pyridostigmine and exposure to chemical weapons.⁵ Illness in British veterans has been associated with vaccination against biological warfare agents (e.g., anthrax and plague) or multiple vaccinations at the time of deployment, but not with exposure to pesticides or pyridostigmine.^{4,6} In this context, whole cell pertussis vaccines were given concomitantly with anthrax vaccine as an adjuvant, despite the fact that their use is normally contra-indicated for adults. The British anthrax vaccine is an alum precipitated sterile culture filtrate (AVP) of avirulent *Bacillus anthracis* (Product Licence 01511/0058), and contains predominantly protective antigen (PA) and trace amounts of lethal factor (LF), oedema factor (EF) and other bacterial proteins.^{7,8} LF and EF combine with PA to form lethal toxin, a protein kinase kinase cytotoxin, or oedema toxin, an adenylate cyclase, respectively.^{9,10} Antibodies to PA, LF and EF are present in recipients of AVP.⁷ British and American anthrax vaccines are safe and efficacious but recent concerns about side effects led to a lower vaccine uptake among the armed forces.^{7,11,12} Whole cell pertussis vaccines are effective and safe for infants, although minor adverse effects can occur.¹³ These vaccines contain pharmacologically active components, among them lipopolysaccharide (LPS) and pertussis toxin (PT).¹⁴⁻¹⁶ PT is an ADP ribosylating toxin with adjuvant activity and a range of biological effects; LPS and PT both contribute to lethal toxicity in mice.¹⁷⁻¹⁹ Information on the safety of the anthrax and pertussis vaccine combination is not available. In 1991, the National Institute for Biological Standards and Control tested the toxicity of anthrax and pertussis vaccine combination used for immunisation of military personnel serving in the first Gulf War. Mice and guinea pigs were given up to 4 standard human doses (SHD) of AVP and 1 SHD of adsorbed whole cell pertussis vaccine (PWCA) via the intraperitoneal route [MJC Personal communication]. Weight loss was observed in ~80% of mice, with some deaths but no effects were seen in guinea pigs. A recent study confirmed the lack of side effects in guinea pigs.²⁰ The current study is part of a programme of research funded by the Gulf Veterans' Illnesses Unit, now Veterans Policy Unit, of the Ministry of Defence.

Table 1 Mouse strains used in this study

Strain (Supplier)	H-2 haplotype	Bodyweight	Experimental group size			Sensitivity of mouse strain to:	
			I	II	III	LPS ²¹	Anthrax toxin ²²
A/J (Harlan)	H2 ^a	16–20 g	5	na	na	Susceptible	Susceptible
Balb/c (Charles River)	H2 ^d	16–20 g	15	15	5	Susceptible	Resistant
NIH (Harlan)	H2 ^q	15–20 g	5	15	5	Susceptible	Not known

Three experiments were carried out to determine the effects of vaccines on appearance, body temperature, body weight and the immune system. Three mouse strains were chosen as an approximation of the biological variety in outbred populations (Table 1). Female mice were given intraperitoneal injections of AVP, PWCA, plain whole cell pertussis vaccine (PWC), the vaccine combinations or aluminium salt. Mice given saline served as strain control. Immunogenicity was determined by measurement of serum IgG response to anthrax or pertussis antigens, and the production by spleen cells of nitric oxide (NO), a marker for macrophage activation, and Th1 and Th2 type cytokines.^{23,24} All animal experiments were approved by the local ethical review process and conducted in accordance with Home Office (Scientific Procedures) Act 1986. AVP batch 378 and PWC batch W208/10/99, containing 10^{10} formalin inactivated *Bordetella pertussis* ml⁻¹, were produced by the Centre for Applied Microbiology and Research now the Health Protection Agency (HPA, Porton Down, United Kingdom) in 1998 and 1999 respectively. PWCA batch G0019, containing 1.5×10^{10} *B. pertussis* and 0.50 mg aluminium SHD⁻¹, was manufactured by Institut Mérieux (Lyon, France) in 1991. PWC and PWCA met pharmacopoeial specifications²⁵ and AVP passed the abnormal toxicity test.²⁶ The aluminium salt solution contained 2.5 mg aluminium ml⁻¹ and consisted of 3.6 mg alhydrogel ml⁻¹ (Superfos) and 45 μ mol AlK(SO₄)₂ ml⁻¹ in saline. An IPTT-200 microchip transponder was used for identification and measurement of body temperature in all mice, except Balb/c mice receiving neat vaccines, data were transferred to a DAS 5002 data acquisition notebook (Plexx). The body weight was measured by an automatic balance linked to the unit. Body weights of Balb/c mice without IPTT-200 were recorded manually. Analysis of variance incorporating Dunnett's test was used to make comparisons between vaccine groups and the saline control group, with results considered significant at the 5% level ($p < 0.05$). Data of cytokine and NO assays were log-transformed prior to analysis.

Figure 1. Change in bodyweights of Balb/c A/J and NIH mice three days after injection with saline, aluminium salt, anthrax vaccine (AVP), whole cell pertussis vaccine (PWC), whole cell pertussis vaccine adsorbed (PWCA) or the vaccine combinations. Significant differences compared to the saline group are indicated by * ($p < 0.05$). Error bars indicate standard deviation.



Experiment I. Mice received one SHD of neat vaccine, the vaccine combinations or aluminium salt via the intraperitoneal route. It is at this dosage level that potential adverse effects of whole cell pertussis vaccines in children are noticeable in mice.²⁷ Mice were observed daily for four weeks. Signs of ill health and loss of bodyweight were most pronounced in the first week after exposure to aluminium salt, pertussis vaccines or vaccine combinations. Lethargy, arched backs, squinted eyes and ruffled fur were observed within a few hours of injection. On day 3, Balb/c mice and A/J mice had lost weight ($p < 0.05$, (Fig. 1). Weights of Balb/c mice exposed to the vaccine combinations remained significantly lower than saline controls until day 14. A/J mice exposed to aluminium salt or the combination of PWCA and AVP had significantly lower weights than saline controls for the test duration. The weights of NIH mice exposed to PWCA or the vaccine combinations decreased in the first week ($p < 0.05$, Fig. 1), however from day 7 onwards the body weight of NIH mice exposed to pertussis vaccines or the combinations increased. These mice developed ascites, two died and six more were culled by day 12. The weights of surviving mice remained high. The weights of NIH mice exposed to aluminium salts increased from day 14 onwards and severe ascites developed requiring a cull on day 24 (data not shown). Mice exposed to pertussis vaccines or the combinations, including NIH mice that were culled, developed splenomegaly ($p < 0.05$). Mice exposed to aluminium salt had enlarged spleens ($p < 0.05$) except for A/J mice (data not shown). By contrast, exposure to AVP did not cause ill health, changes in bodyweight or splenomegaly in

Table 2 IgG response of mice exposed to neat vaccines

Mouse strain and inoculation	PT	Geometric mean (range) of IgG in EU/ml FHA	PRN	Anthrax
Balb/c				
Saline	1 (1–4)	7 (3–45)	3 (2–11)	0
Aluminium salt	0	0 (0–1)	2 (2–3)	1 (0–1)
AVP	1 (1–2)	4 (1–11)	4 (3–5)	8 (1–42)
PWCA	2 (1–5)	66 (0–247)	69 (3–226)	0
PWC	12 (1–59)	222 (2–1092)	69 (2–192)	0
AVP + PWCA	1 (1–2)	73 (1–264)	69 (4–159)	1270 (69–3084)
AVP + PWC	6 (2–37)	415 (143–619)	111 (21–52)	243 (1–1315)
A/J				
Saline	0	0	0	0
Aluminium salt	0	0	1 (0–1)	1 (0–1)
AVP	0	2 (1–3)	0	8 (2–15)
PWCA	1	1319 (829–2503)	144 (130–162)	0
PWC	1 (0–2)	3473 (2631–5046)	152 (84–275)	0
AVP + PWCA	1	209 (55–626)	75 (49–165)	1460 (1071–1759)
AVP + PWC	1 (1–2)	6898 (2619–10461)	126 (62–304)	912 (566–1424)
NIH				
Saline	0	0	0 (0–1)	0
Aluminium salt	0	0	0 (0–1)	0
AVP	0	1	0 (0–2)	22 (12–51)
PWCA	34 (1–653)	153 (19–1389)	328 (171–569)	0
PWC	317 (235–437)	1352 (851–1998)	155 (124–190)	0
AVP + PWCA ^a	1 (1–4)	964 (709–1312)	46 (34–61)	5714 (3007–10857)
AVP + PWC ^b	–	–	–	–

^aData represent two mice. ^bNo mice survived at week 4.

mice. The body temperature was not affected in all mice. Serum IgG responses are given in Table 2. ELISA plates were coated with PT, filamentous haemagglutinin (FHA), pertactin (PRN) as described by Gaines-Das et al., or anthrax vaccine antigens (batch 388, HPA) desorbed with 100 mM sodium citrate and diluted 1:100 in carbonate coating buffer.^{7,28} IgG was detected with goat anti-mouse IgG peroxidase (Sigma) and TMBTM (Biovest Inc.). WHO Pertussis Reference Reagent 97/642 and pooled sera from mice inoculated with a combination of AVP and PWC, with an arbitrary anti-anthrax IgG level of 100 EU ml⁻¹, were included as reference on each plate.²⁸ The unitage of anti-pertussis and anti-anthrax specific IgG in test serum was calculated by comparing the slope ratio of the test sample to the reference sample in an 8-point parallel line assay. Pertussis vaccines were immunogenic, whereas AVP induced a poor IgG response and inoculation of vaccine combinations resulted in higher levels of anti-anthrax IgG. Anti-PRN and anti-FHA IgG levels were high, whereas anti-PT antibodies were only detected in NIH mice. IgG subclasses were determined using biotinylated rat anti-mouse IgG1 and IgG2a (Pharmingen BD International). IgG1 was the dominant subclass, with the exception of A/J mice exposed to PWC or PWC and AVP, which produced anti-PRN IgG2a, IgG1 and IgG2a directed to anti-FHA and anti-anthrax IgG2a (data not shown).

Experiment II. NIH mice and Balb/c mice were exposed to three injections of 1:5 diluted vaccines at three weekly intervals to reflect more closely the vaccination schedule used for military personnel. Sera were collected on the day of the booster and at the end of the experiment. Mice were observed daily for ten weeks. Five mice of each group were killed on the day of the booster injection and at the end of the experiment.

Exposure to diluted pertussis vaccines or the vaccine combinations caused only mild symptoms of illness. After each vaccination, Balb/c mice developed ruffled fur and became lethargic for one to five days. NIH mice displayed ruffled fur after the first and second booster of the vaccine combinations or the pertussis vaccines for two days and one day respectively. The weights of Balb/c mice dropped on day 3 after inoculation with diluted pertussis vaccines or the combinations, but returned to normal quickly. The weights of NIH mice also dropped after primary inoculation but remained significantly higher than saline controls for the duration of the experiment. Only Balb/c mice developed splenomegaly ($p < 0.05$) after exposure to primary injections or boosters of diluted pertussis vaccines or the combinations. The body temperature of mice was not affected (data not shown).

The highest IgG levels were found after three inoculations. Pertussis vaccines acted as an adjuvant for the anti-anthrax IgG response in both strains. Levels of anti-FHA and anti-PT IgG were highest in NIH mice and the anti-PRN response was highest in Balb/c mice. Primary and booster inoculations with PWCA produced IgG1 directed against PRN, PT and FHA in both strains. Exposure to PWC alone or in combination with AVP produced a balanced IgG1/IgG2a response against FHA and PRN. Anti-PT antibodies were predominantly of the IgG1 class. Inoculation with AVP produced anti-anthrax IgG1; after the second booster both IgG1 and IgG2a were present (data not shown).

Experiment III. Balb/c and NIH mice were exposed to neat vaccines and spleens were harvested at week four and two respectively. Spleens representing one experimental group were pooled and suspended in RPMI 1640 culture medium (Gibco BRL). Two milliliters of cell suspension (2×10^6 cells ml⁻¹) was stimulated with 2 µg desorbed AVP antigens ml⁻¹ or 10^7 heat killed *B. pertussis* ml⁻¹ or

both. Cells in RPMI 1640 and saline served as the nonstimulated control. Supernatants were collected after 48 hrs and stored at -20°C .¹⁶ The production of NO was determined using the method of Torre et al.²⁹ Interleukin [IL]-4, IL-5, IL-10 (Th2 markers) and IL-2 and interferon (IFN)- γ (Th1 markers), IL-1 β , IL-6 and tumour necrosis factor- α were measured in the supernatant by capture ELISA kit as described by the manufacturer (Pharmingen BD & Biosource Ltd). Only results for IL-6 and NO production by Balb/c spleen cells are shown in Figure 2. Nonstimulated spleen cells from Balb/c mice inoculated with PWCA or the vaccine combinations produced higher levels of IFN- γ , IL-6 and NO than spleen cells from the control group ($p < 0.05$). Non-stimulated spleen cells from NIH mice inoculated with PWC produced high levels of IL-6 and NO ($p < 0.05$). Spleen cells of mice inoculated with pertussis vaccines or the combinations and stimulated with pertussis antigens produced the highest levels of IL-6 and NO ($p < 0.05$), in addition Balb/c spleen cells produced IFN- γ , IL-2 and IL-10 ($p < 0.05$). Anthrax antigens stimulated IL-6 production by spleen cells of mice inoculated with AVP albeit at lower levels ($p < 0.05$; Fig. 2). Levels of other cytokines did not increase above background level (data not shown).

A variant of the mouse weight gain test was used to determine the toxicity of vaccine combinations used to protect military personnel against the threat of anthrax during the first Gulf War. In this model, mice exposed to pertussis vaccines or aluminium salt showed a rapid deterioration of general health. Exposure to AVP alone did not affect mice, but it exacerbated the toxicity of pertussis vaccines in NIH mice. The toxicity of diluted pertussis vaccines or the combinations was greatly reduced by dilution. These findings confirm the outcome of the 1991 toxicity study, which found that deaths occurred in mice injected with up to 4 SHD of AVP and 1 SHD of PWCA but not among mice exposed to single vaccines. Exposure of mice to these higher doses, at the time allowed by the 1986 Act, explains the more moderate reactions seen in the current study. Thus whole cell pertussis vaccines and not AVP are the main cause of toxicity in mice. Exposure to LPS and PT cause weight loss in mice and exposure to PT only increases body weight.³⁰⁻³³ Aluminium, present in hepatitis and diphtheria vaccines, has been implicated as the cause of myofasciitis, musculoskeletal pain and fatigue in a small group of recipients (reviewed in ref. 34). However, individuals inoculated with anthrax vaccine or a paediatric diphtheria-tetanus-pertussis vaccine had no aluminium-related pathology other than a local transient erythema at the injection site.^{14,35} The exacerbated toxicity of the vaccine combinations at 1 SHD level in our model may be explained by the presence of PT combined with a high level of aluminium.

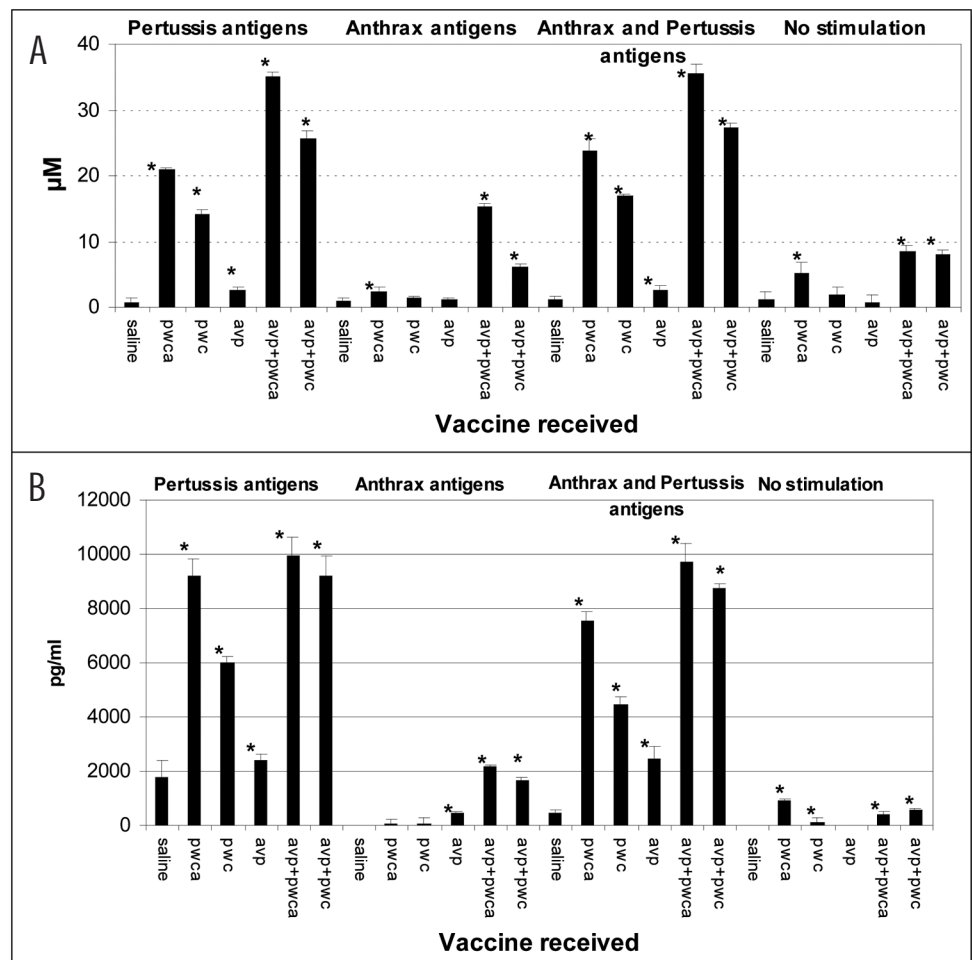


Figure 2. Production of NO (A) and IL-6 (B) by Balb/c spleen cells. Mice injected with saline, anthrax vaccine (AVP), whole cell pertussis vaccine (PWC), whole cell pertussis vaccine adsorbed (PWCA) or the vaccine combinations. Significant increases in NO and IL-6 production compared to the saline group are indicated by * ($p < 0.05$). Error bars indicate standard deviation.

One hypothesis identified a skewed Th2 type immune response triggered by vaccination as the cause of illness among Gulf War veterans.³⁶ This has been contradicted by a study of Danish Gulf War veterans that found no shift towards a Th2 type response.³⁷ In our model, neat pertussis vaccines had a superior immunogenicity and induced predominantly IgG1. However combinations of diluted PWC and AVP produced equal amounts of specific IgG1 and IgG2a after boosting. Spleen cells of Balb/c mice stimulated with pertussis antigens produced a mixture of Th1 and Th2 cytokines as well as NO and IL-6, which are indicators of macrophage activation.^{16,24} These findings agree with previous studies on whole cell pertussis vaccines: plain vaccines elicited a potent Th1 type response and adsorbed vaccines generated a balanced Th1/Th2 type response.^{16,38-40} They do not support the hypothesis of an exclusive Th2 type immune response in Gulf War veterans.

Our findings show that pertussis vaccines act as an adjuvant for AVP and are the major cause of toxicity of the vaccine combination in mice. Our results lend some support to the association of pertussis vaccination with illness in Gulf War veterans as first proposed by Wessely and colleagues although they need to be interpreted with caution.^{4,6} It is difficult to extrapolate observations made in one species to another and this is further complicated by the enormous differences in dosage. On the one hand, a deliberate overdose,

equivalent to 300 human doses, is used in mice to disclose a worst case scenario. On the other hand, the human dosage is selected to provide a protective immune response while minimising toxic effects. Although we showed that at high levels aluminium salts can make a significant contribution to toxicity, a large part of the toxicity of pertussis vaccines for mice can be attributed to PT and LPS whereas the anthrax vaccine components were not toxic for mice. In humans, the reverse situation probably exists because this species tends to be relatively resistant to PT but more sensitive to anthrax toxin. The relevance of the results obtained in mice to the explanation of illness in Gulf War veterans is therefore open to question.

Part of this work can be found at <http://www.mod.uk/issues/gulfwar/research/interact.htm> and was presented at the Conference on Dangerous Pathogens in September 2002 held in Bath UK and the 5th International conference on Anthrax (abstract P646) held in March 2003 in Nice, France.

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