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Multiple vaccine and pyridostigmine interactions: Effects on cognition, muscle function and health outcomes in marmosets ☆

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Abstract

Following active service during the 1990/1991 Gulf Conflict, a number of UK and US veterans presented with a diverse range of symptoms, collectively known as Gulf Veterans Illnesses (GVI). The administration of vaccines and/or the pretreatment against possible nerve agent poisoning, pyridostigmine bromide (PB), given to armed forces personnel during the Gulf Conflict has been implicated as a possible factor in the aetiology of these illnesses. The possibility that long-term health effects may result from the administration of these vaccines (anthrax, pertussis, plague, yellow fever, polio, typhoid, tetanus, hepatitis B, meningococcal meningitis and cholera) and/or PB, have been investigated using a non-human primate model, the common marmoset.

This paper reports the results from three aspects of the study, cognitive behaviour (performance of a touchscreen mediated discrimination task), muscle function (performance of a simple strength test) and general health.

There were no marked long-term changes in cognition, muscle function or health that could be attributed to vaccines and/or PB administration. Statistical differences related to treatments were only observed in two aspects of cognition and one of clinical chemistry. These changes were transient in nature and their magnitude were minor and, in consequence, was not regarded as having long-term biological significance.

Keywords: Vaccines; Pyridostigmine bromide; Non-human primate; Cognition; Muscle function; Health; AChE

1. Introduction

Following active service in the Persian Gulf during the first Gulf Conflict of 1990/1, a number of UK and US veterans presented with a diverse range of symptoms which have become collectively known as Gulf Veterans Illnesses (GVI). A document published by the Parliamentary Office of Science and Technology provides an overview of research relating to these conditions (Border and Norton, 1997), the majority of which have been epidemiological in nature. As well as the complex environment in which they were deployed in the Middle East, UK troops were vaccinated with two anti-biological warfare agent (anti-BWA) vaccines (selected on the basis of contemporary military intelligence), an additional vaccine adjunct and a range of health and hygiene (H&H) vaccines appropriate for deployment to that region. In addition, pyridostigmine bromide (PB) was taken as a pretreatment to help preserve life in the event of nerve agent poisoning.

The study reported here was part of a multi-stage programme of work commissioned by the Veterans Policy Unit Gulf Veterans' Illnesses (VPU GVI) of the Ministry of Defence with advice from an independent, cross-disciplinary panel of experts who have overseen its progress. The work programme was designed specifically to assist in the interpretation of data emerging from the epidemiological studies and addressed the effects of multiple vaccines and PB. This included a mouse study undertaken at the National Institute for Biological Standards and Control (NIBSC) (Rijpkema et al., 1995) and a study of Dstl staff members who were vaccinated against anthrax in the course of their employment. The findings reported here are drawn from a study designed to assess whether administration of the range of vaccines administered to UK military personnel

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during the 1990/91 Gulf conflict, with and without PB, gives rise to long-term adverse effects in a non-human primate model. Results from preparatory phases of this study in guinea pigs and marmosets, which identified dose levels and dose combinations of vaccines and PB that induced measurable responses without producing unacceptable short-term effects, have been reported previously (Griffiths et al., 2001).

The study aimed to investigate whether indices that reflect the most frequently reported signs and symptoms by Gulf veterans, e.g. impaired cognition, sleep disturbances and fatigue (Coker et al., 1999; Unwin et al., 1999; Lee et al., 2001, 2002; Cherry et al., 2001), are observed following administration of multiple vaccines and/or PB. Thus, any changes in physiology, immune response and central function over 18 months following administration could be detected and interpreted and this paper reports the results of cognitive testing, muscle function and general health elements of the marmoset study. The effects on other indices (including sleep, brain electrical activity, pathology and immunology) will be reported in separate publications.

1.1. Cognitive behaviour

Cognitive problems were frequently reported by veterans of the 1990/91 Gulf conflict and these were generally manifested as subjective deficits in concentration ability and memory. The incidence of cognitive dysfunction among Gulf veterans varies between epidemiological studies reported to date. In a questionnaire-based study, Cherry et al. (2001) noted 'frequently' reported cognitive symptoms which occurred twice as often in Gulf veterans than matched controls. Unwin et al. (1999) also found self-reported symptoms occurring at a rate twice that of Bosnia veterans and undeployed Era veterans. No evidence, however, of any major neuropsychological impairment in Gulf veterans was found by David et al. (2002) although Lange et al. (2001) found impairments in attention, concentration and information processing in veterans complaining of fatigue, after taking into account post-war morbidity.

Haley et al. (1997a) suggested 3 syndromes among his study group of 249 veterans, where 175 veterans were complaining of ill health; impaired cognition, characterised by problems with attention, memory and reasoning; confusion-ataxia and central pain. Subsequently, indications of decreased functional neuronal mass in the basal ganglia and brainstem were reported (Haley et al., 2000a). These findings were reported to be associated with altered levels of plasma homovanillic acid and the suggestion was made that 'Gulf War Syndrome' was a neurological illness, in part, related to injury to dopaminergic neurons in the central ganglia (Haley et al., 2000b). Moreover, an NAA/creatine ratio, indicative of hippocampal dysfunction, has also been reported although none of the symptomatic Gulf veterans reported problems with memory or attention (Menon et al., 2004). In other questionnaire based studies of US Gulf veterans, there were 11–33% self-reported cognitive problems compared to 2–11% of military controls (Fukuda et al., 1998; Kang et al., 2000; Murphy et al., 1999; Gray et al., 1999; Proctor et al., 1998).

Other than some assessments of the effects of reversible or irreversible cholinesterase inhibitors, there have been relatively few preclinical studies (e.g. Scremin et al., 2003; Pearce et al., 1999; Muggleton et al., 2005) which have concentrated on effects following recovery to baseline levels of cholinesterase (ChE) levels.

In the present study, an attentional set shifting task, with an additional component to investigate aspects of memory, was used to investigate cognitive changes which could be related to the reported symptoms of "difficulty concentrating" and "forgetfulness" in man. The tests were selected from the Cambridge Automated Neuropsychological Test Automated Battery (CANTAB), a computer based system which has been extensively used in the characterisation of cognitive function in both non-human primates and man. The attentional set shifting test employed was the intradimensional/extradimensional (ID/ED) shift, an analogue of the Wisconsin Card Sort Test (Grant and Berg, 1948). It involves a number of rule changes which have been shown to be differentially sensitive to CNS disruption e.g. brain lesions (Roberts et al., 1992) and pharmacological interventions (Sahakian and Coull, 1993). In order to facilitate long-term repeated presentation of the test, the sequence of stages was adapted from previous human (Sahakian et al., 1990) and non-human primate (Roberts et al., 1988) studies. The home cage approach to testing, which has previously been shown to be practicable (Crofts et al., 1999) and conducive to rapid training and task acquisition (Muggleton et al., 1997), was employed. We have previously used this approach to study the effects of a low dose of sarin (Pearce et al., 1999) and a range of doses of diazinon (Muggleton et al., 2005).

1.2. Muscle function

Muscle weakness has been reported by Gulf veterans, although less frequently reported than attentional deficits. Cherry et al. (2001) found that "feeling too weak to complete what you start" was self-reported twice as often by Gulf veterans than the non-Gulf control group. Amongst 3000 veterans attending the MOD's Gulf Veterans' Medical Assessment Programme, an average of just under 40% reported joint and muscle aches and pains but not weakness per se. There were no objective findings of neuromuscular pathology either in veterans who self-reported muscle weakness, fatigue and myalgia (Amato et al., 1997) or in a comparative study of Gulf veterans and veterans from the Bosnian Conflict (Unwin et al., 1999). It has been hypothesised that some of the symptoms may be related to minor impairments of nerve conduction e.g. carpal tunnel syndrome (Sharief et al., 2002) or mitochondrial insufficiency (Rose et al., 2004) which may not be specifically related to service in the Gulf conflict.

The effects of chronic PB administration in a simulated desert environment were examined by giving healthy soldiers PB for 7 consecutive days (30 mg orally, every 8 h) (Cook et al., 1992). Whilst there was a trend towards decreased grip strength while receiving PB, chronic administration did not negatively impact the soldiers' ability to perform physical work over repeated days in a desert environment. Gray et al. (1999), however, found a significantly lower mean handgrip strength measurement in Gulf veterans who complained of muscle weakness compared to other Gulf veterans.

In vitro studies by Drake-Baumann and Seil (1999) on the effects of PB on neuromuscular junctions have shown that acute

exposure to PB caused potentiation of neuromuscular activity and continuous exposure produced progressive decrease in contractile ability of muscle fibres.

As part of the present investigation, an index of muscle function was assessed using a novel technique with behavioural and motivational elements (Stevens et al., 2005).

1.3. Weight and general health

In the present study, marmosets were weighed on a weekly basis in keeping with sound animal husbandry practices. Changes in bodyweight are invariably a good indicator of underlying illness or poor condition. The animals were observed on a daily basis to monitor general wellbeing and identify adverse clinical signs.

Weight changes have been reported by Gulf veterans (Coker et al., 1999, Lee et al., 2001, 2002). "A loss in weight" was reported as a symptom by both Gulf veterans and non-Gulf controls (Cherry et al., 2001). In a questionnaire completed by US Air Force Gulf veterans and a non deployed control group, 17% of Gulf veterans reported having 'Unintended weight gain ≥10 lb', and 15% reported that this had lasted for over 6 months. 8% of non-deployed military personnel also reported this and 6% said that it had lasted for over 6 months (Fukuda et al., 1998).

Blood samples were analysed for key clinical chemistry markers at baseline and two points during the study. These markers reflect liver, muscle and bone (isocitrate dehydrogenase (ICDH), alkaline phosphatase (ALP) and alanine transaminase (ALT)) as well as kidney function (urea and creatinine). Samples were also taken during the first period to monitor the PB induced inhibition of acetylcholinesterase (AChE).

2. Materials and methods

The study was carried out in accordance with the UK Animals Scientific Procedures Act 1986.

2.1. Animals

Forty-eight adult common marmosets (24 vasectomised males and 24 females, weighing 331–565 g and aged 2–5.5 years at the start of the study) were used. All animals were housed in long-term, male–female pairings. Each pair of animals was housed in 4 interlinked cage units, each of 72(H)×47(W)× 60(D) cm^3, connected by one vertical and two horizontal rigid linkers. Animal rooms were maintained at 25 °C on a 12 h light/dark cycle with 30 min 'dawn' and 'dusk' periods. The room light level during the light phase was 350–400 lux at 1 m above the floor. Animals were fed 20 g of primate pellets (Special Diet Services, Witham, Essex, UK) each day with a supplement of fruit. Water was available at all times and, apart from periods of muscle function testing, animals had unlimited access to forage material (small items of preferred food dispersed in woodshavings).

2.2. Study design

Behavioural, physiological and immunological parameters were monitored over 21 months. The experimental period was divided into seven 3-month periods (Fig. 1); prior to the first (baseline) period, each animal was trained to perform a number of behavioural tasks and was implanted with a radiotelemetry transmitter (for monitoring the electroencephalogram, EEG). The body of the single channel transmitter was placed, under appropriate anaesthesia and postoperative analgesia, within the peritoneum and the electrodes placed over selected points of the dura using the methods described by Pearce et al. (1998). At the end of this training period, animals were assigned to one of four treatment groups (Fig. 1; $n=12$ animals per group) based on, firstly, their gender (equal numbers of males and females), then cognitive performance and finally, bodyweight. The treatment groups received a combination of vaccines (or saline controls) and PB (or saline control) as follows: saline+saline (S+S); saline+PB (S+P); vaccines+saline (V+S) and vaccines+PB

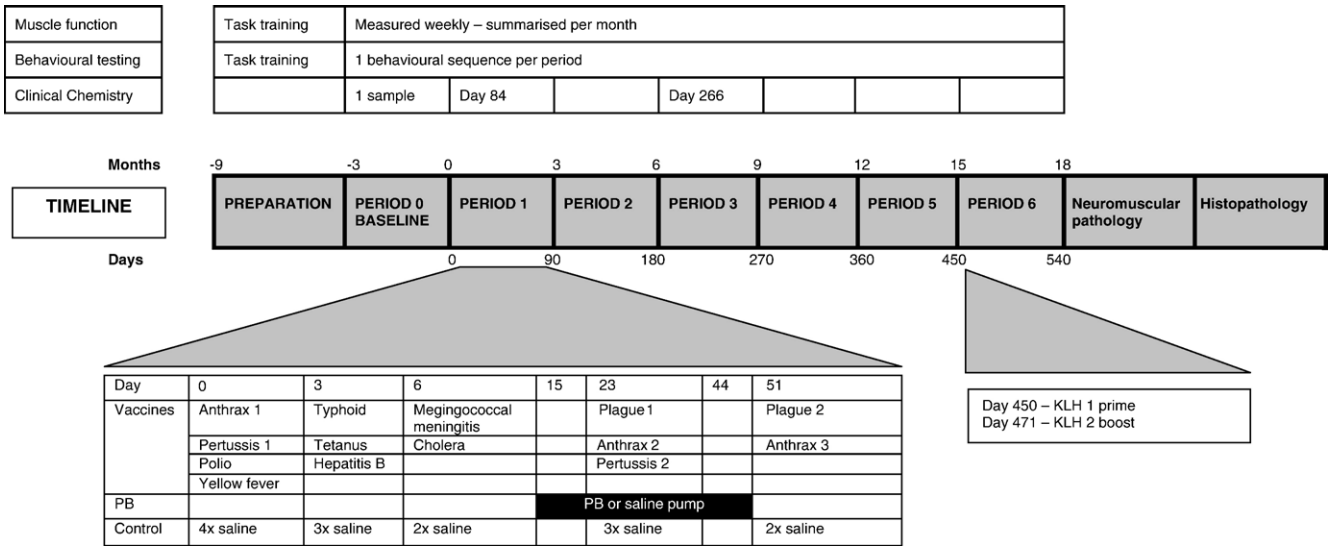

Fig. 1. Outline of experimental protocol. There were four treatment groups which received a combination of vaccines (or saline controls) and PB (saline control): saline+saline (S+S); saline+PB (S+P); vaccines+saline (V+S) and vaccines+PB (V+P).

(V+P). Both animals in each pair received the same treatment. For logistical reasons, it was necessary to stagger the starting times of the study. Animals were monitored in 6 sets of 8 animals and each set consisted of 2 animals from each treatment group.

Over the first 51 days of the second experimental period (Period 1), animals were vaccinated with 1/5th human dose, or administered vehicle, according to the schedule in Fig. 1. The dose chosen was based upon results obtained in preparatory phases of this study in guinea pigs and marmosets, which identified dose levels and dose combinations of vaccines and PB that induced measurable humoral responses without producing unacceptable short-term effects (Griffiths et al., 2001, submitted for publication). Other than yellow fever (0.05 ml) administered subcutaneously and polio (0.027 ml) given orally on maltloaf, vaccines were administered as intramuscular injections at various sites in volumes of 0.1–0.2 ml.

The first day of vaccine administration was designated as day 0. On day 15, animals were anaesthetised (0.2 ml/kg i.m. diazepam followed by 1.0 ml/kg i.m. Saffan) and implanted subcutaneously below the right scapula with primed mini-osmotic pumps containing PB (500 μg/kg/day) (groups S+P and V+P) or sterile saline (0.9%) (groups S+S and V+S). Pumps were removed under anaesthesia on day 44. At the commencement of period 6, animals were challenged with keyhole limpet haemocyanin (KLH). (This challenge, along with the markers of immunological function throughout the study, assessed immune status and tested responsiveness to this hitherto unseen antigen. These data will be reported elsewhere.) Animals were culled 18 months after the first vaccinations when pathology and a series of ex vivo electrophysiology studies were carried out. Throughout the study, all members of the research team carrying out the practical aspects of the work, including data collection, were blind to the treatment administered to each animal.

2.3. Cognitive behaviour

2.3.1. Equipment

The cognitive test system was supplied by Cambridge Cognition (now Campden Instruments Ltd., Loughborough, UK) and utilised either a CRT high resolution colour visual display unit (VGA resolution) or a 12.1 TST flat screen. Both were fitted with Intasolve Microvitec touch arrays and were mounted on a movable platform, the height of which allowed the screen to be positioned against a rigid cage extension or horizontal linker. Bars forming the mesh on the cage extension were at least 1 cm apart and did not impede the animals' ability to see or touch the screen. A metal licker was attached centrally to the system in front of the visual display unit to dispense the reward of banana flavoured milkshake for making a correct response during CANTAB test sessions.

Visual stimuli were generated either by a 486 slc 33 MHz PC or a Pentium 233 MHz PC and consisted of either blue filled shapes (maximum 32 mm×32 mm) or white lines (maximum height and width 38 mm). The groups were counterbalanced such that animals using different systems were divided equally among the treatment groups and there were no statistically significant differences between treatment groups at baseline.

2.3.2. Testing cognitive performance

The touchscreen mediated task was presented to animals in their home cage whilst they were temporarily separated from their partner. In this way, visual and vocal contact with all the other animals was maintained whilst the individuals being tested had sole access to the test equipment (animals were housed in rooms of 16). Animals were first trained to respond to the visual stimuli by a well established method (Pearce et al., 1998; Crofts et al., 1999). The behavioural task was based on the attentional set-shifting paradigm from CANTAB, with an additional factor incorporated into the task (a 'new learning' (NL) and retention (RT) component). A single multistage sequence was presented during each period using new stimuli on each occasion (with the exception of the retention stage) and the design was counterbalanced such that different sequences of rewarded stimuli were employed for each animal within each test sequence (a typical example of which is shown in Fig. 2). Animals were tested daily and performed the behavioural task for either 15 min or 60 trials or until the particular stage of the sequence was completed (whichever came first). Icons remained on the screen until touched i.e., they did not disappear after a predetermined time. Correct responses were indicated by a 4 kHz tone and rewarded by access to up to 0.2 ml banana milkshake for up to 5 s following the response. There was an intertrial interval of 3 s between the end of delivery of reinforcer and presentation of the next stimuli. No tone was sounded for incorrect responses, no reward was available and a 2 s time out was introduced. In order to enable a new sequence to begin at the start of each three month period, each sequence was designed to be completed by the slowest animal within 13 weeks, such that each animal completed 7 different sequences over the experimental timeline (baseline and 6 post-treatment periods).

A criterion of 8 successive correct responses in the same session was used to determine that a stage had been successfully completed. Other than in the baseline sequence (period 0), where a new discrimination was learnt (NL), the sequence began with a retention (RT) of this previously learned discrimination from the NL stage using blue shapes. Pairs of stimuli appeared simultaneously and randomly to the left and right of the centre of the screen. The stimuli pairs were balanced so that half the animals had one stimuli correct and the other half had the other stimuli correct. This was designed to avoid any effects caused by the stimuli themselves (i.e. one stimuli being more appealing than the other to the animals). After the retention component, each sequence consisted of a simple discrimination (SD) (blue shapes) followed by a reversal (SR), then a compound discrimination (CD) (white lines randomly overlaid but irrelevant) which was then reversed (CR). This was then followed by an intradimensional shift (IDS) and reversal (IDR) (using all novel stimuli, shapes relevant), which was followed by a probe stage (novel white lines but still irrelevant). The sequence finished with an extradimensional shift (EDS) (4 novel stimuli but lines now correct and shapes irrelevant) and then a reversal (EDR). After this final stage no further testing was carried out on individual animals until the start of the next period. Completing a stage automatically started the next stage within the same session, except in the case of NL/RT, CR and the probe stage when completion ended the session. In these cases the next stage was introduced in the next session.

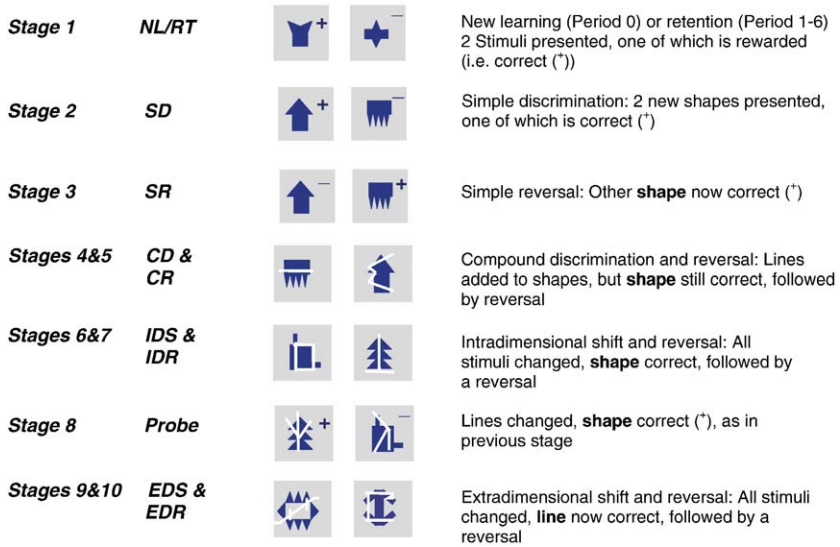

Fig. 2. An illustration of typical cognitive sequence – one performed per period. Icons appeared randomly either side of the screen and, in stages where they occurred, white lines randomly overlayed blue shapes. All icons changed for each sequence, apart from the first two icons which remained the same throughout the study, one of which always being correct (retention element). Stages progressed within each sequence when 8 successive correct responses were made within a single session. Sessions terminated at 60 responses or at 15 min, whichever was sooner. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4. Muscle function

2.4.1. Strength testing equipment

The test equipment was designed and constructed in house at Dstl, Porton Down. It was largely constructed from clear Perspex™ and was attached to the front of the home cage during training and testing. Marmosets were trained to displace a T-bar, connected to variable weights via a pulley system, to open a simple gravity fed dispenser containing a food reward (a piece of chopped nut) which was then retrieved from a chamber below (Stevens et al., 2005).

After training, the task was presented once a week and involved animals displacing increasing weights until they were unable or unwilling to displace additional loads. The starting point for each session was determined by an individual's performance at the end of the previous session. If animals were unable to pull this amount, the weight was lowered until success was achieved. There were no more than five weight increases per session which comprised a maximum of 18 trials. The maximum weight pulled per test session for each animal was compared across groups.

2.5. Acetylcholinesterase analysis

Blood samples for AChE analysis (0.5 ml into EDTA) were collected from conscious, minimally restrained animals on at least two occasions during the baseline period (period 0) and subsequently on days, 15, 29 and 79. All samples were stored at −70 °C prior to assay.

AChE activity in whole blood and plasma was determined by a modified method of Ellman et al. (1961) and erythrocyte cholinesterase activity calculated by subtraction.

2.6. General health/clinical chemistry

2.6.1. Blood sampling

Clinical biochemical markers were measured on whole blood samples (0.5 ml) drawn by femoral venepuncture from conscious animals. Sampling occurred at three points in the study. These were intended to be representative of early (baseline period), mid (day 84) and late stages (day 266), relative to the treatment protocols (final vaccinations were administered on day 51). Blood was stored in heparin at 4 °C until analysis.

2.6.2. Analysis of blood samples

All parameters were assayed using a Technicon RA1000 analyser which measured ALP, ALT, ICDH, urea and creatinine (CREAT).

2.7. Statistical analysis

The overarching strategy for statistical analysis was devised in collaboration with the Statistical Services Department of the University of Reading, who undertook the analysis on an independent consultancy basis. Individual variables were analysed using a mixed model approach for repeated measurements. The mixed model included fixed effects for set, gender, treatment group, time and the treatment × time interaction. An unstructured (i.e. completely general) variance–covariance structure was used to model the variances over time and the covariances amongst the time points. The baseline value of the variable was also included in the model. Set, treatment and gender were all factors, whereas the baseline value was a covariate. Where appropriate, if the profiles over time for a variable showed no evidence of non-linearity, time was incorporated in the model as

a continuous variate rather than as a factor. In the elements of the study reported here, this applied only to the new learning/retention, simple discrimination and extra dimensional reversal elements of the cognition part of the study. The treatment main effect and the treatment×time interaction were of key interest, since together they investigate a null hypothesis of no difference between the treatment profiles over time.

Variables in the study that did not have a repeated measurement structure or only had data at two or three time points – for example ChE activity – were analysed by fitting a general linear model to the data (separately for each time point) with effects for set, gender and treatment. As above, these were all included in the model as factors. Baseline data were incorporated into the analysis as a covariate.

In order to investigate the effects of the treatments in more detail, particularly the effect of PB and vaccines, the analysis also included the following two components which further explored the overall treatment effect (and the treatment × time interaction):

Factorial treatment structure (the effects for vaccine and PB and their interaction)
- the main effect of vaccines (V+S, V+P)
- the main effect of PB (S+P,V+P)
- the vaccines × PB interaction

(plus their interactions with time, when the data were repeated measurements).

Comparisons with control (S+S)
- comparison of S+P versus S+S
- comparison of V+S versus S+S
- comparison V+P versus S+S

(plus their interactions with time, when the data were repeated measurements).

The treatment groups were also compared at baseline to determine whether there was a difference amongst them prior to the start of treatment. Where data were not normally distributed, they were transformed prior to analysis and the natural log transformation was usually employed. The results of all analyses were presented as least squares means for the four treatments (over time), plus standard errors. If no main effect or interaction was apparent in the initial ANOVA analysis ($P<0.1$), any significant effects in the factorial analysis and comparisons with control were discounted. Selection of this level of significance ensured consideration of major potential treatment effects whilst preventing undue emphasis on effects which might occur by chance in a study where over 200 comparisons in total were being made.

Subsequently, a critical examination of the statistically significant differences observed was undertaken to determine whether any differences observed were of biological significance.

3. Results

3.1. General observation

Throughout the study there were no obvious changes in the animals' behavioural repertoire as gauged by their interactions with humans and conspecifics. At no stage were there any localised reactions to the injections or overt clinical signs which could not be explained by factors relating to the radiotelemetry implants or the blood sampling regime.

3.2. Cognitive behaviour

Fig. 3 shows data obtained for each of the measures of cognitive behaviour, expressed as mean number of errors made before a criterion of 8 successive correct responses in a single session was achieved. Of the 48 animals used in the study, only 2 (1 from S+S and 1 from S+P) did not undergo behavioural testing because these individuals were added to the study at a relatively late stage to replace two others removed for non-study related reasons. There were no statistically significant differences between treatment groups at any measure during baseline assessment.

The general pattern of response showed that, in all groups, relatively few errors were made on the retention stage because the same icons had previously been seen in the NL stage and only one icon was correct throughout the study. On the other stages, observation of the data suggests that more errors were made during the reversal element when animals were required to unlearn a previously learnt rule, than on the initial discrimination. In addition, the extradimensional shift, on average, induced more errors than the intradimensional shift.

As testing was animal-paced, there was variability between individuals; some animals completed the ten stage sequence within 3 weeks whereas others required the full 3 months available. Overall, 322 sequences were presented and on a total of 21 occasions, 12 of the 46 animals did not complete the full sequence within the allotted time. These animals came from all groups, 2 from each of S+S and V+S and 4 from each of S+P and V+P groups. On the majority of these occasions only the EDR sequence remained uncompleted and the statistical model was sensitive to this, in that it excluded data only from those aspects of the individual's performance.

3.2.1. New learning/retention

In all groups, fewer errors were made as the period of study progressed ($F(1,35)=23.7$, $P=0.001$). Overall, the control (S+S) group made fewer errors than any of the treatment groups (S+P: $F(1,35)=3.95$, $P=0.055$, S+V: $F(1,35)=6.06$, $P=0.019$, V+P: $F(1,35)=4.05$, $P=0.052$), although the magnitude of the total number of responses required to complete this stage was relatively low.

3.2.2. Simple discrimination

There was a time effect, in that the number of errors increased during the study period, irrespective of treatment (F 1,35)=7.88, $P=0.008$). No other significant effects were observed.

3.2.3. Simple reversal

There were some short-term effects in that during the middle part of the study all groups of animals made fewer errors (F (5,35)=6.85, $P=0.0002$). There were no treatment related effects.

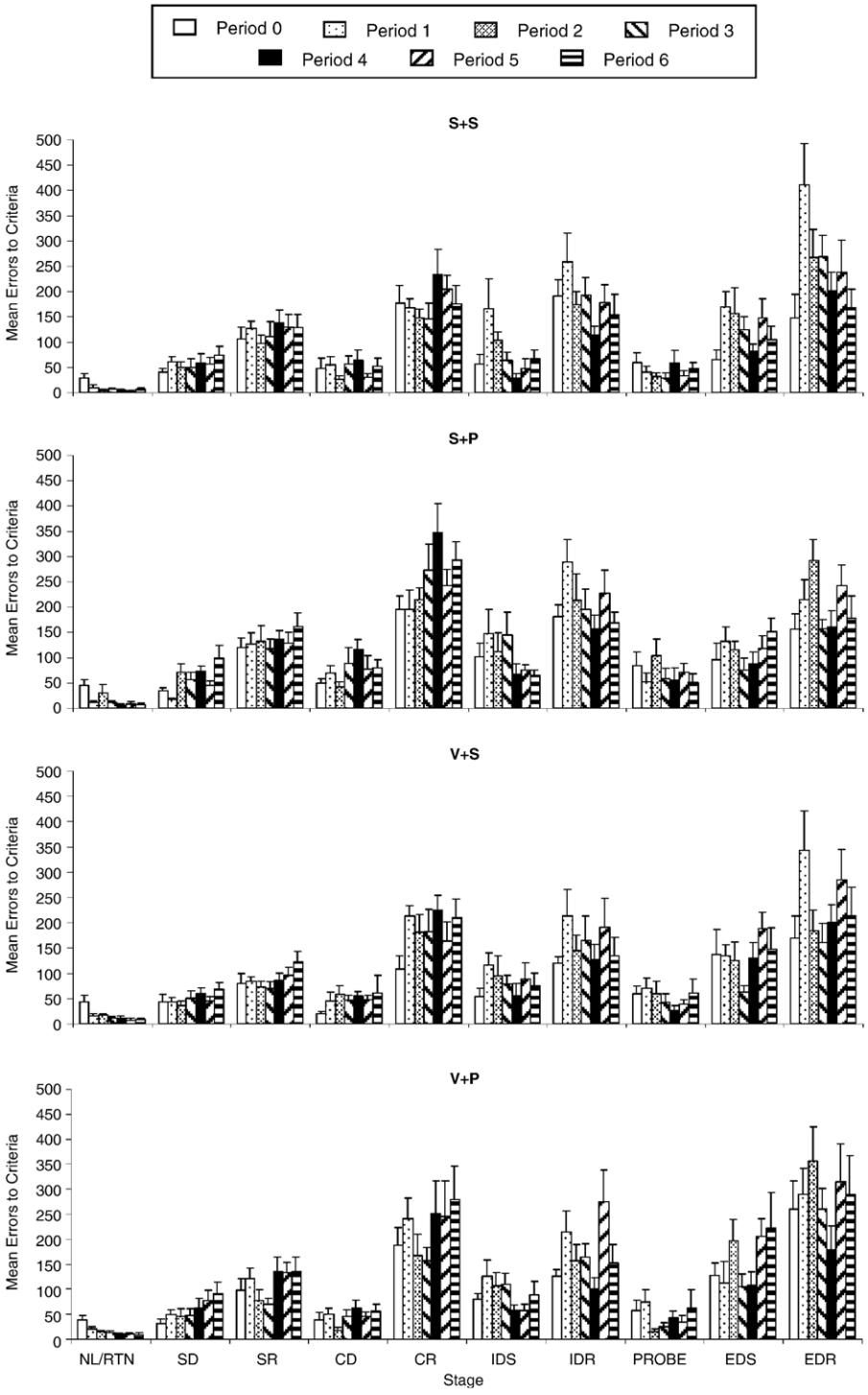

Fig. 3. Performance of cognitive tests for each treatment group. Each block of bars represent 1 stage across time. NL refers only to period 0 (baseline). Error bars = S.E.M.

3.2.4. Compound discrimination

There were some mixed effects over time which applied to all groups ($F(5,35)=4.79$, $P=0.002$) but no effects of treatment.

3.2.5. Compound reversal

There were overall effects of treatment ($F(3,35)=3.09$, $P=0.04$) and period ($F(5,35)=2.88$, $P=0.028$) which were independent of each other. Pyridostigmine treated animals (S+P) and vaccinated animals (V+S) made more errors during the middle part of the study than controls (S+S) ($F(1,35)=6.03$, $P=0.019$ and $F(1,35)=5.80$, $P=0.022$, respectively). No effects were seen in the (V+P) group, when compared with control (S+S).

3.2.6. Intradimensional shift and intradimensional reversal

There was an overall effect of period, independent of treatment, in that all animals made fewer errors with time ($F(5,35)=7.59$, $P=0.0001$ and $F(5,35)=4.96$, $P=0.001$, respectively).

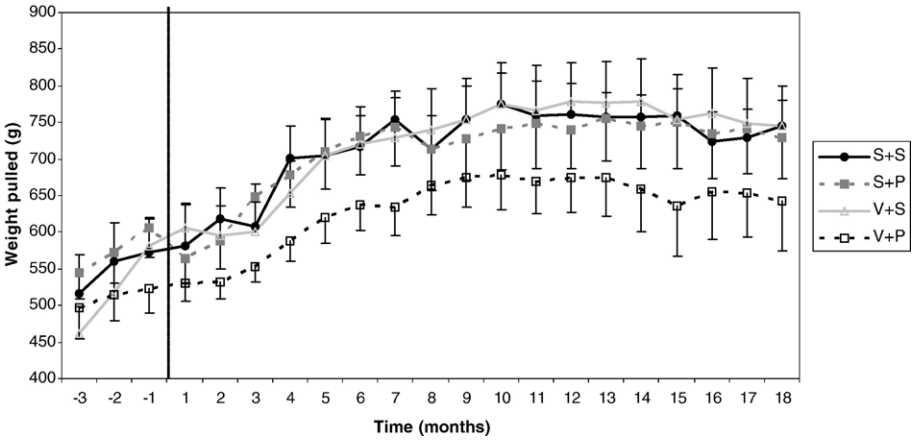

Fig. 4. Performance of strength test for each group across time. Error bars = S.E.M.

3.2.7. Probe, extradimensional shift

There were no significant effects of either period or treatment.

3.2.8. Extradimensional reversal

There was a statistically significant effect of time ($F(1,31) = 12.71$, $P = 0.001$), in that all groups made fewer errors with time, which was independent of treatment.

3.3. Muscle function

For logistical and technical reasons, a number of animals either did not perform this task or were excluded from the results if they had undergone an additional surgical intervention during the course of the study. Analysis of data was carried out on $n = 32$ animals (S+S = 8, S+P = 6, V+S = 10, V+P = 8).

During the first 9 months (−3 to +6) the mean weight pulled by all treatment groups increased to a steady state of approximately 600–750 g ($F(5,21) = 11.57$, $P = 0.0001$) (Fig. 4). There was no evidence of any relationship between weight pulled and bodyweight at baseline and, in consequence, this factor was not included as a covariate. There were no effects relating to treatment.

3.4. General health

3.4.1. Bodyweight

Bodyweight increased over the period of the study in all groups by 10–30 g ($F(5,36) = 26.37$, $P = 0.0001$) (Fig. 5). There were no significant effects on bodyweight which were related to treatment. The V+P group had lower bodyweight throughout the study (including the baseline).

3.4.2. AChE

Erythrocyte AChE activity was inhibited by 28.9% (range = 15.3–47.9%) in the PB only group (S+P) and by 30.0% (range = 10.7–44.5%) in the V+P group on day 28 (i.e. during the period of PB infusion). Saline infusion (groups S+S and V+S) had no effect on levels of AChE activity. AChE activity had returned to control levels when tested on day 98.

3.4.3. Clinical chemistry

The majority of the markers of clinical biochemistry studied remained stable over the 3 time points measured. There was a decrease in ALP on day 84 in the group receiving PB (S+P) compared with control (S+S) ($F(1,3) = 5.77$, $P = 0.02$) and groups

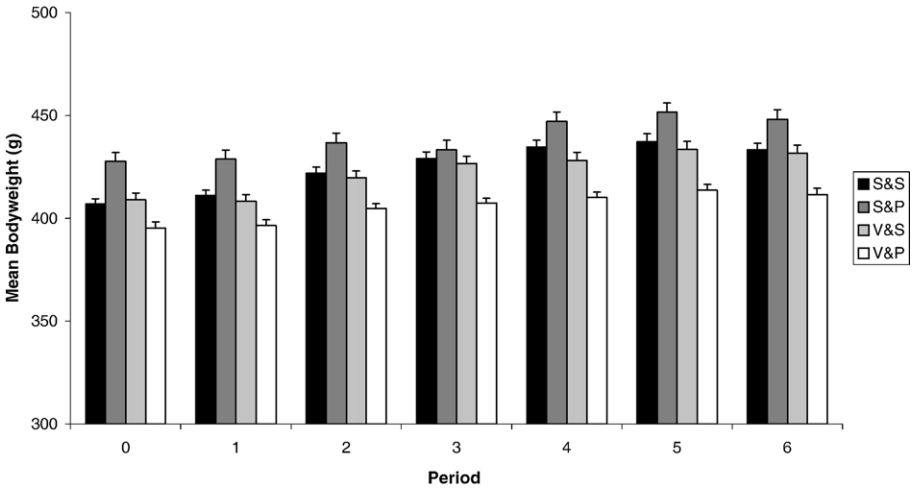

Fig. 5. Mean body weight during each time period for each treatment group. Error bars = S.E.M.

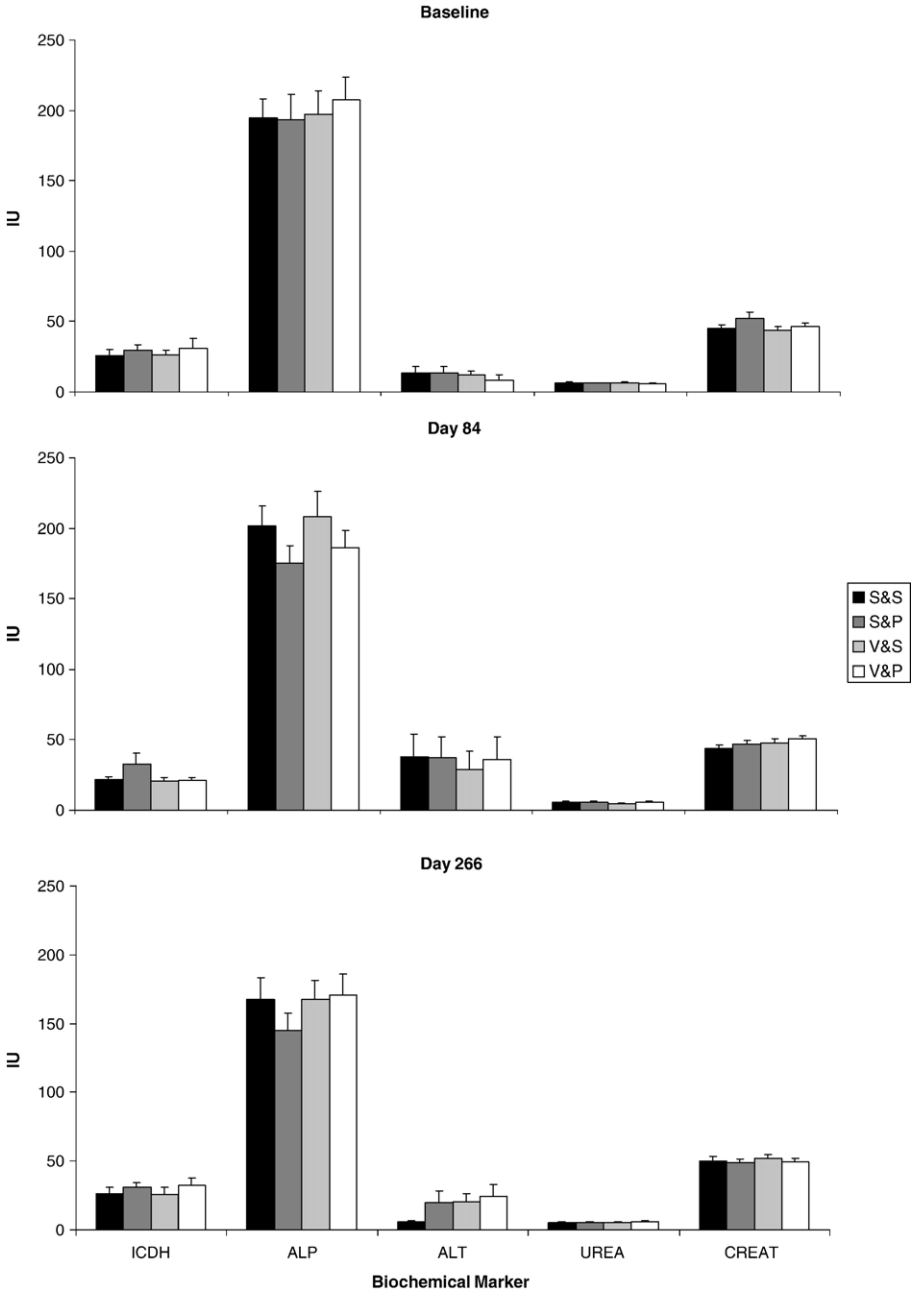

Fig. 6. Changes in clinical biochemistry markers at baseline and days 84 and 266 for each treatment group. Error bars = S.E.M.

receiving PB (S+P and V+P) had lower values than those not receiving PB (V+S and S+S) ($F(1,34)=7.79$, $P=0.009$). There were no other time or treatment related changes in the selected biochemical markers (see Fig. 6).

4. Discussion

In general, all animals remained healthy throughout the 21 months of this study, as assessed by the subjective and objective measures reported here. This period included an intensive schedule of blood sampling (fortnightly in periods 0–3, monthly thereafter), surgical procedures (telemetry device implantation plus osmotic pump implantation and removal), administration of vaccines or saline (9 given by 13 injections and 1 orally), regular handling, cognitive testing (daily) and strength testing (weekly).

4.1. Cognitive behaviour

Data obtained in the present study show no indication of major long-term disruption of behavioural performance. The overall pattern of response to the series of cognitive tasks was consistent with previous studies in this laboratory (Pearce et al., 1999; Crofts et al., 1999; Muggleton et al., 2005;) and others (Roberts, 1996; Spinelli et al., 2004). Changes in cognitive behaviour which have resulted from cholinergic manipulation have been shown following administration of a range of

compounds e.g. scopolamine, arecoline, (Ridley et al., 1986), physostigmine, tacrine (Sahakian and Coull, 1993) organophosphates (Muggleton et al., 2003) as well as in lesion studies (Roberts et al., 1992). In the present study, PB inhibited erythrocyte AChE by approximately 30%, the target level of inhibition for nerve agent pretreatment, and control levels of ChE were restored when tested on day 98. Any PB related effects beyond this time would not be concurrent with ChE inhibition.

There has been much discussion about the role of PB in Gulf health (see Golomb and Anthony, 1999 (Rand Report)). Whilst PB has been reported to have direct CNS effects in the presence of stressors (Friedman et al., 1996, Kaufer et al., 1999), others have found no evidence to support this claim (Lallement et al., 1998; Sinton et al., 2000; Grauer et al., 2000). In addition, any long-term effects reported in humans are not likely to be a result of a current AChE inhibition.

Changes in performance which were long term but unrelated to treatment were likely to be a result of animals becoming familiar with the test sequence and gradually increasing their performance levels with time. Any differences, which were apparent at only selected periods, and independent of treatment, could have been a function of certain icons being easier to differentiate from each other than other combinations.

There was a significant effect on the memory component of the study, as evidenced by RT performance, in all treatment groups compared to control although this effect appears more pronounced in the early stages of the study. By the end of the study period, however, there were no differences across groups and so the possible short-term deficit in memory is unlikely to correlate with the long-term memory problems reported by some veterans.

Despite intensive iterative analysis to explore the measures of cognitive performance, there is no compelling evidence to suggest long-term detrimental effects that could be attributable to either vaccines, PB or the combination thereof.

4.2. Muscle function

The relatively simple, novel method used to assess in vivo "muscle function" in this study has generated quantifiable data, although its interpretation is somewhat limited because it has not yet been widely used in other pharmacological investigations. While it is clearly not ideal to use such a relatively unproven approach in such a challenging study, it was felt to be essential to include a measure of muscle function and motivation to reflect reported neuromuscular sequelae.

The design of the apparatus used in this study did not preclude animals from developing different strategies and techniques for performing the task (e.g. use of one or both arms, with or without the use of one or both legs) and therefore the test should perhaps be considered a general measure of muscle strength and motivation rather than a direct index of muscle function per se. Factors, such as health status and appetitive drive would also be expected to affect performance. Moreover, in the analysis of muscle function, it was also decided to account for additional surgical interventions because inspection of the raw data showed that some animals did not engage in the task during the recovery period and preoperative levels of performance were generally not restored until several weeks later. In some cases performance was never fully restored. It is interesting to note that these surgical interventions did not interfere with the animals' motivation to perform the cognitive task which required much less physical effort.

Administration of high doses of PB (inducing ca 90% AChE inhibition) to animals has previously been shown to produce damaging effects in muscle tissue (Bowman et al., 1989). PB has also been shown to accumulate in muscles after chronic dosing (Somani, 1983) and cause neuromuscular damage (Hudson et al., 1985, 1986). Despite this, PB has long been used safely for the treatment of myasthenia gravis; patients have few side effects, albeit in the context of an already compromised nervous system, despite taking much higher doses of PB (up to 500 mg, three times per day) than is recommended as a nerve agent pretreatment (30 mg, three times per day). There is conflicting evidence of neuromuscular problems from studies conducted in veterans. The majority of studies that have shown muscle weakness or fatigue to be present in Gulf veterans have used self-reporting methods (Cherry et al., 2001; Coker et al., 1999; Lee et al., 2001, 2002; Unwin et al., 1999). Some studies have found evidence of a higher proportion of abnormalities in muscle strength in the lower extremities of ill veterans (Haley et al., 1997b), whilst others could find no objective evidence for peripheral neurological disorders in Gulf veterans (Sharief et al., 2002). Kaiser et al. (2000) found no association between reported PB intake and handgrip strength in a large group of US Gulf veterans when compared with a non-deployed group. Others have found a higher incidence of self-reporting of fatigue in veterans from other conflicts where PB was not administered to troops (De Vries et al., 2000).

Previous studies on muscle function and fatigue have also investigated the effects of AChE inhibitors on 'jitter', a measure of the variability of response when single fibre electromyograph (SFEMG) potentials are recorded from a muscle fibre stimulated via its afferent nerve. This can be increased in conditions which feature disorders of the neuromuscular junction, such as myasthenia gravis. It has also been observed to reversibly increase following exposure to organophosphate AChE inhibitors in animals (Kelly et al., 1990; Smith, 1993) and man (Baker and Sedgwick, 1996). This increase can be blocked by pretreatment with PB (Smith, 1993), which has no effect when given alone (Kelly et al., 1992). It is not known how vaccines and PB may have interacted in the marmoset and in any future studies where muscle fatigue may be a factor, it would be of considerable interest to monitor SFEMG and/or electromyogram in conjunction with muscle function testing.

4.3. Clinical chemistry and general health

There were no unexpected findings in the clinical biochemistry results. The mean bodyweight of all treatment groups increased throughout the study. Minor fluctuations in weight were recorded in individual animals but these were generally related to events outside the treatment regimens. These fluctuations were generally transient in nature and did not affect the period averages taken as measures for the statistical analysis.

The marmosets were young adults (2–5.5 years of age) at the outset of the study and it is likely that the weight gain is not far from the expected range of animals maintained under these husbandry conditions. The growth curves of guinea pigs administered vaccines and PB, using a schedule identical to the one employed in this study, were also unaffected (Griffiths et al., 2001). Evidence of consistent change in bodyweight in veterans reporting Gulf related illnesses remains equivocal.

5. Conclusion

Overall, there were no long-term adverse effects on general health, cognitive performance or muscle function that could be associated with administration of either vaccines or PB or the combination thereof.

On occasion, statistically significant differences between treatment groups were detected. The magnitude and transient nature of the majority of these differences, however, lead to the conclusion that they are not likely to be of clinical significance. Based on the results reported here, cognitive and muscle function deficits reported by Gulf War veterans are unlikely to be attributable to the pretreatments administered. These outcomes, however, must be considered alongside those from other elements of the study, when these have been published.

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