# Factors affecting the immunogenicity and protective efficacy of routine childhood immunisations

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# Abstract

This thesis has examined the effect of adverse storage on the immunogenicity of pertussis, diphtheria and tetanus vaccines, the protective efficacy of pertussis vaccines and the effect of premature birth on antibody responses to routine childhood immunisations.

**Methods**

**Murine Immunogenicity studies**

Female Swiss outbred and Balb/c mice eight weeks of age were immunised intraperitoneally with whole cell pertussis vaccine (DTPw), acellular pertussis vaccine (DTPa) or normal saline on day 0 of each experiment. Blood was collected to determine IgG antibody responses to pertussis toxin (PT), filamentous haemagglutinin (FHA) and pertactin (PRN) on days 0 and 28. Vaccines were stored under ideal conditions ($2^0$C to $8^0$C) or at $-3^0$C for 24 hours prior to immunisation for both strains of mice and also at $-3^0$C for 14 days or at $-6^0$C for 14 days for Swiss outbred mice.

**Murine Protective efficacy studies**

The murine model was extended to include a second immunisation at 28 days, and an intranasal challenge of live pertussis organisms two weeks after the second immunisation, using Balb/c mice. Vaccines were stored under the same conditions as for the immunogenicity studies, and also at $-6^0$C for 24 hours. At serial time points after challenge mice were sacrificed to determine lung clearance of organisms and IgG antibody responses to PT, FHA, PRN and diphtheria and tetanus toxoids. Bronchoalveolar lavages (BAL) for cell count and cytokine measurement and preparation of lungs for histopathological analysis were also performed in subsets of mice.

**Human studies**

Two studies were undertaken. In the first study, term and premature infants were recruited from two months of age to participate in a longitudinal study to determine IgG antibody responses to DTPa and Hib vaccine antigens before and after primary and 18 month booster immunisations. Comparisons were made between both term and premature infants and term infants and premature infant subgroups determined on the basis of gestational age at birth (extremely premature infants: gestational age at birth = 27 weeks, very premature infants: 28-32 weeks, premature infants 33-36 weeks).

In the second study, stored sera from premature and term infants enrolled in a previous study were used to perform longitudinal comparisons of the effect of immunising with DTPa or DTPw in the primary series and DTPa at 18 months on antibody responses to bacterial vaccine antigens. Cross sectional analyses were performed to confirm 18 and 19 month antibody concentrations in premature infants with different primary DTP immunisation schedules using the stored sera and sera from a second cohort of premature infants newly recruited at 18 months. In addition, further cross-sectional analyses were performed, by combining the two newly recruited premature infant cohorts and comparing term and premature infant and term and premature infant subgroup antibody responses to vaccine antigens at 18 and 19 months.

**Results**

**Murine immunogenicity studies**

In Swiss outbred mice, storage at $-3^0$C for 24 hours significantly reduced vaccine immunogenicity in all cases except to PRN in DTPa. Other adverse vaccine storage conditions either had no effect on, or paradoxically produced higher antibody concentrations than ideally stored vaccines. Antibody responses to DTPa and DTPw in

Swiss outbred mice were higher than those in Balb/c mice. In both mouse strains, antibody responses to DTPa were greater, but more variable, than those to DTPw.

**Murine protective efficacy studies**

In general, adverse vaccine storage did not alter lung clearance rates of *B. pertussis* or murine IgG antibody responses to vaccine antigens in DTPa or DTPw or serological responses to immunisation. However, mice immunised with DTPw did not develop antibody responses to PT and demonstrated greater concentrations of diphtheria and tetanus antibodies and lesser concentrations of pertussis antibodies than mice immunised with DTPa. Mice immunised with normal saline did not demonstrate pertussis responses until 14 days after infection and these were of a very small magnitude. No significant correlations between serological responses and lung clearance were demonstrated in these mice when analyses were performed by vaccine and storage condition. However, some of the comparisons tended towards significance with moderately negative correlations demonstrated. Hence, larger numbers of mice may be necessary achieve adequate power for these comparisons.

Immunisation with DTPa resulted in slightly more rapid lung bacterial clearance, less inflammation in stained lung sections, and fewer inflammatory cells in BAL fluid than mice immunised with DTPw or saline. Mice immunised with DTPa and normal saline demonstrated a Th-2 type cytokine response in BAL fluid, whereas immunisation with DTPw conferred a Th-1 cytokine profile. None of these parameters was affected by adverse vaccine storage.

**Human studies**

The general pattern of IgG antibody concentrations at different study time was consistent in both term and premature infants, with low level of maternal antibodies detected at 2 months, increased responses after primary immunisation measured at the 7 month study time, a decline in antibody concentrations at the 18 month study time with increased booster responses measured at the 19 month study time.

Premature infants demonstrated lower concentrations of IgG antibodies to all vaccine antigens throughout the cohort survey period. These were significantly lower than term infants in response to pertussis vaccine antigens at all study times with one exception (PRN at 19 months). In contrast, premature infant responses to diphtheria and tetanus toxoids and Hib PRP were not, in general, significantly lower than those of term infants. Extremely premature infants demonstrated the lowest antibody concentrations, and the magnitude of antibody responses in premature infants increased with increasing gestational age at birth.

Premature immunised with DTPa from two months of age in general demonstrated significantly higher antibody responses to pertussis antigens after primary and 18 month booster immunisations, but significantly lower responses to diphtheria and tetanus toxoids than premature infants immunised with DTPw in the primary series and with DTPa at 18 months regardless of gestational age at birth. Term infant comparisons were similar, at 7 months, but there were no statistical differences in PT, FHA or diphtheria antibody concentrations between the two groups of term infants at 19 months.

Combining the two newly recruited premature infant cohorts confirmed the term and premature infant comparisons of the first study with regard to PT, FHA, PRN and Hib PRP

antibody concentrations at 18 and 19 months. However, comparisons between term infants and premature infant subgroups were altered: very premature infant pertussis antibody concentrations became more comparable with those of term infants. Extremely premature infant pertussis antibody concentrations remained significantly lower than those of term infants. Diphtheria and tetanus antibody concentrations at 18 and 19 months were affected unpredictably by the addition of a second cohort of premature infants in the analyses.

**Conclusions**

Although adverse storage does not appear to diminish vaccine immunogenicity or protective efficacy in the short term, vaccines should be stored according to the manufacturers instructions until long term efficacy studies can be performed.

Premature infants demonstrated lower IgG antibody concentrations to all vaccine antigens, but were able to mount protective antibody responses to diphtheria and tetanus toxoids and Hib PRP. Premature infant responses to pertussis vaccine antigens were, in general, significantly lower than those of term infants at all study times, despite evidence of increased antibody concentrations after primary and 18 month booster immunisations. Extremely premature infants demonstrated the lowest antibody concentrations to all vaccine antigens, and in particular to pertussis vaccine antigens. There is no single serological correlate of protection against pertussis infection, however, and antibody avidity and cell-mediated immune responses were not examined in these infants. Therefore these parameters should be explored before changes are made to the immunisation schedule of premature infants.

# Publications arising from this thesis

**1. Boros CA, Hanlon M, Gold MS, Roberton DM.**

Storage at -3 degrees C for 24 h alters the immunogenicity of pertussis vaccines. Vaccine. 2001 May 14;19(25-26):3537-42.

**2. Conference abstracts**

1. Abstract: Does adverse storage affect the immunogenicity of pertussis vaccines? Presented at the A.S.M. conference, Hobart, October 1998 and at the PHA conference in Melbourne, November 1998 and published in conference proceedings of both meetings.

2. Abstract: Adverse storage affects the immunogenicity of pertussis vaccines. Published in conference proceedings of and presented at the Royal Australasian College of Physicians Annual Scientific Meeting, Perth, May 1999.

J Paeds Child Health, October 1999;35:A5.

3. Abstract

Antibody responses to immunisation in premature and term infants. Published in conference proceedings of and presented at the Royal Australasian College of Physicians Annual Scientific Meeting, Sydney, May 2001

J Paeds Child Health, December 2001 ;35:A5.

4. Abstract

The effect of adverse storage on the immunogenicity and protective efficacy of pertussis vaccines. Published in conference proceedings of and presented at the Royal Australasian College of Physicians Annual Scientific Meeting, Sydney, May 2001

J Paeds Child Health, December 2001;35:A5.

## Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent for the availability of this copy of my thesis for loan and photocopying

Christina Ann Boros

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ELISA methods for the diphtheria and tetanus assays which were adapted for use in both the human and murine studies in this thesis.

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# Abbreviations and Standard Units

| | |
|---|---|
| ABTS | 2.2.-azino-di-3-ethylbenzthiazoline sulphonate |
| ACIR | Australian Childhood Immunisation Registry |
| ANOVA | analysis of variance |
| APC | antigen presenting cell |
| BAL | bronchoalveolar lavage |
| BG | Bordet-Gengou agar |
| *B pertussis* | *Bordetella pertussis* |
| BSA | bovine serum albumin |
| $^0C$ | degrees centigrade |
| CC | charcoal with cefalexin agar |
| CFU | colony forming units |
| CPBS | casaminoacid phosphate buffered saline |
| CSL | CSL Ltd |
| DTPa | acellular pertussis triple antigen vaccine |
| DTPw | whole cell pertussis triple antigen vaccine |
| EIA | Enzyme immunoassay |
| EU | Elisa Units |
| FHA | Filamentous Haemagglutinin |
| G | geometric mean concentration |
| gm | grams |
| $H_20_2$ | Hydrogen peroxide |
| HSA | human serum albumin |
| Hib | *Haemophilus influenzae* type b |
| HRP | horseradish peroxidase |
| IU | international units |
| Lf | flocculation units |
| LPS | lipopolysaccharide |
| MSCII | microbiological Safety Cabinet Type II |
| mg | milligrams |
| ml | mililitre |
| M | Molar |
| nm | nanometre |
| N | Normal |
| $NaHCO_3$ | Sodium bicarbonate |
| NaOH | Sodium Hydroxide |
| NFM-PBS | non-fat milk phophate buffered saline |
| NH&MRC | National Health and Medical Research Council |
| NIBSC | National Institute of Biologic Standards and Controls |
| PBS | phosphate-buffered saline |
| PBS-BSA | phosphate buffered saline-bovine serum albumin |
| PBST | phosphate-buffered saline-tween |
| PRN | Pertactin |
| PRP | Polyribosylribitol phosphate |
| PT | Pertussis Toxin |
| QC | quality control |
| RPM | revolutions per minute |
| SB | SmithKline Beecham |

| | |
|---|---|
| SHD | standard human dose |
| TcR | T cell receptor |
| TdT | Terminal deoxynucelotidyl transferase |
| USFDA | United stated Federal Drug Authority |
| µgm | micrograms |
| µl | microlitres |
| WCH | Women's and Children's Hospital |
| w/v | weight per volume |
| WHO | World Health Organisation |

# Chapter 1 Literature Review

## 1.1 Pertussis infection: the scope of the problem

Pertussis, or whooping cough is caused by the gram negative bacterium *Bordetella pertussis*. It is a highly contagious acute respiratory tract infection which can cause significant morbidity and mortality especially in infants and young children. In industrialised countries, outbreaks occur every 3-4 years (1) and 4 out of every 10 000 infected children die of the disease (2). In unimmunised populations, epidemics also occur every 3-4 years (1). The World Health Organisation has calculated that 600 000 children, mostly less than 12 months of age, die of this disease annually (2).

Pertussis infection is spread by respiratory droplets from a coughing individual to the respiratory tract of a susceptible individual. There is also evidence to suggest that indirect spread may occur via hand contact with respiratory secretions from an infected individual (3). Susceptible household contacts have attack rates between 57-100% (3-5). The incubation period of pertussis varies between 6-20 days, although most infected individuals are symptomatic 7-10 days after exposure.

### 1.1.1 The spectrum of clinical features of pertussis with age

Classical infection occurs in unimmunised infants and children over 12 months of age. The illness usually lasts 6-8 weeks and has three recognizable stages. The initial catarrhal phase is characterized by symptoms suggesting a viral upper respiratory tract infection: mild cough, rhinorrhoea and lacrimation. This period lasts for 1-2 weeks and is followed by the paroxysmal phase of infection during which the coughing occurs in paroxysms in a single expiration. Coughing paroxysms are often followed by a massive inspiratory effort which results in the characteristic "whoop" of inspiration against a closed glottis.

Exhaustion and weight loss due to paroxysms are caused by a combination of the frequency with which they occur and post-tussive vomiting. During this phase, complications such as pneumonia (primary or secondary), otitis media (secondary), seizures, encephalopathy, or subarachnoid haemorrhage may occur. The third or convalescent stage lasts for 1-2 weeks and is characterized by gradual diminution on the frequency and severity of coughing paroxysms.

The clinical manifestations of this disease in infancy depend on age, immunisation status, and the presence or absence of transplacentally acquired antibody (6, 7). Although cough can occur in infants, the classical inspiratory whoop is usually absent. Leukocytosis is often marked, with total white cell counts ranging from 30, 000-60 000 cells/microlitre. In neonates the infection is particularly severe with a death rate of 1.3% (6, 8). Coughing is rarely observed, and apnoea is a frequent clinical presentation, often accompanied by hypoxic seizures.

Previously vaccinated children and older unvaccinated children often develop milder disease, with cough illnesses lasting less than 28 days in most culture-positive cases (7). A recent study utilising both polymerase chain reaction (PCR) and culture for diagnosis found that many mild cases were PCR positive but culture negative (9). Sixty-eight percent of these cases had a cough illness lasting four or more weeks, 57% had a paroxysmal cough, but only 32% had whoop (9).

There is growing awareness that pertussis infection also occurs in adolescents and adults and that these individuals are a source of infection in unimmunised or partially immunised infants and children (10, 11). The severity of infection in adults and adolescents can be surprisingly severe with up to 90% having a paroxysmal cough (12) and post-tussive

vomiting occurring in up to 53% of cases (13). Severe weight loss, inguinal hernia and rib fracture have also been reported as complications of infection in this age group (13).

In Australia between 1993 and 1998, the highest pertussis notification rate was reported in infants less than 12 months of age (105.9/100 000 notifications per annum or 5% of all notifications). Children less than five years of age accounted for 13% of notifications (52.9/100 000) and 71% of hospitalisations. Forty percent of notifications and 15% of hospital admissions were reported in children from 5-14 years of age, whereas notifications in adolescents and adults (= 15 years) accounted for 46% of notifications (14).

## 1.2 Microbiology of Bordetella pertussis

The genus *Bordetella* contains six species, classified by phenotype. Two species, *Bordetella pertussis* (*B. pertussis*) and *B. parapertussis* are closely related with a genetic homology of 98.5% (15). Both cause whooping cough in humans. *B. pertussis* is responsible for 95% of cases and infects humans exclusively (15).

### 1.2.1 B. pertussis virulence factors: control of expression

*B. pertussis* has several biologically active and/or antigenic components, most of which are regulated at one gene locus (*bvgAS*) (16-18): table 1.1). This locus encodes a two-component regulatory system consisting of a sensor protein BvgS and a transcriptional activator, BvgA. (16-19). BvgS is capable of autophosphorylation at a histidine residue (20). This phosphate group is then relayed to an aspartate on BvgA which then directs transcription in *bvg*-dependent genes (21). Phenotypic modulation at this locus produces two phenotypes, *Vir+* and *Vir-* both of which initiate the expression of different virulence factor genes (virulence–activated or virulence-repressed genes (22). A *Vir-* phenotype is produced *in vitro* when bacteria are exposed to magnesium sulphate or nicotinic acid and

when culture temperatures are less than $25^0$C (23, 24). Mutations in the linker region of BvgS abolish these environmental responses by affecting transcriptional promoters at the *bvg* locus (25). The BvgS gene also contains a C-terminal transmitter domain which also appears important in the regulation of the *bvg* locus, as mutations at this locus or substitutions at the BvgS C terminal domain with related domains from other bacterial species caused the inactivation of BvgS (26, 27). However, *in vivo* mechanisms responsible for activation and suppression of this locus have not yet been identified (16).

Virulence factors also appear to be regulated differentially by BvgA (28). In previous studies, temperature shifts from $25^0$C to $37^0$C caused activation of some virulence factor genes within 10 minutes, and others not until 2-4 hours later (29). In addition, Prugnola et al demonstrated that the amount of BvgA produced increased with increasing temperature, implying that production of BvgA is temperature dependent and that the concentration of BvgA determines the expression of different virulence factor genes (29). Steffen et al have postulated that this differential activation of virulence factors reflects differences in the phosphorylation state of BvgA (21). Other investigators have demonstrated that mutations in the BvgA gene can also affect the regulation of virulence factor gene expression (30). Therefore, BvgA activation and function clearly are complex processes which require further investigation.

A second class of genes repressed by the *bvg* locus had also been identified (22). The function(s) of these genes has not yet been identified. However, this locus has been mapped to a region immediately downstream of the *bvg* locus, and appears to require an intact *bvg* locus for transcriptional activation (22).

### 1.2.2 *B. pertussis* virulence factors: description

#### 1.2.2.1 Pertussis Toxin (PT)

Pertussis toxin is a five unit, hexameric protein with two subunits arranged in the A protomer, B oligomer structure common to bacterial toxins (31). The A protomer or S1 subunit is enzymatically active, and acts by ADP ribosylating the a-subunit of regulatory trimeric G-proteins (32). This prevents inhibition of host cell membrane adenylate cyclase, thus increasing intracellular levels of cyclic AMP, and causing the effects of the toxin, namely, lymphocytosis, histamine sensitisation and islet-cell activation (33). The B oligomer is a pentamer containing four subunits: S2, S3, S4 (two copies) and S5. It binds to glycoconjugate receptors on eukaryotic cells facilitating entry of PT into the host cell by endocytosis (31). Pertussis toxin also inhibits the migration and function of phagocytes (34). *B. parapertussis* and *B. bronchiseptica* also contain the gene encoding PT. However, it is transcriptionally silent in these species (35).

#### 1.2.2.2 Filamentous haemagglutinin (FHA)

Filamentous haemagglutinin is a 220 kilo-Dalton (kDa) monomeric cell membrane protein, folded in the shape of a hairpin, so named because of its ability to agglutinate erythrocytes from several species (36). Its role is as a principal adhesin to respiratory epithelial cell cilia (34). It also mediates attachment of *B. pertussis* to neutrophils (37). Despite its size, it is efficiently secreted by *B. pertussis* due to the action of an accessory peptide encoded by a gene located 3' to the structural FHA gene (38). Filamentous haemagglutinin contains the amino-acid triplet arginine-glycine-aspartic acid (RGD), which enables it to bind to the leukocyte integrin CR3 (39) after stimulation of the leukocyte by PT (40). This triggers phagocytosis of the bacterium by macrophages without an oxidative burst, thus favouring intracellular survival of the organism (41). In addition, the RGD sequence is probably also

responsible for the adhesive properties of FHA, as mutant strains deficient in the RGD sequence have 20% reduced binding capacity to respiratory epithelial cells *in vivo* (41).

#### 1.2.2.3 Pertactin (PRN)

Pertactin is a 69-kDa outer membrane protein which acts as an adhesin and an agglutinogen (42). It is produced in the bacterium as a 910 amino acid precursor and undergoes proteolysis to produce a 69kDa fragment located at the cell surface and a 30kDa fragment located in the outer membrane (43, 44). The *prn* gene contains two regions (named I and II) composed of repeat sequences which have both been demonstrated to vary through recombination (45, 46). Pertactin also contains the RGD amino acid sequence which enables it to bind to a number of mammalian cell surface proteins (47). However, the interaction of this sequence in PRN with CR3 has not been reported.

#### 1.2.2.5 Fimbriae

*Bordetella* species express fimbriae of at least four serotypes, Fim2, Fim3, FimX and FimA, encoded by unlinked *fim2, fim3, fimX* and *fimA* genes, which have approximately 60% sequence homology at the amino acid level (48). Fimbriae are important agents in the adhesion of bacteria to respiratory epithelium (49), are required for initial and persistent colonisation of the trachea and are strong humoral immunogens (50). The *B. pertussis* serotypes are determined by fimbrial antigens. Three serotypes exist: type 1, 2, 3, type 1, 2 and type 1, 3 (1)

#### 1.2.2.6 Adenylate cyclase toxin/haemolysin

Adenylate cyclase (ACT) is an outer membrane protein of *B. pertussis.* It acts as a catalyst for the conversion of adenosine triphosphate (ATP) to cyclic adenosine

monophosphate (cAMP) and as a haemolysin. This toxin is activated by the protein calmodulin, which is not produced by *B. pertussis* and therefore must be supplied by host cells (51). Both the adenylate cyclase and haemolytic activites of the toxin are required to initiate infection (52). There are five genes encoding the ACT protein: *cya*A, -B, -C, -D and –E. The gene product of *cya*A produces the active protein. C*ya*B, -C, -D and –E are required for the secretion of ACT (53). Adenylate cyclase blocks the phagocytosis of bacteria by neutrophils (37). Paradoxically, it also inhibits bacterial invasion of epithelial cells *in vitro* (52, 54) and causes apoptosis in macrophages (55). However, on entry into human macrophages, expression of ACT is downregulated, due to the modulation of the bacterium to a Vir- phenotype, providing a possible mechanism for the intracellular survival of *B. pertussis* (53).

#### 1.2.2.7 Tracheal cytotoxin

Tracheal cytotoxin is produced by both virulent and avirulent strains of *B. pertussis* (34). It is a glycopeptide with a molecular weight of 1400kD which causes ciliostasis, thus preventing the natural clearance of infection by the host (56, 57).

#### 1.2.2.8 Dermonecrotic toxin

Dermonecrotic toxin is a protein monomer with a molecular weight of 190 000 daltons, so named for the production of vasoconstrictive necrotic skin lesions in laboratory animals after subcutaneous injection (34, 58). It may have a role in local tissue damage to the respiratory tract (58).

#### 1.2.2.9 Lipopolysaccharide

Lipopolysaccharide (LPS) forms an integral part of the outer membrane of gram negative bacteria. Lipopolysaccharides consist of a highly conserved and strongly immunogenic lipid A component, which has endotoxic effects (59), and a variable carbohydrate moiety, which appears to be species specific (60). *B. pertussis* contains two forms of LPS: LPSA and LPSB, which differ only by the addition of a distal trisaccharide moiety to LPSA (61). Lipopolysaccharide-A appears to have a role in the entry of organisms in to host cells (62). Lipopolysaccharide production in *B. pertussis* is not controlled at the *bvgAS* locus (63), but at a separate locus, *wlb*G, with LPSB being formed by the bacterium only when this locus is inactivated (64).

## 1.3 Pertussis vaccines: historical overview

### 1.3.1 Whole cell vaccines

The *B. pertussis* bacterium was first isolated in 1906 by Bordet and Gengou (65). Although the possibility of pertussis vaccination was considered earlier, the first pertussis vaccine consisting of suspended organisms in phenolysed saline was developed by Madsen in the early 1930's (66). Thereafter, several types of vaccines were experimented with, which demonstrated very variable clinical protective efficacy and toxicity (3, 67). These included washed or unwashed whole cell preparations, vaccines also containing other respiratory tract flora and extracted acellular products (15).

Current whole cell vaccines (DTPw) consist of *B. pertussis* grown in bulk culture, harvested, concentrated by centrifugation, killed by heat and/or detoxified by various methods (including glutaraldehyde or formaldehyde inactivation), suspended in a buffered saline solution and combined with alum-adsorbed diphtheria and tetanus toxoids (3). The

World Health Organisation (WHO) recommends that whole cell vaccines contain all three fimbrial antigens and have not less than four International Units of activity (68). However, despite similar basic methods of formulation, striking differences in antibody responses to various antigens have been reported (15). In addition, childhood immunisation schedules vary significantly between different countries. Immunogenicity data suggest that schedules which include three primary doses in the first year of life with booster doses at approximately 2 and 4-6 years are most efficacious (15). There is also evidence to suggest that whole cell vaccines protect better against infection than severe disease (69), although this finding has not been accepted universally (70).

In the 1970s, significant dissatisfaction arose regarding DTPw vaccines regarding real and perceived adverse events after immunisation which led to decreased vaccine usage in several countries including Japan, Sweden and Great Britain (71). Adverse events which were more frequent in DTPw recipients than diphtheria-tetanus vaccine (DT) recipients included local reactions such as redness, swelling and pain at the site of vaccination, and systemic reactions, including fever (=$38^0$C), drowsiness, irritability, vomiting, persistent crying or a high-pitched unusual cry (72). These adverse events are thought to be related to the amount of endotoxin contained in the vaccine (68). Pertussis vaccine encephalopathy, which was the apparent cause of infantile seizures post immunisation, was also of great concern during this period, and was probably the most significant factor influencing cessation of pertussis immunisation programmes (3). Unfortunately, the cessation of pertussis immunisation programmes caused by concerns regarding DTPw vaccine reactogenicity led to a resurgence of whooping cough in countries where governments ceased pertussis immunisation programmes (71).

Several recent epidemiological studies have demonstrated that DTPw can cause the first febrile seizure in prone children (73-77) and is associated with an earlier onset of seizures in infantile epilepsy, particularly infantile spasms (78). Acute encephalopathy may be caused by immunisation with DTPw at a rate of approximately 10-10.5 cases per million vaccinees (79). A more recent study investigating the immunogenicity and reactogenicity of a reformulated DTPw preparation given to infants at the 18 month booster reported no serious adverse events (80).

### 1.3.2 Acellular vaccines

In 1981, the first acellular pertussis vaccines (DTPa) containing purified pertussis antigens were licensed for use in children from 2 years of age in Japan. They were subsequently licensed for Japanese infants from three months of age in 1989 (1). Adsorbed antigens are suspended in sodium chloride and water with added preservative. In the USA, DTPa was recommended for 18 month booster immunisations in 1992 (81), and for primary immunisation from 1997 (82). These vaccines contain between one and five purified or recombinant pertussis antigens (PT, FHA, PRN and fimbriae 2 and 3) as well as diphtheria and tetanus toxoids adsorbed onto aluminium adjuvant. They were developed primarily because of high rates of local and systemic reactions caused by DTPw preparations, because of concerns regarding whole cell vaccine efficacy, and also because of the continued perception that immunization with DTPw was more dangerous than natural infection (83).

There has been a dramatic decline in disease prevalence in Japan since the introduction of DTPa, and vaccine-induced protection has been reported to last for up to ten years (68). However, the first trials of acellular vaccines in Sweden in the 1980's utilising a monocomponent (PT) or a bicomponent (PT and FHA) acellular vaccine demonstrated

respective efficacies of 54% and 69% (84). Since then, acellular vaccines have been refined and have been demonstrated to be as immunogenic as DTPw (or more so), and less reactogenic (85-95). They are also associated with levels of cell mediated immunity as those after natural infection (68). Not all acellular vaccines contain fimbrial antigens, but there is evidence to suggest that five-component pertussis triple antigens (containing fimbriae) are more immunogenic than three component vaccines (96). However, the differences in clinical protective efficacy between three component pertussis vaccines (containing PT, FHA and PRN) and five component vaccines (also containing fimbrial antigens) have not been found to be significantly different (71).

## 1.4 History of pertussis and pertussis vaccines in Australia

In the early 1900s, there were high rates of notifications of infection and death due to *B. pertussis* infections. An example of this for South Australia is shown in figure 1.1. The first pertussis vaccine was used in Australia in 1942. This was a whole cell preparation manufactured by the Commonwealth Serum Laboratories containing suspensions of heat killed *B. pertussis* organisms (97). A mass pertussis immunization campaign was then introduced in 1953 using a reformulated, more effective vaccine. The first whole-cell triple antigen vaccine containing diphtheria and tetanus toxoids as well as killed *B. pertussis* (DTPw) was recommended by the National Health and Medical Research Council (NH&MRC) for use in children in 1954 (1). However, prior to this, pertussis notifications had already started to decline, probably as a result of public health measures.

A period of approximately 40 years ensued during which notifications and deaths due to pertussis were negligible. During this period, there were several changes to the NH&MRC childhood immunisation schedule. The initial schedule included three doses in the primary series (2, 4 and 6 months of age) and a fourth dose at 18 months of age. The fourth dose

was omitted from the schedule in 1978 after concerns were raised regarding the safety of the vaccine, but required reintroduction (in 1986) due to an increased incidence in the disease. To prevent transmission of pertussis from school-aged children to their younger siblings, a fifth dose was introduced at five years of age in 1994 (1).

Since 1997, acellular triple antigen preparations (DTPa) have been recommended by the NH&MRC for use in Australia from 2 months of age instead of DTPw. The two acellular vaccines in use in Australia are *Infanrix*®, marketed by SmithKline Beecham containing PT, FHA and PRN, and *Tripacel*®, marketed by CSL Ltd, containing all five antigens.

Notification rates of pertussis infection rose again from 1993. Every year since then there have been over 4000 notifications of pertussis infection in Australia with 6432 notifications reported in 1998, 4530 in 1999 and 5795 in 2000 (98). In 1997, 10 669 cases of infection were reported, and six children between the ages of two weeks and four months died. These notification rates were 10 times those of the United States of America and three times those of England and Wales for the same period (99).

There are several factors to consider when accounting for an increased rate of pertussis notifications:

### 1.4.1 Compulsory notification of disease

Pertussis is now a notifiable disease in all Australian states. Obligatory reporting of cases may have led to increased testing for pertussis as well as an increased reporting rate. Unfortunately there are no agreed national standards for laboratory diagnosis in Australia, making estimates of incidence via report difficult to interpret accurately. The Communicable Disease Network, Australia and New Zealand recommends that laboratory diagnosis should only be made on the basis of positive culture and/or polymerase chain

reaction (PCR) (1). However, other diagnostic criteria are acceptable for the purposes of notification. These are: elevated *B. pertussis* specific IgA in serum or the detection of *B. pertussis* antigen in nasopharyngeal aspirates using immunofluorescence with a history consistent with pertussis infection; an illness lasting two weeks or more associated with any one of coughing paroxysms, inspiratory whoop or post-tussive vomiting; or an illness characterised by cough lasting two weeks in a patient who is epidemiolgically linked to a laboratory confirmed case (14).

### 1.4.2 Persistent disease reservoir

Waning vaccine induced immunity after immunisation with whole cell pertussis vaccines has produced a reservoir of infection in the adolescent and adult populations (10, 11). Infection in this group is often unrecognised as pertussis. This combined with the highly contagious nature of the infection increases the risk of transmission to susceptible populations, namely infants and children who have not completed their course of immunisations (5, 100-103).

### 1.4.3 Vaccine coverage rates

A survey conducted by the Australian Bureau of Statistics in 1995 found that only 58% of children were fully immunized against pertussis and only 51% were fully immunised for all vaccines excluding Hib by the age of 2 years (104). In this cross-sectional population survey, information was obtained by parental report for 6768 children aged 0-6 years in 5000 households across Australia. All interviews were conducted by trained interviewers. Parentally held child health records were consulted for validation of parental reporting in 60.6% of interviews. Therefore, almost half of the data collected were unvalidated. Hence there are concerns regarding the accuracy of the survey results (105). Further investigation

demonstrated that compliance rates for immunisation were probably satisfactory for the primary series, but that there was less compliance for the fourth and fifth doses of pertussis vaccine (106). In comparison with overseas data from a similar time period, 92.8% of infants at 12 months and 95.8% of infants at 24 months in the UK were immunised against pertussis (107). At 35 months (after 4 doses of vaccine) 81% of infants in the USA were fully immunised against pertussis (108). In New Zealand, 84.1% of infants 12-18 months of age were fully immunised with DTP (109) and 87% of Canadian infants less than 2 years of age had been immunised with 4 doses of DTP vaccine (110). Although these overseas surveys were performed using different methodologies, the results indicate much lower immunisation coverage rates in Australian children during this period.

In 1996, as a result of the National Immunisation strategy in 1993, (111) the Australian Childhood Immunisation Register (ACIR) was established in order to obtain a more accurate representation of vaccine coverage rates. All vaccine providers are now required to report immunisations administered to children up to 7 years of age to a national database either by completion of immunisation encounter forms or by data entry onto an online database. Linkage of maternity allowance and childcare assistance payments to documentation of immunisation by the ACIR and payment to vaccine providers for documentation of immunisation on the ACIR were introduced by the Australian government in 1997 to increase vaccination coverage and rates of notification (14). Additional payment to general practitioner vaccine providers for immunisation, achievement of practice immunisation coverage targets and notification of vaccine preventable diseases was introduced in 1998 (14). Initial reports of vaccine coverage rates have underestimated true vaccine coverage rates because of late notifications and the exclusion of data from children receiving "catchup" immunisation after 12 months of age (112). Published rates of immunisation coverage from the ACIR on 31st December 1998

report 85% coverage for all vaccines in the first year of life and 86% for Measles-Mumps-Rubella immunisation coverage assessed at 24 months of age (14).

ACIR data for vaccine coverage rates in 4-5 year olds have not yet been published. The 1995 Australian Bureau of Statistics survey indicated coverage rates of 21.5% in this age group (104). Individual state and territory surveys varying in methodology have found rates of vaccine coverage between 67-89% (105). However, those individual state and Territory surveys differed widely in design, implementation and responses rates, and some demonstrated different characteristics between respondents and non-respondents, making data interpretation difficult (105).

Therefore, 4-5 year olds are a potential source of infection for children less than 18 months of age. Vaccine coverage in this age group may be a contributor to the persistence of pertussis in our community.

### 1.4.4 Changes in pertussis serotypes and virulence factors

#### 1.4.4.1 Serotype

The serotype of pertussis organisms is determined by fimbrial antigens. Serotype 1, 2 appears to predominate in unimmunised communities. It is also the serotype used in some of the early vaccines. Serotype 1, 3 is now the predominant serotype in immunized communities (113, 114) and a shift occurred in Australia from 1987 from serotype 1, 2 to 1, 3 probably because of changes to the immunisation schedule (reintroduction of the fourth dose of vaccine in 1986) and to vaccine formulation (addition of aluminium adjuvant) (68).

#### 1.4.4.2 Virulence factors

In 1996, Mooi et al found evidence for vaccine driven evolution in the virulence factors of Dutch isolates of *B. pertussis* between 1949 and 1996 (45). Results of DNA analyses from this study demonstrated that the PT S1 subunit and region I of the *prn* gene were polymorphic and that there were time related shifts in their variation. Epidemiological data during this period demonstrated divergence between vaccine strains and clinical isolates of the organism (45). These data have not been supported by subsequent investigators, however (115, 116).

## 1.5 Epidemiology of other vaccine preventable diseases

### 1.5.1 Diphtheria

Diphtheria is caused by the exotoxin of *Corynaebacterium diphtheriae,* a gram positive, non-sporulating, non-encapsulated bacterium. The exotoxin acts locally on the respiratory tract to cause an obstructing pseudomembrane. The heart, nervous system and adrenals can also be affected by the neurotoxin (117).

In the 1990's, the largest outbreak of diphtheria since World War II occurred in the Newly Independent States (formerly the Union of the Soviet Socialist Republics), with 140 000 cases and 4000 deaths recorded (118). The majority of cases were reported in people aged 15 years and over (119). This was due to the presence of a population of unimmunised adults who were born around the time that diphtheria vaccine was introduced, and, in those that were immunised, a lack of exposure to the organism, thereby preventing boosting of immunity (118). Additional factors contributing to this outbreak included a reduced vaccine coverage rate in children, lower antigen concentration in the vaccine formulation

and a decline in the standards of living associated with the breakup of the Soviet Union (118).

There have been no reported cases of diphtheria in Australia since 1992 (120). The level of immunity in the Australian adult population is relatively low due to waning vaccine induced immunity and a lack of exposure to the organism (120-122). However, there is not the reservoir of infection in susceptible children as occurred in the outbreak in the Newly Independent States (118). To improve immunity in adults, the NH&MRC recommends a booster dose of adult tetanus-diphtheria vaccine in adults 50 years of age and over and a catch-up schedule for unimmunised adults of three vaccinations, each two months apart, followed by two boosters at ten year intervals (123).

### 1.5.2 Tetanus

Tetanus is a disease caused by the exotoxin of the bacterium *Clostridium tetani,* an anaerobic organism that is found in soil and grows in contaminated wounds. It is not an eradicable disease. Infection is characterised by painful muscle spasms primarily of the face and neck, but which can also occur in the trunk and respiratory muscles, causing respiratory paralysis. Between 1993 and 1998 there were 47 notifications of disease in Australia and between 1993 and 1997, 7 deaths attributable to the disease, 86% of which occurred in persons aged 70 years or over (14). Prior to mass immunisation in Australia, tetanus infection was most common in infants and the elderly (14). More recently, females over 55 years of age appear to be most at risk both in Australia and in other industrialised countries (14). This is thought to be due to low vaccination coverage in these women at a time when their male counterparts would have been receiving tetanus boosters for military service (124-126). A booster dose of tetanus immunisation at 50 years of age in men and women is now recommended by the NH&MRC (117).

### 1.5.3 Haemophilus influenzae type b infection (Hib)

*Haemophilus influenzae* type b, a gram negative encapsulated organism causes meningitis, epiglottitis, cellulitis, septic arthritis, pneumonia, pericarditis, osteomyelitis and septicaemia in children. Prior to the introduction of immunisation, meningitis was the most common infection caused by the bacterium, occurring in 60% of reported cases (123). Infants less than 18 months of age were most at risk of meningitis, which had a case fatality rate of 5% and caused neurological sequelae 15% of survivors. Epiglottitis was more common in children over 18 months of age. The incidence of disease was considerably higher in Aboriginal than non-Aboriginal children (117).

Vaccines to prevent this disease became publicly funded in Australia for all infants from 2 months of age in 1993 (14). All licensed vaccines comprise a capsular polysaccharide component of the bacterium, poly-ribosyl ribitol phosphate (PRP), conjugated to a carrier protein (a meningococcal protein or diphtheria or tetanus toxoids) to enhance immunogenicity in infants. Between 1992 and 1998 the number of notified cases in Australia dropped from 502 to 35 per year (127). Fifteen deaths due to infection were recorded between 1993 and 1998, 13 occurring in the 0-4 year age range (14). Six of these deaths occurred in 1993. No deaths due to Hib infection were recorded in 1997 or 1998 (14).

In summary, it is clear that immunisation has had more of an impact on the rates of diphtheria, tetanus and *Haemophilus influenzae* type b infection than on those of pertussis. Factors other than those already mentioned may also impact upon the rates of pertussis in our community. By comparing ACIR data of vaccine coverage rates with the notification rates of pertussis it would appear that even in fully immunised children vaccines may fail to protect against infection. The reasons for primary vaccine failure are not well

understood, but may relate to difficulties with vaccine manufacture or storage. Alternatively, the host may fail to mount a protective immune response despite the use of an apparently immunogenic vaccine. The following sections will discuss these factors in more detail.

## 1.6 Vaccine storage

### 1.6.1 Vaccine cold chain

Manufacturers instructions for all vaccines used in the Australian childhood immunisation schedule state that vaccines should be stored between $2^0$C and $8^0$C to maintain potency. The term "cold chain" describes the storage of vaccine from manufacturer to vaccine provider to immunisation recipient within these temperature limits.

The most common error in vaccine storage in developed countries is exposure to temperatures below $-1^0$C, often for long periods of time (128). This occurs even in Australia where environmental temperatures can be very high (129). In developing countries, however, adverse vaccine storage implies exposure to heat, usually because of the lack of electrical power and access to refrigeration, particularly in peripheral facilities (130).

Lack of adherence to vaccine cold chain guidelines occurs often (128, 129, 131-138). A recent study employing repeated monitoring of individual vaccine provider sites using electronic temperature loggers (see section 1.6.1.1.3) demonstrated that temperature storage conditions may vary considerably at any one site over time, that initial correct storage conditions may not persist on subsequent monitoring, but that educational intervention at sites where storage is suboptimal can correct storage errors in most cases (139).

Adverse vaccine storage is usually due to lack of knowledge regarding appropriate procedures, for example, failure to monitor vaccine storage temperature, failure to check storage temperatures regularly (134, 140-143), failure to have a designated vaccine refrigerator (131, 136), or failure to discard vaccines stored at length at room temperature (141).

### 1.6.1.1 Vaccine temperature monitoring devices

There are many vaccine temperature monitoring devices available currently, most of which have limitations associated with their use. It is important to understand their applications and limitations.

#### *1.6.1.1.1 Vaccine vial monitors*

Vaccine vial monitors are recommended by the World Health Organization (WHO) and United Nations Children's Emergency Fund (UNICEF) for use during vaccine storage and transport (144). The monitor is attached directly to the vaccine vial and comprises a circle with an inner square which is initially lighter than the outer ring. As time passes and the temperature of the vial increases, the inner square gradually becomes darker. Once the inner square becomes darker than the outside ring, the vaccine should be discarded. However, these monitors only measure exposure to heat over time, and not to cold, thus limiting their usefulness. Vaccine vial monitors are not used in Australia.

#### *1.6.1.1.2 Freezewatches*

Freezewatches (3M®) consist of a highly sensitive indicating liquid inside an especially designed ampoule. When exposed to freezing temperatures the ampoule fractures releasing the liquid dye, which stains the cardboard paper behind the ampoule giving a

visual indication of adverse vaccine storage. They are available at two temperatures (-4$^0$C and 0$^0$C) and have a pressure sensitive adhesive on the back of the cardboard. (145). They are usually used to monitor temperature during vaccine transport. Freezewatches should be stored at 20% to 60% humidity, at 5$^0$C, and have a shelf life of three years from the date of manufacture (145).

#### *1.6.1.1.3 Time Temperature integrators*

Time temperature integrators (Monitor Mark 3M®) consist of a high contrast indicator which turns blue as a result of exposure to rising temperatures. This is indicated by the appearance and progressive migration of blue colour from left to right through a series of viewing windows on a piece of cardboard into which the dye is impregnated. There are several monitors of this type in manufacture, which have different specifications regarding the time periods and storage temperatures which will make the indicator turn blue. These include –15$^0$C and 48 hours; 5$^0$C and 48 hours; 10$^0$C and 48 hours; 10$^0$C for one week; 26$^0$C for 48 hours, one week or two weeks; and 31$^0$C and one week. Dual temperature indicators are also available which include a second indicator for temperature exposure above a secondary level (145). These monitors have the advantage of being able to give an indication of the duration of exposure to a certain temperature, but only within defined time limits. Therefore their best use is for short-term vaccine storage or transport. They cannot record variations in temperature over time.

#### *1.6.1.1.4 Electronic minimum-maximum thermometers*

These electronic devices (EMT 88: Temperature Technology ®) consist of a temperature probe on a long sensor lead and a liquid crystal temperature readout display. Minimum and maximum temperatures as well as the current temperature are recorded on the display

(145). These monitors are usually used in storage refrigerators at vaccine provider sites. They have the advantage of a continuous temperature display as well as the temperature range but cannot record the duration of exposure to a particular temperature.

#### *1.6.1.1.5 Electronic monitors*

There are a number of electronic temperature monitoring devices on the market which have the ability to monitor temperatures over a very wide range. They consist of battery operated silicon chips attached to an internal or external sensor which sample temperature at preset intervals and can give an instantaneous readout of temperature storage conditions over time either onto a graphical printout attached to the monitor or after downloading of data onto a computer programme. These monitors have the advantage of exact quantification of the time and temperature of adverse storage, thus enabling more informed decisions regarding the disposal of adversely stored vaccine.

## 1.6.2 Methods of determining the effect of adverse temperature storage on triple antigen (DTP) vaccines

### 1.6.2.1 Effects on the adjuvant

Triple antigen vaccines contain an aluminium-based adjuvant (aluminium hydroxide or aluminium phosphate) to which vaccine antigens are adsorbed. Storage between $4^0$C and $10^0$C over a five-year period will cause gradual changes in the structure of the adjuvant observable by electron microscopy and roentgenographic studies, and by gradual diminution in its ability to adsorb Congo Red dye (144). The gel structure of the adjuvant is also destroyed by freezing (144). It is thought that freezing the adjuvant causes the vaccine to develop agglomerates and floccules which will increase the sedimentation rate of the vaccine after shaking (146, 147). These observations formed the basis of the "shake test", to determine if an aluminium-adsorbed vaccine had been frozen. The vaccine vial is

shaken vigorously, after which the vial is examined by the naked eye for floccules and granules and the extent of sedimentation recorded over a 30-minute period. If floccules and/or granules are apparent after shaking the vial or sediment appears at the bottom of the vial with clear liquid above (or both), the vaccine has been frozen (147). This method has obvious problems with standardisation and reproducibility. In addition, not all adsorbed vaccines will demonstrate these changes after freezing (147).

### 1.6.2.2 Effects on vaccine antigens

#### *1.6.2.2.1 Methods of measuring the efficacy of pertussis vaccines*

Determination of the effects of adverse high or low temperature storage on DTP vaccines is not as straightforward as for live attenuated viral vaccines for which viral titres can be calculated in tissue culture assays. Whole cell DTP vaccines contain a mixture of adsorbed protein, polysaccharide and glycoprotein antigens, whereas acellular DTP vaccines contain only adsorbed protein antigens. As the aluminium adjuvant is more stable at higher than lower storage temperatures, it might be expected that the antigen to which it was adsorbed would also be less stable at lower storage temperatures, especially after freezing (144). However, there is no simple assay to determine the amount of antigen affected or the way in which it is affected by adverse storage conditions, if at all.

The first tests of DTP vaccine protective efficacy were designed to compare different whole cell pertussis vaccines against a reference preparation prior to licensing. Kendrick et al developed a mouse model using intracerebral challenge of Swiss outbred mice with a bacterial suspension containing 200-fold the lethal dose in 50% mice ($LD_{50}$) after intraperitoneal immunisation with different dilutions of the test vaccine (148). The end-point was the determination of the number of deaths 48 hours to 14 days after challenge.

Despite being technically demanding and requiring a large number of mice to complete, this test became adopted worldwide for the pre-licensure testing of whole cell pertussis vaccines after good initial correlation between the clinical protective efficacy of whole cell vaccines and the Kendrick mouse protection test (149).

More recently, concerns have been expressed regarding the relevance of intracerebral infection in mice to human infection (150), the poor differentiation of this test for different levels of whole cell vaccine potency (151), the inter- and intra-laboratory variation and lack of reproducibility of the intracerebral model (152) and the ethical implications of intracerebral challenge in mice (153). In addition, most acellular pertussis vaccines do not provide protection against intracerebral challenge (115), possibly because antigens contained in these vaccines are purified and/or detoxified and may have reduced biological activity in this context (154).

Therefore, respiratory challenge models of mouse protection have recently been re-investigated as potential replacements for prelicensure testing of both whole cell and acellular vaccines. *Bordetella pertussis* is not a natural mouse pathogen and transmission between infected and non-infected mice does not occur (115). However, younger mice are more susceptible to the disease, infection is restricted to the respiratory tract, and histamine sensitisation, lymphocytosis, hyperinsulinaemia, bronchopneumonia and alveolitis occur, all of which are pathophysiological features very similar to those found in humans (155).

Murine intranasal challenge models of pertussis infection have been explored by several investigators since the 1930's (156-158). In 1980, the first aerosol challenge murine model of pertussis infection was described by Sato et al, who demonstrated that this method would cause lung infection as well as respiratory epithelial cell colonisation (159). DDY

or ICR mice of varying ages (10, 18 or 49 days) were exposed to an aerosol of $2x10^9$-$10^{10}$ bacteria/ml for 30 minutes. Deaths occurred 10-14 days after exposure, and occurred earlier in younger than older mice. The increase in bacterial numbers in the lungs of infected mice correlated with an increase in lung and spleen weights and with the degree of leucocytosis in peripheral blood.

Subsequent investigators have used a modified protocol using non-lethal challenge doses, and have incorporated immunisation prior to challenge to assess vaccine protective efficacy. Intranasal challenge models have been re-investigated in other recent studies (115). Both methods use the number of colony forming units of *B. pertussis* in cultures of lung homogenates at various time points after challenge with the organism after priming with intraperitoneal immunisation as the method of comparing protective efficacy of vaccines.

Considerable controversy has existed regarding the appropriate method of respiratory challenge for prelicensure testing for acellular and whole cell pertussis vaccines. Proponents of the aerosol challenge method have stated that it is produces a more uniform infection of lung tissue, does not depend on inoculum volume and is more consistent in causing decreased weight gain of infected mice (160), whereas proponents of the intranasal method have found that the infection of large numbers of mice could not be performed within the 2.5 hour time-limit imposed by the WHO Expert Committee on Biological Standardisation (161) required for experimental reproducibility using aerosol challenge. In addition, although a recent study demonstrated significant correlations between lung bacterial clearance in mice and the efficacy of individual DTPa and DTPw vaccines used in clinical trials in relative terms (162), it was not sensitive enough to differentiate between some of the vaccines which had demonstrated markedly different efficacies in those

clinical trials (115). More recently, the WHO has recommended the intranasal method for the testing of pertussis vaccine efficacy (N. Guiso, personal communication, 2000).

The murine intracerebral method of challenge has been used in all published studies as the standard for determining the effect of adverse temperature storage on the efficacy of the pertussis component of triple antigen vaccines. Data regarding the effects of adverse storage on the efficacy of diphtheria and tetanus toxoid components of DTPw vaccines have been generated using mouse or guinea pig intracerebral challenge tests or the guinea pig antitoxin response test, which estimates antitoxin titres in immunised guinea pigs. The acceptability criteria are the production of at least 2.0 IU/ml of antitoxin in the serum pool of immunised animals (163). There are no known published data regarding the effects of adverse storage on acellular triple antigen preparations.

### 1.6.2.3 Review of previous studies investigating the effect of adverse storage on the efficacy of DTPw vaccines

#### *1.6.2.3.1 The pertussis component of DTPw preparations*

1.6.2.3.1.1 Higher temperature storage

At storage between 4-6$^0$C, DTPw and DTPw-polio vaccines have been reported to retain adequate efficacy for up to two years (164, 165). However, there is a gradual loss of efficacy in the pertussis component of DTPw vaccines even under ideal storage conditions: the estimated average annual loss is 0.35IU of opacity per dose (to a minimum if 4IU) with an average initial efficacy of 8.5IU per dose (166-168). British Pharmacopoeia specifications state that the *B pertussis* concentration of the final bulk vaccine should not exceed that corresponding to an opacity of 20IU per human dose. At higher storage

temperatures, loss of efficacy becomes increasingly rapid until at storage temperatures between $50^0$C to $56^0$C, immediate and complete loss of efficacy occurs (144).

1.6.2.3.1.2 Storage at lower temperatures

In 1980, the WHO performed a study examining the protective efficacy of the pertussis component (as determined by the Kendrick mouse protection test), and the protective efficacy of diphtheria and tetanus components (using mouse or guinea pig intracerebral challenge tests or the guinea pig antitoxin response test) of eight adsorbed and three unadsorbed DTPw preparations after storage at $+4^0$C, between $-5^0$C and $-15^0$C for 12 hours or between $-20^0$C and $-35^0$C for 12 hours (147). The freezing point for DTPw vaccines in this study was reported to be between $-5^0$C and $-10^0$C. Two of the five adsorbed vaccines demonstrated a significant loss of efficacy at storage between $-5^0$C and $-15^0$C for 12 hours (17-43% and 50% respectively). The same two vaccines demonstrated 38-56% and 100% loss of efficacy respectively after storage between $-20^0$C and $-35^0$C for 12 hours. One adsorbed vaccine was completely unaffected by adverse storage and two were not tested for loss of efficacy. Of the unadsorbed vaccines, two demonstrated no significant diminution in efficacy after adverse storage at either temperature range, and the third demonstrated greater loss in efficacy after storage between $-5^0$C and $-15^0$C for 12 hours (53%) than between $-20^0$C and $-35^0$C for 12 hours (34%) (147).

### *1.6.2.3.2 Diphtheria and tetanus toxoids*

1.6.2.3.2.1 High temperature storage

The protective efficacy of these toxoids stored at temperatures higher than $8^0$C determined by murine intracerebral challenge has been shown to depend on the vaccine formulation. However, longer storage and higher temperatures do seem to cause a more accelerated

decline in efficacy (144). Tetanus and diphtheria toxoids contained in several DTPw and DTPw-polio vaccines were shown to have no decline in efficacy after storage for 1.5 years at $18^0$C by one investigator (169). Storage at higher temperatures appears to have produced much more variable results, with efficacy estimates after storage at $37^0$C varying from no change after storage for 2-6 months (170) to 50% loss of efficacy at 22 weeks (165). Storage above $45^0$C will cause rapid denaturing of the proteins, further accelerating loss of efficacy (144).

1.6.2.3.2.2 Storage at lower temperatures

There are fewer published data regarding the effect of storage of DTPw below zero. The WHO study referred to in section 1.6.2.3.1.2 also investigated the effect of the same adverse storage conditions (between $-5^0$C and $-15^0$C for 12 hours or between $-20^0$C and $-35^0$C for 12 hours) on the protective efficacy of the pertussis component of five adsorbed and three unadsorbed DTPw preparations (147). The tetanus toxoid in two of five DTPw vaccines stored between $-20^0$C and $-35^0$C for 12 hours demonstrated a loss of efficacy of approximately 30%, while none of the vaccines stored between $-5^0$C and $-15^0$C demonstrated a loss of tetanus toxoid efficacy. The efficacy of diphtheria toxoid was tested in only two of the eight adsorbed vaccines and was unaffected by storage between $-5^0$C and $-15^0$C for 12 hours or between $-20^0$C and $-35^0$C for 12 hours. Freezing did not appear to affect the efficacy of tetanus or diphtheria toxoids compared with those stored under ideal conditions (147).

In summary, there is little information in the literature documenting the effect of adverse storage on the protective efficacy of DTP vaccines. In addition, most of the available information is from data collected 20 years ago or more and all of the published studies

have investigated effects on whole cell vaccines only, using methods that do not necessarily relate to human infection or responses to immunisation.

The cost of disruption in the vaccine cold chain is potentially immense, not only in monetary terms (171) but also in terms of morbidity and mortality from vaccine preventable diseases in children immunised with vaccine with reduced protective efficacy. Therefore, there is a need for further clarification regarding adverse storage of DTP vaccines used in current childhood immunisation schedules and its effects on vaccine immunogenicity and protective efficacy.

## 1.7 Mechanisms of immunity against pertussis infection

In addition to problems with vaccine storage or manufacture, vaccine failure also may be due to factors intrinsic to the recipient, for example, vaccination in an immunodeficient host. The following section will discuss the mechanisms of acquired immunity to pertussis after natural infection and immunisation and then focus on the development of neonatal immune function and the potential implications for term and premature infants regarding immunity to pertussis infection.

### 1.7.1 Humoral immunity

The mechanisms by which immunity against pertussis infection is acquired are still poorly understood (172). Pertussis–specific antibodies are produced both in response to infection and to immunisation with DTPa or DTPw vaccines (173). Clinical trials investigating the efficacy of whole cell and acellular pertussis vaccines have failed to find a single serological correlate of protection against pertussis infection and its effects (84, 93, 174). However, recent investigators have postulated that fimbriae 2 and 3 and pertactin antibody

concentrations may be suitable candidates, as their presence conferred protection after household exposure to infection (175, 176).

Acellular vaccines containing pertussis toxin alone have been shown to confer protection against pertussis infection in humans (84) and against intracerebral, intranasal and aerosol challenge in mice (177). The proposed role of FHA has only been studied in animal models but immunisation with FHA has been shown to protect mice against aerosol challenge with *B. pertussis* by preventing lung and tracheal colonisation (178). Sato et al demonstrated that adult (179) and/or suckling mice (180) were protected from a lethal aerosol challenge by passive immunisation with anti-FHA and/or anti-PT murine antibodies, and that anti-PT alone appeared to protect better than anti-FHA alone. However, immunisation with both afforded the best protection. In addition, there appeared to be a strong correlation between IgG anti-FHA and anti-PT mouse antibody concentrations and the percent survival of passively immunised, aerosol challenged suckling mice (179). Immunisation with FHA alone did not protect adult mice from intracerebral challenge, but once again, in combination with PT, it conferred better protection than immunisation with PT alone (179).

Studies in gene knockout mice have demonstrated that B cell deficient mice (with a homozygous deletion in the immunoglobulin μ chain: Ig-/-) developed persisting infection after non-lethal aerosol challenge and failed to develop an IgG antibody response, but survived to the termination of the experiment at 20 weeks post challenge (181).

However it is clear that humoral mechanisms are not solely responsible for protection against pertussis infection, as it has been demonstrated that although antibody levels decline after immunisation with acellular and whole cell vaccines (although more rapidly

after immunisation with DTPa), most children remain protected against infection even when serum antibody concentrations are negligible (182, 183) and this is thought to be due to persisting cell-mediated immunity between primary and booster immunisations (172, 182).

### 1.7.2 Cell-mediated immunity

#### 1.7.2.1 Murine studies

Mills et al first demonstrated a role for cell-mediated immunity in the protection against pertussis infection using a murine aerosol challenge model (184). Athymic mice were unable to clear infection after a non-lethal challenge. However, adoptive transfer of immune spleen cells or purified CD4+ cells into athymic or sublethally irradiated mice prior to challenge allowed them to clear infection within 14-21 days, whereas pre-challenge immunisation with immune serum from convalescent mice only marginally decreased the lung bacterial load in the early stages of infection (184). In contrast, normal adult non-immune mice cleared infection 35 days after aerosol challenge. However, normal adult mice demonstrated no antibody response to infection until bacteria had been cleared from the lungs, although they demonstrated antigen-specific T-cell responses throughout the course of infection (184).

Using the same model, this group compared cell-mediated immune responses to pertussis infection in unimmunised mice and in mice immunised with a whole cell or an acellular pertussis vaccine (185). Spleen cells from convalescent, unimmunised mice demonstrated high levels of tritiated-thymidine incorporation after stimulation with heat-killed pertussis sonicate and purified PT, FHA and PRN, and secreted high levels of IFN? and IL-2, but no IL-4 or IL-5. This is indicative of a Th1 immune response, in which cell-mediated

immune responses predominate. Immunisation with whole cell vaccine also produced a Th1 immune response in mice. In contrast, spleen cells from mice immunised with an acellular vaccine demonstrated high levels of IL-5 secretion with no IFN?, indicative of a Th2 or predominant humoral immune response. Serum concentrations of IgG PT, FHA and PRN antibodies in unimmunised mice were negligible 15 days after challenge, whereas antibodies were produced in mice immunised with DTPa and whole cell vaccine five days after aerosol challenge. In addition, mice immunised with DTPa cleared bacteria after aerosol challenge more slowly than mice immunised with whole cell vaccine (185).

Further investigation determined that although CD4+ Th1 cells were important in immunity to pertussis infection, the genetic background of mice, the route of immunisation, the nature of the immunogen and the method of cytokine detection could alter the apparent dominance of one Th cell type over another (186). For example, cytokine production from T cell lines and clones from unimmunised mice recovering from infection demonstrated a dominant Th1 cytokine profile, but Th0 and Th2 cytokines were also detected. Intraperitoneal immunisation of mice produced a predominant Th1 cytokine response in cell culture supernatants, whereas subcutaneous immunisation produced a predominant Th1 cytokine response (186). However there is also evidence to suggest that the classification of T cell subsets into Th1 and Th2 may be an oversimplification of true *in vivo* immune responses to infection and immunisation (187).

More recently, it has been demonstrated in mice that *B. pertussis* has multiple protective antigens and antibody and T-cell responses against several antigens may have an additive effect in the protection against pertussis infection (162), suggesting complementary roles for cell-mediated and humoral immune responses, and an explanation for the difficulty in finding a single immunological correlate of protection against pertussis infection.

### 1.7.2.2 Human studies

There are limited data in the literature regarding human cell-mediated immune responses to pertussis infection. Ryan et al demonstrated, as in mice, that recovery from pertussis infection in children was associated with a Th1 immune response as demonstrated by T cell proliferative responses to pertussis antigens and measurement of cytokines in cell culture supernatants in infected individuals (188). This would indicate that Th1 cells have a role in protection against natural infection.

In contrast to the previous findings in mice, in a recent study immunisation of human infants with DTPa induced peripheral blood mononuclear cells which secreted IFN?, IL-2 and IL-5 but not IL-4, whereas spleen cells from immunised mice produced high levels of IL-5, no detectable IFN?, and low levels of IL-2 (188). Similar findings were also demonstrated by Zepp et al (172). Possible explanations for this observation were that murine and human T cells produce different cytokine profiles, that the specificities of human and murine T cells to pertussis antigens differ after pertussis infection (human T cells demonstrated a more prolific response to PT than murine T cells) and that the mice were "adult" and most of the human subjects were less than one year of age and may not have had fully mature immune systems (188). Zepp et al also demonstrated that cell-mediated immune responses persisted, whereas antibody concentrations declined between primary and booster immunisations (172).

Ryan et all demonstrated a mixed Th1/Th2 cytokine profile in the stimulated peripheral blood mononuclear cells of children 3-4 years of age immunised with DTPa, and a Th1 cytokine profile in children 12-13 months of age after immunisation with DTPw (189). Although these findings appear to contradict those of the previous study (172), Zepp et al

measured IL-10 as the representative Th2 cytokine and not IL-4 or IL-5. Il-10 is not produced by all T cell clones and is produced by cells other than Th2 cells and therefore may not be the best marker for Th2 responses (189).

Cassone et al reported that the stimulated T cells of children immunised with DTPa demonstrated more proliferation than those of children immunised with DTPw at 2, 4 and 6 months of age (182). This study also confirmed the persistence of cell-mediated immunity in both groups even when antibody concentrations had declined.

#### *1.7.2.2.1 Studies in human adults*

Because of the growing awareness that adults and adolescents are a reservoir of pertussis infection, studies investigating immune responses and reactions to pertussis immunisation in adults have been undertaken. A study of Italian adults with no record of previous immunisation against or history of pertussis demonstrated T-cell proliferative responses after stimulation with pertussis antigens as well as a Th1 cytokine profile in cell culture supernatants after stimulation, suggesting repeated exposure of these adults to pertussis infection with a natural acquisition of cell-mediated immunity (190). Edwards et al found that adults immunised with an acellular vaccine demonstrated a strong initial antibody response which declined by about 50% after 12 months (191). Lin and Chiang demonstrated that FHA specific T cell proliferative responses and Th1 cytokines increased significantly in adult medical personnel immunised with DTPw in childhood after immunisation with DTPa containing PT and FHA (192).

Therefore it would appear that both humoral and cell-mediated immunity are important in the protection aginst pertussis infection and that a Th1 response appears to be produced by natural infection and by immunisation with whole cell vaccines. It is likely that cell-

mediated immune responses to DTPa vaccines are more heterogeneous, thus explaining the differing results in the described studies.

## 1.8 Neonatal immune function

Healthy term neonates have a well recognised degree of immunodeficiency. This reflects a lack of immune stimulation *in utero*, but also appears to be developmental in nature, as immune function improves over time (193).

### 1.8.1 Neonatal Humoral Immunity

During fetal life and in the neonatal period, the peripheral B cell population is much less diverse than in adults (194). The majority of B cells are of an immature phenotype: CD5+. This phenotype is associated with low affinity, self reactive and cross reactive antibody responses (195).

Responses to antigens are restricted by limited recombination events at the immunoglobulin V, D and J gene loci, therefore reducing the diversity of the antibody response and the development of affinity maturation (196). This is probably due to a lack of coding ends (N-regions) in neonatal heavy chain genes to which non-templated bases are usually added by terminal deoxynucleotidyl transferase (197). Despite this, somatic mutation of immunoglobulin genes does occur in human neonates from as early as 10 days after birth, albeit at a much reduced rate than that observed in adults (198).

Antibody produced by neonatal B cells is predominantly IgM, as neonatal B cells have a limited ability to switch to IgG or IgA responses (199). Switching is an antigen-driven, T cell dependent process and involves translocation of a V-D-J gene from its location upstream of the IgM heavy chain constant region gene (Cμ) to a new site in the 5' flanking

region of another $C_H$ gene (200). In addition, expression of membrane IgG or IgA on neonatal and fetal B cells always involves co-expression of membrane IgM with or without membrane IgD, in contrast to adult cells which only express one isotype (201, 202).

Neonatal B cells also produce lower antibody concentrations than adult B cells after antigen stimulation (194). The reasons for this have not yet been elucidated fully. However, possible explanations include low levels of T cell cytokines needed for proliferation or differentiation of antibody secretion, or functional impairment of antigen presenting cells (e.g. macrophages) (203). It would also appear that neonatal B cells proliferate in response to a restricted range of stimuli and have limited proliferative responses once stimulated in comparison with adult B cells (194). This is probably due to the differences between neonatal and adult B cells in signal transduction pathways which occur at B cell surface receptors after binding of antigen (204).

### 1.8.2 Neonatal cell-mediated immunity

Cell-mediated immunity is important for the protection against intracellular pathogens. Like B cells, T cells express membrane receptors (TcR) which undergo variability due to recombination events of V, D, J and C segments of the TcR genes. For appropriate function the TcR and cell surface co-receptors such as CD3, CD4, CD8 and CD 28 are required for activation and immune effector function (205). The density of TcR complexes on neonatal T cells is reduced (206). Although human fetal T cells are functionally alloreactive by mid-gestation, the complementarity determining region 3 (CDR3), which is important in the binding of antigen to the TcR, is one amino acid shorter in neonates than in adults (207). In addition, terminal deoxynucleotidyl transferase (TdT) is required to facilitate the addition of random nucleotides between rearranged V(D)J gene segments. In mice this enzyme is not present until four days after birth (208), but in humnas, TdT+ T

cells are present by 19-20 weeks gestation, although not in adult numbers (209). These factors limit the diversity of T cell receptors in murine and human neonates.

Neonatal T cells are also predominately of an immature phenotype, expressing CD45RA rather than CD45RO. This indicates antigenic naïvety (205). The number and distribution of T cells also differs between neonates and adults. There are fewer T cells in the neonatal spleen and peripheral blood, and approximately the same number of T cells in neonatal lymph nodes compared with adults (210-212).

Human cord blood and murine neonatal T cells proliferate poorly and produce only small amounts of IL-2 or IFN? in the presence of endogenous antigen presenting cells or mature adult spleen cells (203, 213). However, although human neonatal T cells respond weakly when stimulated via the TcR under normal circumstances, they can produce adult levels of IL-2 in response to T cell independent stimulation (214). In addition, it has recently been demonstrated that by increasing the amount of co-stimulatory signals promoting Th1 function, adult levels of cytokine production can be attained (214).

#### 1.8.2.1 Implications for responses to immunisation

As indicated in section 1.8.1, neonatal antibody responses to immunisation are of low magnitude, low affinity, predominantly IgM and relatively transient. Immunisation schedules have thus been developed such that multiple doses are administered in the first 6-12 months of life, while humoral immunity undergoes progressive maturation (215). Administration of one dose of diphtheria-tetanus vaccine or Hib conjugate vaccine in the first few days of life is associated with very low antibody responses (216, 217). Antibody titres are higher when vaccines in the primary immunisation schedule are administered at 2, 4, and 6 months or at 3, 5 and 7 months in comparison with a 2, 3, 4, months schedule

(218). However, it should be noted that immunisation with the first Hepatitis B vaccine frequently elicits strong humoral immune responses even when administered at birth (215). The reasons for this have not yet been elucidated fully.

Also as expected, progressive affinity maturation of antibody responses to immunisation has been demonstrated during the primary schedule (219). Finally, although antibody responses to the first dose of vaccine are, in general, poor, they do provide adequate priming for significant increases in antibody responses to subsequent doses of vaccine (215).

Neonatal T cells have a "default" Th2 response, which skews immune responses to vaccination towards humoral rather than cell-mediated responses. However, vaccine formulation and adjuvant can affect the profile of cytokine responses (215). Murine studies have demonstrated that DNA vaccines encoding the sequences of tetanus or measles vaccine antigens can produce Th1 responses at adult levels (220), possibly due to prolonged in vivo antigen production and hence prolonged immune stimulation (215). Vaccine adjuvants other than aluminium (which is the only adjuvant licenced for use in humans), for example, the Sigma TiterMax® formulation, induced adult levels of Th1 responses in one week-old mice (221).

In humans, antigen-specific T cell responses to parasites and allergens have been demonstrated to occur *in utero* (222, 223) and to tetanus toxoid and enteroviral infections in the neonatal period (224). The slow development of infant T cell responses was demonstrated in a recent study which compared 6, 9 and 12 month infant and adult IFN? and IL-12 responses to measles vaccination (225). It is postulated that the limited Th1 responses in the neonatal period and during infancy are due to diminished cytotoxic T cell

function (226) although this remains to be proven, as it also has been demonstrated that adequate APC function/activation is necessary for the development of Th1 cytokine responses (215).

### 1.8.2.2 Implications for the immunisation of premature neonates

Little is known regarding the immune responses of premature infants to immunisation or infection. Most of the available data relate to term neonates in clinical vaccine trials. The few published studies investigating premature infant responses to immunisation have concentrated on humoral immunity and have also conflicted in their results, particularly in response to acellular pertussis vaccines (see table 8.16). None of the studies has assessed immune responses to all vaccine antigens at once.

In general terms, however, it is well documented that premature infants have lower concentrations of maternally derived IgG, as transplacental transfer occurs during the latter part of the second trimester onward. Many premature neonates are also extremely unwell and are given immunosuppressive medications such as corticosteroids, for a variety of reasons, which suppress both humoral and cell-mediated immune responses. Administration of dexamethasone has been demonstrated to diminish reposes of premature infants to Hib immunisation (227). Maternal ingestion of alcohol causes a diminished fetal T cell population and T cell proliferation. Transplacental passage of opioids causes a decrease in total lymphocyte and CD4+ cell count.

Therefore, it would appear that premature infants are at greater risk of vaccine preventable diseases and other infections even from the limited knowledge available regarding the functional maturity of the premature infant immune system.

## 1.9 Rationale for studies presented in this thesis

It is clear from the above discussion that vaccine storage and prematurity are two factors which could impact upon the immunogenicity and/or protective efficacy of routine childhood immunisations.

There are no recent data regarding the effect of adverse storage of currently used DTP vaccines below $0^0$C on vaccine immunogenicity or protective efficacy. In addition, previous studies have all utilised methods of determining vaccine protective efficacy which are no longer recommended by the WHO, and adverse storage conditions which are not relevant to those documented to exist at vaccine provider sites. Therefore there is a need to provide more accurate information in this area.

Similarly, no studies exist which have investigated the responses of premature and term infants to all bacterial antigens contained in vaccines used in current childhood immunisation schedules. In addition, few studies have followed premature infants beyond primary immunisation, therefore missing the opportunity to address the question of premature infant immune maturation. The following hypotheses and aims were thus developed based on these research questions.

## 1.10 Hypotheses

1. That adverse storage of acellular and whole cell pertussis triple antigen below $0^0$C may affect subsequent pertussis antibody responses.

2. That adverse vaccine storage below $0^0$C may affect the protective efficacy of acellular and whole cell pertussis triple antigen vaccines

3. That premature infants have significantly lower concentrations of antibodies than term infants after immunisation with DTP and Hib vaccines both in the primary series and after the 18 month booster immunisations

4. That immunisation with different DTP preparations in the primary series of immunisations affect responses to primary immunisation and to subsequent 18 month booster responses to DTPa.

## 1.11 Aims

1. To develop a murine model to investigate the effects of adverse storage of acellular and whole cell pertussis triple antigen vaccines below $0^0$C on their subsequent immunogenicity

2. To develop enzyme immunoassays to measure murine single antigen pertussis, diphtheria, tetanus antibody concentrations

3. To perform definitive experiments which investigate the effects of adverse storage of acellular and whole cell pertussis triple antigen vaccines below $0^0$C on their immunogenicity

4. To develop a murine model to investigate the protective efficacy of pertussis vaccines stored under ideal conditions and below $0^0$C

5. To investigate the effect of adverse storage of pertussis vaccines below $0^0$C on their protective efficacy

6. To compare the effect of adverse storage below $0^0$C and ideal storage ($2^0$C to $8^0$C) on the protective efficacy of pertussis vaccines

7. To investigate and compare the effect of adverse pertussis vaccine storage on the cytokine profiles and cytospin cell counts in bronchoalveolar lavage fluid, lung histopathology and pertussis serology in mice challenged with *B. pertussis*

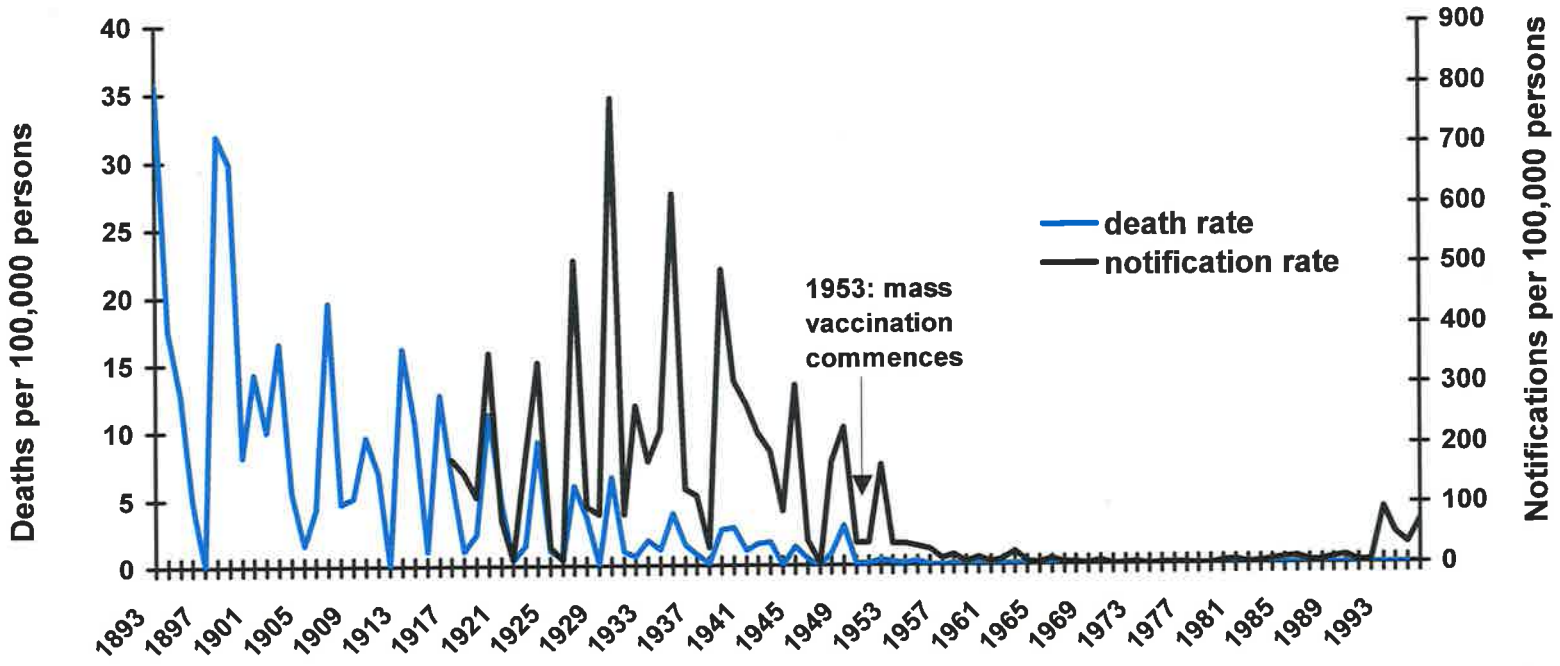
8. To determine the difference in antibody responses to routine childhood immunisations (DTP and Hib vaccines) between premature and term infants followed longitudinally from two to 19 months of age

9. To compare the effect of immunisation with DTPa or DTPw in the primary series on primary immunisation antibody responses and upon subsequent 18 month booster antibody responses to DTPa

**Table 1.1 Virulence factors of *B. pertussis***

| VIRULENCE FACTOR | FUNCTION |
|---|---|
| Pertussis toxin | -adhesion and entry into host cell<br>-? intracellular levels of cAMP (systemic effects of infection)<br>-inhibition of phagocytes |
| Filamentous haemagglutinin | -principal adhesin<br>-intracellular survival of organism<br>-agglutination |
| Pertactin | -adhesion<br>-agglutination |
| Fimbriae | -adhesion<br>-tracheal colonisation |
| Adenylate cyclase toxin/haemolysin | -initiation of infection<br>-inhibition of bacterial invasion<br>-macrophage apoptosis<br>-intracellular survival |
| Tracheal cytotoxin | -ciliostasis |
| Dermonecrotic toxin | -local tissue damage |
| Lipopolysaccharide | -endotoxic effects<br>-entry into host cell |

**Figure 1.1 Pertussis notification and death rate, South Australia: 1893-1997**



# Chapter 2 Murine immunogenicity studies: outbred mice

## 2.1. Introduction

The effect of low temperature storage on the subsequent immunogenicity of pertussis vaccines appears not to have been well documented in the available scientific literature. Previous studies have examined changes in sedimentation rate visible to the naked eye after adverse storage (147), and the efficacy of adversely stored DTPw vaccines as measured using the Kendrick mouse protection test (148). The effects of storage of vaccines between $0^0$C and $-5^0$C (the most prevalent adverse storage condition at vaccine provider sites (128)) have not been reported. In addition, the effect of adverse storage on the immunogenicity or protective efficacy of DTPa preparations has not been reported.

The immunogenicity of adversely stored pertussis vaccine cannot be studied easily in humans but can be studied readily in a murine model. The aims of this study were to develop an appropriate methodology and examine the immunogenicity of pertussis vaccines in mice, following storage under adverse conditions similar to those which have been documented at some vaccine provider sites (128, 139).

## 2.2 Methods

### 2.2.1 Mouse strain, age and sex

Swiss outbred mice were chosen for initial experiments as they exhibit an average heterozygosity reflective of that in humans (228). Therefore antibody responses to pertussis antigens in an outbred murine model may exhibit similar variability to those found in humans. Mice approximately 20 gm in weight and 8 weeks of age at the commencement of each experiment were selected to ensure mature immune status and adequate blood sample size collection. Female mice were chosen to avoid unexpected death during housing, as male mice can become aggressive even when housed appropriately.

### 2.2.2 Mouse housing

Mice were bred in the University of Adelaide Animal Care Facility and transferred to the Women's and Children's Hospital (WCH) in filter cages. On arrival, mice were transferred to standard cages with no more than 15 mice per cage and were housed in the general rodent room in the WCH animal care facility for one week prior to the commencement of each experiment. This time period prevented the stress caused by transport from confounding the experimental results. After experimental procedures, mice were returned to the rodent room for housing during each experiment.

### 2.2.3 Vaccines

The vaccines used in this study were SB Infanrix DTPa (SB Belgium Infanrix® batch numbers 856A2, 857A2, 14867B9B, 14887B9B) and CSL DTPw (CSL Ltd Melbourne, Australia, batch number 043401609). The CSL DTPw preparation had been used universally

in Australia until March 1997. SB Infanrix® was the first DTPa preparation used in Australia, and has been provided statewide in South Australia as part of the universal immunisation programme since March 1997. This DTPa vaccine contains not less than 25Lf of diphtheria toxoid, 10Lf of tetanus toxoid, 25 μg pertussis toxoid, 25μg filamentous haemagglutinin, 8 μg pertactin, sodium phenoxyethanol 2.5 mg as preservative, aluminium hydroxide adjuvant (equivalent to 0.5mg aluminium), sodium chloride 4.5mg and water for injections to a volume of 0.5 ml. CSL DTPw vaccine contains no more than $20x10^9$ formalin-inactivated *Bordetella pertussis* organisms, 30Lf diphtheria toxoid, 6Lf tetanus toxoid, with thiomersal 0.01% w/v as a preservative, aluminium phosphate as the adjuvant and phosphate buffered saline to a volume of 0.5 ml.

### 2.2.4 Vaccine transport

Vaccines were transported at between $2^0$C and $8^0$C from the suppliers (SmithKline Beecham Biologicals, Melbourne, Australia and CSL Ltd, Melbourne, Australia) to the South Australian vaccine distribution centre with a "freezemark" indicator which changes colour if vaccine storage temperatures fall to $-1^0$C or below. To record that the cold chain was maintained during transport from the South Australian vaccine distribution centre to our laboratory, HOBO Temp-INT® electronic temperature loggers were transported with the vaccines and data were downloaded to a computer programme immediately upon arrival of vaccines in the laboratory.

### 2.2.5 Temperature loggers

HOBO Temp-INT® temperature loggers with a temperature recording range of $-37^0$C to $+46^0$C were used in this study (refer to section 1.6.1.1.3). These loggers are reported by the

manufacturer to record air temperature with an accuracy of +/- 0.2$^0$C. Recording times of between 15 minutes and 360 days could be selected using appropriate software. Recording intervals could be varied between 0.5 seconds to 4.8 hours and depended upon the recording time selected. Data from the logger could be printed and/or transferred to other programmes for statistical analysis.

### 2.2.6 Vaccine storage

Vaccines were stored on receipt in an especially designated laboratory vaccine refrigerator, using a HOBO Temp® electronic temperature logger to ensure that ideal conditions (2$^0$C to 8$^0$C) were maintained. Prior to immunisation, some vaccines were stored in the closed freezer compartment of a separate 40L refrigerator under stipulated adverse temperature storage conditions. Adverse storage conditions selected were: –3$^0$C for 24 hours, and –3$^0$C or –6$^0$C for 14 days. These conditions were selected as they were demonstrated to be the most common adverse vaccine storage conditions by vaccine providers in a recent study using electronic temperature loggers (128). To maintain the freezer temperatures within controlled limits, a temperature servo-controller was connected to the refrigerator compressor (Elliwell® EWPC 902/T/RP) and its temperature probe was placed in the freezer compartment of the study refrigerator to provide feedback. During vaccine storage under experimental conditions, an electronic logger recorded storage temperatures, confirming that vaccine had been stored within +/- 1.5$^0$C of the desired temperature.

### 2.2.7 Anaesthesia

Mice were anaesthetised using halothane inhalation. This involved placement of mice, one at a time, in a glass jar with a screw-top lid containing cotton wool balls soaked with 1-2 millilitres (mls) of liquid halothane. The halothane quickly vaporised in the closed container at room temperature. To protect the study investigator, anaesthesia was performed in a laminar flow cabinet.

The depth of anaesthesia was judged by observation of respiratory rate and the amount of whisker movement. After an initial phase of rapid respirations, the respiratory rate slowed and mouse whisker movement ceased, indicating an appropriate level of anaesthesia to perform blood collection.

Occasional unexpected deaths occurred using this method of anaesthesia due to the difficulty in judging the depth of anaesthesia accurately.

### 2.2.8 Blood collection

Blood was collected from the retro-orbital sinus using a Pasteur pipette immediately prior to immunisation (average sample volume 250µl), and via cardiac puncture 28 days post immunisation (average sample volume 1000µl). Samples were allowed to clot at room temperature for 2 hours and were then centrifuged in a microfuge at 13 000rpm (16 060g) for 10 minutes before being aliquotted and frozen at $-70^0$C until testing.

### 2.2.9 Immunisation

Vaccines were equilibrated to room temperature immediately prior to administration. Mice were randomised to receive 0.125 ml DTPa, (which is 0.25 the standard human dose {SHD}) 0.1 ml DTPw (0.2 SHD) or 0.1 ml normal saline via intraperitoneal injection. Table 2.1 summarises the antigenic content of these vaccine doses.

As no recommendations for standard dose administration using murine models are available in the published literature, the vaccine doses used were derived from the work of previous investigators performing experiments to compare the protective efficacy of DTPa and DTPw preparations using respiratory challenge models. The doses and composition of acellular preparations vary in these studies as outlined in table 2.2 (115, 185, 186, 229-231) and only one study used Infanrix®, the DTPa preparation used in this study (115). Hence the dose used in the latter study was chosen for DTPa. None of the published studies used the CSL DTPw vaccine: most used the WHO international whole-cell reference preparation 88/522 containing 50IU per ampoule. The 88/522 doses used varied between studies from 0.125 to 0.5 of the standard human dose recommended for formulation of DTPw preparations in the British Pharmacopoeia.

### 2.2.10 Mouse numbers

For all experiments, there were between 11 and 15 mice in each of the three treatment groups.

### 2.2.11 Measurement of pertussis IgG antibody responses

Specific IgG antibody concentrations for antibodies to pertussis toxin (PT), filamentous haemagglutinin (FHA), and pertactin (PRN) were measured using enzyme-linked immunoassays (EIA), adapted from standardised EIAs used to measure human anti-pertussis antibodies. The assays used for the initial murine immunogenicity studies were developed and performed by Mr M Hanlon, in the Department of Immunology and Infectious diseases (New Children's Hospital, Westmead, New South Wales, Australia). The mouse numbering code for each experiment was unknown to the assay laboratory prior to analysis of serum samples and was unblinded only after completion of assays for each experiment.

Ninety-six well microtitre plates (Greiner, Cat 655101) were coated overnight at 4°C with PT (List Biologicals, USA, Cat No. 181) at 0.5µg/ml, FHA (List Biologicals, USA, Cat. No. 170) at 1.0µg/ml, or PRN (batch 42/RS: a generous gift from Dr. Rino Rappuoli, IRIS, Siena, Italy) at 1.0 µg/ml in carbonate-bicarbonate coating buffer, pH 9.6.

The purity of the List Biologicals PT preparation was assessed using electrophoresis on 12% polyacrylamide SDS urea gels. Only preparations which had five identifiable bands when run on this gel system were accepted as pure. The purity of FHA was assessed using 7.5% polyacrylamide SDS urea gel electrophoresis. This demonstrated a major band of 200 000 Daltons and several smaller minor bands of lower molecular weight related to the larger protein. The details of the purity and reparation of PRN supplied by Dr Rappuoli were not available.

After washing five times with 300 µl of phosphate buffered saline (PBS tablets: Sigma cat P4417 lot 20K8205) with 0.05% Tween 20 (PBST), coated and uncoated wells were blocked with 250 µl PBS containing 1% bovine serum albumin (Sigma, Cat A-7638, PBST-BSA), and were incubated at $37^0$C for one hour. One PBS tablet was dissolved in 200ml water to provide a solution of pH 7.4 at $25^0$C, and was equivalent to 0.01 Molar (M) phosphate buffer, 0.0027 M potassium chloride (KCl) and 0.137 M sodium chloride (NaCl). During the blocking step, samples were diluted 1:50 and 1:100 in PBST-BSA. After washing as above, 100µl of the 1:50 dilution was added to the PT coated wells and 100µl of the 1:100 dilution was added to the FHA and PRN coated wells, in triplicate. PBST-BSA was then added to 12 wells, three uncoated and three each coated with PT, FHA and PRN to provide a reagent blank.

The plates were incubated at $37^0$C for 90 minutes, and then washed five times with PBST. HRP-labelled anti-mouse IgG (Serotec, Cat AAC10P) was diluted 1:1000 in PBST-BSA, and 100 µl was added to all wells. After incubation at $37^0$C for 90 minutes, the plates were washed again, five times, as before.

O-PD substrate (Dako, Cat S 2045) was prepared 10 minutes before the end of the incubation, according to the manufacturer's instructions. One hundred microlitres were added to each well, and the plates were incubated at $37^0$C for 30 minutes at which time the reaction was stopped with 50 µl/well of 2N $H_2SO_4$. The plates were read at 490nm in a microtitre plate reader (Biotek EL312).

#### 2.2.11.1 Reference Sera

To provide reference sera, samples were collected from 42 female Swiss outbred mice three weeks after intraperitoneal immunisation with 0.125ml DTPa (SB Infanrix) which had been stored between $2^0$C and $8^0$C. Sera were separated and stored as described in section 2.2.8. The samples were separated into two approximately equal groups: those with generally higher and those with generally lower concentrations of anti-pertussis antibodies to PT, FHA and PRN. Sera in the high titre group were pooled to provide a high-positive control, and sera in the low titre group were pooled for a low-positive control. These two pools of sera were included as quality controls in each assay. A further control sample was obtained from a mouse immunised in a pilot study. This control was given an arbitrary value of 100 EU/ml (EIA units) of IgG specific for PT, FHA and PRN and was diluted serially eight times (two-fold) from 1:50 to 1:6400 in PBST-BSA and plated in triplicate, to construct a standard curve for each batch of assays.

#### 2.2.11.2 Determination of antibody concentrations

Results were analysed using KinetiCalc software (Biotek). The "serum blank" optical densities (OD) and the triplicate sample ODs were averaged, and then the serum blank OD was subtracted from the corresponding sample OD. The net OD was then substituted in a four parameter regression equation generated by the software from the eight point standard curve, giving a concentration relative to the assigned titre of 100 EU/ml for the mouse control sample, after Reizenstein et al (232). Only results which fell on the "straight line", central section of the sigmoid four-parameter regression curve were used to calculate titres. Coefficients of variation (CVs) for the assays were all <15% for inter-assay variation, and <12% for intra-assay variation.

For some samples the net OD was near zero, or in the low, flat section of the regression curve. These results were recorded as below the minimum level of detection, and were assigned the value of the minimum level of detection for that antigen for the purpose of statistical analysis.

### 2.2.12 Statistical analysis

Raw data were transformed to $\log_{10}$ values prior to statistical analyses. One-way analysis of variance (ANOVA) was used to compare all four storage conditions and two sample t-tests were used to determine differences between two storage conditions. P values =0.05 were deemed to be statistically significant.

## 2.3 Results

### 2.3.1 Vaccine storage

No vaccine had been exposed to a temperature below $+1^0$C prior to receipt from the suppliers. Vaccine stored in the temperature range "2-$8^0$C" was recorded by the electronic loggers as never being outside the range $1.5^0$C to $8.5^0$C for either DTPa or DTPw. Vaccine stored at "-$3^0$C" for 24 hours or 14 days was recorded by the electronic loggers as never being outside the range $-1.5^0$C to $-4.5^0$C for DTPa and DTPw. Storage of vaccine at "$-6^0$C" for or 14 days was recorded as never being outside the range of $-4.5^0$C to $-7.2^0$C for both vaccines.

### 2.3.2 IgG antibody concentrations

#### 2.3.2.1 Pre immunisation responses (day 0)

On day 0 of each experiment (i.e. prior to the first immunisation), IgG antibody responses to pertussis antigens in immunised mice were no different from those of the saline controls. The day 0 Geometric Mean Concentrations (GMCs) with 95% confidence intervals of IgG antibodies to PT, FHA and PRN for all experiments combined are presented in table 2.3. Although no significant differences were found between vaccine treatment groups for each antigen prior to immunisation, GMCs of PT, FHA and PRN antibodies were all above the lower limit of detection of the EIAs.

### 2.3.2.2 Post- immunisation antibody responses (day 28)

#### *2.3.2.2.1 Mice immunised with normal saline*

Mice immunised with normal saline did not demonstrate a post-immunisation elevation in IgG antibody concentration in response to PT or FHA (p=0.178, p=0.874 respectively), but did demonstrate a significant post-immunisation elevation in IgG antibody concentration to PRN (p=0.015, table 2.4). Comparison of post-immunisation antibody concentrations between mice immunised with DTPa and mice immunised with normal saline demonstrated significantly higher antibody responses in DTPa–immunised mice in all cases (table 2.5). However, mice receiving DTPw stored at–3$^0$C for 24 hours had antibody responses no different from those immunised with normal saline (PT p=0.203, FHA p=0.397, PRN p=0.733). There was a trend to a higher antibody concentration at 28 days post immunisation in response to PRN in DTPw stored at 2-8$^0$C in comparison with saline (p=0.087) and a lower response to PRN in DTPw stored at –3$^0$C for 14 days in comparison with saline (p=0.090), but the differences did not reach significance.

##### 2.3.2.2.1.1 Investigation of high levels of background activity in EIAs

Because of the high levels of background activity demonstrated in the PT and PRN assays, investigation into possible causes was required. The background activity was thought to be due to non-specific binding of substances in mouse serum.

A subset of samples (n=30) was assayed for the presence of IgG antibodies to lipopolysaccharide (LPS), a component of the outer membrane of all gram-negative bacteria. The CSL DTPw vaccine contains whole *B. pertussis* organisms and hence was a possible source of LPS.

IgG antibody concentrations to LPS were much higher in samples from DTPw than DTPa immunised mice indicating a possible source of cross-reactivity in those samples (figure 2.1).

Heterophile antibodies are a well-recognised cause of high backgrounds due to cross-reactivity in EIAs (233-238). Therefore, the same samples (n=30) were also assayed to determine if IgG heterophile antibodies were present by pre-incubating serum samples with 20% sheep serum overnight and performing the assays using standard methods the following day. This had the effect of increasing IgG responses to PT and FHA in DTPa, and slightly reducing IgG responses to PRN in DTPw (figures 2.2a and 2.2b). Therefore, heterophile antibodies were not thought to be a significant contributing factor to the presence of low-level activity in the day 0 samples and the day28 samples in mice immunised with normal saline.

#### *2.3.2.2.2. Mice immunised with DTPa or DTPw*

Figures 2.3-2.6 document murine IgG PT, FHA and PRN post immunisation IgG antibody concentrations. Results are represented as GMCs in Enzyme Units per millilitre (EU/ml). The error bars represent the 95% confidence intervals of the GMCs. P values are also documented. The numerical values for the GMCs and confidence intervals are also presented in table 2.5 for convenience.

#### *2.3.2.2.3 Comparisons between all storage conditions*

Statistically significant differences were demonstrated overall between the four storage conditions for both vaccines using a one-way ANOVA (figures 2.3a and 2.3b). Although not analysed statistically, as the concentrations of individual pertussis antigens in the CSL DTPw

preparation were not available from the manufacturer, responses to DTPa were clearly higher than those to DTPw for almost all antigens and for all storage conditions.

#### *2.3.2.2.4 Comparisons between ideal storage and individual adverse storage conditions*

##### 2.3.2.2.4.1 Comparisons between storage at $2^0$C to $8^0$C and $-3^0$C for 24 hours

Mice immunised with both DTPa and DTPw stored at $-3^0$C for 24 hours demonstrated post immunisation responses to FHA which were significantly lower than those in mice receiving vaccine stored under ideal conditions ($p=0.001$ and $p=0.006$ respectively, figures 2.4a and 2.4b). Responses to PRN in DTPw were also significantly reduced by adverse storage ($p=0.046$). Responses to PT in either vaccine were not significantly different. However, the IgG responses of mice immunised with DTPw stored adversely were no different from those of mice immunised with normal saline (section 2.3.2.2.1).

##### 2.3.2.2.4.2 Comparisons between storage at $2^0$C to $8^0$C and $-3^0$C for 14 days

IgG antibody responses to PT were significantly higher in mice receiving either DTPa or DTPw stored at $-3^0$C for 14 days than in those immunised with vaccine stored between $2^0$C and $8^0$C (DTPa $p=0.007$, DTPw $p=0<0.001$). Responses to FHA and PRN were significantly lower in mice immunised with adversely stored DTPw (FHA $p=<0.001$, PRN $p=0.002$: responses to PRN were no different from mice immunised with normal saline) but only to FHA for adversely stored DTPa ($p<0.001$), although responses to PRN demonstrated a similar trend (figures 2.5a and 2.5b).

2.3.2.2.4.3 Comparisons between storage at $2^0$C to $8^0$C and $-6^0$C for 14 days

Mice immunised with DTPw stored at $-6^0$C for 14 days had significantly increased antibody responses to all three antigens in comparison to mice immunised with correctly stored vaccine (PT $p<0.001$, FHA $p=0.003$, PRN $p=0.001$, figures 2.6a and 2.6b). The same trend was demonstrated for responses to PT and PRN in DTPa. However, only responses to PRN were significantly increased ($p=0.001$). The response to FHA was marginally lower for DTPa stored at $-6^0$C for 14 days.

## 2.4 Conclusions

This study is the first to examine systematically in an animal model the effects on immunogenicity of storage of both whole cell and acellular pertussis vaccines between $0^0$C and $-6^0$C, conditions which have been documented to occur frequently at vaccine provider sites.

Using Swiss outbred mice as the model, IgG antibody responses to all antigens in both vaccines were paradoxically significantly increased after storage of vaccines at $-6^0$C for 14 days with the exception of FHA in DTPa. Similarly, storage of DTPa and DTPw at $-3^0$C for 14 days significantly increased the immunogenicity of PT. Although the same trend was demonstrated for PRN in DTPa after storage at $-3^0$C for 14 days, the difference was not significant. In contrast, storage of vaccines at $-3^0$C for 24 hours reduced vaccine immunogenicity in all cases except to PRN in DTPa. These results indicate that shorter periods of adverse temperature storage of vaccines may be more damaging in terms of antibody response than longer periods at the same temperature. In addition, longer periods of adverse storage may even result in elevation of IgG antibody responses in the outbred murine model. The reasons for this are unclear.

Previous investigators have suggested that ultra-low temperature storage of DTPw vaccines ($-20^0$C to $-30^0$C) causes a change in the gel structure of the aluminium adjuvant (144). This, in turn, is thought to alter the appearance, sedimentation rate, and protective efficacy (as measured by the Kendrick mouse protection test) of these vaccines (147). However, as mentioned in section 1.6.2.1, not all DTPw preparations tested demonstrated these changes. In addition, the study reported here did not use ultra-low temperature storage conditions.

An alternative hypothesis is that there may be changes in the secondary, tertiary or quaternary structure or even fragmentation of the protein antigens in both DTPa and DTPw after adverse storage. These altered proteins may also desorb from the aluminium adjuvant. Any of these mechanisms could cause a change in the manner in which antigen is presented to antigen presenting cells and hence the specificity of antibody produced by mice for the coating antigen in the EIA. Testing these hypotheses would require the use of complex techniques such as x-ray crystallography for accurate determination of protein structure and degree of aluminium adsorption.

IgG antibody responses to DTPw were lower than those to DTPa in almost all experiments. This may relate to the dose of vaccine used, as mice receiving DTPa were immunised with a greater proportion of the human dose than those receiving DTPw. However, the individual antigen content of the whole cell vaccine is unknown and is not available from the manufacturer. The DTPw formulation used in this study contains formaldehyde-inactivated whole pertussis organisms, with all bacterial antigens represented. The DTPa contains known quantities of purified and formaldehyde-inactivated PRN and FHA and formaldehyde- and glutaraldehyde-inactivated PT extracted from *B. pertussis* organisms. However, in general, similar patterns of change in antibody concentrations after adverse storage were demonstrated in response to all three pertussis antigens.

Higher post immunisation antibody titres in inbred mice to DTPa in comparison with DTPw have been demonstrated previously in the context of an aerosol challenge with pertussis after immunisation of mice with correctly stored vaccine (115, 186). The total doses of DTPa and DTPw used in these studies were higher than those used in the study reported here. The study of Barnard and Mills also examined the cytokine profiles of *B pertussis* specific T cells in

immune inbred mice (186). Antigen-specific T cells from these inbred mice immunised with DTPa were almost exclusively of the Th2 subtype and those from mice receiving DTPw were predominantly Th1. The route of immunisation and the genetic background of the inbred mouse strain used also affected the relative proportion of Th1 or Th2 cells produced. Higher antibody responses to DTPa vaccines also were seen in the majority of clinical trials in infants and children comparing DTPa and DTPw, regardless of formulation (88, 91, 93, 174, 239, 240). Hence, in general, the murine model may reflect the human situation.

The predictive value of antibody responses in protection against *B pertussis* infection is unclear. Recent evidence from clinical trials suggests that antibodies to PRN, fimbrial antigens and PT may be useful as serological correlates of protection against *B pertussis* infection (175, 176). However, investigators testing the protective efficacy of correctly stored DTPa and DTPw vaccines in inbred mice after intranasal or aerosol respiratory challenge have not found a direct relationship between antibody responses and protective efficacy (115, 184). Antibody responses in mice after respiratory challenge tend to be greatest after organisms have been cleared from the lung (184). In addition, protection from infection with *B pertussis* involves both cellular and humoral immunity (162, 184).

There may also be a synergism between antibodies to the three vaccine antigens. Guiso et al demonstrated that a monocomponent PRN vaccine protected mice against a lethal intranasal pertussis challenge. However, at 10 days post challenge there were still significant numbers of organisms present in the lungs of infected mice (115). After a non-lethal intranasal challenge, antibody concentrations to PRN were increased post-immunisation by the addition of a bicomponent (PT, FHA) or tricomponent (PT, FHA and PRN) acellular vaccine. In addition, clearance of organism after intranasal challenge of mice immunised with PRN alone was

significantly slower than in mice immunised with PRN and a bicomponent acellular vaccine at separate sites (115). Therefore, it is unlikely that a single correlate of protection against *B. pertussis* infection and its effects will be found.

IgG antibody concentrations in Swiss outbred mice in response to vaccines stored under all experimental conditions demonstrated very wide 95% confidence intervals. In studies investigating the immunogenicity of acellular DTP vaccines in humans, GMCs, where reported, generally demonstrated narrower 95% confidence intervals (90, 174, 241-244). However, the numbers of participants in those studies were significantly greater than in the study reported here. Therefore, the heterogeneity in responses reflected by the wide confidence intervals is probably also consistent with the numbers of mice in each treatment group.

Mice from all treatment groups demonstrated a pre-immunisation concentration of IgG antibody slightly above the lower limits of assay detection as documented in table 2.3. Mice immunised with normal saline demonstrated a significant elevation in antibody concentration to PRN on day 28, although this response was generally much lower than for mice immunised with DTPa or DTPw (table 2.5). These observations most likely reflect a level of background activity inherent in the EIAs used, as further investigation for the presence of heterophile antibodies was inconclusive. IgG antibodies to Lipopolysaccharide may explain the background activity of samples from mice immunised with DTPw, but not with DTPa. An alternative explanation is subclinical infection of mice with *B. bronchiseptica.* However, this could only explain the presence of background activity in the PRN and FHA assays, as *B. bronchiseptica* does not express PT, despite the presence of the gene in the DNA of this

organism. In addition, routine monitoring of the WCH animal care facility for potential contaminating bacterial and viral pathogens has not detected *B. bronchiseptica* to date.

In summary, longer periods of adverse vaccine storage may increase pertussis vaccine immunogenicity. However, the mechanisms causing these changes are yet to be determined. Because of the heterogeneity of antibody response of mice in the same treatment group and because of the low but significant concentrations of antibody in mice immunised with normal saline, these results must be interpreted with caution.

**Table 2.1 Antigenic content of study vaccines: comparison of standard human dose and dose administered to mice**

| Vaccine | Antigen | Standard Human dose | Murine dose |
|---|---|---|---|
| **Infanrix DTPa** | -Pertussis toxoid | 25µg | 6.25µg |
| | -Filamentous haemagglutinin | 25µg | 6.25µg |
| | -Pertactin | 8µg | 2µg |
| | -Diphtheria toxoid | 25Lf | 6.25Lf |
| | -Tetanus toxoid | 10Lf | 2.5Lf |
| **CSL DTPw** | -Heat inactivated *B. pertussis* organisms/ml | $20x10^9$ | $4x10^9$ |
| | -Diphtheria toxoid | 30Lf | 6Lf |
| | -Tetanus toxoid | 6Lf | 1Lf |

## Table 2.2 Summary of pertussis vaccine doses used in protective efficacy experiments

| Study | Challenge method | Acellular preparation | Pertussis antigen dose | Whole cell preparation | Whole cell dose |
|---|---|---|---|---|---|
| **Boros et al**<br><br>2001, current study | Intranasal | Infanrix DTPa | 0.125 SHD | CSL DTPw | 0.1 SHD |
| **Canthaboo et al**<br><br>Vaccine 2001, 19:637-643 | Aerosol | Triple antigen vaccine: pertussis components as for Infanrix® | 0. 5 SHD* | Vaccine not specified | 0.25 "human dose" |
| **Guiso et al**<br><br>Vaccine 1999, 17:2366-2379 | Intranasal | a)Infanrix DTPa<br><br>b)Bicomponent DTPa | a) 0.25 SHD*<br><br>b) 0.25 SHD‡ | No DTPw used | |
| **Syukuda et al**<br><br>Tokai J Clin Exp Med 1998, 13(supp), 71-77 | Aerosol | a)Takeda L-30 DTPa PT, FHA, fimbrial antigens 9:90:1 ratio<br>b)JNIH-3 DTPa PT, FHA 50:50 ratio | a) 0.05 SHD<br><br>b) 0.05 SHD | WHO reference preparation 88/522 | 0.5IU (0.125 SHD†) |
| **Mahon et al**<br><br>Infection and Immunity 1996, 64(12):5955-5301 | Aerosol | Purified antigens (PT, FHA, PRN) | 5µg of each antigen (0.2 SHD* PT, FHA, 0.625 SHD* PRN) | WHO reference preparation 88/522 | 0.8 IU (0.2 SHD†) |
| **Barnard et al,**<br><br>Immunology 1996, 87:372-80. | Aerosol | Purified antigens (PT, FHA, PRN) | 12.5 µg each of PT (0.5 SHD*) ,FHA (0.5 SHD), and PRN (1.56 SHD) | WHO reference preparation 88/522 (50 IU/ampoule) | 2.0 IU (0.5 SHD†) |
| **Redhead et al**<br><br>Infection and Immunity 1993, 61(8): 3190-3198 | Aerosol | Purified antigens (PT, FHA, PRN) | 12.5 µg each of PT (0.5 SHD*), FHA (0.5 SHD), and PRN (1.56 SHD) | WHO reference preparation 88/522 | 2.0 IU (0.5 SHD†) |

*: standard human dose of Infanrix® containing PT and FHA 25µg and PRN 8µg, diphtheria toxoid 25 Lf, tetanus toxoid 10Lf

†: standard human dose of heat-killed pertussis organisms in UK DTPw preparation

‡: standard human dose of SB bicomponent DTPa preparation containing 25µ g of PT and FHA, diphtheria toxoid 25Lf, tetanus toxoid 10Lf

**Table 2.3 Day 0 GMCs of IgG antibodies (EU/ml) in Swiss outbred mice**

(combined from all four experiments: pre-immunisation results, n=60)

| | PT | FHA | PRN |
|---|---|---|---|
| **DTPa** | 7.06 (3.83-13.02) | 3.68 (3.28-4.16) | 8.24 (6.44-10.56) |
| **DTPw** | 4.98 (2.56-9.71) | 3.61 (3.26-3.99) | 6.38 (5.56-7.23) |
| **Saline** | 5.02 (2.62-9.59) | 3.82 (3.24-4.49) | 9.23 (6.96-12.24) |
| **ANOVA** | p=0.690 | p=0.831 | p=0.078 |
| **Lower limit of assay sensitivity (EIAU/ml)** | 0.1 | 3 | 5.6 |

**Table 2.4 Comparison of day 0 and day 28 IgG GMCs (EU/ml) in saline-immunised Swiss outbred mice**

(combined from all four experiments, n=60)

| | PT | FHA | PRN |
|---|---|---|---|
| Saline pre- immunisation GMCs | 5.02 (2.62-9.59) | 3.82 (3.24-4.49) | 9.23 (6.96-12.24) |
| Saline post- immunisation GMCs | 6.92 (3.88-12.32) | 3.92 (3.32-4.62) | 14.94 (10.00-22.32) |
| Two sample t-test | p=0.178 | p=0.874 | p=0.015 |

**Table 2.5 Statistical comparisons between post-immunisation IgG antibody concentrations in mice immunised with DTPa vs saline and mice immunised with DTPw vs saline**

(GMCs and 95% confidence intervals also documented: saline post immunisation GMCs combined for all 4 storage conditions)

| | 2-8$^0$C | | | -3$^0$C/24 hours | | | -3$^0$C/14 days | | | -6$^0$C/14 days | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **PT** | **FHA** | **PRN** | **PT** | **FHA** | **PRN** | **PT** | **FHA** | **PRN** | **PT** | **FHA** | **PRN** |
| **DTPa** (GMCs with 95% CIs) | 181.26 (110.26-327.69) | 330.59 (142.83-765.16) | 150.07 (59.44-378.98) | 87.94 (41.87-184.71) | 36.77 (16.65-81.26) | 228.59 (119.65-436.72) | 414.05 (303.26-565.32) | 21.40 (17.3-25.54) | 175.87 (123.06-251.34) | 234.74 (141.19-390.26) | 291.47 (134.28-632.67) | 1034.97 (441.64-1425.47) |
| **DTPw** (GMCs with 95% CIs) | 16.01 (8.78-29.54) | 46.08 (26.30-80.76 | **25.60*** (15.70-41.76) | **12.53*** (6.25-25.11) | **6.46*** (2.10-19.90) | **13.54*** (9.08-20.19) | 356.19 (267.82-473.72) | 7.01 (4.38-11.23) | **9.30*** (6.50-13.30) | 299.92 (151.10-721.87) | 130.36 (82.60-205.73) | 176.34 (72.00-419.07) |
| **Saline** (GMCs with 95% CIs) | 6.92 (3.88-12.32) | 3.92 (3.32-4.62) | 14.94 (10.00-22.32) | 6.92 (3.88-12.32) | 3.92 (3.32-4.62) | 14.94 (10.00-22.32) | 6.92 (3.88-12.32) | 3.92 (3.32-4.62) | 14.94 (10.00-22.32) | 6.92 (3.88-12.32) | 3.92 (3.32-4.62) | 14.94 (10.00-22.32) |
| **DTPa vs saline** | p<0.001 | p<0.001 | p<0.001 | p<0.001 | p<0.001 | p<0.001 | p<0.001 | p<0.001 | p<0.001 | p<0.001 | p<0.001 | p<0.001 |
| **DTPw vs saline** | p<0.001 | p<0.001 | **p=0.087** | **p=0.203** | **p=0.397** | **p=0.733** | p<0.001 | P=0.036 | **p=0.090** | p<0.001 | p<0.001 | p<0.001 |

*Comparisons between DTPw and combined Saline IgG GMCs for which p> 0.05 (two sample t-test)

**Figures 2.1a and b LPS IgG antibody concentrations in mice immunised with DTPa (2.1a) or DTPw (2.1b)**

**Figure 2.1a (n=30)**

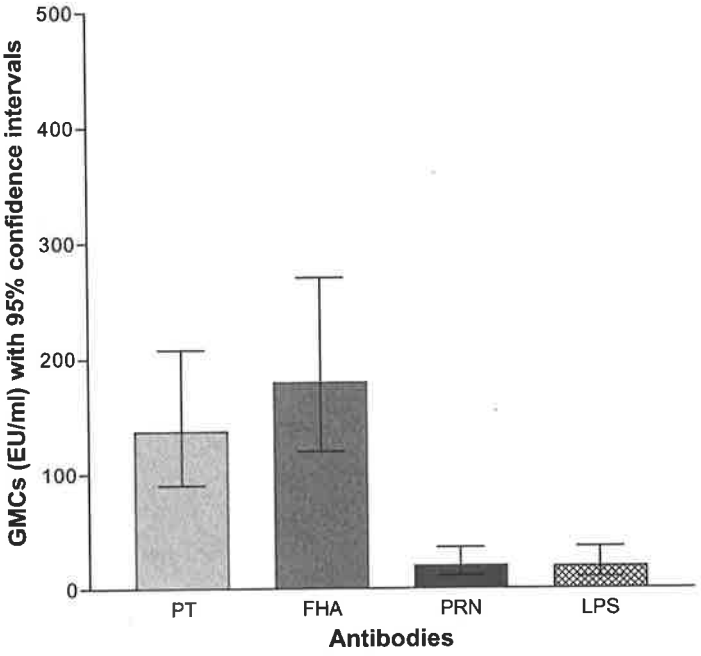



**Figure 2.1b (n=30)**

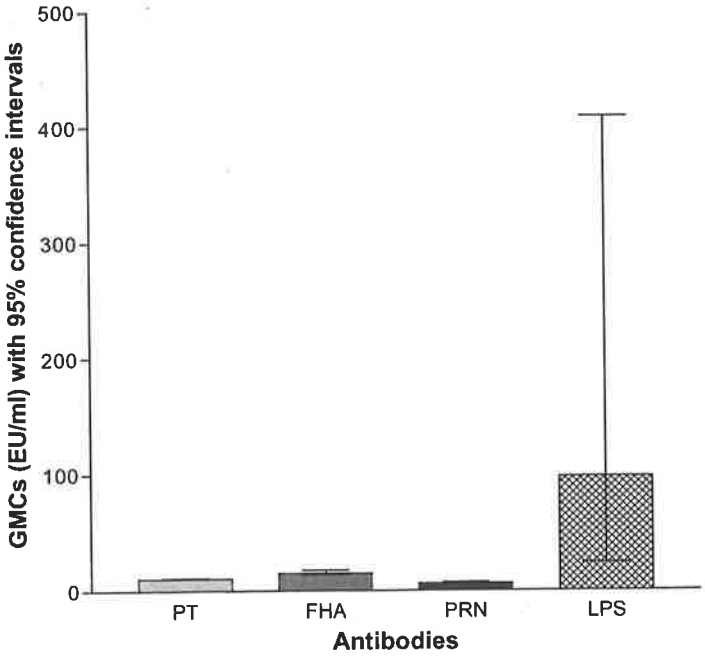


**Figure 2.2 Comparison between normal assays and samples pre-incubated with 20% sheep serum in Swiss outbred mice immunised with DTPa (Fig 2.2a) or DTPw (Fig 2.2b).**

**Figure 2.2a (n=30)**

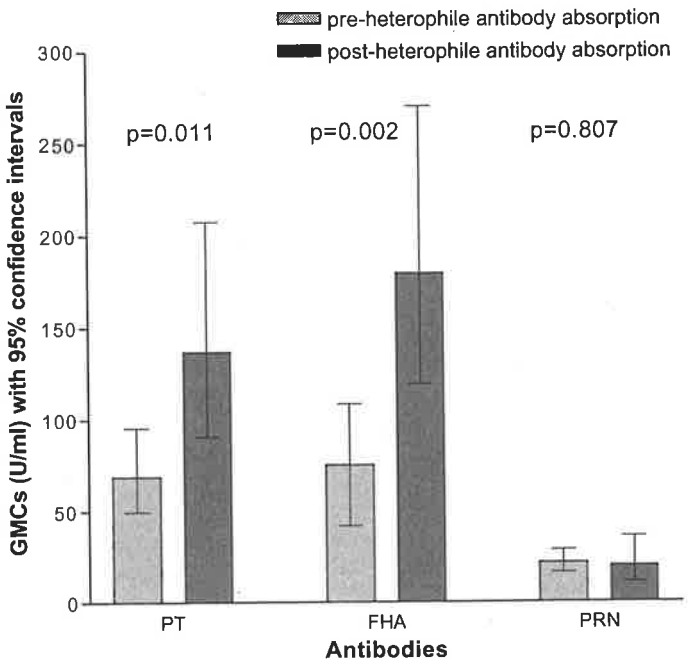
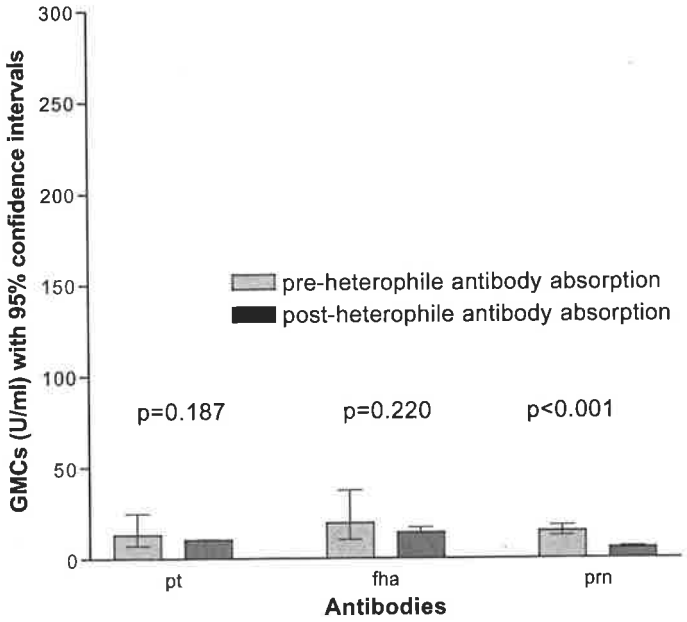



**Figure 2.2b (n=30)**



## Figure 2.3 Swiss outbred IgG antibody responses to pertussis antigens: four storage conditions (DTPaFig 2.3a, DTPw Fig 2.3b)

**Figure 2.3a (n=83)**
**statistical comparisons between storage conditions using one-way ANOVA**

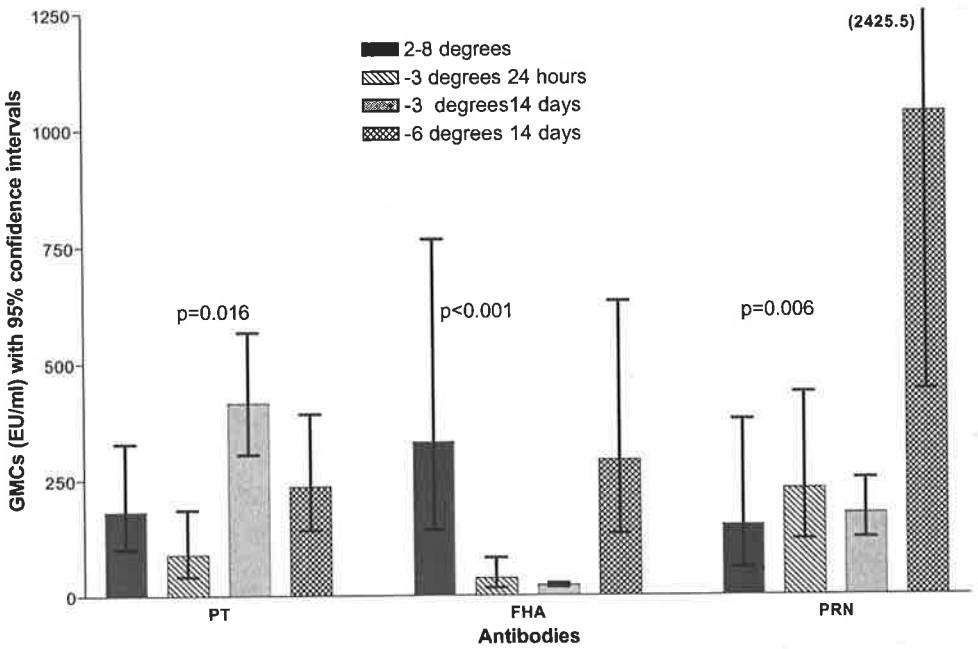
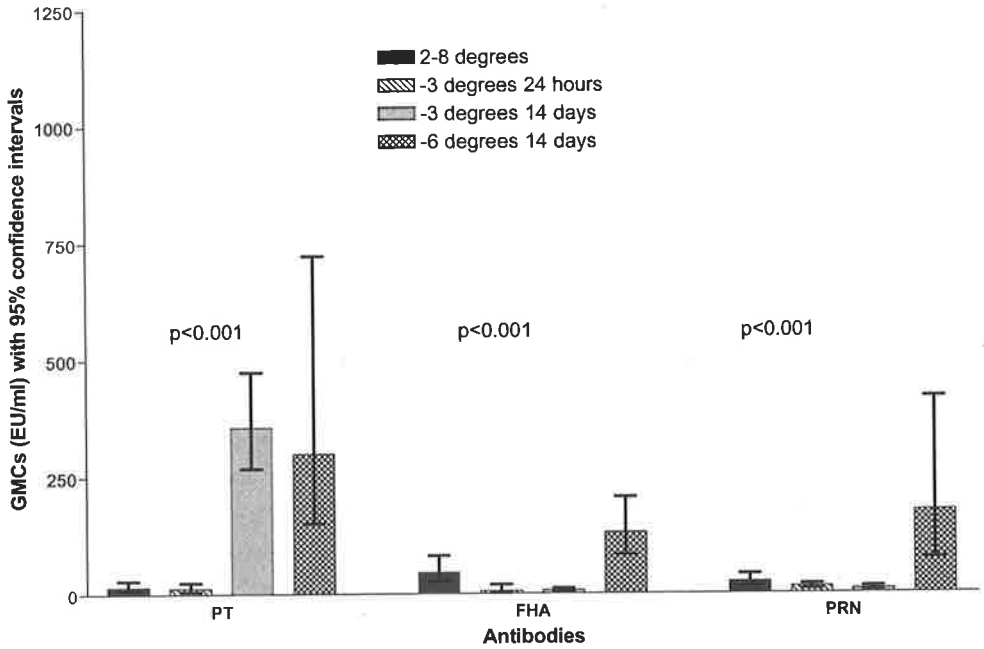



**Figure 2.3b (n=81)**
**statistical comparisons between storage conditions using one-way ANOVA**



## Figures 2.4, 2.5 and 2.6 IgG antibody responses: comparisons between ideal storage and individual adverse storage conditions

Figure 2.4a
IgG antibody responses to DTPa pertussis antigens in Swiss Outbred mice
2-8°C vs -3°C 24 hours (n=54)

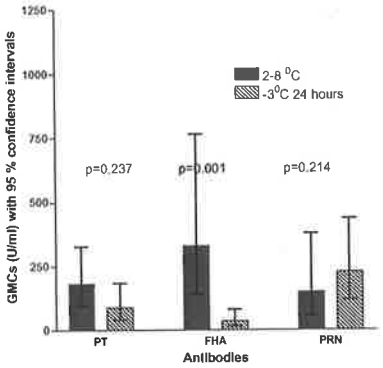



Figure 2.4b
IgG antibody responses to DTPw pertussis antigens in Swiss Outbred mice
2-8°C vs -3°C 24 hours (n=53)

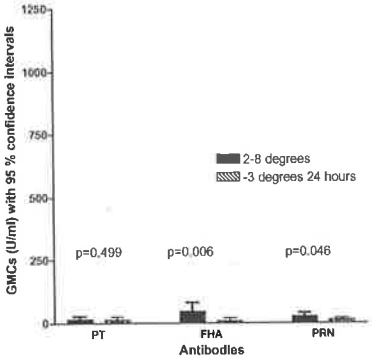



Figure 2.5a
IgG antibody responses to DTPa pertussis antigens in Swiss outbred mice
2-8°C vs -3°C 14 days (n=43)

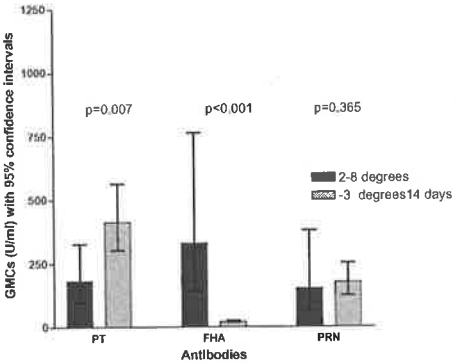



Figure 2.5b
IgG antibody responses to DTPw pertussis antigens in Swiss outbred mice
2-8°C vs -3°C 14 days (n=42)

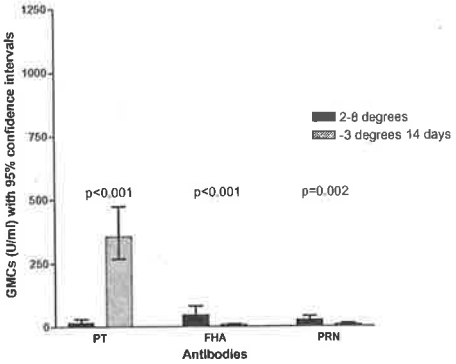



Figure 2.6a
IgG antibody responses to DTPa pertussis antigens in Swiss outbred mice
2-8°C vs -6°C 24 hours (n=42)

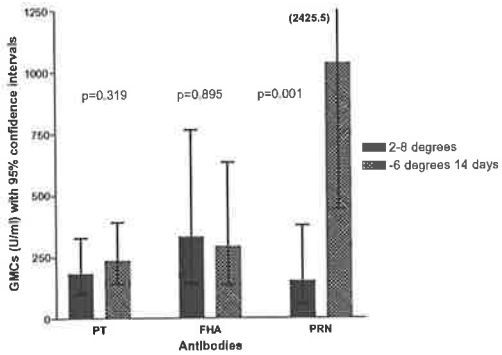
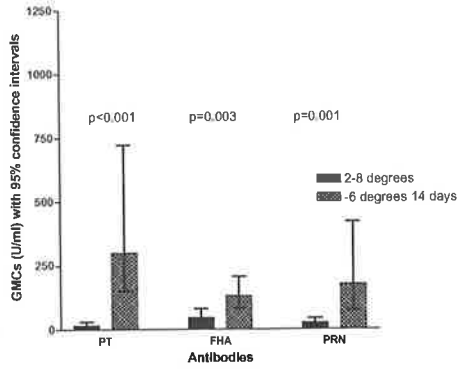



Figure 2.6b
IgG antibody responses to DTPw pertussis antigens in Swiss outbred mice
2-8°C vs -6°C 14 days (n=42)



# Chapter 3 Immunogenicity studies: inbred mice

## 3.1 Introduction

Wide confidence intervals and relatively high background activity were observed in assay results from experiments utilising Swiss outbred mice in the previous chapter. Therefore, further experiments were performed in inbred mice to determine if this variability was inherent in the experimental model, or whether choice of mouse strain would influence assay results.

## 3.2 Methods

### 3.2.1 Mouse strain, age and sex

Female Balb/c inbred mice 8 weeks of age at the commencement of each experiment were used. Th2 responses after antigen challenge are known to predominate in this strain (245), thus favouring antibody production. The age and sex of these mice were chosen to be consistent with the methods described in section 2.2.

### 3.2.2 Vaccine transport and storage

The same vaccine preparations were used in these experiments as for the studies using outbred mice (SB Infanrix® DTPa batch 14887B9B, CSL Ltd DTPw® batch 0434 01609). Vaccine transport and storage conditions were identical to those described in section 2.2.4.

Two storage conditions were investigated: ideal storage conditions ($2^0$C to $8^0$C) and $-3^0$C for 24 hours. Storage of vaccines at $-3^0$C for 24 hours was chosen as the single adverse storage condition for these experiments, as changes in antibody response to both DTPa and DTPw were the most consistent in Swiss Outbred mice immunised wth vaccines stired under the same conditions. Adverse vaccine storage was performed in the same manner as

described in section 2.2.6 using an electronic temperature logger to document the conditions of adverse storage and a temperature servo controller in the freezer compartment of the designated 40L refrigerator to generate adverse temperature storage conditions.

### 3.2.3 Mouse numbers

In each experiment 15 mice were immunised with DTPa, 15 were immunised with DTPw, and 5 mice were immunised with normal saline. To gain the necessary information with the minimum use of mice (as recommended by the NH&MRC animal ethics guidelines), a lower number of negative controls was used in these experiments.

### 3.2.4 Mouse housing

Mice were bred in the University of Adelaide animal care facility under specific pathogen free (SPF) conditions, and were transported in filter cages to the WCH at the age of 7 weeks, in order to allow one week of recovery prior to the commencement of each experiment. On arrival at the WCH animal house mice were transferred to standard cages with no more than 15 mice per cage, and were housed in the general rodent room.

### 3.2.5 Anaesthesia, blood collection and immunisation

Mice were anaesthetised by halothane inhalation. Blood collection and immunisation were performed on days 0 and 28 of each experiment in the same manner as in sections 2.2.8 and 2.2.9.

### 3.2.6 Measurement of IgG antibody responses to pertussis antigens

Antibody measurement for these experiments was also performed in the Department of Immunology and Infectious Diseases Laboratory at the NCH as in section 2.2.10.

### 3.2.7 Statistical analysis

All data were $\log_{10}$ transformed prior to statistical analysis using SYSTAT10®. Statistical comparisons were performed using one-way ANOVA or the Student's two sample t-test.

## 3.3 Results

### 3.3.1 Vaccine storage

The temperature ranges of vaccine storage as recorded by the electronic temperature loggers were between 1.8$^0$C and 8.5$^0$C for ideal storage and between –1.8$^0$C to -4.5$^0$C for storage at "-3$^0$C for 24 hours".

### 3.3.2 Pertussis IgG antibody responses

#### 3.3.2.2 Pre-immunisation antibody responses (day 0)

There were no significant differences in day 0 IgG antibody concentrations between experiments utilising different vaccine storage conditions, therefore these results were combined to compare responses between vaccine storage groups (table 3.1: PT p=0.438, FHA p=1.000, PRN p=0.611). In addition, the level of background activity demonstrated for PT and PRN in experiments in outbred mice was much reduced.

#### 3.3.2.3 Post immunisation antibody responses (day 28)

##### *3.3.2.3.1 Mice immunised with normal saline*

Although post immunisation IgG concentrations were marginally higher than pre-immunisation concentrations, particularly in response to PRN, no statistically significant differences were detected between concentrations on days 0 and 28 to PT or FHA (PT p=0.624, FHA p=1.000) but responses to PRN were, once again marginally increased on day 28 (p=0.047).

#### *3.3.2.3.2 Mice immunised with DTPa or DTPw*

IgG antibody responses to PRN in mice immunised with correctly stored DTPa and DTPw were higher than those in mice immunised with vaccine stored at $-3^0$C for 24 hours (figures 3.1a and 3.1b). However, this was only significant in mice immunised with DTPa (DTPa p=0.014, DTPw p=0.313). There were no significant differences in IgG antibody concentrations between mice in the two experiments for PT or FHA for either vaccine. However, the trend in mice immunised with DTPa was for IgG antibody concentrations to be higher after immunisation with adversely stored vaccine in comparison with mice immunised with vaccine stored under ideal conditions (between $2^0$C and $8^0$C).

Antibody responses to PT were significantly lower in magnitude in mice immunised with DTPw than in those immunised with DTPa ($p<0.001$ for storage between $2^0$C and $8^0$C and $-3^0$C for 24 hours). Responses to FHA were only significantly lower in mice immunised with DTPw stored at $-3^0$C for 24 hours ($2^0$C-$8^0$C p=0.505, $-3^0$C/24 hours p=0.039). Similar PRN IgG antibody concentrations after immunisation with DTPa and DTPw were demonstrated in both experiments ($2^0$C-$8^0$C p=0.953, $-3^0$C/24 hours p=0.147).

Mice immunised with DTPa or DTPw demonstrated significantly higher IgG concentrations to FHA and PRN than mice immunised with normal saline in both experiments (table 3.3). However, IgG antibody responses to PT were significantly higher in mice immunised with DTPa only, indicating reduced responses to PT in the whole cell vaccine in Balb/c mice.

#### *3.3.2.3.3 Comparisons between inbred and outbred mice*

Swiss outbred mice immunised with correctly and adversely stored DTPa had significantly higher antibody responses than Balb/c mice to FHA and PRN (figs 3.2a and 3.2b; 2-$8^0$C:

FHA p<0.001, PRN p<0.016, –3$^0$C 24 hours: FHA p=0.002, PRN p<0.001). Responses to PT in DTPa did not differ significantly between the two strains (2-8$^0$C p=0.057, –3$^0$C 24 hours p=0.868). Outbred mice immunised with DTPw demonstrated significantly higher antibody responses to PT than inbred mice after immunisation with vaccine stored under ideal or adverse conditions (2-8$^0$C p=0.001, –3$^0$C 24 hours p=0.009) but only to FHA in DTPw stored at 2-8$^0$C (p=0.009).

For outbred mice, the differences in GMC between the two storage conditions tended to be greater, if not always statistically significant. However, after immunisation with DTPa or DTPw, inbred mice demonstrated greater consistency in antibody responses to correctly and adversely stored vaccines as well as demonstrating narrower 95% confidence intervals for the GMCs of antibody responses.

As with Swiss outbred mice, the rise in antibody titres on day 28 in Balb/c mice immunised with normal saline was only significant in response to PRN (table 3.2). However, the antibody concentrations in Balb/c mice immunised with normal saline were much closer to the lower limits of detection for each assay than those observed in outbred mice.

Balb/c mice immunised with DTPw stored correctly or adversely had significantly higher antibody responses to and FHA and PRN than those immunised with normal saline (table 3.3), whereas responses to DTPw PRN in outbred mice were no different from those immunised with normal saline for three of the four storage conditions (2$^0$C to 8$^0$C, -3$^0$C 24 hours, -3$^0$C 14 days). In addition, Swiss outbred antibody responses to FHA and PT in DTPw stored at –3$^0$C for 24 hours were no different from those in mice immunised with

saline. In inbred mice, only antibody responses to PT were no different between the DTPw and saline immunised mice.

## 3.4 Conclusions

In general, Balb/c mice demonstrated no significant differences in antibody response to pertussis antigens in DTPa or DTPw after immunisation with correctly or adversely stored vaccine (the exception being responses to PRN in DTPa). In addition, narrower 95% confidence intervals of GMCs were found than in previous experiments with Swiss outbred mice, using similar numbers of mice.

Inbred mice immunised with normal saline did not demonstrate the same degree of antibody change after immunisation as Swiss Outbred mice. Antibody concentrations in inbred mice were also much closer to the lower limits of sensitivity for each assay. As a consequence, mice immunised with DTPw had significantly higher antibody responses than those immunised with normal saline except in response to PT. Although murine antibody responses to pertussis immunisation are not well reported in the published literature, lack of murine antibody response to PT in whole cell pertussis vaccines has not been reported by other investigators. However, most of these investigators used the WHO pertussis reference preparation, which contains killed pertussis organisms only, with no adjuvant and no diphtheria or tetanus toxoid added (table 2.2, (115, 185, 186, 229-231). Therefore it is possible that the addition of these agents to the vaccine may alter antibody responses in Balb/c mice to PT by attaching to the antigenic site of PT or altering it such that it is not recognised by APCs.

In general, Balb/c mice demonstrated significantly lower responses to DTPa antigens and moderately lower responses to DTPw than Swiss Outbred mice despite the tendency to Th2 responses documented to occur in the Balb/c (245). No previous investigators have reported comparison of the antibody responses of inbred and outbred mice to correctly or adversely stored pertussis vaccines. However, data exist regarding the comparative

antibody responses of inbred and outbred mice to correctly stored diphtheria and tetanus toxoids as determined by toxin neutralisation and haemagglutination assays (246). Outbred mice (ddy{SPF}] and ddy{conv}) demonstrated higher antibody responses to both toxoids than 11 tested inbred mouse strains. The C57BL/6 C57BL/10 and BALB/c inbred strains (in descending order of magnitude) demonstrated higher responses than other inbred strains. In addition, the H-2 haplotype (the murine equivalent of the HLA loci) also affected antibody responses. Outbred strains (H-2 haplotype variable) and the inbred strains with $H\text{-}2^b$ haplotype (C57BL/6 and C57BL/10) demonstrated high antibody responses to both antigens. Inbred mice with $H\text{-}2^d$ haplotype (including Balb/c mice) demonstrated high or intermediate antibody responses to both antigens. $H\text{-}2^k$, $H\text{-}2^a$ or $H\text{-}2^m$ conferred high responses to diphtheria toxoid and low responses to tetanus toxoid, and $H\text{-}2^{h4}$ conferred low responses to both antigens. Therefore the H-2 haplotype may be a factor influencing the antibody responses to pertussis antigens in the strains of mice used in the experiments described here and in chapter 2.

In addition, Balb/c substrains differ in the Qa2 locus, which governs the expression of cell surface antigens (Mouse Genome informatics website). The substrain used in this study was Balb/c ARC, which was imported into Australia in 1977, before detailed substrain genetic analysis was performed (Mr D. Thomas personal communication, Animal Resource Centre, Western Australia). Swiss outbred mice, however, exhibit an average heterozygosity that is similar to that found in feral mice and humans (228, 247). Hence cell surface antigens are likely to be less restricted in Swiss outbred mice. This difference provides another potential explanation for the diminished antibody responses demonstrated by the Balb/c mice used in this study.

Antibody responses to immunisation in mice can also be influenced by antigen dose. In the experiments performed by Kameyma et al (246) all mouse strains demonstrated linear diphtheria and tetanus toxoid dose: antibody response relationships between $\log_{10}$ –2.0 and –1.0 U/ml of immunising antigen in addition to the previous finding of differential antibody concentrations between individual mouse strains. Hay and Torrigiani demonstrated that Balb/c mice immunised intraperitoneally with $\leq$1000μg alum precipitated human serum albumin (HSA) and $2x10^9$ B pertussis organisms had consistent levels of anti-HSA (defined as total antigen binding capacity) one week post immunisation which remained relatively constant for the next 6 weeks. However, immunising doses $\geq$1000μg induced a slower rise in antibody titres, which did not reach those of mice immunised with lower doses until 5 weeks post immunisation (248). A possible explanation for this was neutralisation of all circulating antibodies due to the high dose of immunising antigen. However, this was refuted after IgG subclass analysis demonstrated a drop in $IgG_1$ subclass antibodies with a paradoxical rise in $IgG_{2a}$ subclass antibodies after immunisation with the higher antigen dose.

In the present studies, mice immunised with DTPa received ¼ of the human dose, whereas mice immunised with DTPw received 1/5 of the human dose. It is interesting to note that although antibody responses to PT and FHA stored at $-3^0$C for 24 hours and PT stored between $2^0$C and $8^0$C in DTPw were significantly lower in mice immunised with DTPw, responses to PRN did not differ significantly between mice immunised with DTPa or DTPw indicating that the difference in vaccine dose did not affect the antigenic content of PRN administered to mice immunised with DTPw. In the light of human studies which have indicated that the antibody response to PRN may be a useful serological correlate of protection against infection (175, 176), this is also reassuring in terms of potential effect on vaccine protective efficacy.

Finally, studies in both humans and mice have demonstrated that natural infection and immunisation with DTPw stimulate a predominant Th1 immune response, whereas infection with DTPa tends to produce either a Th2 or mixed Th1/Th2 response. This could explain the lower antibody responses of both Swiss Outbred and Balb/c mice to DTPw.

Therefore, it is likely that the genetic background of the Balb/c mice used in the present study, the immunising doses of antigen as well as the vaccine used have affected antibody responses to pertussis antigens in both vaccines in these mice.

These experiments have demonstrated that the variability in antibody response demonstrated in experiments utilising Swiss Outbred mice was explicable in terms of mouse strain. Despite lower antibody responses in Balb/c mice, the consistency of results to antigens in both vaccines determined their use in the subsequent studies of vaccine protective efficacy reported here.

**Table 3.1 Pre -immunisation Balb/c IgG GMCs**

| | PT | FHA | PRN |
|---|---|---|---|
| **DTPa (n=30)** | 0.45 (0.23-0.87) | 3 (3-3) | 6.46 (5.83-7.17) |
| **DTPw (n=30)** | 0.30 (0.18-0.49 | 3 (3-3) | 6.17 (5.69-6.70) |
| **Saline (n=10)** | 0.23 (0.09-0.56) | 3 (3-3) | 5.95 (5.31-6.67) |
| **Lower limit of assay detection (EU.ml)** | 0.1 | 3 | 5.6 |
| **ANOVA** | p=0.438 | p=1.000 | p=0.611 |

**Table 3.2 Comparison of day 0 and day 28 saline Balb/c IgG GMCs**

| | PT | FHA | PRN |
|---|---|---|---|
| **Saline pre- immunisation GMCs** | 0.23 (0.09-0.56) | 3 (3-3) | 5.95 (5.31-6.67) |
| **Saline post- immunisation GMCs** | 0.35 (0.09-1.46) | 3.11 (2.89-3.36) | 7.91 (5.69-11.00) |
| **Lower limit of assay detection (EU.ml)** | 0.1 | 3 | 5.6 |
| **2 sample t-test** | p=0.624 | p=1.000 | p=0.047 |

**Table 3.3 Statistical comparisons between day 28 post-immunisation IgG antibody concentrations in Balb/c mice immunised with DTPa vs saline and mice immunised with DTPw vs saline**

| | 2-8$^0$C | | | -3$^0$C/24 hours | | |
|---|---|---|---|---|---|---|
| | **PT** | **FHA** | **PRN** | **PT** | **FHA** | **PRN** |
| **DTPa** (GMCs with 95% CIs) | 43.11 (14.13-131.47) | 5.91 (3.79-9.21) | 27.71 (16.11-47.66) | 70.12 (48.85-100.65) | 8.87 (5.33-14.75) | 11.27 (7.94-16.01) |
| **DTPw** (GMCs with 95% CIs) | **1.11*** (0.30-4.14) | 4.92 (3.62-6.69) | 39.94 (17.63-90.46) | **1.15*** (0.27-4.85) | 4.70 (3.51-6.31) | 17.95 (11.63-27.68) |
| **Saline** (GMCs with 95% CIs)** | 0.35 (0.09-1.46) | 3.11 (2.89-3.36) | 7.91 (5.69-11.00) | 0.35 (0.09-1.46) | 3.11 (2.89-3.36) | 7.91 (5.69-11.00) |
| **DTPa vs saline** | p<0.001 | p=0.004 | p=0.003 | p<0.001 | p=0.001 | p=0.050 |
| **DTPw vs saline** | **p=0.184** | p=0.023 | p=0.005 | **p=0.199** | p=0.007 | p=0.015 |

*Comparisons between DTPw and combined Saline IgG GMCs for which p> 0.05
Saline post immunisation GMCs combined for both storage conditions, as no differences in antibody responses were demonstrated

**Figures 3.1a and 3.1b: IgG antibody responses in Balb/C mice**

**Figure 3.1a**

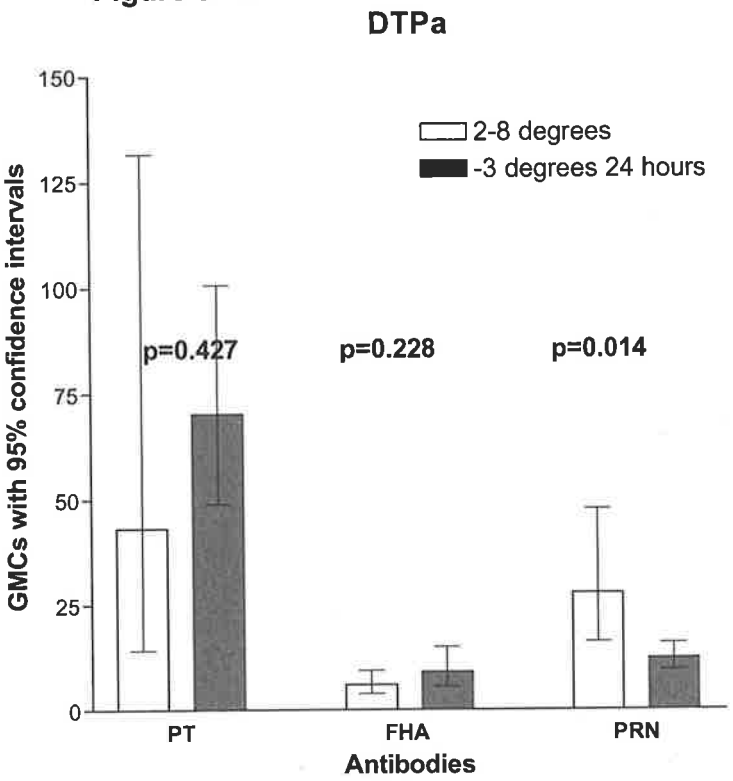
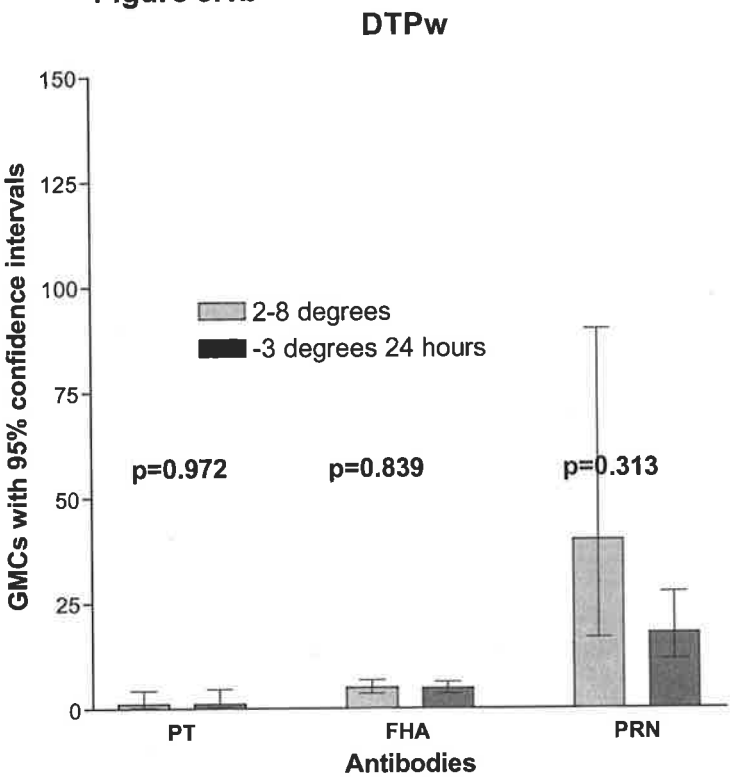



**Figure 3.1b**



**Figures 3.2a and 3.2b:** **Comparison of IgG antibody responses in Swiss outbred and Balb/C mice**

**Figure 3.2a**

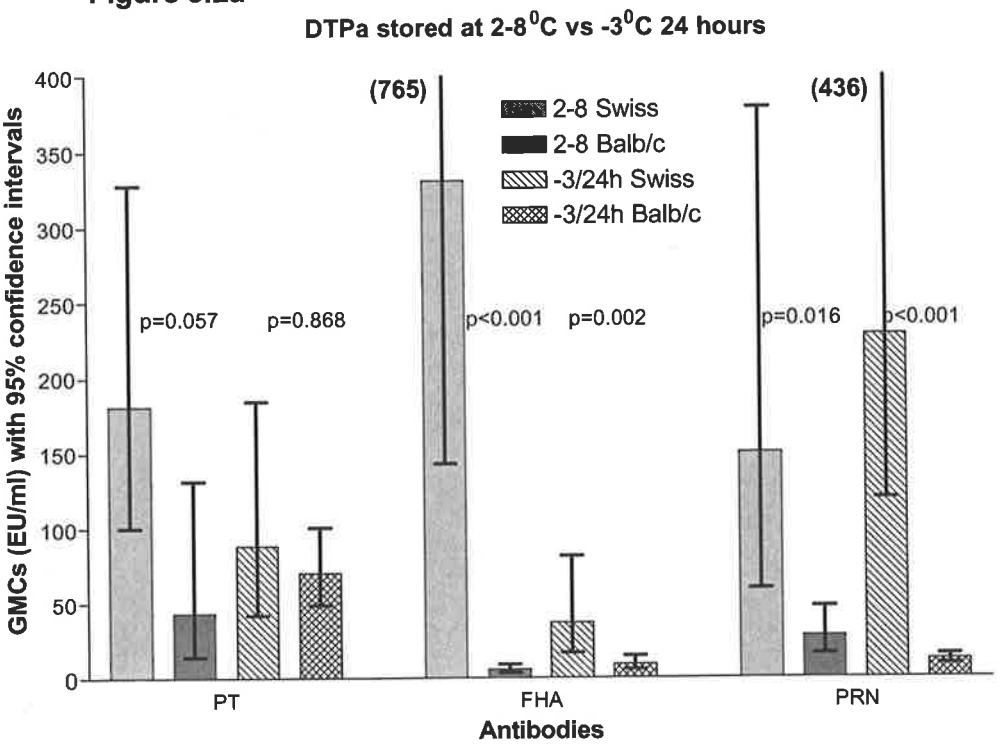
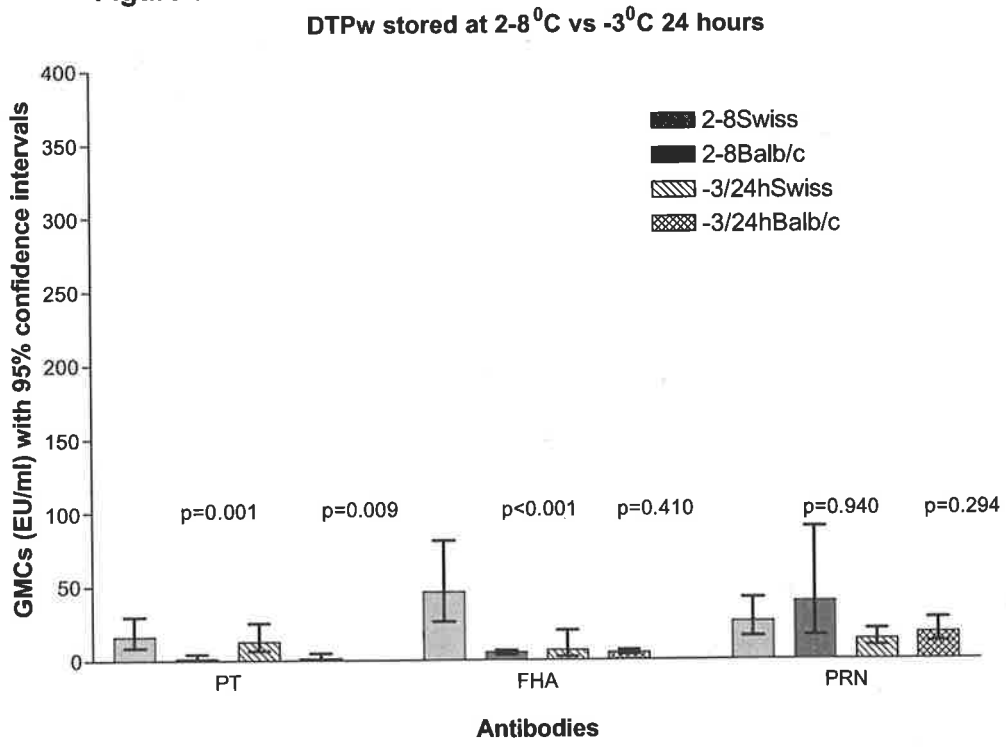



**Figure 3.2b**



# Chapter 4 The protective efficacy of adversely stored pertussis vaccines: development of the mouse model

## 4.1 Introduction

The immunogenicity of pertussis vaccines is altered under certain conditions of adverse storage below $0^0$C (249). However, there is no agreed serological correlate of protection against pertussis infection and its effects. Therefore, only limited information regarding the potential clinical protective efficacy of such adversely stored vaccines can be gained from immunogenicity experiments which use serum antibody concentrations as the end point. In addition, it has been demonstrated that cell-mediated immunity has a definite role in protection against pertussis infection (41, 185, 250), further emphasising the need for a different experimental model to investigate the protective efficacy of adversely stored pertussis vaccines.

Previous experiments performed to investigate the effects of adverse storage on the protective efficacy of pertussis vaccines have used whole cell preparations only (147), and have not utilised adverse temperature storage conditions which have been documented to exist at vaccine provider sites (128). In addition, the experimental model for these studies was the Kendrick intracerebral challenge model of mouse protection (148), which has now been demonstrated to lack correlation with the clinical protective efficacy of both whole cell and acellular pertussis vaccines used in more recent clinical trials (93, 115, 174, 176, 251).

The following experiments were therefore designed to assess the protective efficacy of DTPa and DTPw preparations utilising storage temperatures likely to be found at vaccine provider sites (as in sections 2.2 and 3.2) in a murine model using an adaptation of methods from previous studies (115, 184, 252). The advantage of using such methods for

assessing the protective efficacy of pertussis vaccines is that the main outcome measure, i.e. rate of clearance of organisms from the lungs, reflects the overall efficacy of the immune response and allows a better understanding of the pathological effects of the bacterium in addition to assessment of the role of adverse vaccine storage in these processes. The pilot studies used to develop the definitive protocol for the protective efficacy study are described in section 4.3.

## 4.2 Review of methods of previous investigators

A summary of the methods of a selection of previous studies using murine challenge models to delineate the course of pertussis infection or to measure or compare the protective efficacy of pertussis vaccines is presented in table 4.1. From this table it can be seen that the interval between Kendrick's intracerebral challenge model and the development of respiratory challenge models is over 20 years. The impetus to develop such respiratory challenge models arose from the unsuitability of intracerebral challenge to determine the protective efficacy of acellular pertussis vaccines as described in section 1.6.2.2.1.

These subsequent models demonstrate several differences. Although most investigators used inbred mice, the strains of mice used differed, as did their choice of vaccine and dose (data not presented in table), route of immunisation, timing of immunisation and time of challenge post immunisation.

However, some similarities exist. The first was the timing of sacrifice post challenge: focusing on several time points within two to three weeks of challenge. The second was that all investigators using aerosol challenge (with the exception of one in which the challenge dose was inferred from day 0 lung homogenate colony counts (252) utilised an

inoculum strength between $10^9$ and $10^{11}$ CFU/ml. Only one recent study utilised intranasal challenge (115) and the challenge dose was $10^8$ CFU/ml.

Therefore methods which were a composite of those from the work of these previous investigators were used as a starting point for pilot studies to determine the final methods of definitive protective efficacy experiments.

## 4.3 Development of the mouse model

Four separate pilot studies were conducted to establish a reproducible method for the determination of the protective efficacy of the DTPa and DTPw preparations used in chapters 2 and 3 after ideal and adverse storage.

### 4.3.1 Pilot study #1: general methodology

#### 4.3.1.1 Mouse strain and age, method of challenge

The first pilot study was designed to test general study methods, extending the murine model outlined in section 2.2 to include a booster immunisation on day 28 as well as intranasal challenge with pertussis organisms two weeks after the second immunisation. Day 28 was chosen as the time of the second immunisation to maintain consistency with methods of previous investigators (162) and with the time of blood sampling in experiments described in chapters 2 and 3 so that direct comparisons of immunogenicity could be made with these experiments. Most previous investigators had also allowed a 14 day interval between booster immunisation and challenge (115, 162, 184). Hence this interval was adopted in the following experiments.

Female Balb/c mice 8 weeks of age were chosen to reduce experimental variability and to maintain consistency with the previous immunogenicity experiments. The intranasal

method of inoculation was chosen initially for the ease of administration and maintained because it is now the method recommended by the WHO for prelicensure testing of the protective efficacy o both acellular and whole cell pertussis vaccines (N Guiso, personal communication, 2000).

#### 4.3.1.2 Blood collection and immunisation

Twelve mice were anaesthetised using halothane inhalation and were bled from the retro-orbital sinus using a Pasteur pipette prior to intraperitoneal immunisation with 0.1ml normal saline or CSL DTPw (batch 043401609) or 0.125 ml Infanrix® DTPa (batch numbers 14887B9B, 14940B9B) on days 0 and 28 of the experiment. The doses of vaccine were the same as those used in sections 2.2.9 and 3.2.5. The choice of days 0 and 28 for immunisation allowed comparison of antibody results between immunogenicity and protective efficacy experiments. Four mice were included in each treatment group. However, due to the unexpected death of two mice each on days 0 and 28 under anaesthesia, final numbers were reduced to three mice in the vaccine treated groups and two in the saline group.

#### 4.3.1.3 Preparation of challenge inoculum and intranasal administration

Three days prior to intranasal inoculation of mice (day 39 of the experiment), *B. pertussis* Tohama strain (a generous gift from Dr A Focaretta, University of Adelaide Department of Microbiology) was cultured on Bordet-Gengou (BG) agar plates for three days in a humid chamber at $37^0$C. On day 42, organisms from these plates were suspended in a few drops of sterile PBS in a laminar flow cabinet housed in the Department of Microbiology, WCH. To confirm that bacteria were in phase 1 (i.e. expressing virulence factors), slide agglutination of this suspension was performed using SEIKEN® *B. pertussis* phase 1 immune serum against a saline control. Agglutination of the suspended organisms

occurring within 60 seconds of addition of the serum confirmed that bacteria expressed the *Vir*+ phenotype.

The inoculum was created by suspending live organisms in approximately two millilitres (mls) of PBS. It was calibrated against a Vitek #52-1210® colorimeter to 0.5 MacFarland units (equivalent to approximately $0.5x10^8$ CFU/ml) to approximate the non-lethal inoculum dose used by previous investigators (115). After serial 10-fold dilutions in sterile PBS in the laminar flow cabinet, the suspension was cultured on BG agar (one plate per dilution) before being taken to the animal care facility.

The remaining 8 mice were then anaesthetised with an intramuscular injection of 30-40µl of a 3:2 mixture of xylazine and ketamine. After injection, they were placed in a steel canister (no more than 4 mice at a time), approximately 10cm diameter and 20cm high covered by an opaque cloth, to prevent them escaping during the initial phase of hyper-excitation occurring immediately prior to the onset of anaesthesia.

All mice were then bled from the retro-orbital sinus using a Pasteur pipette and were inoculated intranasally with 50µl of the live bacterial suspension in a class II microbiology safety cabinet (MSCII) using a Gilson® pipette. Mice were held by the nape of the neck and droplets of the suspension were carefully pipetted into the nares, approximately 25µl into each nostril (figure 4.1). Some mice swallowed part of the inoculum. This was corrected by holding the mice more horizontally during intranasal inoculation. Mice were subsequently placed on their sides on a warming blanket in the MSCII under direct observation until they awoke from anaesthesia, approximately 30-40 minutes later.

The time of preparation of the inoculum was approximately 20 minutes. The time from creation of the challenge inoculum to the completion of intranasal challenge in these mice was exactly 2.5 hours, which is the limit imposed by the WHO Expert Committee on Biological Standardisation.

After intranasal challenge, mice were housed in a separate, closed room with separate air circulation to prevent infection of other animals in the facility.

#### 4.3.1.4 Sacrifice, lung homogenisation and bronchoalveolar lavage

Three days after challenge, mice were sacrificed using carbon dioxide ($CO_2$) inhalation. It was anticipated that this would be the time when mice would be most likely to have the greatest number of organisms in the lungs after challenge (186, 252). After sacrifice, the mice were placed onto a corkboard overlaid with plastic sheeting cleaned with 70% alcohol in the MSCII. After pinning the mice to the corkboard by the skin at six points to stabilise the mice, a midline incision was made and the skin was bluntly dissected from the body. The lungs were then excised using aseptic technique after resection of the anterior thoracic cage. The heart, thymus and oesophagus were resected from the lungs, leaving the trachea attached.

Lungs with the trachea attached from five mice (two had received immunisation with DTPa, two with DTPw and one with saline) were weighed outside the MSCII before being homogenised in 1% casaminoacid-PBS (CPBS): the volume of CPBS was two millilitres (mls) minus the weight of lungs in microlitres (µl). Homogenisation of lungs was initially attempted manually with a modified disposable mortar and pestle, but was unsuccessful. Therefore, for the remainder of the mice, an electric homogeniser (Ultra-Turax TP18-10®) was used. Between sets of lungs, the homogeniser was turned on to clean it serially in

sterile 50 ml containers containing 70% alcohol, sterile water or sterile normal saline (one container per solution).

Three mice (one from each treatment group) also had bronchoalveolar lavage (BAL) performed prior to lung resection and homogenisation. Because the process of BAL introduces fluid into the lungs, lung weights could not be assumed to be accurate and were therefore not weighed in these mice. Bronchoalveolar lavage was performed outside the MSCII, by isolating the trachea after midline incision using blunt dissection. Sterile 4.0 silk thread was then passed posterior to the trachea to prepare for stabilisation of an intratracheal cannula.

The parietal peritoneum of the abdomen was separated from the thoracic cage and the diaphragm pierced to deflate the lungs. A sterile 25-gauge needle was used to create a small hole between cartilaginous rings whilst keeping the trachea under moderate tension. A 23-gauge cannula with the sharp end cut off and with a 2-3 millimetre cuff of silicon tubing placed on the end was then carefully inserted into the hole and the trachea was tied above the level of the cuff around the cannula to stabilise and seal it. Two volumes of 1ml of sterile PBS were then slowly injected into the lungs using a one ml syringe and the volumes withdrawn after each injection were placed separately into eppendorf tubes. These aliquots were spun in a microfuge at 13 000 rpm (16 060G) and the supernatants stored at $-70^0$C for future use. Figures 4.2-4.6 illustrate the techniques of lung resection, homogenisation and BAL.

Lung homogenates were diluted serially 10-fold in CPBS and cultured one plate per dilution on BG agar at $37^0$C in a humid chamber for four days.

Spreading of lung homogenate and inoculum dilutions on agar plates was performed using a copper loop which was dipped in 70% alcohol and flame sterilised between plates. Dilutions of homogenate and inoculum were spread by rotating the agar plate in one direction and the copper loop in contact with the plate in the opposite direction until there was no evidence of moisture on the agar plate.

#### 4.3.1.5 Results

##### *4.3.1.5.1 Culture results*

After four days, plates were examined for the number of colony forming units (CFU) of *B. pertussis* per plate. All plates were contaminated with *Bacillus sp, Staphylococcus sp* and/or *Streptococcus viridans.* The degree of contamination was dependent upon the concentration of the homogenate, with plates incubating the most concentrated dilutions demonstrating the most contamination. However, significant contamination was demonstrated on most plates, in some cases completely covering the plate. Colonies of *B. pertussis* were identified by their classical "half pearl" appearance on less contaminated plates and by slide agglutination with anti-*B. pertussis* serum on more contaminated plates.

Cultures of lung homogenates from mice receiving pertussis vaccines had no growth of *B. pertussis*, whereas the mice receiving saline demonstrated a detectable growth of *B. pertussis* at dilutions between $10^{-3}$ and $10^{-5}$ of the initial homogenate, as well as contaminant organisms.

Separate cultures of the preparation used for intranasal inoculation demonstrated no evidence of contamination after 4 days of culture under the same conditions.

#### *4.3.1.5.2 Calculation of CFU/ml of B. pertussis in inoculum and lung homogenates*

All plates demonstrating a growth of *B. pertussis* were counted using a Gallencamp® manual colony counter. Where growth was present at more than one dilution, results were corrected for the dilution factor and averaged to provide a final count expressed as CFU/ml. The inoculum suspension contained 2.3 x $10^8$ CFU/ml. Therefore the amount administered to mice intranansally was 1.15 x $10^7$ CFU. Mice immunised with normal saline averaged 6.0 x $10^4$ CFU/ml of *B. pertussis* in lung homogenates three days after intranasal challenge.

#### *4.3.1.5.3 Lung weights*

Mice receiving DTPa had lung weights averaging 0.272 gm and the average lung weight in mice receiving DTPw was 0.381 gm. The mouse receiving immunisation with saline had a lung weight of 0.391 gm. It is likely that lung weights reflect the amount on inflammatory infiltrate and intrapulmonary exudate. Therefore, although these data are limited, it could be concluded that DTPa is more effective than DTPw (and saline) in clearing *B. pertussis,* and hence inflammation and exudation, from the lungs.

### 4.3.1.6 Summary of first pilot study

The interpretation of results from thus study was hampered by the small numbers of mice used, the further reduction in numbers of mice due to unexpected deaths under anaesthesia and the degree of contamination of cultures of lung homogenates.

#### 4.3.1.7 Alterations to the experimental methods

##### *4.3.1.7.1 Anaesthesia*

After the death of a significant number of mice using halothane anaesthesia, it was decided that the xylazine: ketamine mixture should be used for all procedures requiring anaesthetic

##### *4.3.1.7.2 Contamination of lung homogenate cultures*

To reduce the amount of contamination of the lung homogenate, subsequent experiments were designed to include removal of the mouse trachea prior to homogenisation and used separate mouse lungs for BAL and lung homogenisation.

##### *4.3.1.7.3 Counting colony forming units*

To enable a more accurate quantification of inoculum concentration, inocula dilutions were plated onto charcoal with cefalexin (CC) agar, a selective growth medium for *B. pertussis*.

### 4.3.2 Pilot study #2: Investigation of the natural course of infection in unimmunised mice using two inocula of *B. pertussis* of different concentrations

The principal aim of this experiment was to compare the rate of clearance of organisms from the lungs of mice receiving inocula of *B. pertussis* differing 100-fold in concentration. The proposed suspension strengths were $0.5 \times 10^7$ and $1 \times 10^9$ CFU/ml. Approximate intranasal challenge doses therefore were $2.5 \times 10^5$ and $5 \times 10^7$ CFU respectively.

#### 4.3.2.1 Intranasal inoculum preparation and intranasal challenge

To create inocula, organisms cultured for three days prior to commencement of the experiment were suspended in sterile PBS in the Department of Microbiology as described previously (4.2.1.2). The lower dose inoculum was first calibrated to 0.5 MacFarland Units with the Vitek® colorimeter. As this calibration yielded approximately $0.5x10^8$ CFU/ml and was the lowest possible calibration on the colorimeter, the suspension was then diluted 10-fold prior to intranasal inoculation of mice. The higher dose inoculum was calibrated against the highest available calibration on the colorimeter, equivalent to approximately $1.2x10^9$ CFU/ml. Inocula were diluted serially 10-fold. Each dilution was plated on to a CC agar plate for culture under standard conditions for *B. pertussis*.

Two groups of 20 female Balb/c mice received intranasal challenge with 50µl of the designated suspension after anaesthesia and blood collection as described in section 4.2.1.3. Blood collection, anaesthesia and intranasal challenge were completed 2.25 hours after creation of inocula.

#### 4.3.2.2 Sacrifice post challenge

On days 2, 4, 7 and 14 after intranasal challenge, five mice from each treatment group were sacrificed using $CO_2$ inhalation. These time points were chosen to give a more complete picture of lung clearance over time and were similar to those chosen by other investigators (162, 186). Three mice in each group had lungs resected for homogenisation, and two mice from each group had lungs resected for histopathological analysis.

Whole lung preparations for homogenisation were resected and weighed as described in section 4.2.1.4 with the addition of resection of the trachea prior to homogenisation. They were then homogenised in 2ml CPBS: no subtractions were made to account for differing

lung weights. Homogenates were diluted serially 10-fold in CPBS and plated onto one BG agar, two plates per dilution, and cultured for four days at $37^0$C in a humid chamber.

Preparation of lungs for histopathological analysis was performed in a similar manner to BAL. However, instead of injecting the lungs with PBS, lungs were filled with 10% formalin through the intratracheal cannula. A second silk thread was used to tie off the trachea below the level of the cannula and the lungs were carefully excised from the chest cavity before being placed in a sterile container of 10% formalin. Detailed discussion of staining and interpretation of the lung sections is provided in section 5.2.

#### 4.3.2.3 Results

##### *4.3.2.3.1 Inocula concentrations*

Dilutions of inocula were spread onto CC agar plates only (one plate per dilution) prior to culture. There was no evidence of contamination of CC agar plates after culture. After counting of plates, the higher dose inoculum was shown to have had an actual concentration of $3.2x10^7$ CFU/ml. Therefore mice received $1.6x10^6$ CFU intranasally in the 50µl inoculum. The lower dose inoculum contained an actual concentration of $3.6x10^6$ CFU/ml, providing an intranasal dose of $1.8x10^5$ CFU.

##### *4.3.2.3.2 Lung homogenates*

Dilutions of lung homogenates were spread onto BG agar plates, with two plates for each dilution. Contamination of BG agar plates was prominent again and demonstrated a similar pattern to that observed in the first pilot study with similar contaminating organisms (*Bacillus s.p, Staphylococcus sp. and Streptococcus viridans*). This made interpretation of results difficult. Table 4.2 documents the average results of colony counts

for each day of the experiment (in CFU/ml). Most results were read on plates containing dilutions of homogenates between $10^{-2}$ and $10^{-4}$. Marginally diminished contamination of plates was noted from day 14 homogenates. Colony counts were higher at each time point in mice receiving the higher dose inoculum. In each group, maximum lung growth of organisms was seen at day 4 to day 7, with a reduction by day 14. Overall, however, a significant number of plates grew too many contaminants to be counted. Therefore no firm conclusions could be drawn relating to the natural course of infection in mice receiving different inoculum doses.

#### *4.3.2.3.3 Lung weights*

Table 4.3 documents the lung weights recorded on days 2, 4,and 7 post challenge in this experiment. Recorded values are the mean for three mice in grams (gm) with the ranges in brackets below. After detailed examination of potential causes for contamination of lung homogenate cultures between days 7 and 14 of this experiment, weighing of lungs was abandoned, as this occurred outside the sterile environment of the MSCII before homogenisation and was a potential cause of contamination.

Table 4.3 demonstrates that lung weights were higher in the mice receiving the higher dose inoculum at all three time points, suggesting the presence of a greater inflammatory cell infiltrate and exudate into the lungs, possibly caused by slower clearance of organisms. This difference was only statistically significant at day 4 post challenge, however ($p=0.011$, Student's t-test). Analysis by one-way ANOVA showed there were no significant differences between days 2, 4 and 7 post challenge within each inoculum group (high dose $p=0.114$, low dose $p=0.964$).

#### *4.3.2.3.4 Histopathological analysis of lungs*

Each set of lungs was cut into six sections prior to staining with haematoxylin and eosin. Each section was assessed for the degree of lung inflammation using an arbitrary scale (0-12) developed from murine lung scoring systems used by previous investigators (253, 254). The results are presented in figure 4.4.

At all time points post challenge, mice inoculated with the higher concentration of organisms demonstrated significantly more lung inflammation than those receiving the lower concentration inoculum ($p<0.001$ for all comparisons). No definite pattern of inflammation was demonstrated for mice receiving the higher concentration inoculum over time, as high fluctuating scores were maintained throughout the experimental period. However, the degree of lung inflammation seemed to increase gradually with time in mice challenged with the lower concentration inoculum.

#### *4.3.2.3.5 Summary of second pilot study*

Although contamination of cultures of lung homogenates was improved marginally by the omission of weighing of lungs prior to homogenisation on day 14 of this experiment, contamination still caused difficulty with interpretation of results. Therefore, detailed analysis of the experimental methods was undertaken and the following changes were instituted.

#### *4.3.2.3.6 Alterations to methods after second pilot study*

##### 4.3.2.3.6.1 Mouse housing arrangements

Mice were housed separately from other animals for the duration of each experiment, not just after intranasal challenge. They received sterilised food and water and were housed in filter cages with sterilised bedding.

##### 4.3.2.3.6.2 Preparation of the inoculum

Future experiments included the preparation of the challenge inoculum in the animal care facility rather than in the Microbiology laboratory.

Sterile pipette tips in small packs of four rather than in packs of 100 were used for pipetting the inoculum and diluting the inoculum and lung homogenate prior to plating out for culture.

##### 4.3.2.3.6.3 Preparation and dissection of mice after sacrifice

Separate scissors and forceps were used for external and internal dissection. The skin was wiped with 70% alcohol prior to dissection in sacrificed mice.

##### 4.3.2.3.6.4 Agar plates used for culture of inocula and lung homogenates

All dilutions of inocula and lung homogenates were spread onto one BG agar and one CC agar plate.

##### 4.3.2.3.6.5 Lung homogenisation and cleaning of homogeniser

Mouse lungs were not weighed prior to homogenisation. Lungs were homogenised in sterile PBS and not CPBS. This decision was made after plain sterile PBS, unsterilised CPPBS and filter sterilised CPBS were cultured on BG and CC agar plates. After 4 days'

culture at $37^0$C in a humid chamber, plates culturing sterile PBS demonstrated no growth, but both unsterile and filter sterilised CPBS grew a small number of contaminants on both BG (*Streptococcus viridans, Bacillus sp and Staphylococcus sp*) and CC plates (fungal colonies).

Culture of water and saline used for cleaning the homogeniser between lung homogenisation of several mice on day 14 post challenge grew a small numbers of the same contaminants as those on BG plates of lung homogenates. Therefore, the experimental protocol was altered to include cleaning of homogeniser with two aliquots of each wash solution, making six washes in all, between homogenisation of separate lungs. Two mice receiving no immunisation or challenge were sacrificed in a subsequent experiment to examine for baseline contamination of lung homogenates.

### 4.3.3 Pilot study #3: assessment of outcome of modifications of experimental protocols from pilot study #2.

Before further meaningful experiments to determine a dose-response relationship between inoculum concentration and lung clearance rates could be conducted, it was a requirement that the modifications of the experimental protocol were shown to be successful.

Fourteen female Balb/c mice were used for this experiment. On day 0, 12 mice were anaesthetised and received 50µl of a suspension of *B. pertussis* in sterile PBS made using the recommended protocol modifications, calibrated to 0.5 MacFarland Units. Two mice were inoculated intranasally with 50µl of sterile PBS. The bacterial intranasal inoculum was then diluted 10-fold in sterile PBS and each dilution plated in duplicate on one BG agar plate and one CC agar plate.

Preparation of the challenge inoculum was much more rapid than in previous experiments due to a greater familiarity with the technique. This reduced the time from preparation to completion of inoculation and plating for culture significantly. It also affected the final concentration of the inoculum as measured by counting of colony forming units after culture (see section 4.2.3.1.2 and table 4.4).

A further two mice were not anaesthetised or inoculated but were sacrificed on day 0. Six mice were sacrificed on both days 2 and 4 post intranasal challenge. Five of the sacrificed mice had received bacterial challenge; the sixth mouse had received the saline inoculum. Two of the pertussis-challenged mice were sacrificed for BAL, and the remainder were sacrificed for lung homogenisation.

Bronchalveolar lavage fluids were plated neat onto one BG and one CC agar plate and cultured under the usual conditions. Lung homogenates were diluted serially 10-fold in sterile PBS and were plated in duplicate: one BG and one CC plate per dilution.

#### 4.3.3.1 Results

##### *4.3.3.1.1 Cultures of lung homogenates*

At both time points, cultures of lung homogenates from all mice demonstrated significant contamination at dilutions of $10^{-1}$ and $10^{-2}$ on BG agar plates (*Bacillus sp, Staphylococcus sp, Streptococcus viridans*). With increasing dilution, the degree of contamination diminished markedly, such that there was minimal or no contamination detectable at increasing dilution from $10^{-3}$ onwards. No contamination was detectable on any of the CC agar plates.

Mice receiving bacterial challenge demonstrated significant growth of organisms at both days 2 and 4 post challenge. Colony counts of lung homogenates were, in general higher on day 4 than day 2 post challenge. These results are summarised on table 4.5.

#### *4.3.3.1.2 Inoculum culture results*

Culture of the inoculum demonstrated occasional contaminant colonies with similar organisms to those from previous experiments at dilutions of $10^{-1}$ and $10^{-2}$ on BG agar plates and no contamination at all on CC agar plates. The final inoculum strengths were very similar: $2.195 \times 10^{8}$ CFU/ml from BG agar plates and $1.935 \times 10^{8}$ CFU/ml from CC agar plates. After averaging these results, mice received approximately $1.033 \times 10^{7}$ CFU in the 50µl inoculum.

##### 4.3.3.1.2.1 Bronchoalveolar lavage culture results

On the BG agar plate from one of the two mice sacrificed at each time point there were 2-3 contaminant colonies, but otherwise there was a pure growth of *B. pertussis* on all other BG and CC plates.

### 4.3.3.2 Summary

Contamination of BG agar plates was diminished significantly by the alterations in the experimental protocol introduced in the third pilot study. Contamination was still present, but not on the plates on which individual colonies were discrete enough to be counted (i.e. on plates culturing dilutions of $10^{-3}$-$10^{-5}$ and beyond).

#### 4.3.3.3 Modifications of experimental protocols after pilot #3

Due to the success of previous modifications in significantly improving sterile procedures, the only remaining modification was culture of all subsequent lung homogenate, intranasal inoculum and BAL fluid dilutions in triplicate, using two BG and one CC plate per dilution.

### 4.3.4 Pilot study #4: Investigation of natural course of infection at days 2 and 4 post intranasal challenge in unimmunised mice using two inocula of different concentrations and protocol modifications from pilot studies two and three.

Two groups of 10 mice were anaesthetised and challenged intranasally with inocula either one log above or one log below 0.5 MacFarland units ($0.5 \times 10^8$ CFU/ml). These concentrations were chosen to replicate those from the second pilot study. Inocula were plated out in triplicate (two BG and one CC plate per dilution) after challenge and cultured for 4 days under standard conditions.

Five mice in each group were sacrificed on days 2 and 4 post challenge. Three mice had lungs homogenised for culture (in triplicate for each dilution). Two mice were sacrificed for BAL which were also diluted serially 10-fold in sterile PBS and plated in triplicate for each dilution. The remaining BAL samples were spun at 16 600 G and the supernatants stored at $-70^0$C for later analysis.

### 4.3.4.1 Results

#### *4.3.1.4.1 Inocula concentrations*

The lower dose inoculum concentration was $1.85x10^7$ and the higher dose inoculum concentration was $1.0x10^9$ CFU/ml. Mice therefore received either $9.25x10^5$ or $5.0x10^7$ CFU intranasally. Table 4.5 compares actual inoculum concentrations in CFU/ml from all pilot studies with expected concentrations on calibration. These results indicate an inherent variability in the inoculum challenge concentration even after calibration to the same standard on the colorimeter. This variability was probably the result of inbuilt inaccuracy in the colorimeter combined with differing times of preparation of challenge inocula and completion of intranasal challenge in different pilot studies. This, in turn, was a function of the familiarity of the principal investigator with these techniques.

#### *4.3.1.4.2 Comparison of lung homogenate and BAL colony counts*

Lung homogenate and BAL colony counts on days 2 and 4 post challenge are shown in table 4.6. It can be seen that, in general, there was little difference in colony counts between lung homogenates and BAL supernatants. However, interpretation of these data is hampered by the lack of one "low dose" BAL sample on both days of sacrifice due to technical difficulties with the collection of these samples. In addition, there appears to be little difference between the rates of lung clearance in the two treatment groups on days 2 and 4 post challenge

### 4.3.4.2 Changes to experimental protocol after pilot study #4

Due to technical difficulties obtaining BAL samples, the intratracheal cannula was inserted without the silicon cuff, providing a smaller diameter to be inserted into the trachea and better stabilisation in situ by the silk thread tie. It was also determined that centrifugation

of BAL samples at 13 000 rpm (16 060 G) would be likely to lyse cells in the BAL. Subsequent centrifugations were performed at 2000 rpm or 2140 G.

### 4.3.5 Final protocol for the definitive protective efficacy experiments

The final experimental protocol is summarised in table 4.7.

#### 4.3.5.1 Mouse strain and age

For the same reasons as in the pilot studies, female Balb/c mice 8 weeks of age at the commencement of each experiment were used.

#### 4.3.5.2 Housing of mice

Mice were bred in the University of Adelaide animal care facility under Specific Pathogen Free (SPF) conditions. They were transported to the Women's and Children's Hospital (WCH) animal care facility in filter cages and housed with a maximum of 10 mice per filter cage in a closed room separate from other animals for the duration of each experiment. Mice received sterilised food and water and had sterilised cage bedding. Experiments were performed one week after arrival at the WCH to prevent the stress of transport from confounding the experimental results.

#### 4.3.5.3 Mouse handling

All procedures on mice (blood sampling, immunisation, lung homogenisation, and fixation for histopathology and bronchoalveolar lavage) were performed in a microbiological class II cabinet. Mice were anaesthetised with an intramuscular injection of 30-40µl of a 3:2

mixture of xylazine and ketamine for blood sampling and intranasal inoculation and sacrificed using $CO_2$ inhalation prior to lung homogenisation and (BAL).

#### 4.3.5.4 Day 0 and day 28 Immunisation and blood collection

Balb/c mice were anaesthetised and bled from the retro-orbital sinus on days 0 and 28 of each experiment. After blood collection, they were immunised intraperitoneally with 0.1 ml of DTPw (CSL Ltd batch 043401609) or normal saline, or 0.125 ml DTPa (SB Infanrix batches 14887B9B, 14940B9B) using a 1ml syringe and a 25 gauge needle. On day 42 of each experiment a mice were bled from the retro-orbital sinus prior to the intranasal challenge of all mice. At various time points after challenge, mice were sacrificed for lung homogenisation. Cardiac puncture was performed under direct vision in these mice to collect blood.

#### 4.3.5.5 Vaccine transport and storage

Vaccines were transported to the WCH as in section 2.2.2 and stored either in the especially designated laboratory refrigerator or the WCH pharmacy cool room (refer to section 5.2.1).

#### 4.3.5.6 Preparation and quantification of challenge inoculum

*Bordetella pertussis* organisms (Tohama strain) were stored at $-70^0$C in glycerol until required. Three days prior to preparation of the challenge inoculum, bacteria were thawed at room temperature and cultured on BG agar at $37^0$C in a humid chamber. To confirm that bacteria were in phase 1, slide agglutination was performed as in the pilot studies.

Immediately prior to inoculation, bacteria were suspended in sterile PBS in the MSCII and the suspension calibrated using the Vitek® colorimeter to 0.5 MacFarland units. The

colorimeter was calibrated initially with PBS. In all experiments, the challenge inoculum was prepared within 2 minutes and mice were inoculated within 60-90 minutes of preparation of the challenge inoculum, well within the WHO recommended limit of 2.5 hours. The inoculum was then plated in triplicate at serial 10-fold dilutions in sterile PBS on BG agar (2 plates per dilution) and CC agar (one plate per dilution) and cultured at $37^0$C in a humid chamber for four days.

To determine the inoculum concentration, plates providing discrete colonies after culture were selected for counting using the Gallencamp® manual plate counter. In most cases plates from three dilutions could be counted and averaged after adjusting for the dilution factor to provide the final count.

#### 4.3.5.7 Intranasal inoculation

On day 42 of each experiment, all mice were anaesthetised with xylazine and ketamine as described previously. After blood collection, mice were inoculated intranansally with 50µl of the suspension of *B. pertussis* using a Gilson's pipette. Approximately 25µl was instilled into each nostril. Mice were held horizontally during inoculation and upright at the nape of the neck for 10-15 seconds after inoculation, and then were placed on their sides on a warming plate until they awoke from anaesthesia, approximately 40 minutes later. During the first 5-10 minutes post inoculation on the warming plate, mice were observed to develop a significant rise in respiratory rate. Thereafter the respiratory pattern normalised until mice awoke from anaesthesia.

#### 4.3.5.9 Lung homogenisation

At selected time points after intranasal challenge (refer to section 5.x for details) mice were sacrificed using $CO_2$ inhalation. The lungs were then resected aseptically, separated from the heart, trachea, thymus and oesophagus and then homogenised in two millilitres of sterile PBS for 60 seconds using the Ultra-Turax® tissue homogeniser. Lung homogenates were then serially diluted 10-fold in sterile PBS. Each dilution was plated in triplicate as for the intranasal inoculum as in section 2.2.5. Plates were read after 4 days of culture at $37^0$C in a humid chamber, and counted as in section 4.2.2.6 to calculate the number of CFU/ml in the lung homogenate from each mouse. Two aliquots each of 70% alcohol, sterile water and sterile saline were used to clean the homogeniser after each lung was homogenised.

## 4.4 Conclusions

This series of pilot studies has demonstrated the evolution of a reproducible, non-lethal intranasal challenge model to investigate the protective efficacy of pertussis vaccines in mice. Many of the early problems (e.g. contamination) were solved using relatively simple modifications to the experimental protocol or were a function of lack of familiarity with the required techniques (e.g. preparation of the challenge inoculum).

The following chapters describe a series of definitive experiments investigating the rate of lung clearance of bacteria, associated serological responses, lung histopathological changes and BAL cytospin cell counts and cytokine concentrations in mice using this model

**Table 4.1 Comparison of methods of pertussis mouse protection tests from previous investigators**

| Principal Investigator | Mouse strain(s) | Immunisation schedule and route | Timing of challenge after immunisation | Inoculum strength and route | End point of experiments | Timing of sacrifice post challenge |
|---|---|---|---|---|---|---|
| **KENDRICK** Am J Pub Health, 1957 | Swiss outbred | day 0, day 5 intra-abdominal | 10 days after second immunisation | 40, 000 organisms in 0.4ml inoculum intracerebral | Number of mice dead 0-14 days post challenge | N/A |
| **SATO** Infect, Immun, 1980 | ddY | No immunisation | N/A | $10^9$, $10^{10}$, or $10^{11}$ CFU/ml aerosol | Development of the model, characterisation of infectious course | 3, 6, 9, 12, 14, 18, 21 days |
| **SYUKUDA** Tokai J Exp Clin Med, 1988 | ddY | day 0 intraperitoneal | 21 days after immunisation | -1L of suspension aerosolised over 30 min -suspension strength unspecified | Comparison of lung clearance between mice immunised with: 1.multi-component or mono-component acellular vaccines 2.whole cell/acellular pertussis vaccine or no vaccine | 1, 3, 5, 7, 10, 14 days |
| **KIMURA** Infect, Immun, 1990 | Balb/c | day 0 intraperitoneal | 21 days after immunisation | suspension $\log_{10}$ 9-9.3 CFU/ml aerosolised for unspecified time | Number of CFU/ml in lung homogenates | 0, 1, 5, 10 days |
| **BARNARD** Immunology 1996 | Balb/c, CBA, NIH, C57BL/6 | day 0, day 28 intraperitoneal | 14 days after second immunisation | suspension $\log_{10}$ 10.3 CFU/ml aerosolised for 12-15 minutes | Number of CFU/ml in lung homogenates | variable e.g. 3, 7, 10, 14 days or 0, 2, 5, 8, 15 days |

**Table 4.1 (continued) Comparison of methods of pertussis mouse protection tests from previous investigators**

| Principal Investigator | Mouse strain(s) | Immunisation schedule and route | Timing of challenge after immunisation | Inoculum strength and route | End point of experiments | Timing of sacrifice post challenge |
|---|---|---|---|---|---|---|
| **GUISO** Vaccine 1999 | Balb/c | day 0, day 21 subcutaneous | Aerosol: 14 days after second immunisation Intranasal: 7 days after second immunisation | Aerosol: $2x10^{10}$ CFU/ml at 0.4ml/min for 20 mins Intranasal: $1x10^{8}$ CFU/ml | Comparison of sensitivity of methods to detect differences in protective efficacy of different DTP a preparations | Aerosol: 0, 2,4, 8, 15 days Intranasal: 0, 1, 2, 4, 8, 14, or 0, 1, 2, 5, 7, 14 days |
| **XING** Vaccine, 1999 | NIH female | day 0 intraperitoneal | optimum time determined to be day 21 for DTPa, day 9 or day 15 for DTPw | $\log_{10}$ 5.04 CFU/ml aerosol* | -Development of the aerosol challenge model -Comparison of lung bacterial clearance after immunisation with DTPa or DTPw | 5, 10. 15, 20 days |
| **BOROS (present study)** | Balb/c female | day 0, day 28 intraperitoneal | 14 days after second immunisation | $\log_{10}$ 7.0 CFU/ml intranasal | Number of CFU/ml in lung homogenates | 2, 4, 7, 14 days |

* inoculum dose determined by culturing lung homogenates of mice sacrificed on the day of challenge

**Table 4.2 Average colony counts in lung homogenates from mice in second pilot study expressed in CFU/ml**

| Day post challenge | Low dose inoculum | High dose inoculum |
|---|---|---|
| Day 2 | Too much contamination to count accurately | $4.678 \times 10^5$ |
| Day 4 | $4.333 \times 10^5$ | $5.150 \times 10^6$ |
| Day 7 | $2.217 \times 10^6$ | $7.567 \times 10^6$ |
| Day 14 | $1.825 \times 10^5$ | $3.550 \times 10^5$ |

**Table 4.3 Average lung weights of mice from second pilot study: lungs not weighed on day 14 post challenge**

**(n=3 per group)**

| Day post challenge | Low dose inoculum | High dose inoculum |
|---|---|---|
| **Day 2** | 0.193gm (0.181-0.211) | 0.236gm (0.206-0.281) |
| **Day 4** | 0.210gm (0.199-0.216) | 0.240gm (0.221-0.261) |
| **Day 7** | 0.168gm (0.145-0.202) | 0.236 (0.232-0.241) |

**Table 4.4 Average colony counts (CFU/ml) from lung homogenates in third pilot study**

| Day post challenge | Colony count mouse 1 | Colony count mouse 2 | Colony count mouse 3 |
|---|---|---|---|
| **Day 2** | $4.75 \times 10^6$ | $2.295 \times 10^6$ | $5.545 \times 10^6$ |
| **Day 4** | $4.57 \times 10^6$ | $1.136 \times 10^7$ | $1.81 \times 10^7$ |

**Table 4.5 Comparison of colorimeter calibrations and inoculum concentrations for all pilot studies**

| Pilot study number | Colorimeter calibration | Inoculum concentration | Time taken<br>a) to prepare inoculum<br>b) to inoculate mice |
|---|---|---|---|
| 1 | $0.5x10^8$ | $2.30x10^8$ | (a) 20 minutes<br>(b) 2.25 hours |
| 2 | Low dose $0.5x10^7$<br>High dose $1.2x10^9$ | Low dose $1.6x10^6$<br>High dose $3.2x10^7$ | (a) 20 minutes each<br>(b) 2.10 hours each |
| 3 | $0.5x10^8$ | $2.065x10^8$ | (a) 5 minutes<br>(b) 1.25 hours |
| 4 | Low dose $0.5x10^7$<br>High dose $1.2x10^9$ | Low dose $1.85x10^7$<br>High dose $1.0x10^9$ | (a) 5 minutes each<br>(b) 1.0 hour each |

**Table 4.6 Average colony counts (CFU/ml) in lung homogenates and corresponding BAL supernatants in fourth pilot study**

| Day post challenge | Lung homogenate counts (n=3 mice) | BAL counts (n=2 mice) |
|---|---|---|
| Day 2 high dose | $3.81x10^6$ | $7.9x10^5$<br>(one mouse only*) |
| Day 2 low dose | $5.44x10^5$ | $4.75x10^5$ |
| Day 4 high dose | $4.05x10^6$ | $1.07x10^6$ |
| Day 4 low dose | $4.03x10^6$ | $1.13x10^6$<br>(one mouse only*) |

*technical difficulties were encountered obtaining the BAL sample from the second mouse at both time points.

**Table 4.7 Final experimental protocol for murine intranasal challenge experiments**

| | Day 0 | Day 28 | Day 42 | Day 46 |
|---|---|---|---|---|
| Blood sampling | • | • | • | • |
| Immunisation | • | • | | |
| Intranasal challenge | | | • | |
| Sacrifice | | | | • |

**Figure 4.1 Method of blood collection via cardiac puncture**

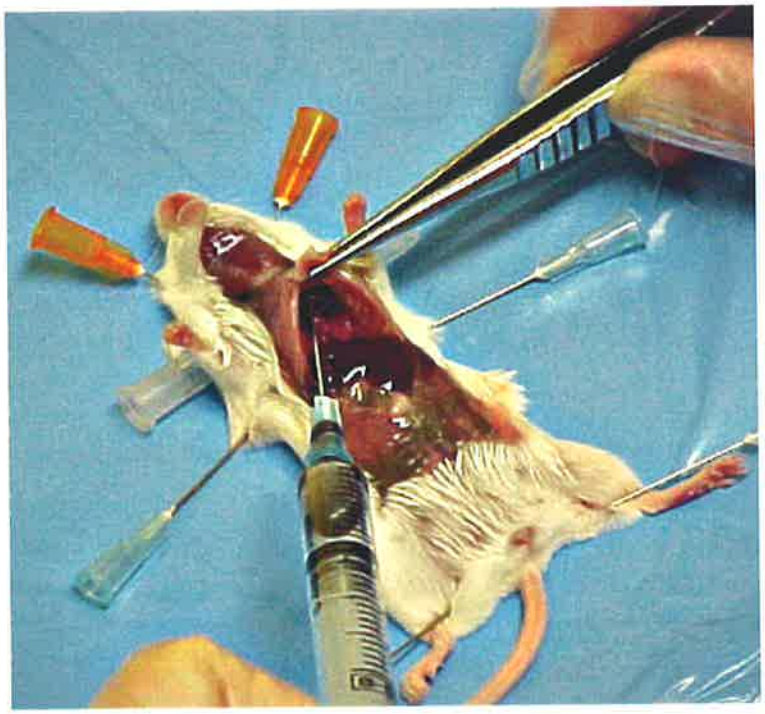


**Figure 4.2 Haemolysis of Bordet-Gengou agar around *B. pertussis* colony forming units**

**Figure 4.3 Method of intranasal inoculation**

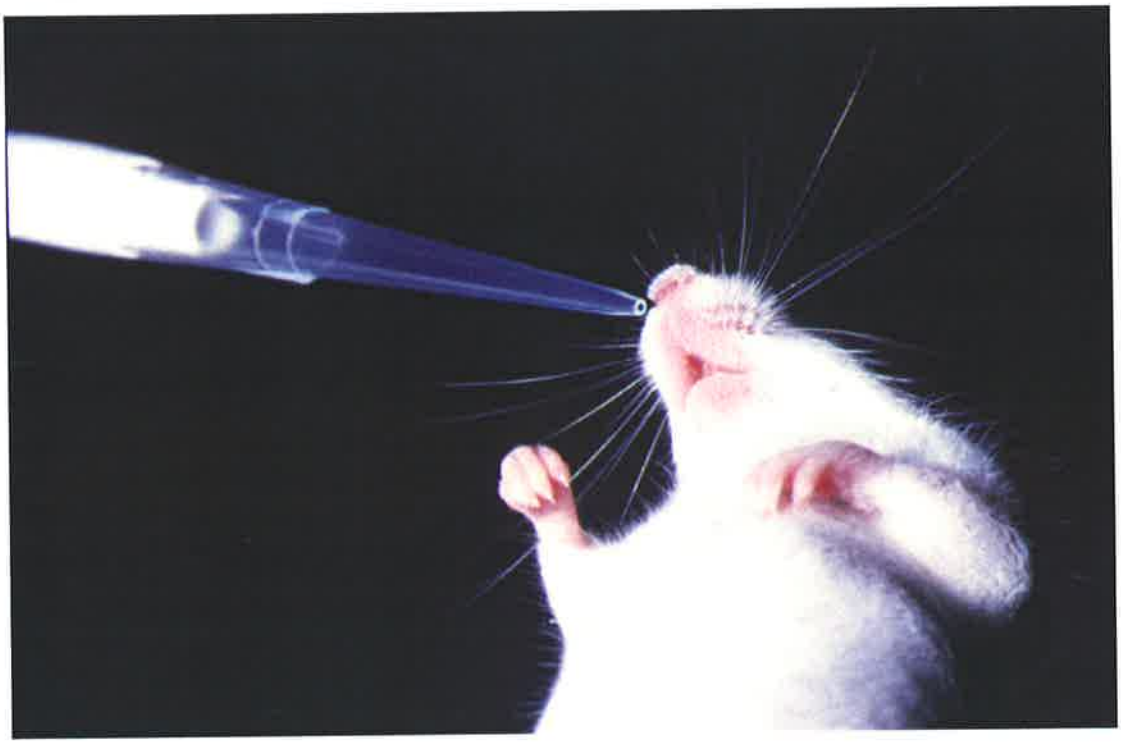
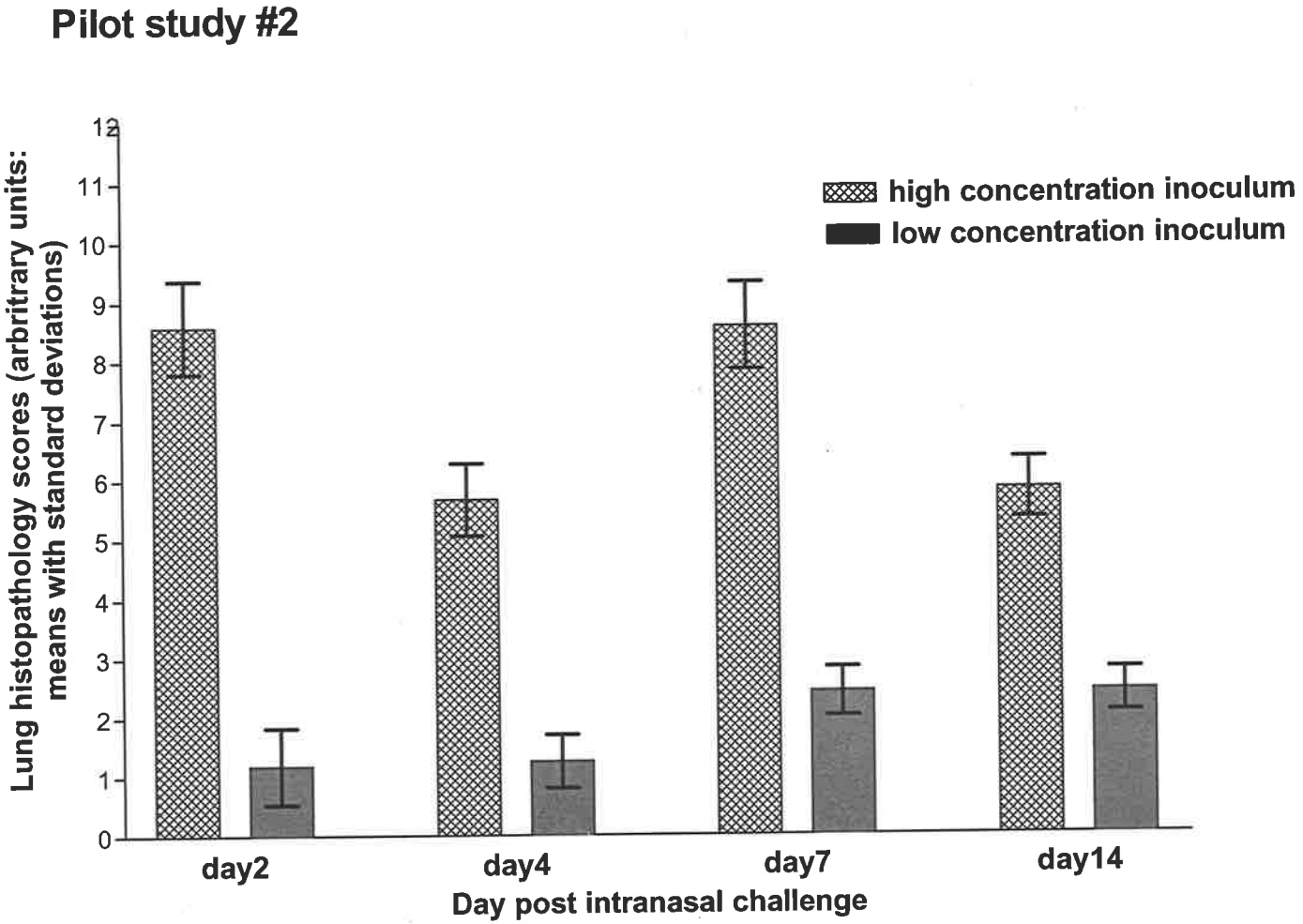


**Figure 4.4** **Pilot study lung histopathology scores by day of sacrifice post intranasal challenge**



# Chapter 5 Definitive protective efficacy experiments: Lung clearance studies

## 5.1 Introduction

This chapter describes the definitive experiments designed to investigate the protective efficacy of correctly and adversely stored pertussis vaccines using the final experimental protocol described in table 4.7. The results presented in this chapter are those from the cultures of lung homogenates.

## 5.2 Additional methods

### 5.2.1 Vaccine storage prior to commencement of experiments

Vaccines were stored in the laboratory refrigerator with electronic monitoring using a HOBO-Temp INT® logger as described in section 2.2.3 or in the alarm monitored WCH pharmacy cool room. Temperatures in the pharmacy cool room are maintained between $4^0$C and $5^0$C. A HOBO-Temp INT® electronic temperature logger was also used in the pharmacy cool room to confirm ideal vaccine storage.

### 5.2.2 Vaccine storage conditions

Two initial experiments were performed to investigate the natural course of infection in mice immunised with vaccine stored under ideal conditions (2-$8^0$C): protection from infection after immunisation with DTPa compared with normal saline was performed in a separate experiment from that comparing the protective efficacy of DTPw and normal saline immunisation. One batch of vaccines stored at +$13^0$C for 48 hours was also used in separate experiments designed in the same manner for separate comparison of immunisation with DTPa or DTPw with normal saline. The remainder of experiments

compared simultaneous adverse storage of both DTPa and DTPw with saline. Vaccine storage conditions for these experiments were: -3$^0$C for 24 hours or 14 days, or –6$^0$C for 24 hours or 14 days.

### 5.2.3 Mouse numbers and time points for sacrifice post challenge

#### 5.2.3.1 Assessment of efficacy at days 2, 4, 7 and 14 after challenge

In experiments investigating immunisation of mice with vaccines stored at 2-8$^0$C and +13$^0$C, 40 mice were used for each experiment: 20 mice were immunised with commercial vaccines and 20 mice were immunised with normal saline. On days 2, 4, 7 and 14 after intranasal challenge, five mice from the vaccine immunised and the saline immunised groups were sacrificed for lung homogenisation or BAL: four mice for lung homogenisation and one for BAL and lung homogenisation in experiments utilising storage at 2-8$^0$C, three mice for lung homogenisation and two for BAL in the +13$^0$C DTPw experiment and four for lung homogenisation and one for BAL in the +13$^0$C DTPa experiment.

#### 5.2.3.3 Assessment of efficacy at day 4 after challenge

In experiments utilising adverse storage conditions of -3$^0$C for 24 hours, –6$^0$C for 24 hours and –6$^0$C for 14 days 18 mice were used for each experiment with 6 mice in each treatment group (saline, DTPa and DTPw immunisation). Four days after intranasal inoculation, all mice were sacrificed: five for lung homogenisation and one for BAL and lung histopathology from each group of six. Day four post challenge was chosen as the single time point for sacrifice as it appeared to be the time when maximum differences in culture of lung homogenates were obtained between mice receiving commercial vaccine and those

receiving saline in experiments investigating storage of vaccine between $2^0$C and $8^0$C and at $+13^0$C as described in 5.2.3.1.

In the final experiment utilising storage of vaccines at $-3^0$C for 14 days, 31 mice were included. Three groups of six mice were immunised with normal saline, DTPa stored at $-3^0$C for 14 days or DTPa stored at 2-$8^0$C. Two further groups of five mice each were immunised with DTPw stored at $-3^0$C for 14 days or DTPw stored at 2-$8^0$C*. Three mice remained unimmunised, and were sacrificed 2.5 hours after intranasal challenge to determine the number of bacteria reaching the lung after intranasal inoculation. The immunised mice (saline, DTPa, or DTPw) were sacrificed at day 4 post intranasal challenge. Five mice from each treatment group were sacrificed for lung homogenisation. In treatment groups containing six mice, one mouse was also sacrificed for BAL collection and lung histopathology.

### 5.2.3.4 Statistical analysis

All statistical analyses were performed using Systat 10®. Colony counts of lung homogenates were $\log_{10}$ transformed prior to statistical analysis. For the purpose of analysis, where no growth at all dilutions of cultured lung homogenate was found, the value of "0.1" was substituted for zero. Statistical comparisons between several experiments utilising adverse storage below $0^0$C were analysed using one–way ANOVA. Comparisons between any two of these experiments were performed using the Student's t-test.

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* These experiments used the last remaining vials of CSL DTPw available in Australia. There was only enough vaccine left to immunise 5 mice in these treatment groups.

## 5.3 Results

### 5.3.1 Lung clearance in mice immunised with correctly stored vaccines

Figures 5.1 and 5.2 demonstrate the $\log_{10}$ transformed colony counts of cultures of lung homogenates for the experiments investigating the rate of lung clearance (days 2, 4, 7 and 14 after challenge) after immunisation with either DTPa or DTPw stored under ideal conditions in comparison with immunisation with normal saline. Results are presented as medians with ranges.

The results of these experiments confirm the validity of the experimental protocol. Mice immunised with DTPa or DTPw cleared the lungs of infection significantly more rapidly than those immunised with normal saline on days 2 and 4 post intranasal challenge (day 2: DTPa vs saline p=0.014, DTPw vs saline p=0.009, day 4: DTPa vs saline p=0.013, DTPw vs saline p=0.009). The lung clearance in DTPa immunised mice was also significantly greater than saline immunised mice on day 7 post challenge (p=0.011). By day 14 post challenge there were no significant differences detected in lung clearance between mice immunised with DTPa or DTPw and those immunised with normal saline, although the mice immunised with saline still had higher lung homogenate colony counts.

Comparison of lung clearance in mice immunised with DTPa or DTPw did not differ significantly at any of the four time points after intranasal challenge, although at days 2 and 4 only one of four mice immunised with DTPa had identifiable colonies in lung homogenates while four of five mice immunised with DTPw had identifiable colonies (Fisher's exact test p=0.0989). On day 7 post challenge no mice had identifiable colonies in lung homogenates regardless of vaccine administered. On day 14 post challenge one mouse immunised with DTPa had identifiable colonies on culture of lung homogenates

whereas none of the mice immunised with DTPw had identifiable colonies (Fisher's exact test p=0.2357).

### 5.3.2 Comparisons between ideal storage and adverse storage of vaccines below $0^0$C

The results of initial experiments comparing 2-$8^0$C storage of DTPa and DTPw with normal saline (section 5.3.2.1) incorporated sacrifice at 4 days after intranasal challenge. These results did not differ significantly from those of the positive controls immunised with vaccines stored at 2-$8^0$C included in the final experiment (-$3^0$C for 14 days). Therefore, these results have been combined for the purposes of comparison between storage conditions.

#### 5.3.2.1 Inoculum concentrations

Inoculum concentrations were calculated by dividing the concentration of the original suspension by 20, as 50µl aliquots were instilled intranasally. The concentrations of the intranasally instilled aliquots varied from $\log_{10}$ 6.5 to $\log_{10}$ 7.2 CFU/ml between the six experiments.

#### 5.3.2.2 Day 0 lung homogenate culture results

The three unimmunised mice sacrificed 2.5 hours after intranasal challenge had lung homogenate colony counts of $\log_{10}$ 6.33, 6.37 and 6.34 CFU/ml respectively. These mice received an intranasal inoculum concentration of $\log_{10}$ 6.81 CFU/ml. These results demonstrate the accuracy of the intranasal inoculation method and the probable small loss of inoculum from infection of the upper airway and/or loss into the stomach after swallowing.

### 5.3.2.3 Mice immunised with DTPa

Figure 5.2 documents the lung homogenate culture results at four days after intranasal challenge from experiments utilising DTPa. Results are presented as $\log_{10}$ CFU *B. pertussis*/ml. Each symbol on the graphs represents the results for one mouse lung calculated from at least three dilutions of homogenate cultured in triplicate (i.e. =9 culture plates). Results for mice immunised with normal saline (combined from all experiments) and initial inoculum concentrations from each experiment have also been presented for comparison. The horizontal bars represent the medians for each group of data. The p values represent the results of statistical comparisons between ideal storage and each adverse storage condition.

Comparison of results from all five experiments was statistically significant (p=0.014) and was due to the comparison between mice immunised with vaccine stored at 2-8$^0$C and –3$^0$C for 14 days. Six of ten mice immunised with DTPa stored at 2-8$^0$C showed complete lung clearance at day 4 after challenge. Immunisation with DTPa stored at –3$^0$C for 24 hours, -6$^0$C for 24 hours and –6$^0$C for 14 days had no significant effect upon the rate of lung clearance of *B. pertussis* in mice (p=0.782, p=0.985, p=0.115 respectively). Immunisation with DTPa stored at –3$^9$C for 14 das was associated with reduced clearance (p=0.046).

### 5.3.2.4 Mice immunised with DTPw

Comparison of results from all five experiments was, once again, statistically significant. (p=0.005, figure 5.3). Only one mouse immunised with DTPa stored at 2-8$^0$C showed complete lung clearance at day 4. Paradoxically, immunisation with DTPw stored at –3$^0$C for 24 hours, -6$^0$C for 24 hours and –6$^0$C for 14 days increased the rate of lung clearance significantly in comparison with mice immunised with vaccine stored at 2-8$^0$C (p=0.041,

p=0.041, p=0.010 respectively). Mice immunised with vaccine stored at $-3^0$C for 14 days demonstrated no significant difference in lung homogenate culture results from mice immunised with vaccine stored under ideal conditions (p=0.341).

#### 5.3.2.5 Mice immunised with normal saline

All mice immunised with saline demonstrated significantly less lung clearance four days after intranasal challenge than mice immunised with either vaccine stored at 2-8$^0$C despite a range of $\log_{10}$ 5 CFU/ml in cultures of lung homogenates between experiments. As demonstrated by the results in section 5.3.3.2, this variability in lung clearance of bacteria is more likely to represent the natural variation in resistance to infection in these mice, as there was little variability in the dose of live organisms delivered intranasally.

#### 5.3.2.6 Comparison between the protective efficacy of DTPa and DTPw in all experiments

Although immunisation with DTPa after ideal and adverse storage appeared to be associated with faster lung clearance of bacteria, one-way ANOVA found this difference to approach statistical significance only (p=0.092). However, direct comparison of DTPa and DTPw stored at 2-8$^0$C showed significantly better clearance rates in mice immunised with DTPa (p=0.024 student's t-test) on day 4 post challenge (figure 5.4).

### 5.3.4 Comparisons between ideal storage and storage of vaccines at +13$^0$C for 48 hours prior to immunisation

The results of comparisons between lung homogenate culture results 2, 4, 7 and 14 days after intranasal challenge in mice immunised with DTPa or DTPw stored at 2-8$^0$C or 13$^0$C are presented in figures 5.5a and b. These graphs represent the results of four experiments,

with the relevant intranasal inoculum concentration included for each experiment. The horizontal bars represent the medians of each group of data.

#### 5.3.4.1 Inoculum doses

Although there was minimal variation between inoculum concentrations in experiments utilising the same storage conditions, a difference of $\log_{10}$ 1.5 CFU/ml existed between the initial inoculum concentrations of experiments utilising 2-8$^0$C vaccine storage and those utilising +13$^0$C storage.

#### 5.3.4.2 Lung homogenate culture results

Statistical comparisons of temperature storage for both DTPa and DTPw detected no differences between 2-8$^0$C and +13$^0$C vaccine storage at any of the four time points post intranasal challenge despite the greater inoculum concentration administered to mice immunised with vaccine stored at +13$^0$C. However, a trend towards significance was demonstrated in the day 4 post challenge comparisons for DTPw stored at 2-8$^0$C and +13$^0$C ($p=0.057$). In addition, no mice had evidence of bacteria in cultures of lung homogenates seven days after intranasal challenge, irrespective of vaccine type or storage condition.

## 5.4 Conclusions

As in experiments by previous investigators, mice immunised with either DTPa or DTPw stored at 2-8$^0$C showed good lung clearance at day 4 post challenge compared with mice immunised with saline, regardless of immunisation dose or schedule and timing or route of bacterial challenge (115, 162, 252). A greater proportion of mice immunised with DTPa stored at 2-8$^0$C demonstrated total lung clearance at day 4 after challenge than those immunised with DTPw stored at 2-8$^0$C. Storage of both vaccines at +13$^0$C for 48 hours had no significant effect on the protective efficacy of DTPa or DTPw. In addition, storage of vaccine at –3$^0$C or –6$^0$C for 24 hours and at –6$^0$C for 14 days also did not affect lung clearance with either vaccine.

Adverse storage of DTPw paradoxically enhanced its protective efficacy in comparison with the saline control in three of four adverse storage experiments. This appeared to be largely due to the lesser efficacy of the DTPw stored at 2-8$^0$C by day 4 after challenge and may have been due to aggregation of bacteria or physico-chemical changes to antigens critical to immune activation and bacterial clearance in the adversely stored vaccines.

Paradoxically, lung clearance was not as good when DTPa and DTPw were stored at –3$^0$C for 14 days. Once again, physicochemical changes to critical vaccine antigens may be responsible. Although these results remain unexplained, it is of note that these results were consistent for both vaccines.

Overall DTPa protected better than DTPw against pertussis infection, regardless of vaccine storage condition. This was statistically significant four days after intranasal challenge, indicating that DTPa may be more robust to adverse storage than DTPw.

These results differ from those of other investigators using similar models to compare the protective efficacy of DTPa and DTPw preparations stored under ideal conditions (162, 186, 252). All used aerosol rather than intranasal challenge, and all found that inbred mice (Balb/c or NIH) immunised with whole cell vaccine cleared lung infection (as determined by CFUs in lung homogenates) faster than those immunised with DTPa. However, the DTPw preparation used in these studies was the WHO reference preparation 88/522 (50 IU/ampoule) (162, 186, 252) or another unspecified commercial whole cell preparation (252) neither of which were used in the present studies. Guiso et al (115) found no difference in lung clearance of organisms between aerosol and intranasal challenge models using DTPa vaccines only, but did not find the aerosol challenge method sufficiently sensitive to compare the protective efficacies of several DTPa preparations. Only Mills et al (162) used the same time points for immunisation and intranasal challenge as in the present study. Hence the better performance of DTPa in the present study could be due to reduced protective efficacy of the CSL whole cell preparation relative to preparations used in other studies, the difference in DTPw immunising dose (table 2.2) the difference in inoculum strength (particularly in relation to intranasal versus aerosol challenge doses: table 4.1) or the different times of sacrifice post challenge (table 4.1).

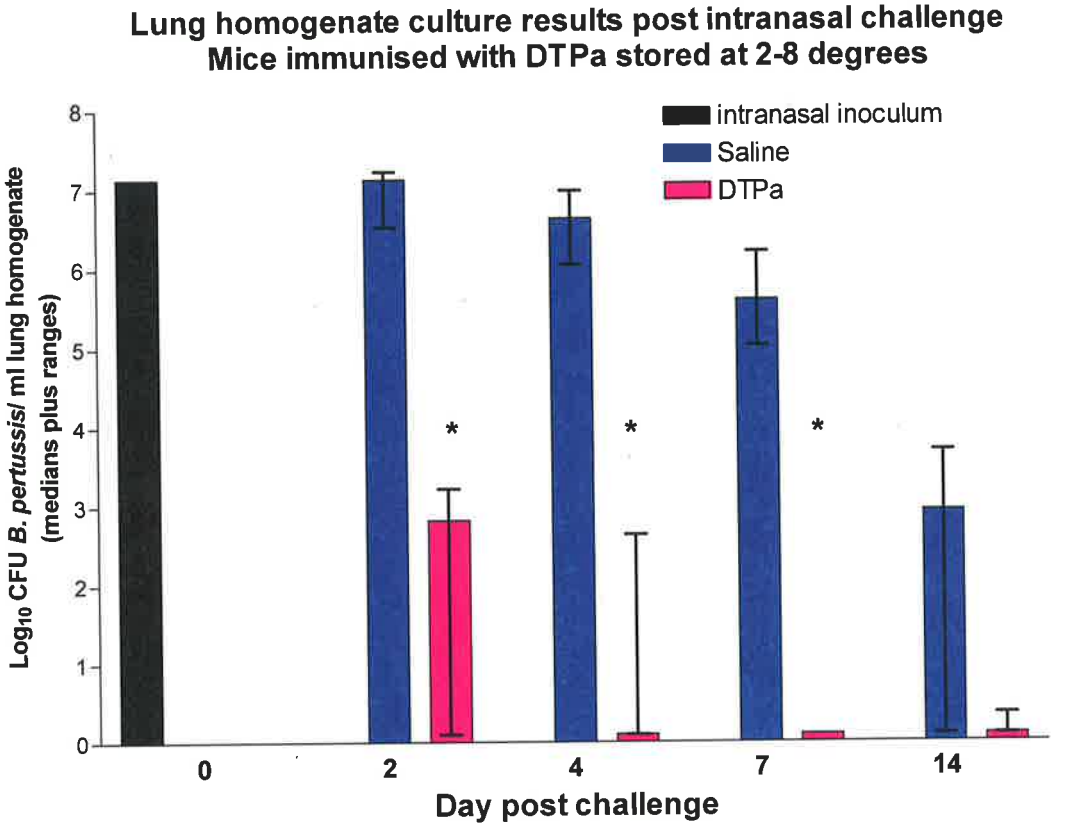
Variability of results within each data group was demonstrated, particularly in mice immunised with normal saline (figures 5.2 and 5.3) and more at days two and four post challenge in experiments ending at day 14 post challenge in mice immunised with DTPa or DTPw. Although variability appears to be less in published studies (utilising ideal vaccine storage conditions only), errors are reported as standard deviations or standard errors of the mean, not as medians with ranges. The number of mice sacrificed varied from three to five mice per time point in the present study. These numbers do not differ from those reported in the published literature (115, 162, 186, 252). In addition, the variability in lung

homogenate CFU counts observed in this study is unlikely to relate to techniques of inoculation preparation or installation, as mice sacrificed within 2.5 hours of challenge demonstrated lung homogenate culture results varying by $\log_{10}$ 0.04 CFU/ml only. Other potential sources of variability include slight differences in the dose of vaccine injected intraperitoneally, although this would not affect saline immunised mice, or genetic variation in the inbred mice due to spontaneous post-conceptional mutations in the mouse genome.

These results indicate that short periods of adverse storage are unlikely to affect the short-term protective efficacy of the vaccines used in this study. However, until the long term effects of adverse vaccine storage on subsequent protective efficacy can be established, vaccines should still be stored according to the manufacturers instructions.

**Figures 5.1a and 5.1b** **Lung homogenate culture results after ideal vaccine storage**

(* indicates statistically significant comparisons between vaccine and saline:$p<0.05$)



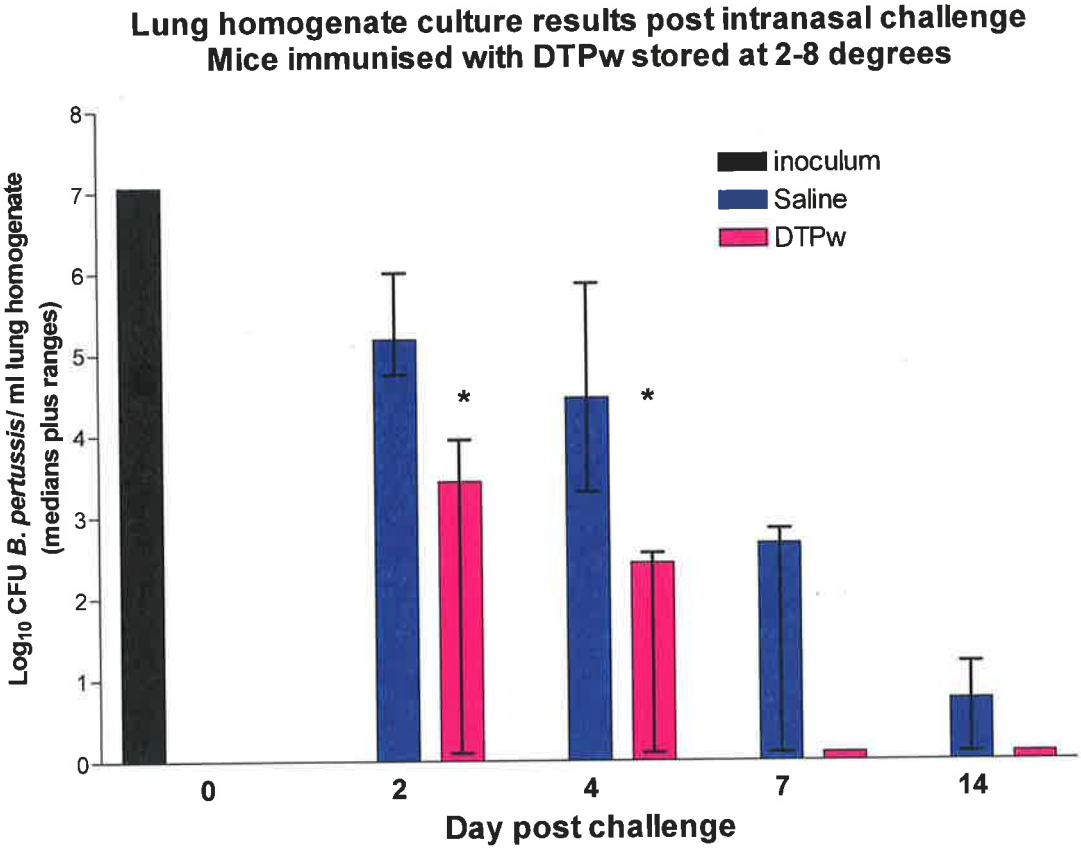



**Figure 5.2** **Comparison of lung homogenate culture results after immunisation with correctly and adversely stored DTPa**

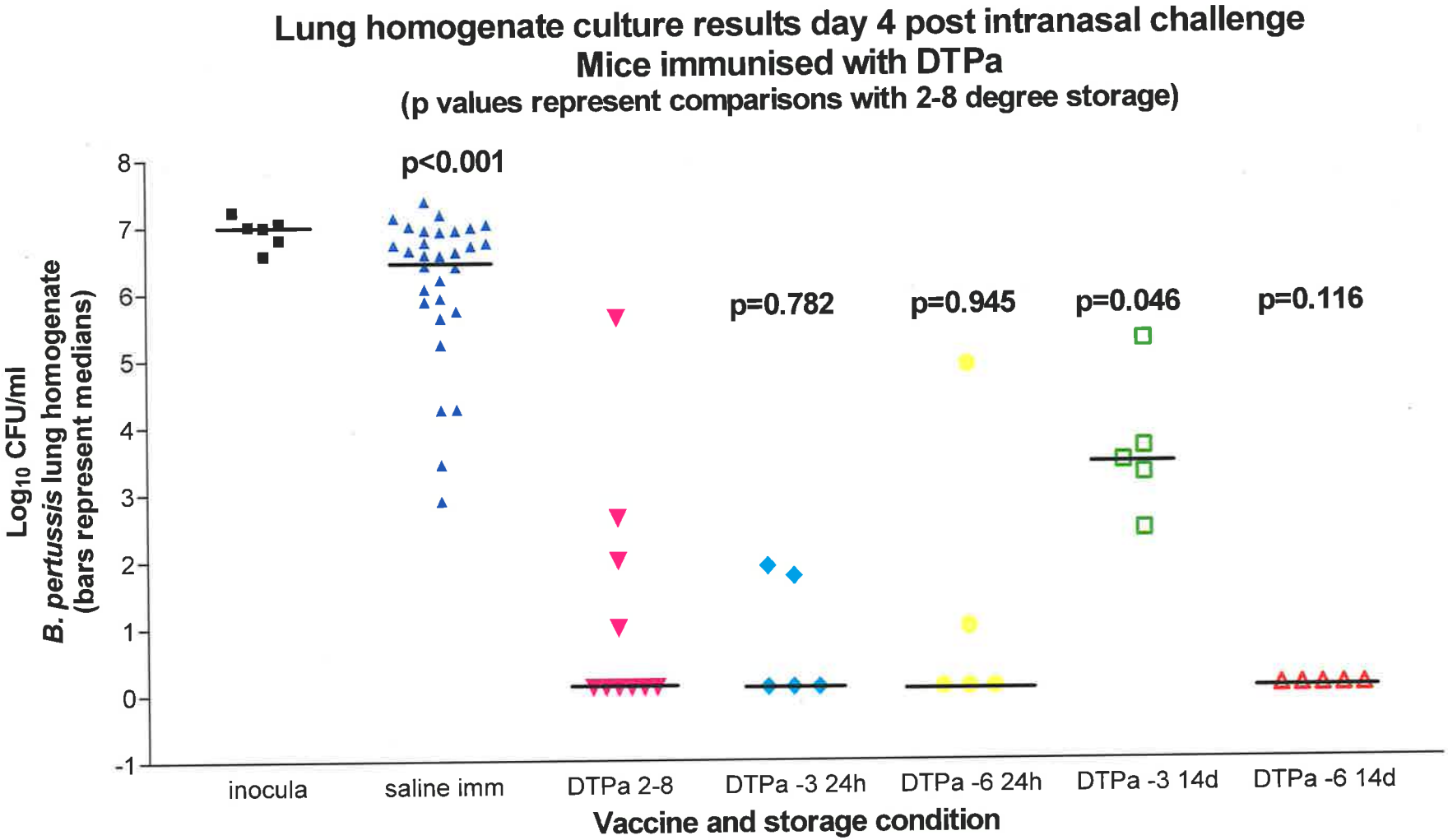


**Figure 5.3** **Comparison of lung homogenate culture results after immunisation with correctly and adversely stored DTPw**

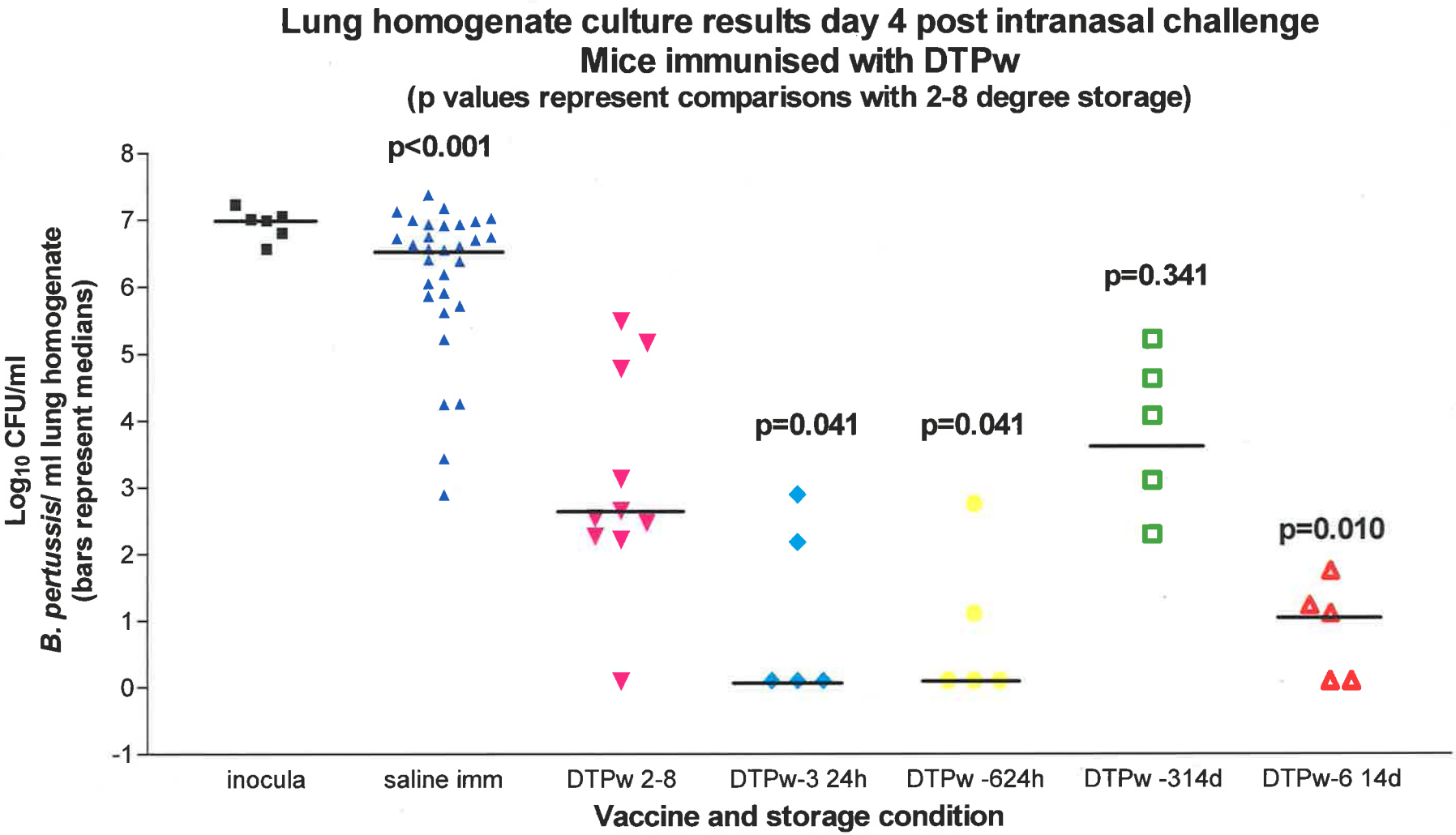
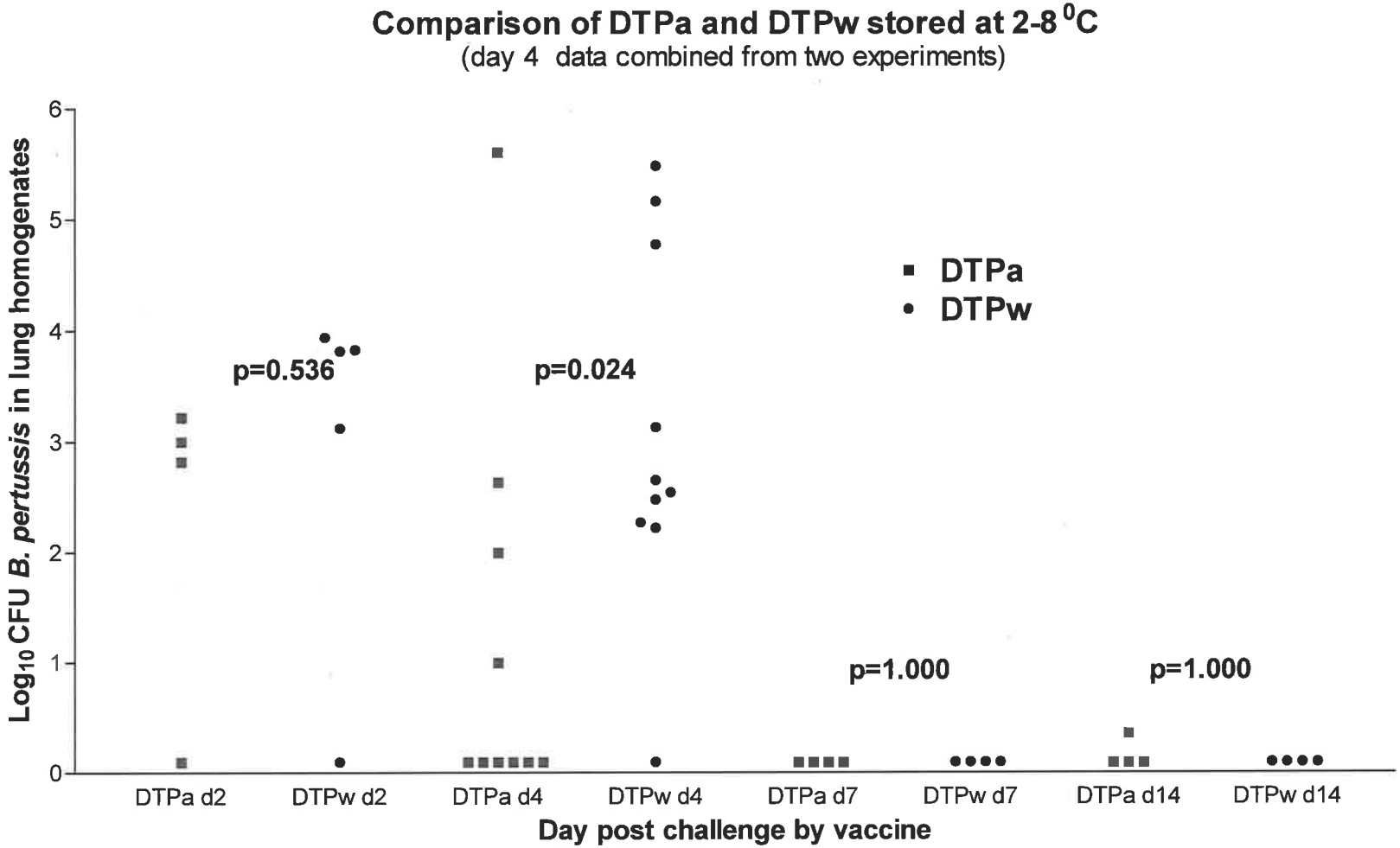
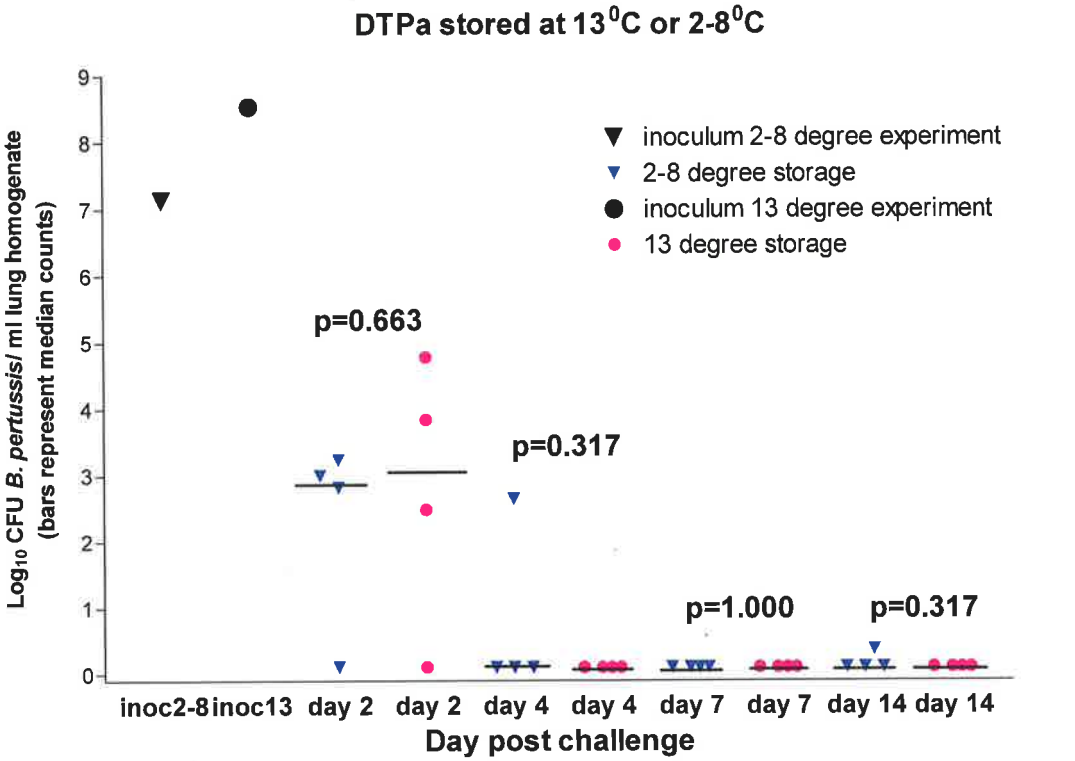
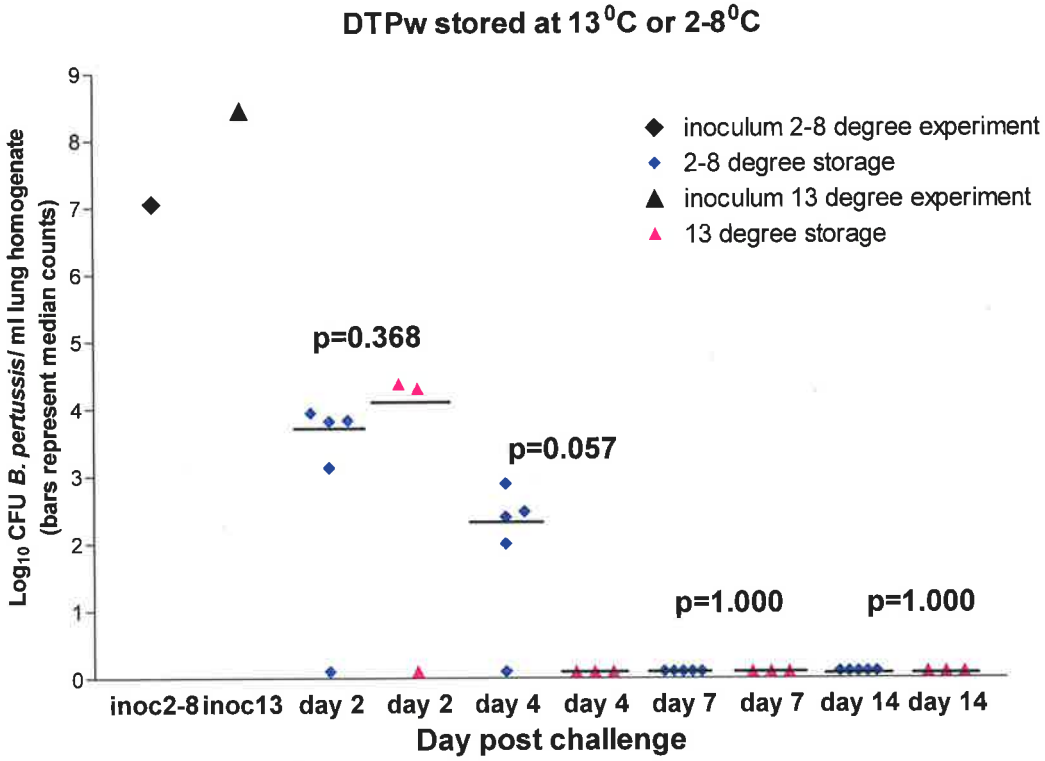


**Figure 5.4** **Comparison of lung clearance between mice immunised with DTPa or DTPw stored at 2-8$^0$C from 2 to 14 days post intranasal challenge**



**Figures 5.5a and 5.5b** **Comparison of lung homogenate colony counts after immunisation with vaccine stored at 2-8$^0$C or +13$^0$C**



# Chapter 6 Definitive protective efficacy experiments: IgG antibody responses

## 6.1 Introduction

Although recent evidence from household contact studies suggests that antibodies to fimbriae, PRN and FHA may be potential serological correlates of protection against pertussis infection (175, 176), no single serological correlate has yet been identified. Cherry et al found that PRN and PT antibodies were significant in preventing infection with *B. pertussis*. In addition, PRN antibody concentrations predicted the probability of infection after household exposure more than PT antibodies (175). Storsaeter et al found that children with lower concentrations of PRN and fimbrial 2/3 antibodies at 12 months of age had an attack rate after exposure similar to that of unimmunised controls (176).

Information from murine protective efficacy studies has also suggested that antibodies to pertussis antigens may act synergistically to prevent infection (115, 179). The aims of this study were to provide more information about the kinetics of pertussis antibody responses after infection in mice immunised with pertussis vaccine, to delineate further the effects of adverse vaccine storage on pertussis antibody responses, to determine if a there is a relationship between lung clearance of organisms and pertussis IgG antibody concentrations, and to assess IgG antibody responses to antigens contained in triple antigen vaccines.

## 6.2 Methods of seroanalysis

### 6.2.1 Measurement of IgG antibodies to PT, FHA and PRN

Enzyme immunoassays to measure the IgG antibody responses to PT, FHA and PRN were conducted in the University Department of Paediatrics Laboratory at the WCH using the

same methods as in sections 2.2.10 and 3.2.6 with some minor modifications. Table 6.1 outlines the differences in serological methods between the two laboratories.

#### 6.2.1.1 Reference sera

Ten female Balb/c mice were immunised intraperitoneally at 8 weeks of age with 0.125 mls DTPa. Blood was collected via cardiac puncture under halothane anaesthesia 21 days later to create a pooled reference serum. This internal reference was calibrated against the reference serum used in assays performed for immunogenicity experiments in the Department of Immunology and Infectious Diseases at the NCH and also against the first WHO international murine anti-*B pertussis* reference serum 97/642. The WHO preparation has been calibrated against the USFDA pertussis reference sera and is now used as the first International Murine Pertussis Reference Preparation. It contains 17 IU of anti-PT, 143 IU of FHA, 30 IU of PRN and 32 IU of Fimbriae 2 and 3 and was collected from NIH inbred mice (255). It became available on the WHO reference standards catalogue after the completion of assays performed in the Dept of Immunology and Infectious Diseases at the New Children's Hospital at Westmead, NSW.

### 6.2.2 Measurement of IgG antibodies to diphtheria and tetanus toxoids

Enzyme immunoassays (EIAs) to measure murine IgG anti-diphtheria and anti-tetanus antibodies were adapted from human EIAs first developed in the Department of Microbiology at the Women's and Children's Hospital by Mr A Lawrence and Dr H MacDonald. These methods were subsequently modified in our laboratory to those described in detail in section 8.2.2. Further modifications were then required to standardise assays for murine sera. Details of reagents used in these assays are outlined in table 6.2.

Briefly, Nunc Polysorp® strips were coated with 100µl/well of a 1:500 dilution of CSL Tetanus toxoid fractionated concentrate (2155 LF/ml, batch # 552602501) or CSL Diphtheria toxoid fractionated concentrate (3600 LF/ml, batch # 082033301) in carbonate-bicarbonate buffer ph 9.6 and incubated overnight at $4^0$C in a humid chamber. After washing four times with a NUNC immunowash-12® manual plate washer using sterile PBS (pH 7.2) Tween (0.5%) {PBST}, strips were blocked with 100µl/well of PBS-1% purified bovine serum albumin (PBS-BSA; Sigma cat A7638 lot 16H1394) and washed again, four times with PBST. One hundred microlitre aliquots of murine serum samples, quality controls and dilutions of the reference serum (to generate a standard curve) were diluted in PBST-BSA were then added to wells and incubated for one hour at $37^0$C in a humid chamber. All unknown and quality control sera were assayed in triplicate at an appropriate dilution. The reference serum was diluted serially eight times in doubling dilutions from 1:100. Each dilution of the reference serum was assayed in duplicate. Further details of reference and quality control sera are described in table 6.3.

Plates were washed again, four times, as previously and 100µl/well of Serotek® sheep anti-mouse IgG horseradish peroxidase conjugate (cat AACI0P, batch 150800) were added before the final incubation step at $37^0$C for one hour in a humid chamber.

After washing, plates were developed by the addition of 100µl of 2.2.-azino-di-3-ethylbenzthiazoline sulphonate (ABTS) and 33% w/v hydrogen peroxide ($H_20_2$) to all wells. When the optical density of the most concentrated samples of the reference curve reached 2.0 (after approximately 10 minutes) plates were read using a Dynex OPSUS MR plate reader at a wavelength of 410nm. Results were then transferred to an immunoassay data management programme, Multicalc®, (Pharmacia: Uppsala, Sweden) to standardise

the immunoassays and to quantitate antibody concentrations of murine samples. For further details of assay standardisation refer to section 8.2.10.2.

### 6.2.3 Statistical analysis

Data were $\log_{10}$ transformed prior to analysis using Systat 10®. Comparisons between antibody responses to each antigen for all storage conditions were made using one-way ANOVA, and those between any two groups of data using the Student's two groups t-test.

## 6.3 Results

All figures present antibody responses as GMCs with 95% confidence intervals. None of the Day 0 preimmunisation results have been presented, as antibody responses were below the level of sensitivity of the EIAs for all antigens in all experiments. Any statistically significant comparison is indicated on the graphs by asterisks (*=p≤0.05, **=p≤0.005).

### 6.3.1 IgG antibody responses in mice immunised with correctly stored vaccines

#### 6.3.1.1 Responses to pertussis antigens

##### *6.3.1.1.1 Mice immunised with DTPa*

IgG antibody responses to PT and PRN rose substantially after the day 28 booster immunisation and rose again 4 and 14 days after intranasal challenge with *B. pertussis* organisms. However, responses to FHA did not increase further after the day 28 booster immunisation (figure 6.1a).

##### *6.3.1.1.2 Mice immunised with DTPw*

No detectable antibody responses were demonstrated in response to PT on day 28, 42 or 46 (day 4 post challenge) in mice immunised with DTPw. These responses did not differ from those of mice immunised with normal saline. However, a substantial rise in PT antibody concentration was detected 14 days after intranasal challenge with *B. pertussis* organisms. Although antibodies to FHA and PRN were detectable on days 28, 42 and day 4 post challenge, there was no significant rise in antibody titre during this time. However, on day 14 post challenge a substantial rise in antibodies to both antigens was demonstrated, particularly in response to PRN (although there were very wide 95% confidence intervals

for the day 14 post challenge DTPw PRN responses). Refer to figure 6.1b for graphical representation of these data.

#### *6.3.1.1.3 Mice immunised with normal saline*

Mice immunised with normal saline had no detectable responses to pertussis antigens on day 28, 42 or 46 (day 4 post challenge). However, by day 14 post challenge small rises in antibody concentrations to PT and PRN were demonstrated (PT: GMC 3.37 EU/ml {CI: 2.94-3.89}; PRN GMC 3.78 EU/ml {CI: 2.00-7.15}). The lower limit of assay detection for both antigens was 2 EU/ml. No antibody responses were demonstrated to FHA.

#### *6.3.1.1.4 Comparison between IgG antibody responses to DTPa and DTPw*

Mice immunised with DTPa had significantly higher PT antibody concentrations on day 42, day 4 post challenge and day 14 post challenge than those immunised with DTPw ($p<0.001$ in all cases). Antibody responses to FHA were significantly higher in DTPa-immunised mice on day 4 post challenge ($p=0.002$), and responses to PRN were higher in mice immunised with DTPa on day 42 and day 4 post challenge ($p<0.001$ and $p=0.003$ respectively).

### 6.3.1.2 IgG antibody responses to diphtheria and tetanus toxoids

#### *6.3.1.2.1 Mice immunised with DTPa*

Responses to diphtheria toxoid increased markedly after the day 28 booster immunisation, but increased no further thereafter. A similar response was demonstrated to tetanus toxoid, except antibody responses appeared to diminish between day 4 and day 14 post challenge (refer to figure 6.2a).

#### *6.3.1.2.2 Mice immunised with DTPw*

Mice immunised with DTPw demonstrated a marked increase in response to both diphtheria and tetanus toxoids after the day 28 booster with a trend towards progressive increases in response to diphtheria toxoid on day 42 and days 4 and 14 post challenge. IgG antibody responses to tetanus toxoid increased no further after day 42, however (refer to figure 6.2b).

#### *6.3.1.2.3 Mice immunised with normal saline*

No detectable IgG antibody responses to diphtheria or tetanus toxoids were demonstrated at any time.

#### *6.3.1.2.4 Comparison between IgG antibody responses to DTPa and DTPw*

Diphtheria and tetanus IgG antibody concentrations were significantly higher in mice immunised with DTPw than those immunised with DTPa on day 14 post challenge, ($p=0.001$ in both cases). However, only the responses to tetanus toxoid were significantly higher in DTPw-immunised mice on day 42 ($p=0.011$).

### 6.3.2 Comparison of antibody responses between mice immunised with correctly stored vaccines and those immunised with vaccines stored below $0^0$C

The p values documented on the figures represent the results of one-way ANOVA. Comparisons between ideal ($2^0$C to $8^0$C) storage and individual adverse storage conditions below $0^0$C which were statistically significant are indicated by asterisks (*=$p \leq 0.05$, **=$p \leq 0.005$). Each graph in figures 6.3-6.7 documents the results for ideal storage and all

adverse storage conditions below $0^0$C by the day of the experiment and the duration of adverse storage. Experiments utilising adverse vaccine storage below $0^0$Cwere completed at day 4 post challenge, therefore, there are no comparisons for day 14 post challenge data.

### 6.3.2.1 IgG antibody responses to pertussis antigens

#### *6.3.2.1.1 IgG responses in mice immunised with DTPa*

On days 0, 28 and 42 of each experiment, no significant overall differences by ANOVA were found between vaccine storage groups for antibody responses to PT, FHA or PRN or on day 46 in response to FHA or PRN. However, IgG antibody responses to PT on day 46 4 days after intranasal challenge with live *B. pertussis*) were significantly different when assessing all vaccine storage groups (ANOVA p=0.040). For the individual storage conditions, the comparison between ideal and adverse storage at $-3^0$C for 24 hours tended towards significance (p=0.076). No other direct comparisons of PT antibody responses between ideal and individual adverse storage conditions on day 46 were statistically significant (refer to figs 6.3a, 6.4a, and 6.5a).

It is interesting to note that antibody responses to all antigens increased after the day 28 booster immunisation regardless of how the vaccine had been stored. In addition, antibodies to PT and PRN increased further in concentration after intranasal challenge, as was demonstrated with antibody responses to vaccine stored between $2^0$C and $8^0$C.

Overall, for DTPa, adverse storage $<0^0$C did not alter the immunogenicity of the vaccine, except in terms of PT response after challenge, when antibody responses were higher if DTPa was stored adversely.

#### *6.3.2.1.2 IgG responses in mice immunised with DTPw*

Comparisons when assessing all vaccine storage groups in combination by ANOVA in mice immunised with DTPw were significant only in response to PRN on day 42 ($p<0.001$) and day 46 ($p=0.004$) and in response to FHA on day 46 ($p=0.009$). (Refer to figures 6.3b, 6.4b, and 6.5b)

##### 6.3.2.1.2.1 Comparisons between ideal storage and individual adverse storage conditions for DTPw in response to PT

As demonstrated in the results of experiments utilising vaccine storage under ideal conditions (6.3.1.1.2), antibody responses to PT in DTPw on days 28. 42 and 46 of each experiment were no greater than those on day 0. That is, no response to PT in DTPw was demonstrated. This is also reported in sections 2.3.2.2.4.1 and 3.3.2.3.3, but only one dose of vaccine was administered in those experiments.

##### 6.3.2.1.2.2 Comparisons between ideal storage and individual adverse storage conditions for DTPw in response to FHA

On day 46, antibody responses in mice immunised with DTPw stored at $-3^0$C for 24 hours, $-6^0$C for 24 hours and $-3^0$C for 14 days all has significantly higher antibody concentrations to FHA than those immunised with correctly stored DTPw ($p=0.003$, $p=0.029$, $p=0.016$ respectively).

##### 6.3.2.1.2.3 Comparisons between ideal storage and individual adverse storage conditions for DTPw in response to PRN

Antibody responses to PRN in DTPw stored at $-3^0$C for 24 hours, $-3^0$C for 14 days and $-6^0$C for 14 days were significantly higher than those to PRN in DTPw stored at $2^0$C to $8^0$C on day 42 ($p<0.001$, $p=0.001$, $p<0.001$ respectively) and day 46 ($p=0.016$, $p=0.024$,

p=0.005 respectively). Mice immunised with DTPw stored at $-6^0$C for 24 hours also had significantly higher responses to PRN than mice immunised with correctly stored vaccines on day 46 (p=0.004).

### 6.3.2.2 IgG antibody responses to diphtheria and tetanus toxoids

#### *6.3.2.2.1 Mice immunised with DTPa*

Comparison of IgG antibody responses to diphtheria toxoid were significantly different between all storage conditions in combination on day 42 (ANOVA: $p<0.001$), with storage of vaccine at $-3^0$C for 24 hours conferring significantly lower antibody concentrations than those in mice immunised with vaccine stored under ideal conditions ($p<0.001$).

IgG antibody responses to tetanus toxoid did not differ significantly between vaccine storage groups in combination or for individual comparisons with $2^0$C to $8^0$C storage in mice immunised with DTPa.

Antibody responses to both toxoids increased after the day 28 DTPa booster (except for vaccine stored at $-3^0$C for 24 hours), but no further increases in antibody concentrations were demonstrated after the day 42 intranasal challenge with live *B. pertussis* organisms. (Refer to figures 6.6a and 6.7a).

#### *6.3.2.2.2 Mice immunised with DTPw*

No significant differences in IgG antibody concentrations to diphtheria toxoid in DTPw were demonstrated between vaccine storage groups assessed in combination by ANOVA on day 28, day 42 or day 46 (day 4 post challenge). However, storage of vaccine at $-3^0$C for 24 hours or $-6^0$C for 14 days resulted in significantly increased antibody responses to

diphtheria toxoid in comparison with mice immunised with correctly stored vaccine (p=0.013, p=0.036 respectively: refer to figures 6.6b and 6.7b).

On day 28, comparison of IgG antibody responses to tetanus toxoid for the adverse storage groups was statistically significant (ANOVA: p=0.010), with all adverse storage conditions individually being associated with significantly higher antibody concentrations to tetanus toxoid in comparison with vaccine stored between $2^0$C and $8^0$C (-$3^0$C/24h p=0.040, -$6^0$/24h p=0.016, -$3^0$C/14d p=0.009,-$6^0$C/14d p=0.027). Responses on day 42 also were different when assessd by ANOVA (p<0.001).

In general, responses to DTPw were higher than those to DTPa for both diphtheria and tetanus toxoids, in contrast to the responses to pertussis antigens. These trends are similar to those reported in section 6.3.1.2.2.

### 6.3.3 Comparison between vaccine stored under ideal conditions and at +13$^0$C for 48 hours

IgG antibody responses in mice immunised with vaccine stored at +$13^0$C demonstrated similar trends to those in mice immunised with vaccine stored under ideal conditions: antibody concentrations to PT and PRN in DTPa and DTPw continued to increase progressively on day 4 and day 14 post intranasal challenge, whereas antibody responses to FHA increased only on day 14 post challenge in mice immunised with adversely stored DTPw. (Refer to figures 6.8-6.11).

### 6.3.3.1 IgG antibody responses to pertussis antigens

#### *6.3.3.1.1 Mice immunised with DTPa*

Mice immunised with DTPa stored at +13$^0$C for 48 hours had significantly greater PRN antibody concentrations on day 42 than mice immunised with vaccine stored under ideal conditions (p=0.002). No other significant differences were found. Tthere was a tendency towards significantly higher responses to FHA 14 days post challenge (p=0.064) and PRN on day 28 (p=0.065) in mice immunised with adversely stored vaccine (refer to figures 6.8a-c). However, given the number of comparisons being made, it is possible that these results are due to chance alone.

#### *6.3.3.1.2 Mice immunised with DTPw*

Mice immunised with adversely stored DTPw had significantly higher antibody responses to PT on day 14 post intranasal challenge (p=0.002). Responses to FHA were also higher on day 4 post immunisation in mice immunised with vaccine stored at +13$^0$C, (p=0.050: refer to figures 6.9a-c). Again, these results may be due to chance alone.

#### *6.3.3.1.3 Comparison between responses to DTPa and DTPw after immunisation with vaccine stored at +13$^0$C for 48 hours*

IgG antibody responses to PT in DTPa were significantly higher than those to DTPw at all time points (day 28 p<0.001, day 42 p=0.011, day 4 post challenge p=0.011, day 14 post challenge p<0.001). Responses to FHA in DTPa were higher at all times except at 14 days post challenge (day 28 p=0.030, day 42 p=0.018, day 4 post challenge p=0.030), and responses to PRN in DTPw were higher than those to DTPw at day 28 (p=0.003) day 42 (p<0.001) and day 14 post challenge (p=0.027).

#### *6.6.3.1.4 IgG antibody responses to diphtheria and tetanus toxoids*

6.6.3.1.4.1. Mice immunised with DTPa and DTPw

No significant differences in antibody responses between mice immunised with vaccine stored between $2^0$C and $8^0$C and mice immunised with vaccine stored at $+13^0$C for 48 hours were demonstrated in response to diphtheria or tetanus toxoids at any time point (refer to figures 6.10a and b and 6.11 a and b).

6.3.3.1.4.2 Comparison between responses to DTPa and DTPw after immunisation with vaccine stored at $+13^0$C

IgG antibody responses to diphtheria and tetanus toxoids in DTPw were significantly higher than those in DTPa on all days except day 42 (day 28 diphtheria and tetanus $p<0.001$; day 4 post challenge diphtheria $p=0.008$, tetanus $p=0.048$; day 14 post challenge diphtheria $p<0.001$, tetanus $p=0.007$).

It is interesting to note that for all storage conditions, the confidence intervals for the geometric mean diphtheria and tetanus antibody concentrations were much wider indicating greater variability in murine responses to these antigens in DTPw.

### 6.3.3.2 IgG antibody responses of mice immunised with normal saline

In contrast to results reported in the immunogenicity experiments in chapters 2 and 3, mice immunised with normal saline did not demonstrate any response to PT, FHA, PRN, diphtheria toxoid or tetanus toxoid during the course of these experiments.

### 6.3.3.3 Correlation between lung clearance and pertussis IgG antibody concentrations four days after intranasal challenge

Pearson correlations were performed to determine if there was any relationship between pertussis antibody concentrations and lung homogenate culture results four days after intranasal challenge. When analyses were performed on the basis of vaccine storage group and separated by vaccine treatment group, because of low numbers in some groups, or identical antibody concentrations or lung bacterial counts, some correlations could not be generated. Mice immunised with normal saline had no detectable responses until 14 days after challenge, making correlations between serology and lung clearance incalculable. Therefore data presented in figures 6.12-6.16 and table 6.4 are from the calculable correlations only.

Strong negative correlations were demonstrated between FHA serology and lung clearance for DTPw stored at 2-8$^0$C (r=-0.7592), -3$^0$C for 24 hours (r=-0.5261), and at –6$^0$C for 14 days (r=-0.6217) and for DTPa stored at -6$^0$C for 24 hours (r=-0.7933). Strong negative correlations were also demonstrated between PRN serology and lung clearance for DTPa stored at –6$^0$C for 24 hours (r=-0.7954) and DTPw stored at –6$^0$C for 14 days (r=-0.8016). However, only the correlation for FHA serology in mice immunised with DTPw stored at 2-8$^0$C was statistically significant (p=0.018). The remainder of the calculable correlations were positive or weakly to moderately negative and none of these were statistically significant.

Table 6.5 presents the results of correlations between murine serology and lung homogenate culture results when mice immunised with saline were included in the analyses and analyses were performed independent of vaccine treatment group (ie all results for each storage experiment were combined prior to analysis).

Although only moderate negative correlations were demonstrated in this second analysis, correlations were significant for PRN antibodies in all cases, and for FHA antibodies in most cases except when vaccines were stored at $-3^0$C for 24 hour or 14 days prior to immunisation. However, PT antibodies were significantly negatively correlated with lung homogenate culture results only when vaccines were stored under ideal conditions or at $-3^0$C for 24 hours. These results indicate the need for larger numbers of mice in such analyses to ensure experimental power.

## 6.4 Conclusions

In general, adverse storage of vaccines below $0^0$C did not affect antibody responses to pertussis antigens in DTPa or DTPw. Significant differences in antibody concentrations between vaccine storage groups were only demonstrated on day 42 (the day of challenge) for PRN in DTPw, and on day 46 (4 days post challenge) for FHA and PRN in DTPw and PT in DTPa. In addition, where significant differences were demonstrated between all vaccine storage groups, antibody concentrations of mice in individual adverse storage groups were significantly greater than those immunised with vaccine stored under ideal conditions only in response to DTPw.

Comparison of antibody responses in mice immunised with correctly stored vaccines or vaccines stored below $0^0$C were significant on one-way ANOVA in response to diphtheria toxoid in DTPa on day 42 and to tetanus toxoid in DTPw on days 28 and day 42. However, significantly higher antibody responses in mice in individual adverse storage groups than those immunised with vaccine stored under ideal conditions were demonstrated in response to tetanus toxoid in DTPw on day 28 only. In addition, in most cases adverse storage of vaccines below $0^0$C was associated paradoxically with increased antibody concentrations to all antigens except PT, although these increases tended to be statistically significant in response to adversely stored DTPw only. These results would indicate that DTPa appears to be the more robust vaccine under conditions of adverse storage below $0^0$C with respect to immunogenicity alone.

As demonstrated in the immunogenicity experiments described earlier, responses to pertussis antigens in DTPa were substantially higher than those to DTPw in most cases. This has also been demonstrated in a previous study investigating antibody responses to correctly stored vaccines (231). Possible explanations including immunising dose of

vaccine and the tendency for DTPw to skew immune responses towards the Th1 phenotype have already been discussed (section 3.4). In contrast, DTPw appeared to be more immunogenic than DTPa with respect to responses to diphtheria and tetanus toxoids, regardless of storage condition, and despite slightly higher doses administered to mice immunised with DTPa (table 2.1). This is most likely due to the adjuvant effect of the killed pertussis organisms in DTPw, but could also reflect differences in purification of these antigens between vaccine manufacturers.

Mice immunised with vaccine stored at $+13^0$C for 48 hours also demonstrated antibody responses to pertussis antigens and to diphtheria and tetanus toxoids that were, in general, no different from those in mice immunised with vaccine stored under ideal conditions. In addition, the same general tendencies for antibody responses to pertussis antigens to be higher in mice immunised with DTPa and responses to diphtheria and tetanus toxoids to be higher in mice immunised with DTPw were demonstrated.

Experiments utilising vaccine storage conditions of $2^0$C to $8^0$C or $+13^0$C provided longitudinal data regarding murine antibody responses to PT in DTPw immunised mice, in which antibody responses were not detectable until day 14 post challenge. This is in contrast to the results of previous investigators reporting high antibody responses to PT at 5 and 15 days after aerosol challenge with live *B. pertussis* in mice immunised with the NIBSC *B. pertussis* reference preparation (88/522) (185, section 3.4) and of investigators documenting antibody responses to immunisation, who demonstrated PT antibody responses from day 20 post immunisation with DTPa or DTPw in NIH mice (231). Results from the present study may therefore indicate that PT in the CSL DTPw is not immunogenic in Balb/c mice, and that the day 14 anti-PT antibodies are a response to infection rather than immunisation. This would, however, represent a relatively slow rise

in IgG anti-PT after challenge which may be explicable either in terms of a predominant early IgA/IgM anti-PT response or binding of all available IgG anti-PT to antigen with subsequent splenic sequestration of immune complexes in the early phase of infection.

Similarly, mice immunised with normal saline did not demonstrate IgG antibody responses to PT, FHA or PRN four days after intranasal challenge, but demonstrated a small rise in antibody concentrations of IgG anti-PT and -PRN 14 days after intranasal challenge. This contradicts the findings of previous investigators (185) who used the same immunisation and challenge schedule as in the present study and found that at day 5 and day 15 after aerosol challenge, unimmunised control mice demonstrated no antibody responses to PT, FHA or PRN. However, unimmunised convalescent mice (aerosol-infected with *B. pertussis* 6 weeks previously at the same time that immunised mice received their first dose of vaccine) had moderate IgG antibody responses to PT with no detectable response to FHA or PRN at the same times that immunised and control mice had antibody quantification performed (5 and 15 days after aerosol challenge (185). Mills et al also investigated the antibody responses of unimmunised Balb/c mice to aerosol infection and found no evidence of antibody response to PT or FHA until 6 weeks post infection and no antibody response to PRN in these mice even 12 weeks after infection (184). Hence IgG production after natural infection is highest after organisms have been cleared from the lungs in relation to both intranasal and aerosol challenge. Although intranasally infected saline immunised mice in the present study have not had antibody responses measured beyond 14 days after challenge, it would appear that intranasal infection without previous immunisation is also associated with an earlier rise in antibodies to PT and PRN in Balb/c mice. Unimmunised humans tend to develop antibodies early in the course of infection with further rises in antibody concentrations noted in convalescent sera. This suggests that

intranasal challenge in mice mimics natural human infection more closely than aerosol challenge with respect to antibody responses.

Mice immunised with DTPa or DTPw stored between $2^0$C and $8^0$C or below $0^0$C demonstrated significant increases in pertussis, diphtheria and tetanus antibody titres after the second immunisation on day 28 (with the exception of PT in DTPw). In addition, continued increases in antibody titres to PRN, diphtheria and tetanus toxoids in both vaccines and to PT in DTPa were demonstrated four days after intranasal challenge. Mice immunised with DTPw stored at $+13^0$C for 48 hours or between $2^0$C and $8^0$C demonstrated further increases in PT, FHA and PRN antibody titres on day 14 post challenge. In contrast, antibody responses to diphtheria and tetanus toxoids in both vaccines tended to decrease between day 4 and day 14 post challenge. Therefore it is likely that the increases in diphtheria and tetanus antibody concentrations four days after intranasal challenge with live *B. pertussis* represents the results of an inflammatory response in these mice, whereas the continued trend towards increased pertussis antibody concentrations probably represents a true further response to infection in addition to the non-specific effects of inflammation on total IgG antibody concentrations.

Results of correlations between antibody concentrations and lung homogenate culture results indicate that although higher FHA antibody titres in mice immunised with DTPw stored at 2-$8^0$C, -$3^0$C for 24 hours, and at $-6^0$C for 14 days appear to be associated with lower lung homogenate colony counts, only results for vaccine stored under ideal conditions are statistically significant. Correlations between PT and PRN antibodies and lung clearance of organisms appear in general to be unaffected by adverse storage in this regard and are not statistically significant. Apart from Sato et al, (179), few previous investigators have found significant correlations between IgG antibody concentrations and

lung homogenate culture results (115, 162, 183, 252). This gives further support to the findings of Mills et al that there may be no single serological correlate of protection against pertussis infection (162).

In summary, it would appear that antibody concentrations to pertussis antigens and to diphtheria and tetanus toxoid are, in general, not reduced by adverse vaccine storage between $-6^0$C and $+2^0$C and between $+8^0$C and $+13^0$C. In addition, the magnitude of IgG antibody concentrations to pertussis antigens does not appear to correlate with the rate of lung clearance of organisms in immunised mice even after adverse vaccine storage.

In the future, investigation of correlations between lung homogenate culture results before day four post intranasal challenge may provide more useful information in immunised mice. Alternatively, as mice immunised with saline did not develop antibody responses to infection until day 14 post challenge, it may be more useful to investigate correlations between IgA or IgM concentrations in serum or IgA concentrations in BAL fluid and lung clearance after challenge to determine if a serological correlate of protection against pertussis infection does exist.

## Table 6.1 Differences between NCH and WCH murine pertussis serology

| | NCH<br>(Swiss outbred and Balb/c mice) | WCH<br>(Balb/c mice) |
|---|---|---|
| **Serology** | 1.PRNfrom Dr R Rappuoli, Italy<br><br>2. Internal reference serum from Swiss outbred mice<br>3. No external reference<br><br>4. Biotek EL312 plate reader<br>5. KinetiCalc® data analysis programme | 1. PRN from SB Biologicals, Belgium<br>2. Internal reference serum from Balb/c mice<br>3. External reference WHO 97/642*<br>4. Dynex® Opsys MR plate reader<br>5. Multicalc® data analysis programme |
| **Lower limits of sesitivity** | PT 0.1 EU/ml<br>FHA 3EU/ml<br>PRN 5.6 EU/ml | PT 2 EU/ml<br>FHA 1.1 EU/ml<br>PRN 2 EU/ml |

* WHO first international anti-*B. pertussis* serum (mouse)

## Table 6.2 Murine IgG diphtheria and tetanus enzyme immunoassay reagents

| Reagent | Recipe/specifications |
|---|---|
| Carbonate-bicarbonate coating buffer (pH 9.6) | 0.8gm $Na_2CO_3$<br>1.5gm $NaHCO_3$<br>500ml distilled $H_2O$ |
| Phosphate buffered saline (pH 7.2) | 0.35gm $NaHPO_4$<br>0.65 gm $Na_2HPO_4$<br>8.5gm NaCl<br>1000ml distilled $H_2O$<br>(adjust pH to 7.2 using NaOH) |
| Wash and diluent buffer | PBS plus 0.5ml Tween 20/1000 ml distilled $H_2O$ |
| Blocking buffer | PBS-1% BSA (purified BSA) |
| Sheep-anti mouse conjugate | Serotek® sheep anti-mouse IgG horseradish peroxidase<br>Cat AACI0P, batch 150800 |
| ABTS | 11.852gm ctric acid<br>12.379gm $Na_2HPO_4$ anhydrous<br>1000ml distilled $H_2O$<br>check pH 4.2 prior to adding ABTS |

**Table 6.3 Details of reference and quality control sera for murine diphtheria and tetanus enzyme immunoassays**

| Serum | Details |
|---|---|
| **Reference serum** | -pooled serum from five Balb/c mice immunised once with 0.125 mls DTPa and bled three weeks later<br>-1:100 dilution given arbitrary value of 1Unit (both assays)<br>-1:12800 dilution assigned value of 0.0078 Units (both assays)<br>-lower limit of assay detection 0.01Units (both assays) |
| **High quality control** | -pooled serum from another four Balb/c mice immunised once with 0.125 mls DTPa and bled three weeks later<br>-diluted 1:100 |
| **Low quality control** | -1:1000 dilution of High QC pooled serum |

**Table 6.4 Results of Pearson correlations to investigate the relationship between lung clearance and IgG pertussus antibody concentrations four days after intranasal challenge**

| | DTPa | | | DTPw | | |
|---|---|---|---|---|---|---|
| | **PT** | **FHA** | **PRN** | **PT** | **FHA** | **PRN** |
| **2-8$^0$C** r value | 0.4358 (p=0.247) | 0.2256 (p=0.560) | 0.0360 (p=0.927) | -0.1157 (0.767) | **-0.7592 (p=0.018)** | -0.4230 (p=0.257) |
| **+13$^0$C** r value | * | * | * | * | * | * |
| **-3$^0$C 24 hours** r value | 0.4963 (p=0.395) | -0.0907 (p=0.885) | -0.0707 (p=0.911) | † | -0.5261 (p=0.363) | 0.3350 (p=0.586) |
| **-6$^0$C 24 hours** r value | 0.2905 (p=0.635) | -0.7933 (p=0.105) | -0.7954 (p=0.108) | † | -0.1330 (p=0.829) | -0.3342 (p=0.583) |
| **-3$^0$C 14 days** r value | 0.1127 (p=0.857) | 0.0422 (p=0.946) | -0.3122 (p=0.609) | † | 0.2026 (p=0.744) | 0.2122 (p=0.732) |
| **-6$^0$C 14 days** r value | * | * | * | † | -0.6217 (p=0.263) | -0.8016 (p=0.103) |

*lung homogenate cultures yielded no growth 4 days post intranasal challenge therefore no correlations were calculable

†no serologic response to PT in the DTPw formulation therefore no correlations were calculable

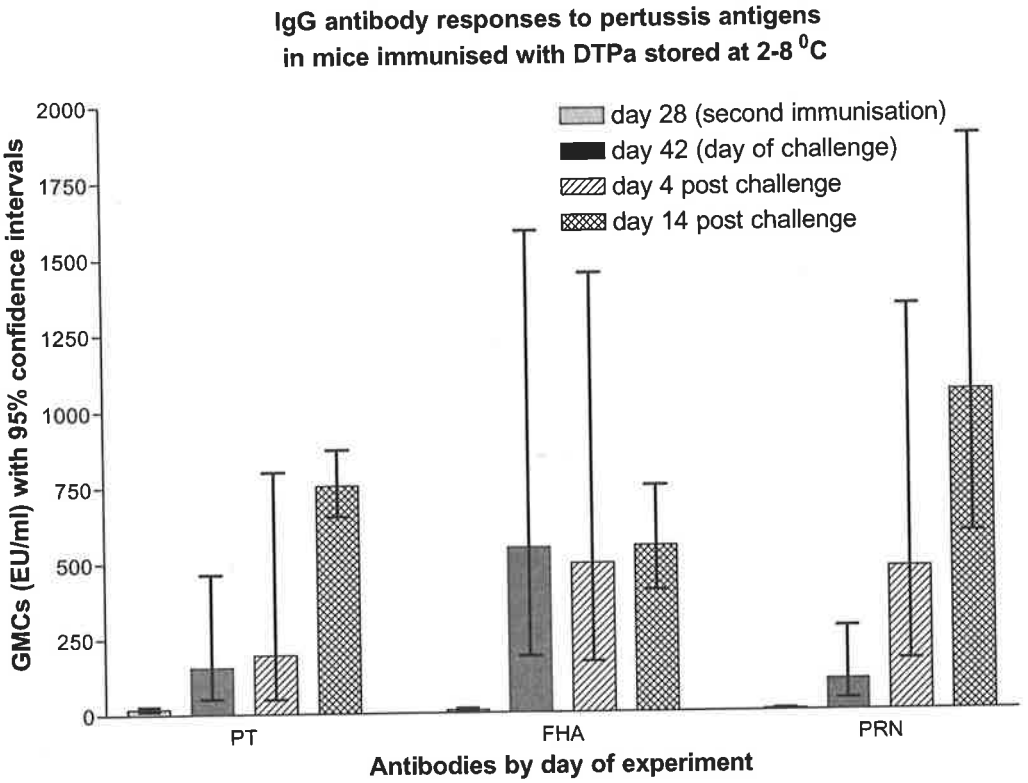
**Table 6.5 Results of Pearson correlations to investigate the relationship between lung homogenate cultures and antibody concentrations**

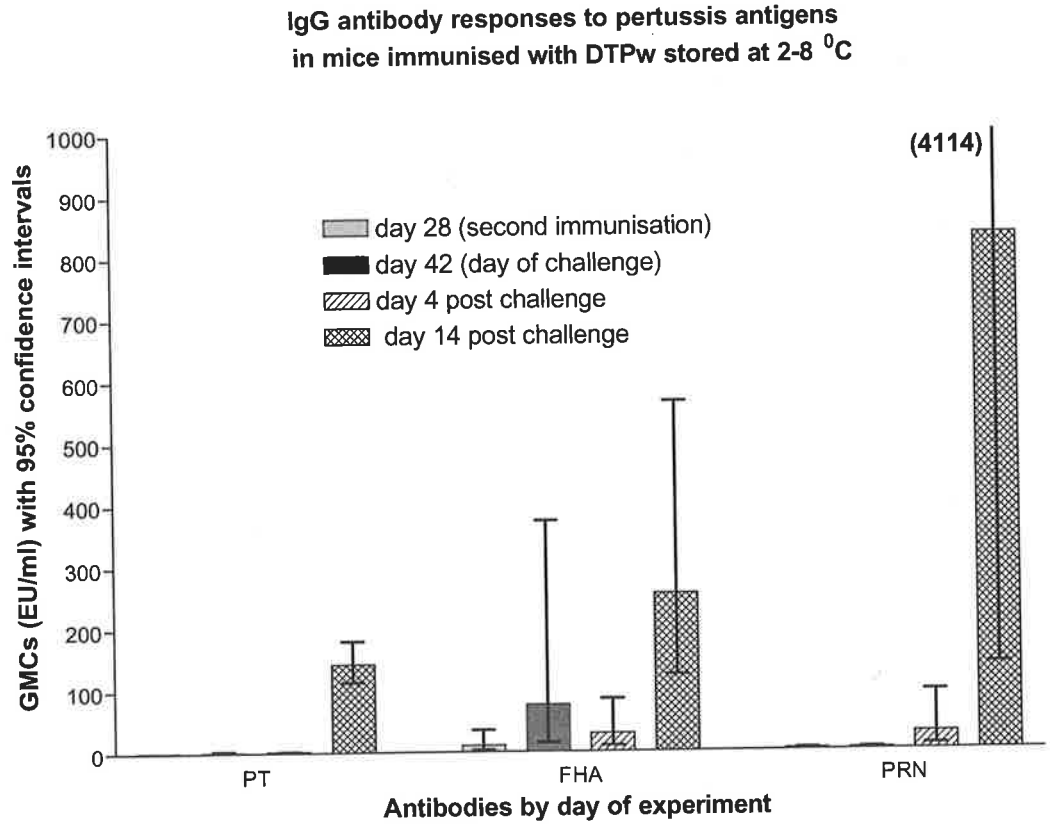
(results of all vaccines combined)

| | PT | FHA | PRN |
|---|---|---|---|
| **2-8$^0$C r value** | -0.413 (p=0.014) | -0.447 (p=0.007) | -0.486 (p=0.004) |
| **+13$^0$C** r value | -0.344 (p=0.108) | -0.500 (p=015) | -0.621 (p=0.002) |
| **-3$^0$C 24 hours** r value | -0.532 (p=0.005) | -0.476 (p=0.085) | -0.641 (p=0.019) |
| **-6$^0$C 24 hours** r value | -0.336 (p=0.212) | -0.583 (p=0.023) | -0.683 (p=0.005) |
| **-3$^0$C 14 days** r value | -0.450 (p=0.092) | -0.407 (p=0.132) | -0.643 (p=0.009) |
| **-6$^0$C 14 days** r value | -0.512 (p=0.061) | -0.607 (p=0.021) | -0.586 (p<0.001) |

**Figures 6.1a and 6.1b** **Pertussis serology results from 14 day protective efficacy experiments utilising correctly stored vaccine**

(Note 2-fold difference in Y-axis scales)

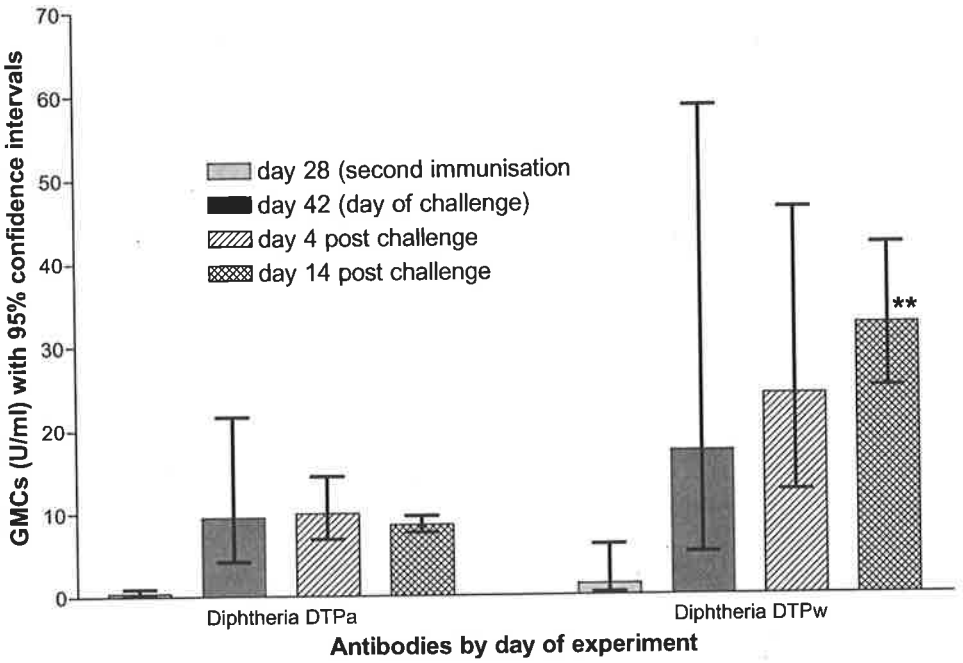


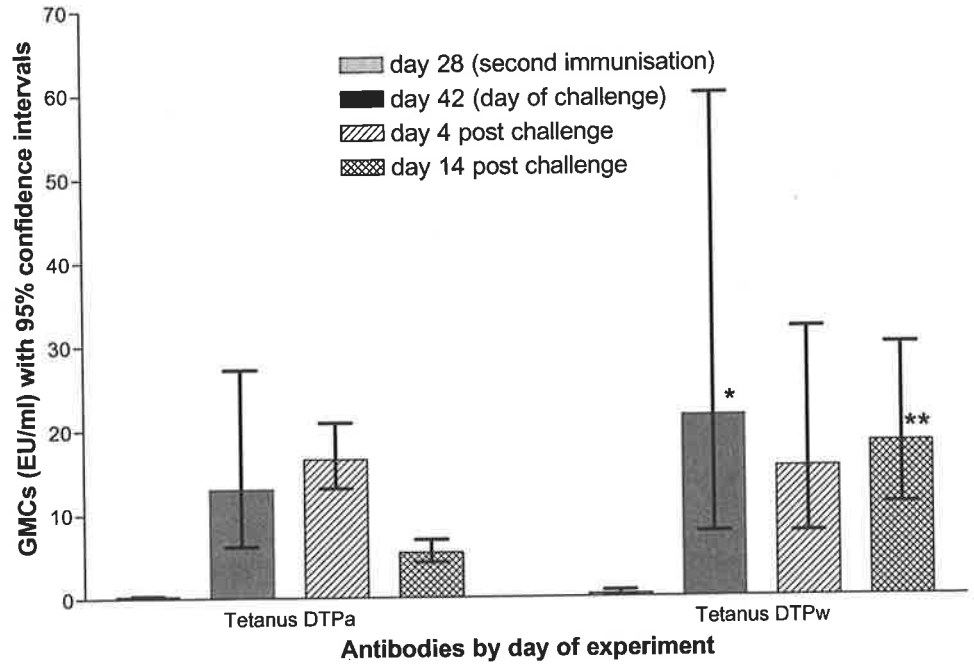
**Figures 6.2a and 6.2b** **Diphtheria and tetanus serology results from 14 day protective efficacy experiments utilising correctly stored vaccine**

asterixes indicate comparisons for which responses to DTPw which were significantly higher than those to DTPa: *=p<0.05, ** p≤0.005

**IgG antibodies to diphtheria toxoid in mice immunised with DTPa or DTPw stored at 2-8 $^0$C**

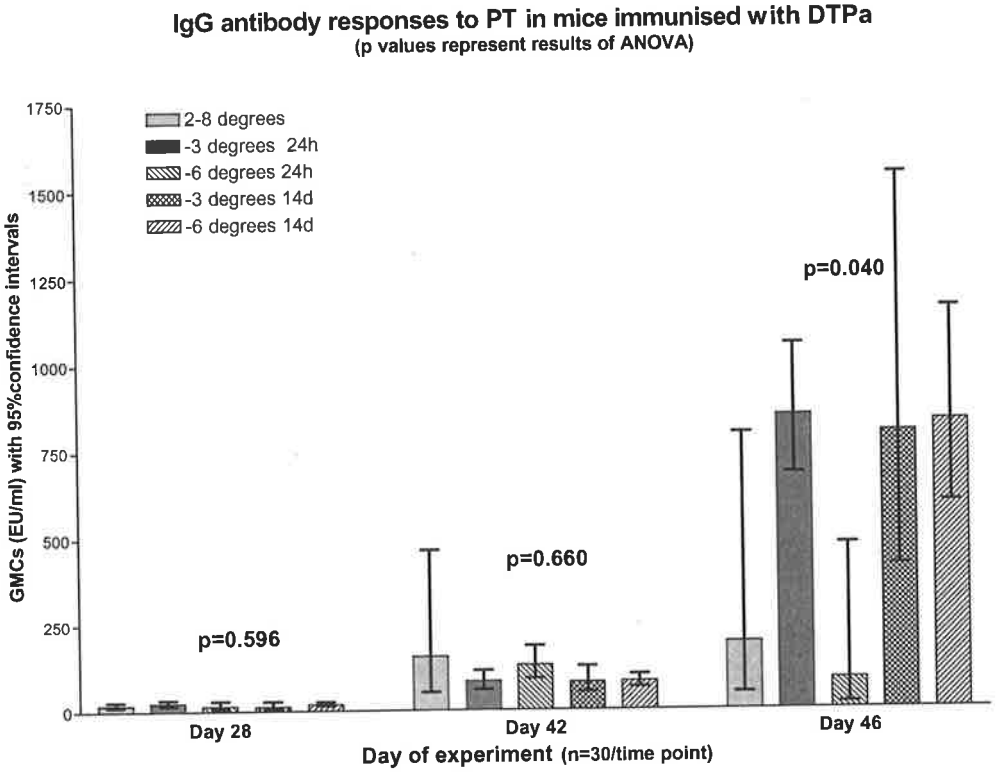


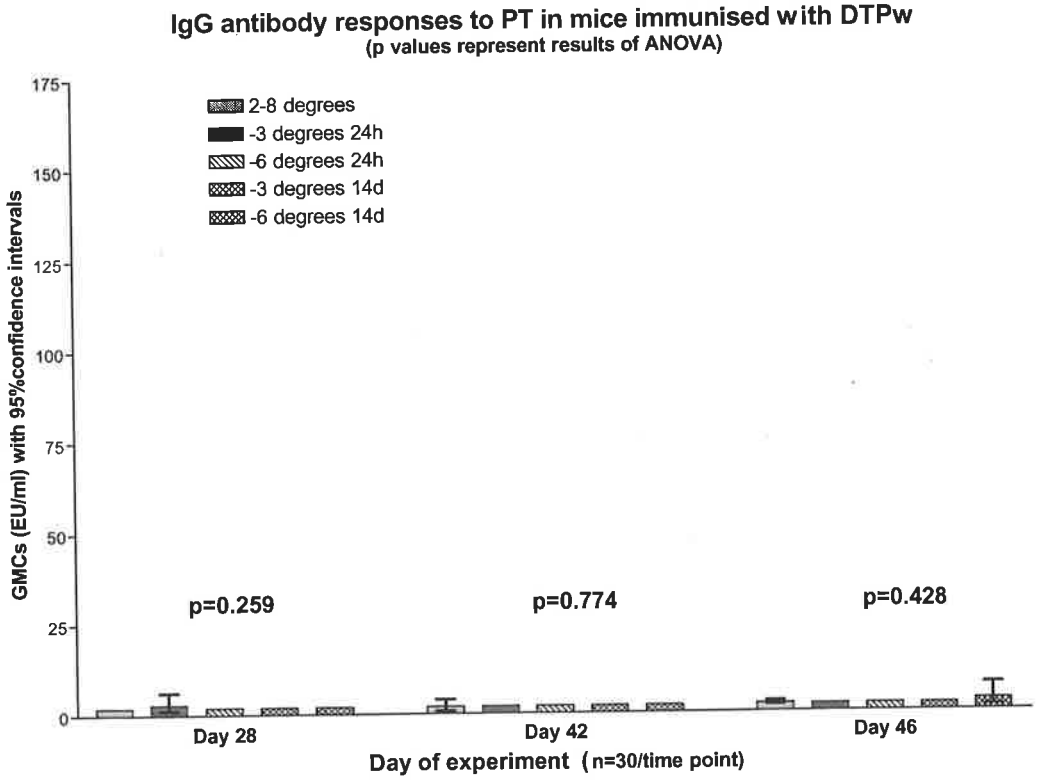

**IgG antibodies to tetanus toxoid in mice immunised with DTPa or DTPw stored at 2-8$^0$C**



**Figures 6.3a and 6.3b. Comparative PT serology results from all vaccine storage experiments**

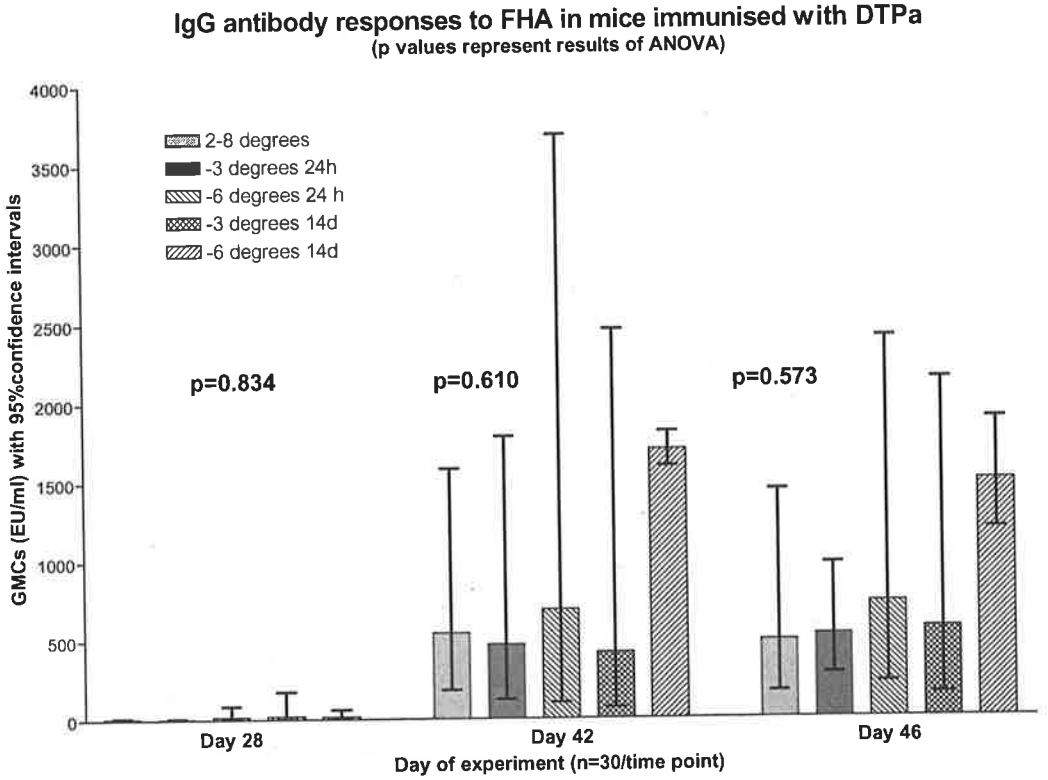
(Note 10-fold difference in y-axis scales)
asterixes indicate significant individual comparisons with 2-8$^0$C storage only *=p<0.05, ** p≤0.005

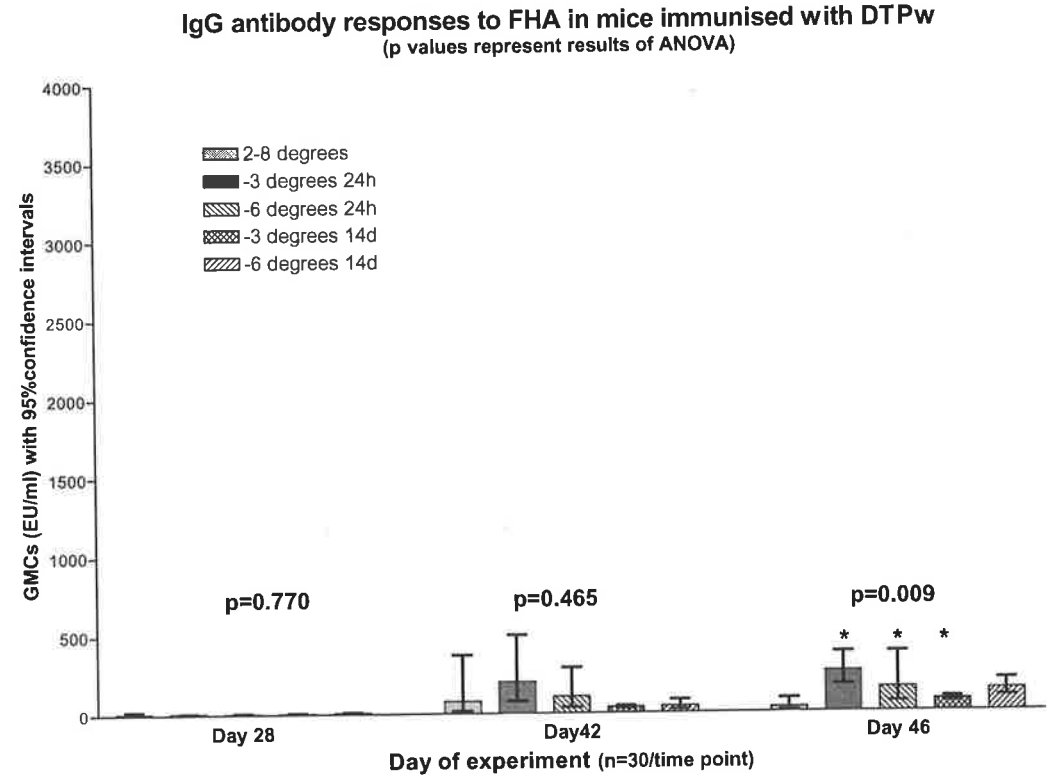


**Figures 6.4a and 6.4b** **Comparative FHA serology results from all vaccine storage experiments**

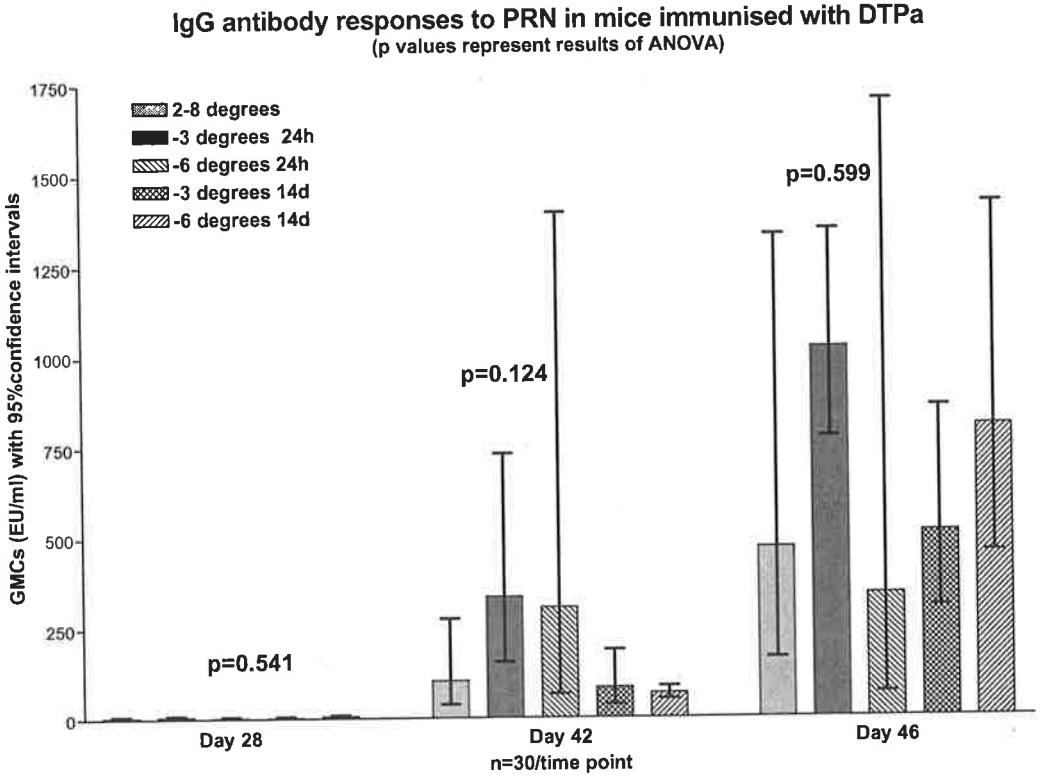
asterixes indicate significant individual comparisons with 2-8$^0$C storage only *=p<0.05, ** p≤0.005

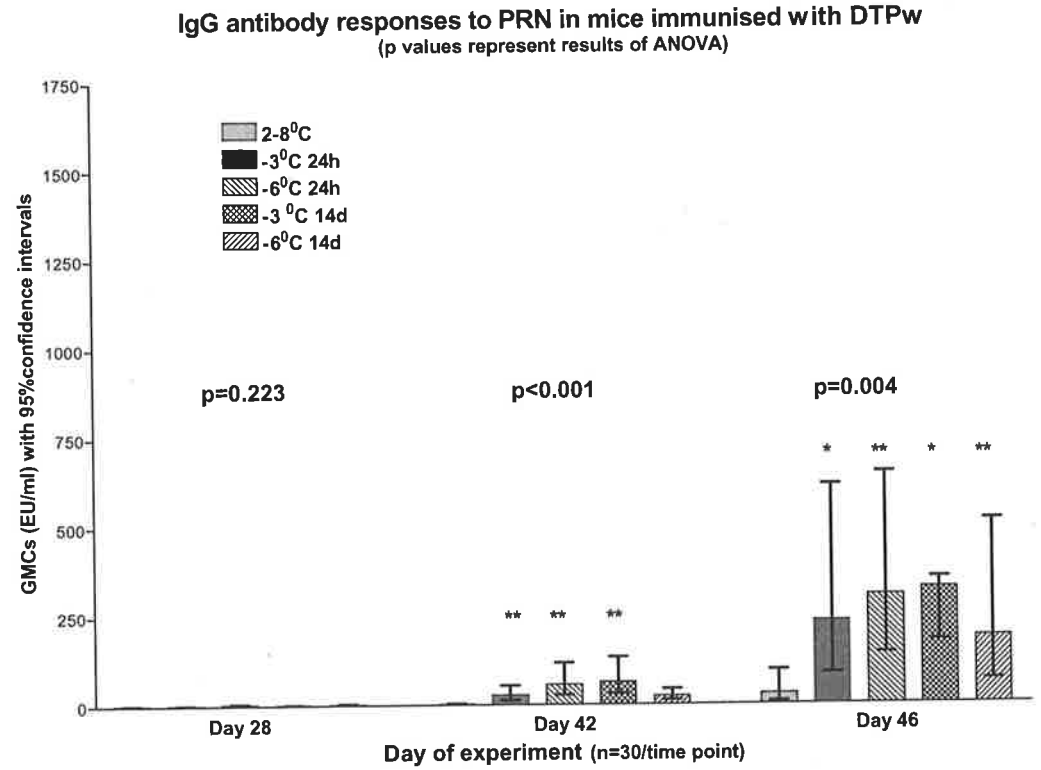


**Figures 6.5a and 6.5b** **Comparative PRN serology results from all vaccine storage experiments**

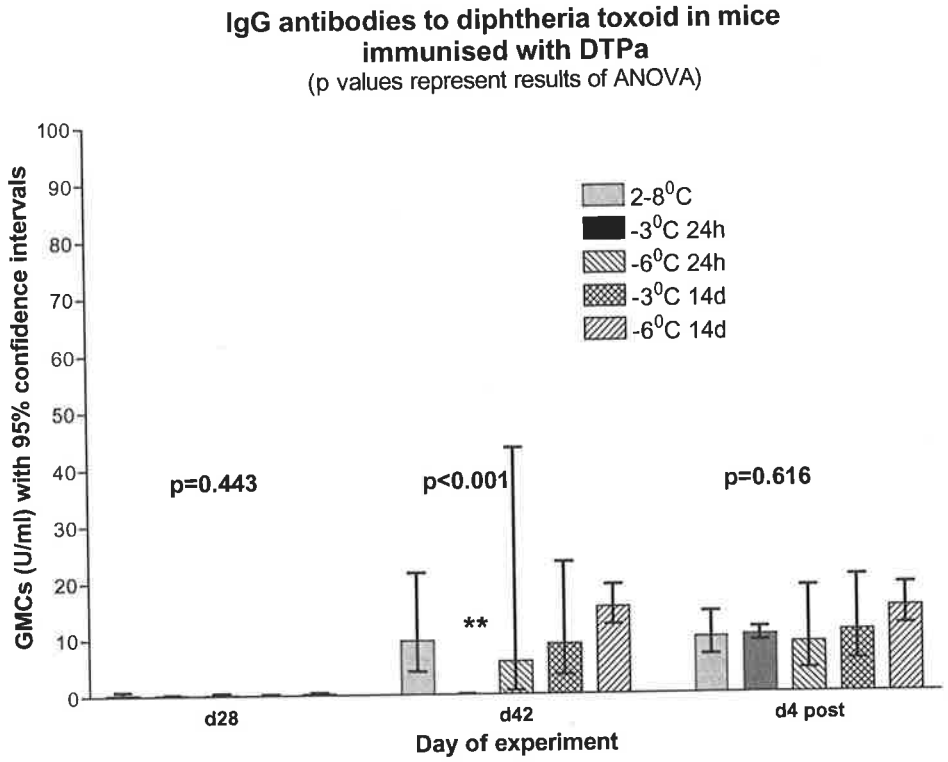
asterixes indicate significant individual comparisons with 2-8$^0$C storage only *=p<0.05, ** p≤0.005

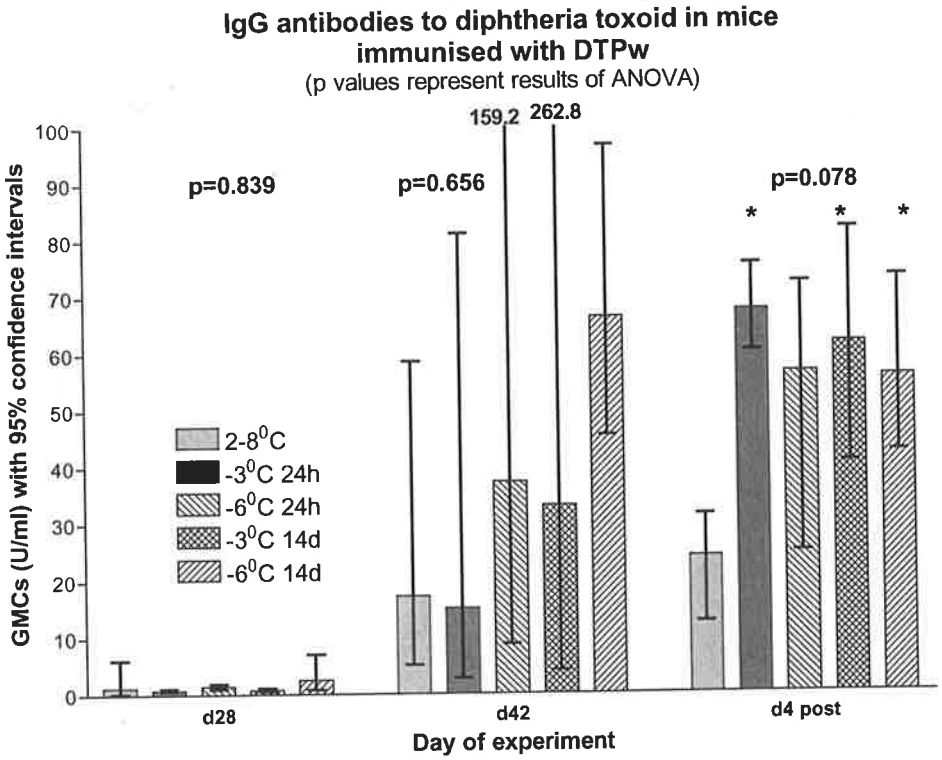


**Figures 6.6a and 6.6b** **Comparative diphtheria serology results from all vaccine storage experiments**

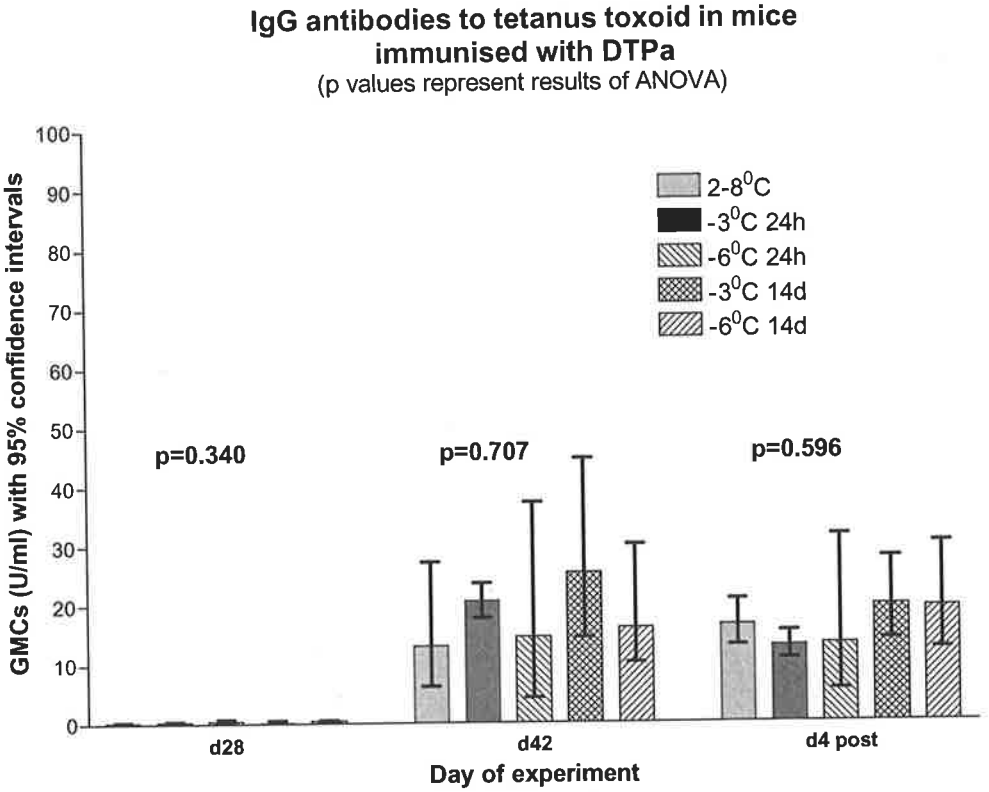
asterixes indicate significant individual comparisons with 2-8$^0$C storage only *=p<0.05, ** p≤0.005

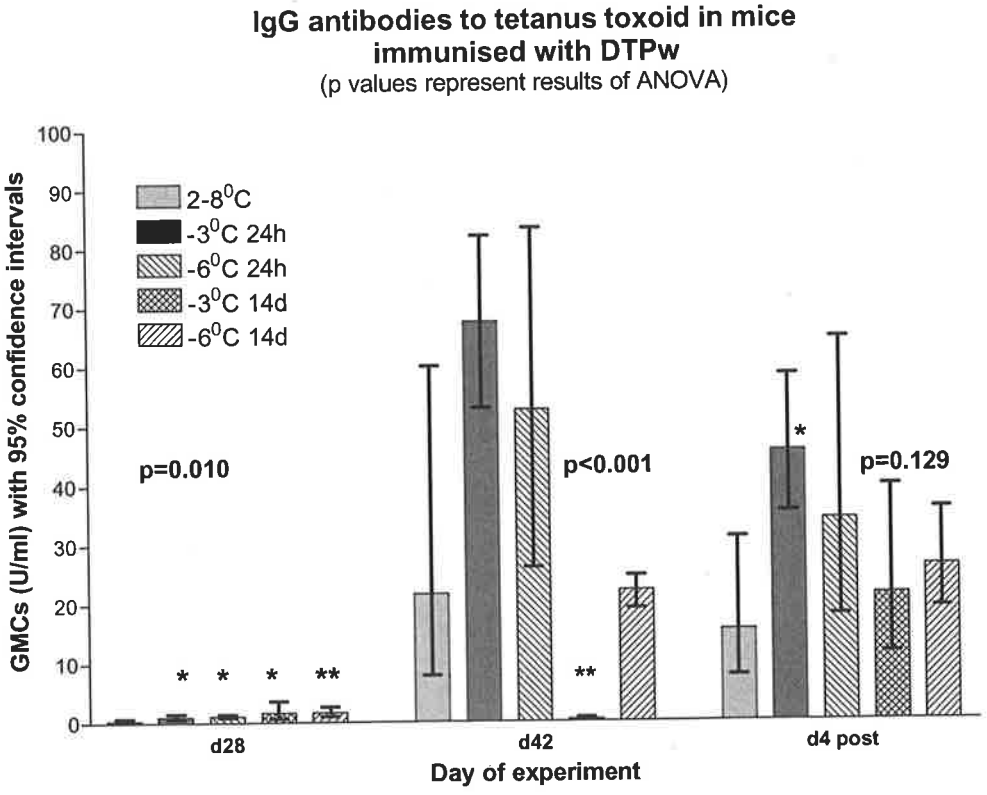


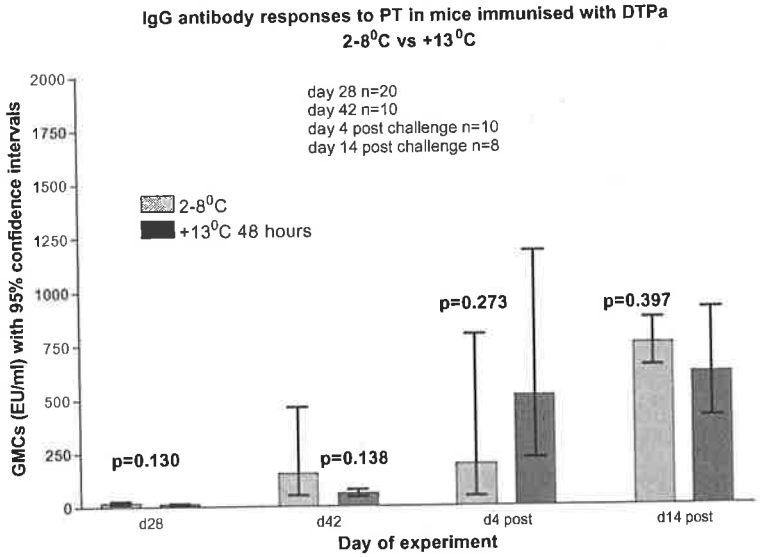
**Figures 6.7a and 6.7b** **Comparative tetanus serology results from all vaccine storage experiments**

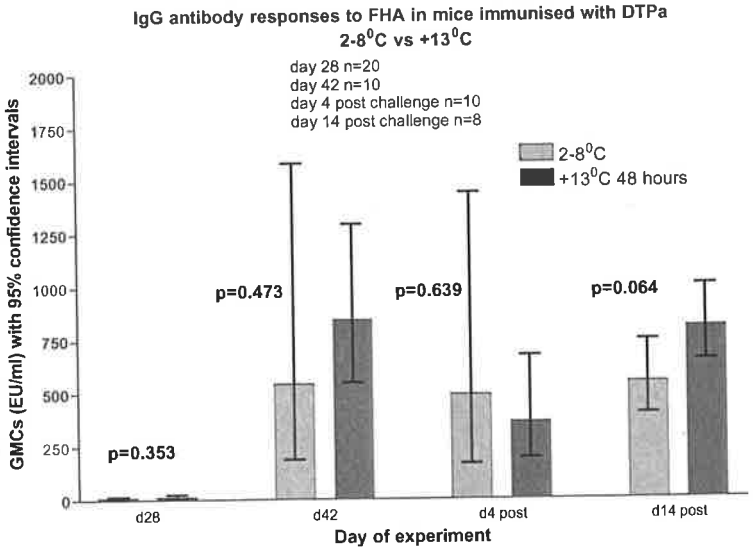
asterixes indicate significant individual comparisons with 2-8$^0$C storage only *=p<0.05, ** p≤0.005



**Figures 6.8a-c** **Comparative pertussis serology results after vaccine storage at +13$^0$C for 48 hours and 2-8$^0$C in mice immunised with DTPa**



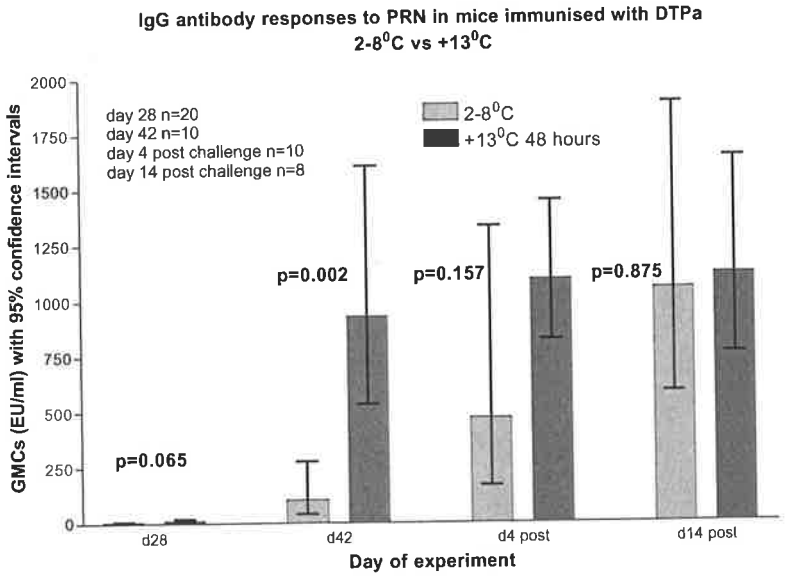
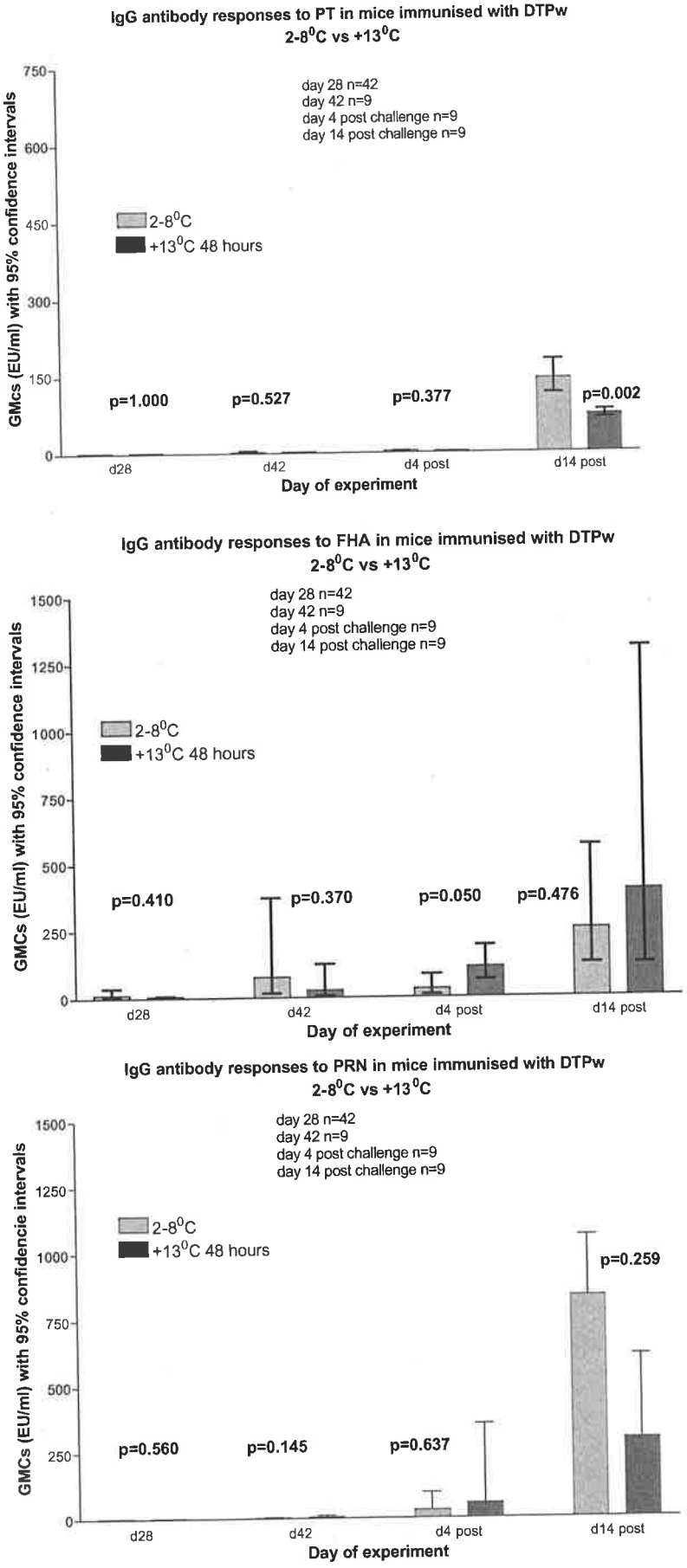
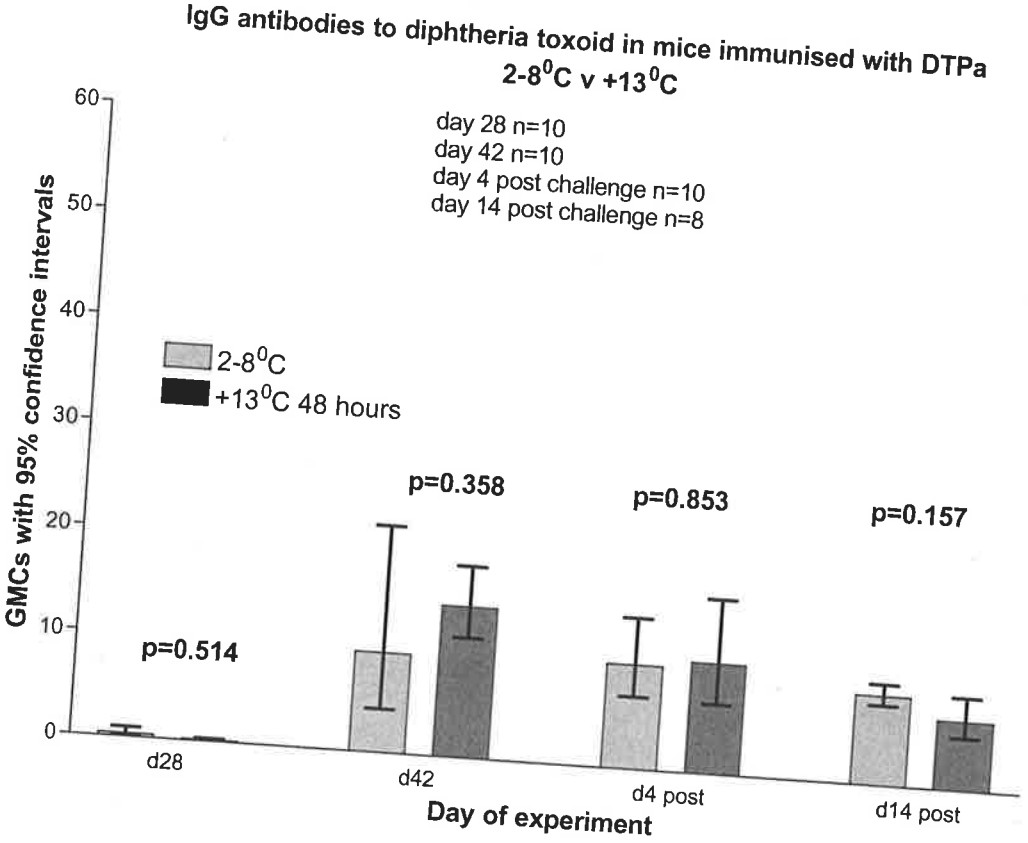
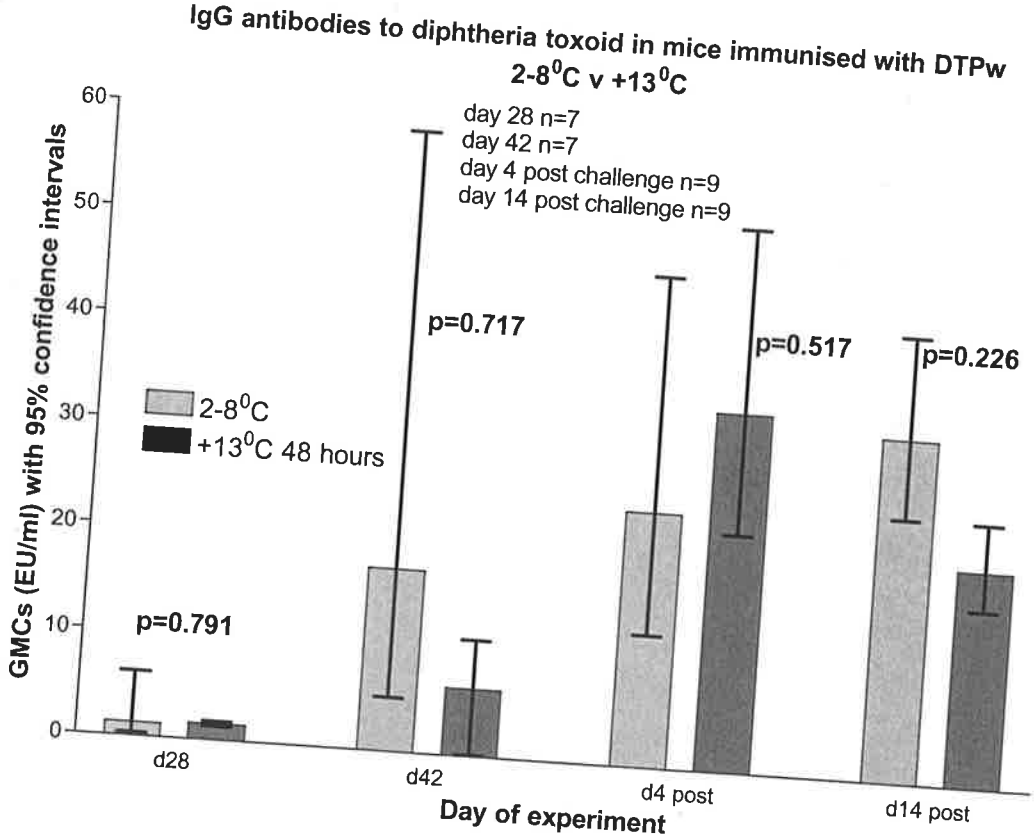



**Figure 6.9a-c** **Comparative pertussis serology results after vaccine storage at +13$^0$C for 48 hours and 2-8$^0$C in mice immunised with DTPw**

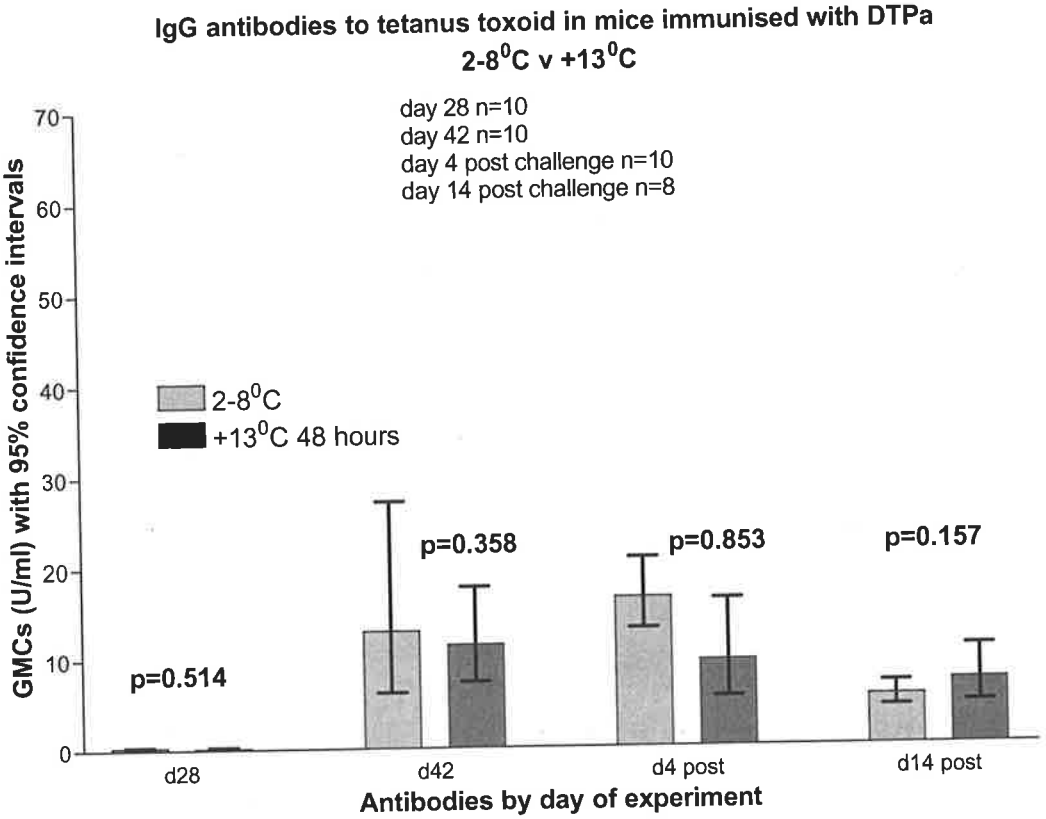


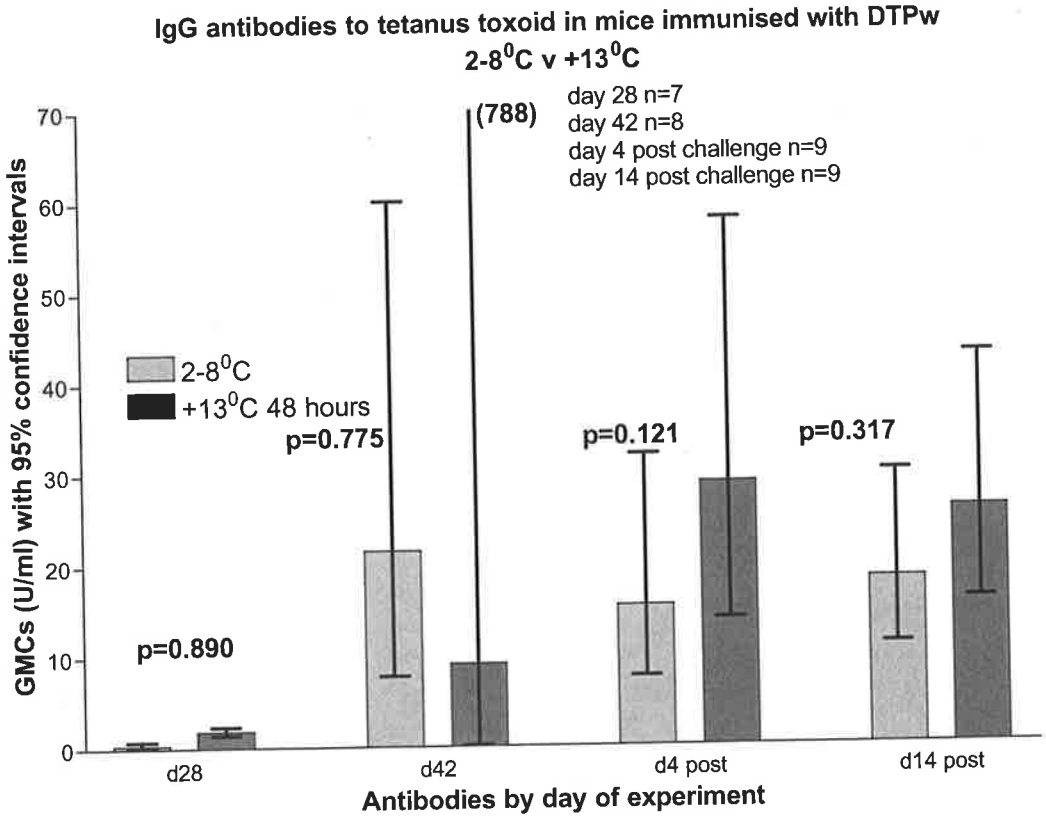
**Figures 6.10a and 6.10b** **Comparative diphtheria serology results after vaccine storage at +13$^0$C for 48 hours and 2-8$^0$C**



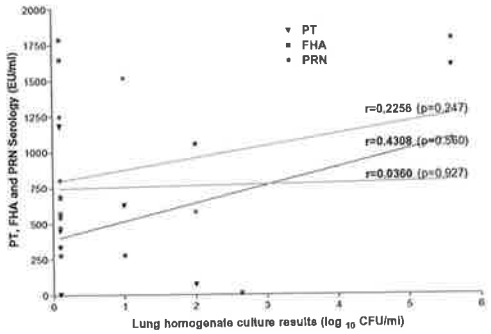
**Figures 6.11a and b Comparative tetanus serology results after vaccine storage at +13$^0$C for 48 hours and 2-8$^0$C**



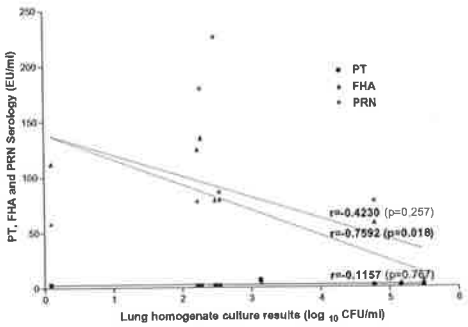



## Figures 6.12a-f Correlations between serology and lung homogenate culture results

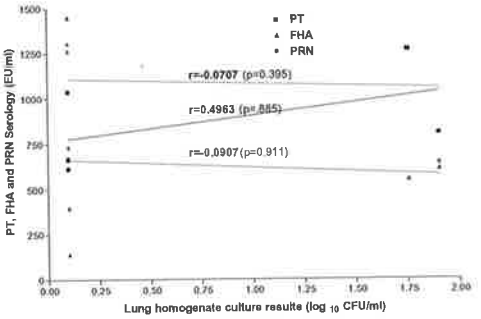
DTPa 2-8$^0$C: Serology/Lung homogenate culture correlations



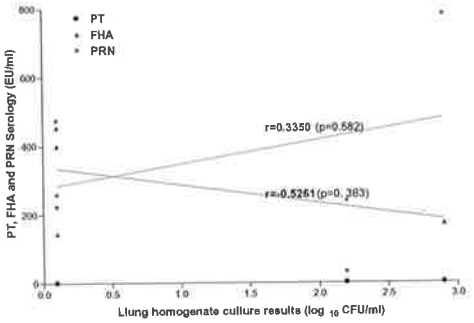

DTPw 2-8$^0$C: Serology/Lung lung homogenate culture correlations



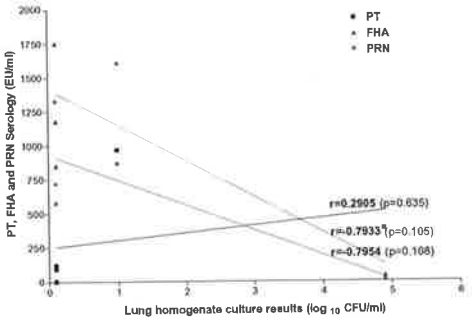

DTPa -3$^0$C/24 hours: Serology/Lung homogenate culture correlations



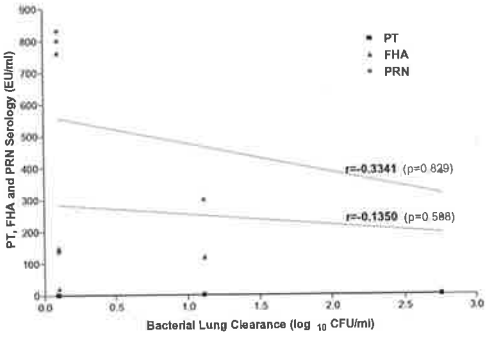

DTPw -3$^0$C/24 hours: Serology/Lung homogneate culture correlations




DTPa -6$^0$C/24 hours: Serology/Lung homgenate culture correlations

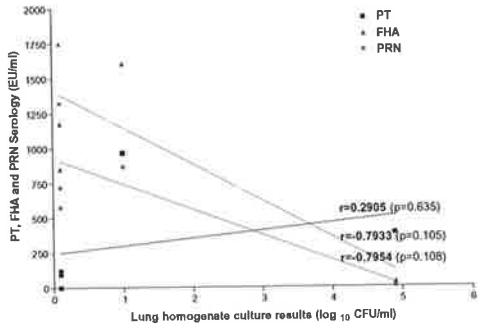



DTPw -6$^0$C/24 hours: Serology/Lung clearance correlations

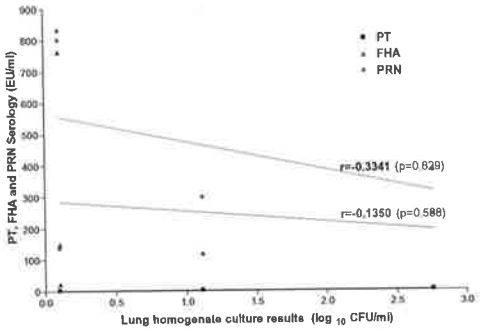


## Figures 6.12 g-k Correlations between serology and lung homogenate culture results

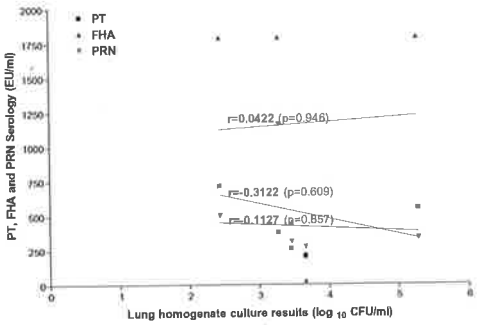
DTPa -6 $^0$C/24 hours: Serology/Lung homogenate culture correlations



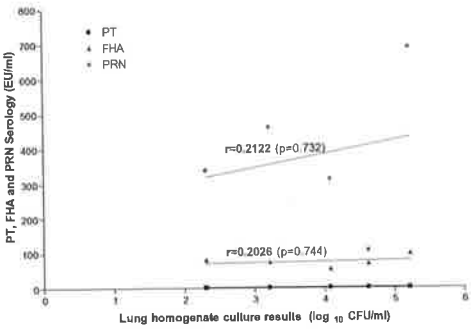

DTPw -6 $^0$C/24 hours: Serology/Lung homogenate culture correlations



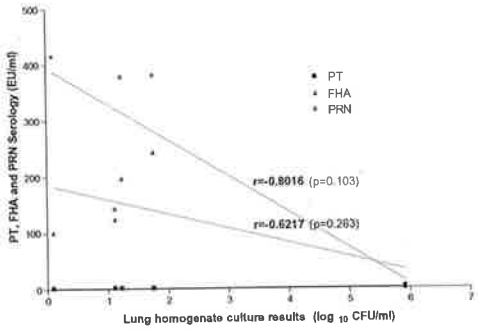

DTPa -3 $^0$C/14 days: Serology/Lung homogenate culture correlations




DTPw -3 $^0$C/14 days: Serology/Lung homogenate culture correlations




DTPw -6 $^0$C/14 days: Serology/Lung homogenate culture correlations



# Chapter 7 Definitive protective efficacy experiments: Lung histopathology and bronchoalveolar lavage fluid analysis

## 7.1 Introduction

It has already been demonstrated that adverse storage of pertussis vaccines, in general, does not diminish lung clearance of *B. pertussis* or the magnitude of antibody responses to pertussis vaccine antigens in the experiments described in chapters 5 and 6. There is no information available in the published literature regarding how immunisation with adversely stored vaccine may affect lung inflammatory responses to pertussis infection. Therefore the aims of this study were to delineate the lung inflammatory processes associated with pertussis infection in immunised and unimmunised mice and to compare responses between mice immunised with correctly and adversely stored vaccine using lung histopathology, and BAL fluid cytokine analysis and cytospin cell counts as surrogate markers of inflammation.

## 7.2 Methods

### 7.2.1 Preparation of lungs for histopathological analysis

Lungs were preserved for histopathological analysis after bronchoalveolar lavage by the intratracheal injection of 10% formalin as described in section 4.2.2.2.

Mouse lungs were then embedded in paraffin after which horizontal sections were cut in the upper, middle and lower section of both left and right lungs, each section having a thickness of four microns. Therefore each pair of lungs from individual mice provided six sections. Sections were placed on slides and stained with haematoxylin and eosin using methods described by Bancroft and Stevens (256).

#### 7.2.1.1 Histopathological assessment of lung inflammation

All lung sections were scored using a histopathological grading system developed and modified from those of previous investigators (253, 254). Slides were examined blind and in random order under 40x magnification, therefore blinding for vaccine storage condition, vaccine treatment group and lung section. Four parameters were assessed: acute lung inflammation (0-4), chronic lung inflammation (0-4), the degree of respiratory epithelial dysplasia (0-2) and number and location of alveolar macrophages (0-2). For each score, 0 represented no inflammation and the upper number of the range represented marked abnormality. Thus the maximum possible inflammatory score was 12.

### 7.2.2 Analysis of bronchoalveolar lavage fluid

#### 7.2.2.1 Collection and storage of BAL fluid

On day 4 post challenge in each experiment, BAL fluid was collected from the lungs of one mouse from each treatment group as described in section 4.2.1.4. Due to the shortage of CSL DTPw, no mice immunised with DTPw stored at $-3^0$C for 24 hours of $-6^0$C for 24 hours were available for collection of BAL fluid. Technical difficulties prevented collection of BAL fluid from one of the saline-immunised mice. For each mouse studied. lavage was performed twice with sterile PBS, and the fluid from each collection was placed into separate Eppendorf tubes on ice before being spun in a microfuge at 2000 rpm (263.85G) • for 10 minutes. The BAL fluid supernatant from each collection was then separated and stored immediately at $-70^0$C. BAL collections from the same mouse were stored separately.

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• conversion between RPM and G described by the equation:
relative centrifugal force (G)=11.18x $(n/100)^2$x radius of centrifugation (8.5cm), where n= speed in RPM

### 7.2.2.2 Enzyme immunoassay cytokine analysis

Supernatants of the BAL were later assayed for levels of the cytokines IL-2 and IL-4 concentrations using Endogen® commercial EIA kits (cat numbers EM-IL2 and EM-IL4 respectively).

#### *7.2.2.2.1 Murine IL-2 and IL-4 EIA methods*

Plates were precoated with anti-murine IL-2 or IL-4. Fifty microlitres of BAL fluid supernatant sample or relevant standard were then added to wells in duplicate, and incubated for two hours at $37^0$C in a humid chamber with an adhesive cover over the plates. The external standard for the IL-2 assay was lyophilised *E. coli* derived recombinant mouse IL-2 and the external standard for the IL-4 assay was the NIBSC/WHO reference lot 91/656 (recombinant murine IL-4).

Plates were washed five times with supplied wash buffer diluted in sterile deionised water after which 100µl of supplied conjugate reagent was added to each well. Plates were then covered as before and incubated for one hour at $37^0$C in a humid chamber and washed five times. TMB substrate solution was then added to all wells (100µl/well) and the plates were covered and incubated for 30 minutes at room temperature after which stop solution was added. Plates were then read at 450nm and 550 nm and the results were calculated by subtracting the absorbances at 550 nm from those read at 450nm. This result was then substituted into the equation describing the slope of the standard curve to calculate the final concentration in picograms/ml, as recommended by the kit manufacturers.

The lower limit of sensitivity of the IL-2 assay was 3 picograms/ml with a standard curve range of 34-850 picograms/ml, and the lower limit of sensitivity of the IL-4 assay was 5 picograms/ml with a standard curve range of 15-375 picograms/ml.

### 7.2.3 Cytospin preparation and analysis

#### 7.2.3.1 Cytospin slide preparation

After the BAL supernatant had been separated, lavage cells were resuspended in 500µl sterile RPMI for cytospin analysis. This was performed using a Shandon Cytospin® cyofuge. During this process, cells were concentrated onto a glass slide by centrifugation. A pencil-labelled glass slide and filter card were fitted into a slide clip. A 250µl aliquot of each BAL sample was pipetted into a sample chamber. The sample chamber was then fitted into the clip, counterbalanced with another and spun at 600 rpm at high acceleration for 5 minutes.

#### 7.2.3.2 Staining of cytospin slide preparations

Cytospin preparations were stained with Giemsa on a Hematek® slide stainer. Giemsa stock solution was made by slowly dissolving 13.5gm of Giemsa powder (Merck® cat 9203) in 900 mls methanol and then 900 mls glycerol. The stock solution was incubated at $37^0$C for three days in a brown glass bottle. Working solutions of Giemsa stain were made on a daily basis by adding 40mls of the stock solution to 30mls phosphate buffer pH 7.0 and 270 mls distilled water.

#### 7.2.3.3 Analysis of cytospin cellular composition

Slides were examined for cell counts blinded for mouse number, experiment number and vaccine treatment group under 40x magnification using a reticulated lens. Each slide was assessed for the numbers of lymphocytes, neutrophils, macrophages and red blood cells on four occasions, each time on a different section of the slide preparation. To determine the final count for each cell type, results of the four individual counts were averaged and expressed as counts per field at 40x magnification.

## 7.3 Results

### 7.3.1 Histopathological staging of mouse lungs

The general pattern of lung inflammation was patchy, predominantly lymphocytic and perivascular and peribronchiolar in nature, that is, indicative of bronchopneumonia. In addition, lung inflammation did not always involve all five lobes. In severe infection, alveolar, perivascular and peribronchiolar abscess formation, extension of lymphocytic infiltration to involve the sub-pleural spaces, and significant respiratory epithelial dysplasia were noted, along with a fibrinous exudate into airways and parenchymal interstitial oedema. (refer to figures 7.1-7.4).

#### *7.3.1.1.1 Comparisons between vaccine storage conditions*

Figures 7.5a-c document the total inflammation scores for lung sections by vaccine treatment group. Each point on the graphs represents the total score for one lung section, with a total of six sections per mouse. Results are presented by vaccine storage condition. The horizontal bars represent the mean score for each group of data. The p values represent the results of one-way ANOVA between vaccine storage conditions. For each storage experiment, a maximum of three mice were sacrificed for BAL and lung histopathology: one immunised with saline and, where possible, one mouse immunised with DTPa and one mouse immunised with DTPw. Therefore, only one mouse immunised with adversely stored DTPa and/or one mouse immunised with adversely stored DTPw were available for BAL and lung histopathology for each experiment. A total of four mice immunised with normal saline (stored under identical conditions) were available for BAL and lung histopathology at the completion of experiments, as there was no saline control for this part of the experiment which stored commercial vaccines under ideal conditions.

7.3.1.1.1.1. Comparison between vaccine treatment groups

One-way ANOVA demonstrated significant differences in total lung inflammation scores between vaccine treatment groups ($p<0.001$). Scores from mice immunised with DTPa were significantly lower than those immunised with DTPw ($p<0.001$) or normal saline ($p<0.001$). However, no significant differences in total scores were demonstrated between mice immunised with DTPw or normal saline ($p=1.000$).

7.3.1.1.1.2 Comparison between vaccine storage conditions

No significant differences were demonstrated between lung inflammation scores for mice immunised with correctly or adversely stored DTPa or DTPw ($p=0.965$ and $p=0.093$ respectively). However, although normal saline for immunisation was stored continuously at room temperature, significant differences in scores between different experiments were documented ($p<0.001$). This was due to the unexpectedly significantly lower scores found in saline-immunised mice in experiment number five, where DTPa and DTPw vaccines were stored at $-6^0$C for 14 days prior to immunisation.

### 7.3.1.2 Histopathology score sub-components

Table 7.1 documents the mean scores for the individual components of the total lung inflammation score separated by vaccine treatment group and storage condition.

#### *7.3.1.2.1 Comparison between vaccine treatment groups*

Significant differences were demonstrated between vaccine treatment group scores in all four histopathology score subcomponents on one-way ANOVA: acute inflammation (A) $p<0.001$, chronic inflammation (C) $p<0.001$, macrophage activation (MA) $p=0.003$, respiratory epithelial dysplasia (RE) $p<0.001$. For all four parameters, scores for lungs of

mice immunised with DTPa were significantly lower than those immunised with DTPw (A:$p<0.001$, C: $p<0.001$, MA: $p=0.009$, RE: $p<0.001$). Acute and chronic inflammatory scores and the degree of respiratory epithelial dysplasia were also significantly lower in DTPa- than saline-immunised mice ($p<0.001$ in all cases). However, DTPa and saline-immunised mice demonstrated the same degree of macrophage activation ($p=0.406$). In contrast, mice immunised with DTPw demonstrated higher lung scores than mice immunised with normal saline with regard to chronic inflammation ($p=0.016$) and macrophage activation ($p=0.024$).

#### *7.3.1.2.1.2 Comparison between vaccine storage conditions*

##### 7.3.1.2.1.2.1 Mice immunised with DTPa

Mice immunised with DTPa demonstrated no significant differences in chronic inflammation, macrophage activation or respiratory epithelial dysplasia scores when comparisons were made between vaccine storage conditions ($p=0.995$, $p=0.222$ and $p=0.537$ by ANOVA respectively). However, acute inflammation scores were significantly different between vaccine storage groups ($p=0.002$). Comparisons of lung subscores between mice immunised with correctly stored vaccine and individual adverse vaccine storage conditions demonstrated significantly less acute inflammation in mice immunised with vaccine stored at $-6^0$C for 24 hours, $-3^0$C for 14 days or $-6^0$C for 14 days than in mice immunised with vaccine stored under ideal conditions ($p=0.019$ in all cases).

##### 7.3.1.2.1.2.2 Mice immunised with DTPw

Due to the shortage of CSL DTPw, no data are available for mice immunised with DTPw stored at $-3^0$C or $-6^0$C for 14 days. However, significant differences in respiratory epithelia dysplasia were demonstrated between vaccine storage groups ($p=0.048$) and this was due to comparisons between ideal vaccine storage and storage at $-6^0$C for 24 hours.

7.3.1.2.1.2.3 Mice immunised with normal saline

Figure 7.1c demonstrates the lung histopathology scores from all four mice immunised with normal saline. Significant differences in scores were demonstrated between individual experiments by ANOVA ($p<0.001$), as shown by the spread of scores between 2 and 10.

## 7.3.2 BAL cytospin counts

Figures 7.6a-c document the cytospin cell counts (lymphocytes, neutrophils and macrophages) according to vaccine treatment group and figures 7.7a-c by individual cell type. Results are shown as the mean of four counts with the bars representing one standard deviation above and below the mean. Contamination of BAL cytospin preparations with peripheral blood was excluded by calculating the red blood cell: white blood cell ratio for all slides. The highest calculated ratio was 2.9:1 which is much lower than the red cell: white cell ratio in peripheral blood of approximately 1000:1.

### 7.3.2.1 Comparison by vaccine treatment group

Significant differences by ANOVA in lymphocyte (L), neutrophil (N) and macrophage (M) counts were documented between vaccine treatment groups ($p<0.001$ in all cases). Mice immunised with DTPa had significantly lower counts of all three cell types than mice immunised with DTPw (L: $p=0.006$, N: $p<0.001$, Ma: $p=0.004$) and significantly lower lymphocyte and neutrophil counts than mice immunised with normal saline ($p=0.016$ and $p<0.001$ respectively). Mice immunised with DTPw had significantly lower lymphocyte and macrophage counts than mice immunised with normal saline ($p=0.009$ and p=0,005 respectively).

### 7.3.2.2 Comparison by vaccine storage condition

#### *7.3.1.2.2 Mice immunised with DTPa*

Significant differences in counts of all cell types were documented between vaccine storage conditions (L:$p<0.001$, N:$p=0.025$, M:$p=0.001$). Lymphocyte counts were higher in mice immunised with vaccine stored correctly than in mice immunised with vaccine stored at $-3^0$C for 24 hours ($p=0.006$), and macrophage counts in mice immunised with vaccine stored under ideal conditions were significantly lower than in mice immunised with vaccine stored at $-6^0$C for 24 hours. Significant differences in neutrophil counts were due to differences between adverse vaccine storage groups, and not due to comparisons between ideal vaccine storage and individual adverse vaccine storage groups.

#### *7.3.1.2.3 Mice immunised with DTPw*

Lymphocyte and neutrophil counts differed significantly between vaccine storage groups ($p=0.001$, $p<0.001$ respectively). In both cases storage of vaccine between $2^0$C and $8^0$C produced significantly lower cell counts than in mice immunised with vaccine stored at $-3^0$C for 24 hours (L:$p=0.002$, N:$p=0.002$).

#### *7.3.1.2.4 Mice immunised with normal saline*

One-way ANOVA demonstrated significant differences in cell counts for all cell types between different experiments for mice immunised with normal saline (L:$p=0.002$, N:$p<0.001$, M:$p=0.001$.

### 7.3.3 BAL cytokine measurements

Figures 7.8a-c document the absolute IL-2, Il-4 concentrations and IL-4: IL-2 ratios (in picograms/ml) by vaccine treatment group. Statistical analysis was not performed on these data, as only one mouse from each vaccine storage group was sacrificed for BAL collection.

Mice immunised with DTPa and normal saline demonstrated IL-2 concentrations below the lower limit of detection (3 picograms/ml) with much higher IL-4 concentrations, indicating a predominant Th-2 immune response in these mice.

Mice immunised with DTPw demonstrated much higher concentrations of both cytokines in BAL fluid. However, relatively greater increases in IL-2 concentrations in comparison with mice immunised with DTPa and normal saline produced lower IL-4:IL-2 ratios in DTPw-immunised mice than in the other vaccine treatment groups, indicating that the Th-1 response was probably predominant mice immunised with DTPw.

### 7.3.4 Correlations between lung histopathology mean scores and serology day 4 post challenge

Calculations of Pearson correlations between histopathology scores and serology at four days after challenge were only possible for DTPa (figure 7.9). There were only three time points for which both sets of data were collected for mice immunised with DTPw, and mice immunised with saline did not develop serological responses to infection until day 14 after challenge, thus no correlations were calculable for these data.

In addition, calculations were performed only on samples for which serology and histopathology were taken from the same mouse. Therefore each data point on the graph represents the matched data for a different vaccine storage condition: one mouse per data point.

A strong negative correlation was observed between IgG FHA concentrations and lung histopathology mean scores ($r=-0.8936$, $p=0.041$). Positive correlations were observed between IgG PT or PRN concentrations and histopathology mean scores, but these analyses were not statistically significant.

### 7.3.5 Correlations between lung histopathology mean scores and mean lung homogenate culture results day 4 post challenge

Histopathological analysis and lung homogenisation on a single were performed separate sets of lungs in each experiment. Therefore, unmatched data were used to perform these correlations. Once again, there were only three time points for which both sets of data were collected for mice immunised with DTPw making correlations for this vaccine incalculable, and again each data point on the graph represents the matched data for a different vaccine storage condition: one mouse per data point.

Although strong positive correlations between mean lung histopathology scores and mean lung homogenate culture results were observed in mice immunised with DTPa or normal saline, only the latter correlation was statistically significant ($r=0.9648$, $p=0.035$: figures 7.10a and b).

## 7.4 Conclusions

Total lung histopathology scores were much lower in mice immunised with DTPa than with either DTPw or normal saline and did not did not differ significantly between vaccine storage groups. However, mice immunised with DTPw had similar scores to mice immunised with normal saline, and although there was a trend towards lower scores in mice immunised with DTPw stored at $-3^0$C or $-6^0$C for 24 hours, these changes did not reach statistical significance. Mice immunised with normal saline did demonstrate significant differences in total lung inflammation scores between experiments, although this was due to significantly lower lung histopathology scores in mice immunised with saline in one experiment. This could indicate some inherent variability in response of these mice to infection, or a problem with the individual experiment (in which commercial vaccines were stored at $-3^0$C for 14 days) as unexpected lung bacterial clearance results were also obtained from mice immunised with commercial vaccine in this experiment (sections 5.3.2.3 and 5.3.2.4).

Analyses of histopathology sub-component scores showed similar findings, with mice immunised with DTPa having lower scores in all four subcomponents than mice immunised with DTPw, and lower scores to all subcomponents except macrophage activation in comparison with mice immunised with normal saline. However, mice immunised with DTPw demonstrated higher chronic inflammatory and macrophage activation scores than those immunised with normal saline.

Analysis by vaccine storage group within each vaccine treatment group indicated significantly lower acute inflammation scores in mice immunised with DTPa stored at $-3^0$C or-$6^0$C for 14 days and $-6^0$C for 24 hours and in mice immunised with DTPw stored at $-6^0$C for 24 hours than mice immunised with correctly stored vaccine. Acute

inflammation scores were also the cause of the significant differences in results between experiments for saline–immunised mice.

Therefore the degree of acute inflammatory infiltrate appeared to be the most important factor affecting lung histopathology scores at day 4 post intranasal challenge. This may be have been affected by the weighting of this sub-component for which scores between 0-4 were possible, in comparison with respiratory epithelial dysplasia and macrophage activation scores for which scores between 0-2 were possible. In addition, it is clear that mice immunised with DTPa demonstrated significantly less lung inflammation on histopathological analysis than mice immunised with DTPw or with normal saline, and that mice immunised with DTPw demonstrated a degree of lung inflammation similar to that demonstrated by saline-immunised mice. Vaccine storage conditions did not appear to influence the degree of lung inflammation as demonstrated by histopathological analysis in DTPa and DTPw immunised mice.

Correlations between mean histopathology scores and pertussis serology were calculable only in mice immunised with DTPa. This was partly due to missing data in DTPw immunised mice caused by a cessation of CSL DTPw production in Australia during the course of these studies and also due to a lack of serological response in mice immunised with normal saline four days after intranasal challenge with *B. pertussis*. An extremely strong and significant negative correlation was demonstrated between IgG anti-FHA concentrations and mean histopathology. This may represent a serological correlate of protection in DTPa immunised mice using this experimental model, although , due to the large number of comparisons made, this result could also have occurred by chance. Examination of these correlations between days 0 and 4 post challenge in mice immunised with DTPa or DTPw and from day 14 post challenge in mice immunised with normal

saline may also be of use in the determination of serological correlates of protection in immunised and unimmunised mice.

Similarly, incomplete data were available for correlations between mean lung histopathology scores and lung homogenate colony counts. However, a strong, significant positive correlation was demonstrated between these two parameters in mice immunised with normal saline, showing that lung inflammation is most prevalent during active infection with *B. pertussis* in unimmunised mice. A moderately strong positive association was demonstrated between histopathology scores and lung clearance for DTPa. However, this was not statistically significant and was probably influenced by the one data point representing an experiment in which lung clearance in mice immunised with DTPa was less than that of other experiments (storage of vaccine at $-3^0$C for 14 days prior to immunisation). The use of larger numbers of mice and examination of such correlations at times other than day 4 after intranasal challenge may help delineate whether significant correlations could exist between these parameters in immunised mice.

The results of BAL cytospin cell counts were similar, as mice immunised with DTPa had significantly lower lymphocyte, macrophage and neutrophil counts than mice immunised with DTPw and significantly lower lymphocyte and neutrophil counts than mice immunised with normal saline. Mice immunised with DTPw demonstrated significantly higher lymphocyte and macrophage counts than mice immunised with normal saline. These results correspond with those from the analyses of histopathology subscores.

Cytospin preparations in mice immunised with DTPa demonstrated a predominance of macrophages, regardless of vaccine storage condition, whereas mice immunised with normal saline demonstrated a predominance of neutrophils in all except the final

experiment (experiment 5, in which vaccines were stored at $-6^0$C for 14 days prior to immunisation). This experiment was also associated with significant differences in histopathology scores in mice immunised with normal saline, and, once again, this was due to differences in the acute inflammation sub-score (i.e. the degree of neutrophil infiltration). Mice immunised with DTPw demonstrated high counts of all cell types, with a predominance of neutrophils, particularly after storage of vaccines at $-3^0$C for 24 hours. However, total and sub-component histopathology scores did not demonstrate significant differences between DTPw stored correctly or at $-3^0$C for 24 hours.

Overall, immunisation with DTPa resulted in less lung inflammation after intranasal challenge with *B. pertussis*.

No previous studies have investigated the relationship between vaccine storage and the pattern and degree of lung inflammation after pertussis infection. However, bronchopneumonic change has been described previously in the literature in studies investigating the effect of intranasal pertussis infection in unimmunised mice (155, 160, 257). The study of Khelef et al also assessed the degree of lung inflammation on histopathology and counted the numbers of inflammatory cells/ml of BAL fluid. Khelef et al demonstrated a constitutive, low macrophage count in the BAL fluid of unimmunised Swiss outbred mice given normal saline intranasally with *B. pertussis* Tohama (the same strain used as in the present experiments). This is similar to the findings demonstrated in mice immunised with DTPa in the present study, and is supportive evidence for the efficacy of DTPa in the prevention of lung inflammation following intranasal challenge.

In the study of Khelef et al, outbred mice demonstrated high numbers of all cell types (lymphocytes, macrophages and neutrophils) in BAL fluid with higher neutrophil counts in

the first three weeks of infection, followed by a gradual replacement with macrophages and lymphocytes (257). Lung parenchymal cellular infiltrates reflected the chronological changes in BAL fluid cell composition. In addition, the general pattern of lung inflammation altered with the progression of infection. In the first week inflammation was restricted to the perivascular and peribronchiolar areas, with extension in the second and subsequent weeks to involve alveoli and subpleural spaces with occasional abscess formation. Lung histopathology did not return to normal until 3 months after infection.

Although histopathological changes were assessed at 4 days post intranasal challenge only in the study reported here, Balb/c mice in the present study immunised with normal saline and, to a certain extent, mice immunised with DTPw demonstrated similar lung histopathological changes and BAL fluid cell composition as did the Swiss outbred mice in the study of Khelef et al (257). However, lymphocytic infiltration of lung parenchyma was also present four days after intranasal challenge in Balb/c mice. The intranasal challenge dose of *B. pertussis* Tohama was $10^7$ CFU/ml in the present study and $10^5$ CFU/ml in the study of Khelef et al, which may have caused the differences in early lung inflammatory cell infiltrate.

Investigation of IL-2 and IL-4 concentrations in BAL fluid in the present study demonstrated absent IL-2 concentrations and relatively higher IL-4 concentrations in mice immunised with DTPa or normal saline regardless of vaccine storage condition, indicative of a predominant Th-2 immune response. Mice immunised with DTPw, however, demonstrated relatively high concentrations of both cytokines in BAL fluid, again regardless of vaccine storage condition. Comparison of IL-4:IL-2 ratios demonstrated higher ratios in DTPa and saline immunised mice, confirming the predominant Th-2

responses in these mice and suggesting that Th-1 responses predominate in DTPw-immunised mice.

Previous studies investigating cytokine profiles in immunised and unimmunised mice after respiratory challenge with *B. pertussis* have used spleen cell supernatants rather than BAL fluid (184, 185). These studies demonstrated that mice immunised with a whole cell vaccine and unimmunised mice produced moderate to large amounts of IL-2 and moderate amounts of IFN-$\gamma$ and low responses to IL-4 and IL-5 in spleen cell supernatants after aerosol infection. These results differed from the present study in which saline immunised mice demonstrated a cytokine profile more similar to that of DTPa- rather than DTPw-immunised mice. Mice immunised with DTPa produced little or no IL-2 or IFN-$\gamma$, low levels of IL-4 and moderate to high levels of IL-5 in spleen cell culture supernatants (184, 185). However, another study by the same group of investigators demonstrated that the cytokine profile of DTPa-immunised mice was influenced by the route of immunisation and the genetic background of the inbred mouse strain (186). Subcutaneous immunisation was associated with a predominant Th-2 cytokine profile and slightly delayed lung clearance of organisms, whereas intraperitoneal immunisation was associated with predominant Th-1 cytokine profile and faster lung clearance of organisms. Similarly, inbred mouse strains producing higher IFN$\gamma$ levels after intracerebral challenge were more likely to survive. The results of the present study differ from these findings, with slightly better lung clearance of organisms after intranasal challenge in DTPa-immunised mice.

A more recent study investigating cytokine mRNA profiles in the stimulated peripheral blood mononuclear cell culture supernatants of human infants one month after primary immunisation with DTPa or DTPw demonstrated constitutive production of IFN-$\gamma$ in all vaccine recipients (258). Infants immunised with whole cell vaccine expressed increased

IFN-? and IL-2 mRNA with no expression of either IL-4 or IL-5 mRNA. Two acellular preparations were investigated, both of which contained PT, FHA and PRN. However, one acellular vaccine contained significantly higher concentrations of all three antigens and produced higher levels of IL-4 and IL-5 mRNA in cell culture supernatants after immunisation. The lower dose acellular vaccine produced no IL-4 mRNA after immunisation.

Therefore, cytokine concentrations in response to pertussis infection and immunisation are clearly complex and depend on the genetic background of the mouse, the particular vaccine formulation and dose, the route of immunisation, the route of challenge and the method of cytokine analysis.

In summary, the present studies of pertussis vaccine protective efficacy have demonstrated that adverse vaccine storage does not, in general, diminish the rate of lung clearance of organisms, antibody responses to pertussis antigens, or the degree of lung inflammation produced by infection. Immunisation of mice with DTPa produces higher antibody concentrations to pertussis antigens after immunisation regardless of vaccine storage condition and better booster antibody responses after infection. Higher FHA antibody concentrations appear to correlate with better lung clearance of organisms in mice immunised with DTPw and with a lesser degree of lung inflammation in mice immunised with DTPa. The degree of lung inflammation is positively correlated with the numbers of of *B. pertussis* organisms in the lungs of mice immunised with normal saline.

Immunisation of mice with DTPa is associated with faster lung clearance of organisms, much lower levels of lung inflammation, BAL inflammatory cell infiltrate and BAL

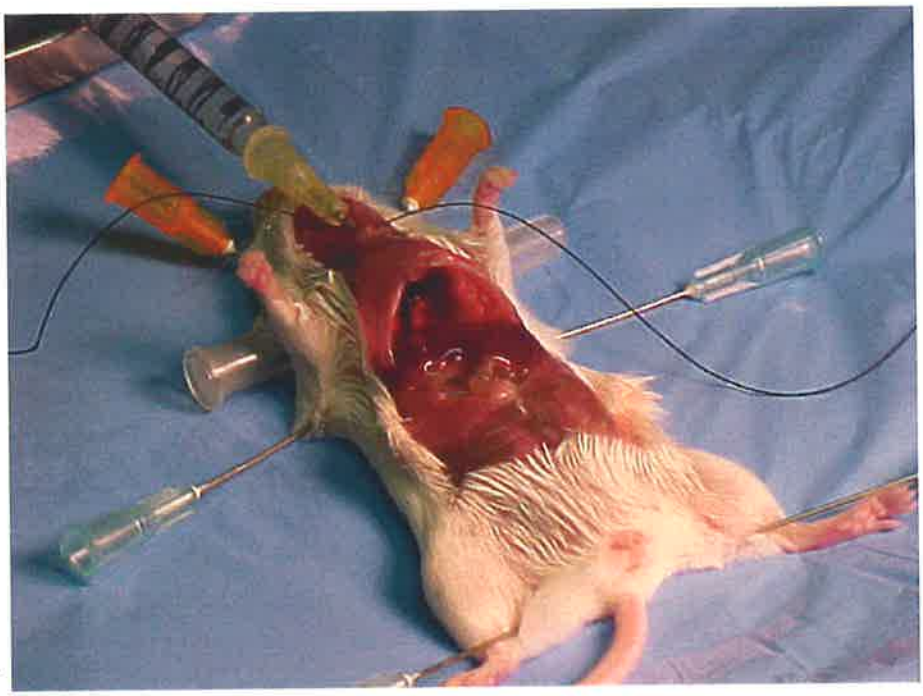
cytokine production four days after intranasal infection than DTPw also regardless of vaccine storage condition.

Future studies should therefore concentrate on the period between intranasal challenge and four days post intranasal challenge to delineate further the differences in lung inflammation and the kinetics of lung clearance of organisms of correctly and adversely stored vaccines and to determine whether mice immunised with DTPa have less lung inflammation *de novo* in the early stages of infection. This, combined with studies of the long term protective efficacy of adversely stored vaccines will enable better decisions to be made regarding the discarding of vaccines which have not been stored according to the manufacturer's instructions.

**Table 7.1 Histopathology individual component mean scores by vaccine and storage condition**

| | Chronic inflammation | Macrophage activation | Acute inflammation | Respiratory epithelial dysplasia | Total Mean Score |
|---|---|---|---|---|---|
| **DTPa** | | | | | |
| 2-8$^0$C | 1.3 | 1.0 | 0.7 | 0.2 | 2.2 |
| -3$^0$C/24h | 1.3 | 0.7 | 0.2 | 0.0 | 2.5 |
| -6$^0$C/24h | 1.5 | 0.8 | 0.0 | 0.0 | 2.7 |
| -3$^0$C14d | 1.3 | 1.0 | 0.0 | 0.3 | 2.3 |
| -6$^0$C 14d | 1.3 | 1.0 | 0.0 | 0.0 | 2.3 |
| | | | | | |
| **DTPw** | | | | | |
| 2-8$^0$C | 3.8 | 1.2 | 3.5 | 1.0 | 9.5 |
| -3$^0$C/24h | 3.5 | 1.3 | 2.5 | 0.7 | 8 |
| -6$^0$C/24h | 3.0 | 1.2 | 1.8 | 0.3 | 6.3 |
| -3$^0$C14d | no data | no data | no data | no data | no data |
| -6$^0$C 14d | no data | no data | no data | no data | no data |
| | | | | | |
| **Saline** | | | | | |
| expt 1 | no data | no data | no data | no data | no data |
| expt 2 | 3.2 | 1.0 | 3.7 | 1.0 | 8.8 |
| expt 3 | 2.8 | 1.0 | 3.0 | 0.8 | 9.3 |
| expt 4 | 3.2 | 1.0 | 4.0 | 1.2 | 7.7 |
| expt 5 | 2.0 | 0.8 | 0.8 | 0.5 | 4.2 |

**Figures 7.1a and 7.1b Bronchoalveolar lavage before and after lung inflation**



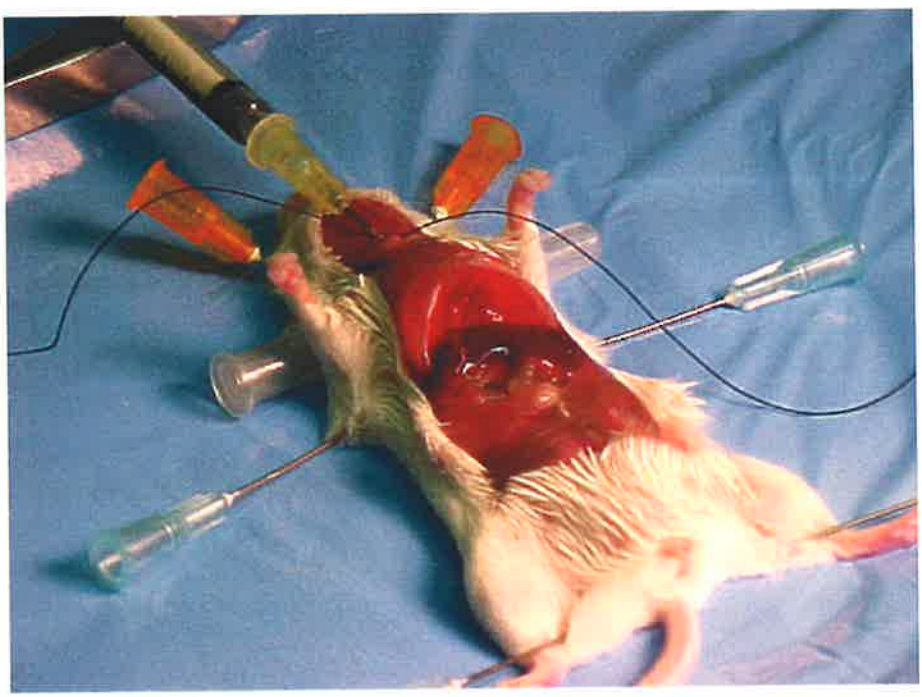
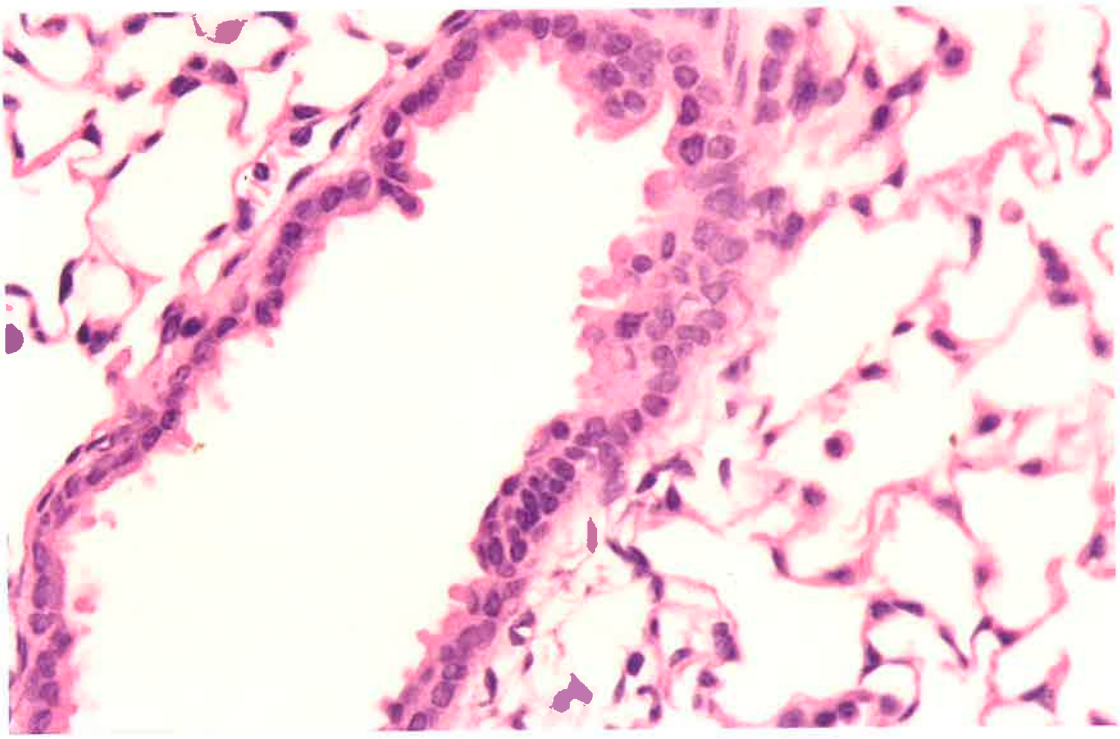
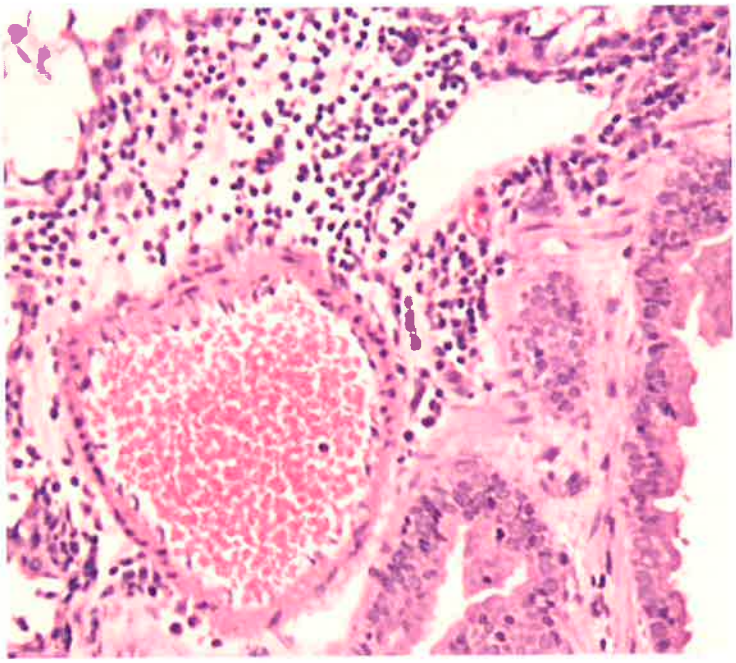


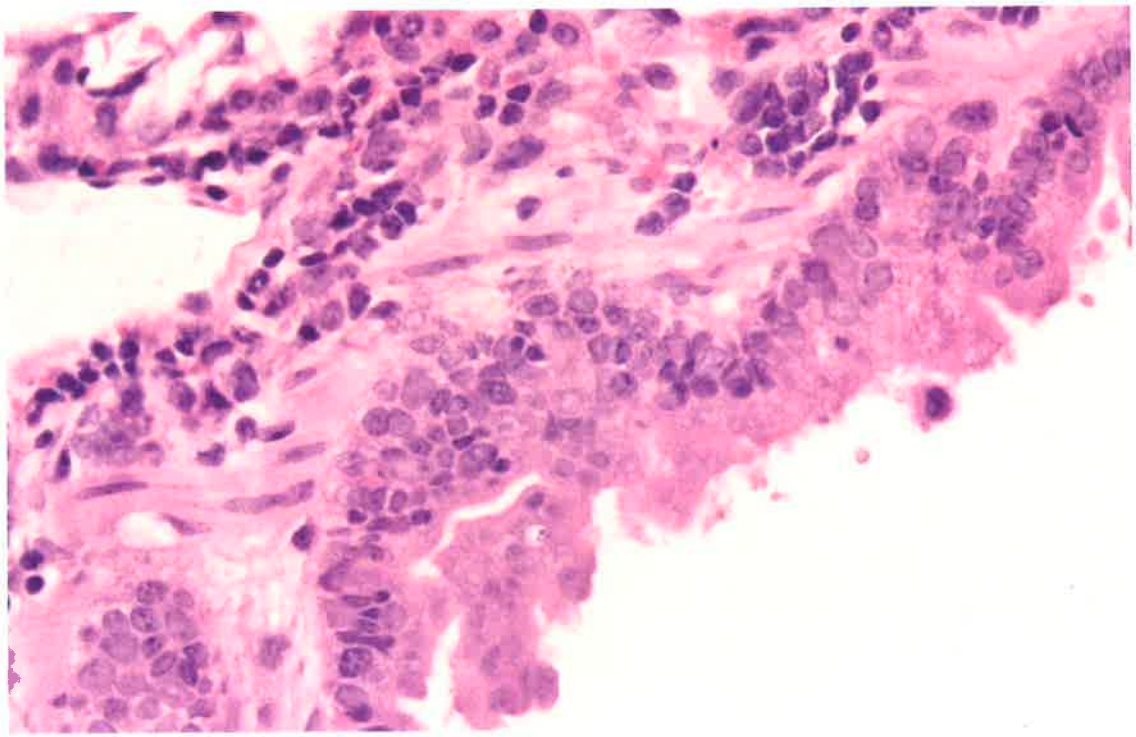
**Figure 7.2 Normal mouse lung**



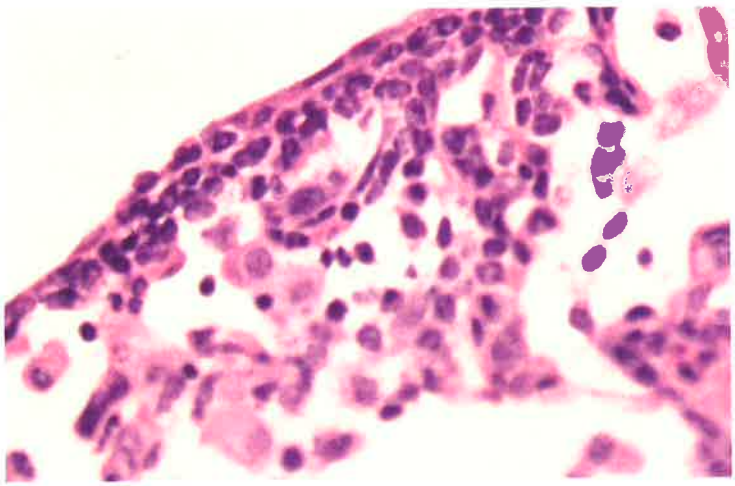
**Figures 7.3a and 7.3b** **Histopathology four days after intranasal challenge demonstrating perivascular lymphocytic infiltration and respiratory epithelial dysplasia**

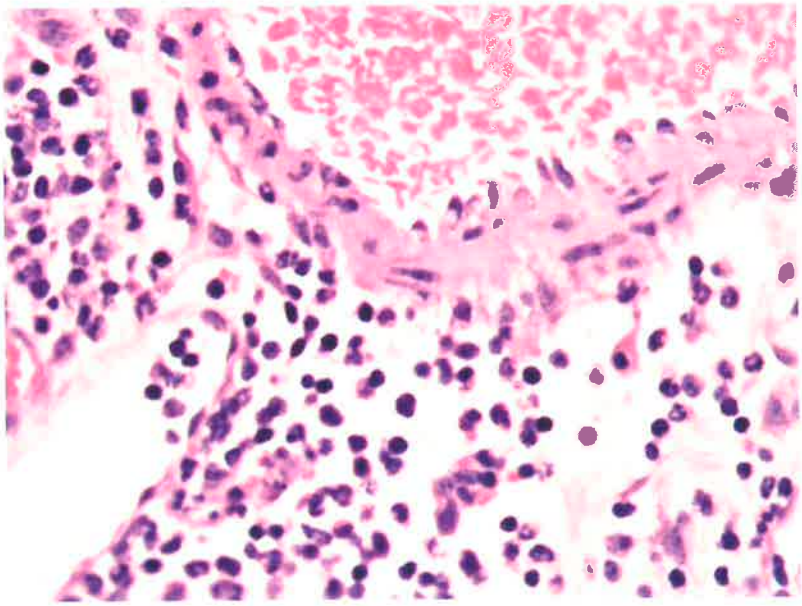
**(low and high power magnification)**



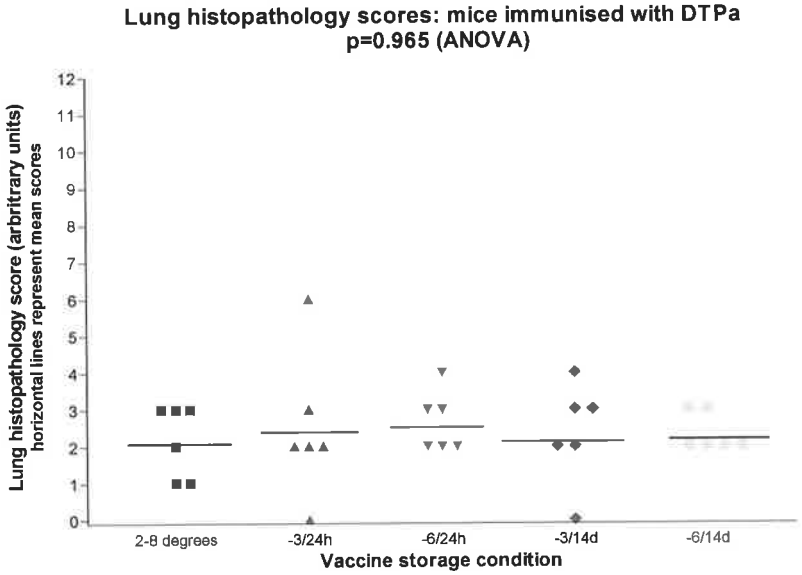


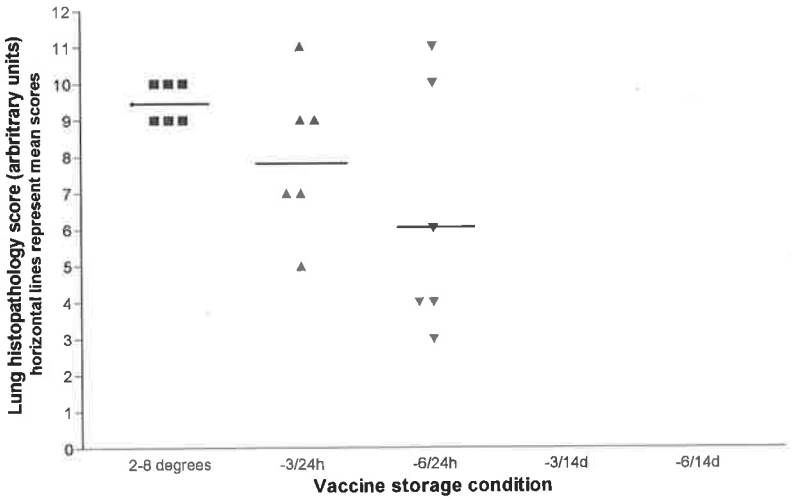
**Figures 7.4a and 7.4b** **Subpleural inflammation and perivascular abscess formation**

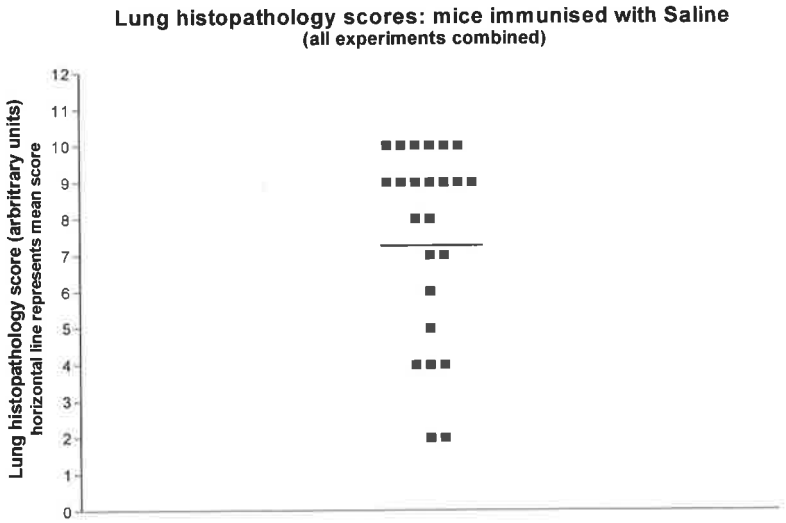




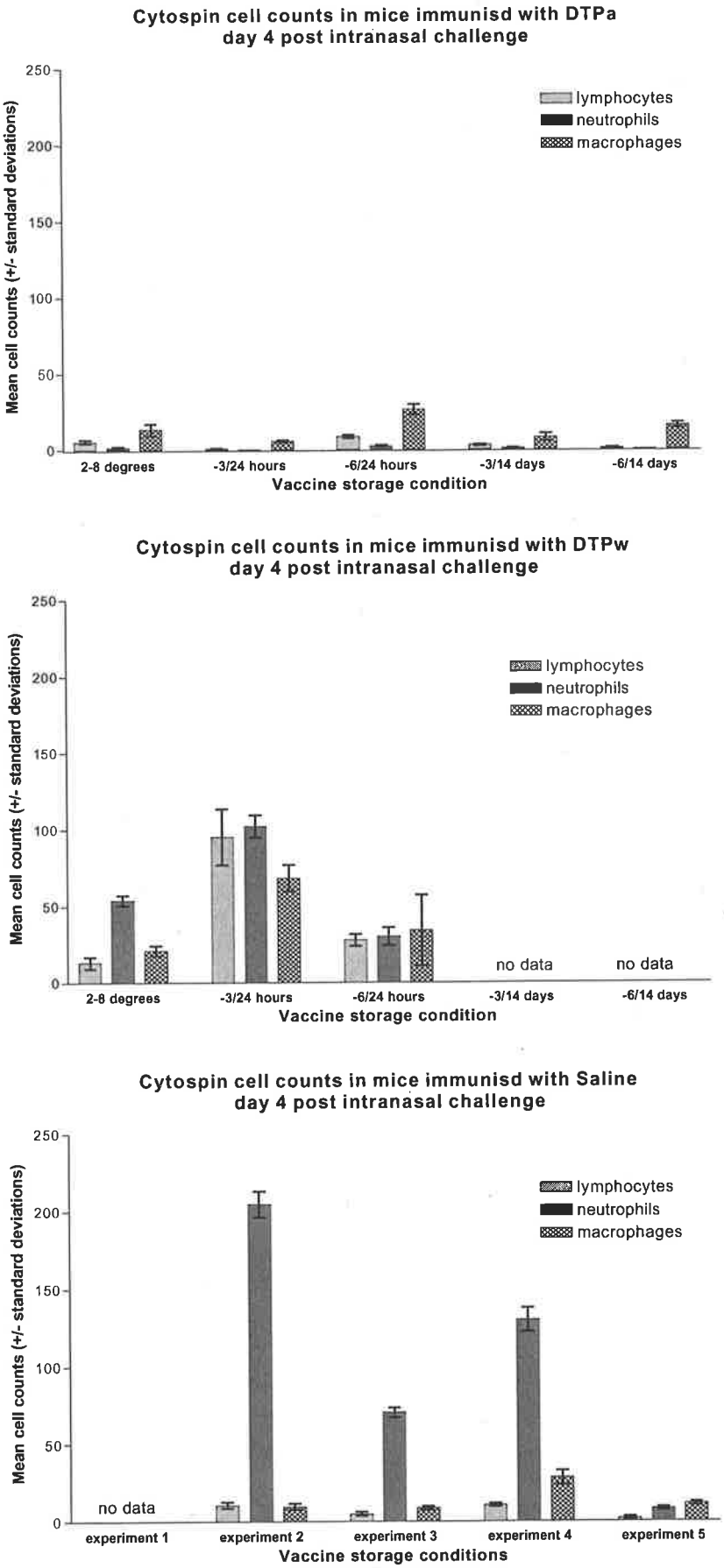
## Figures 7.5a, 7.5b, 7.5c Lung histopathology scores



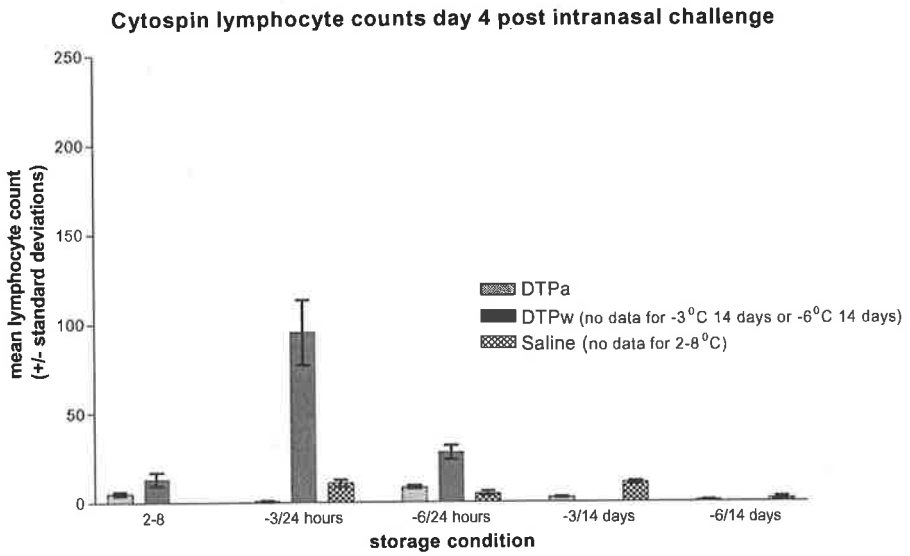



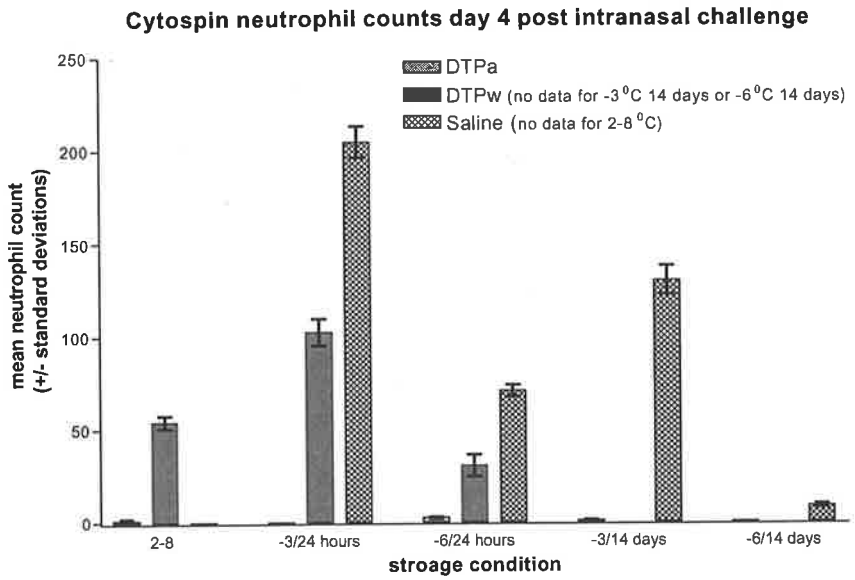



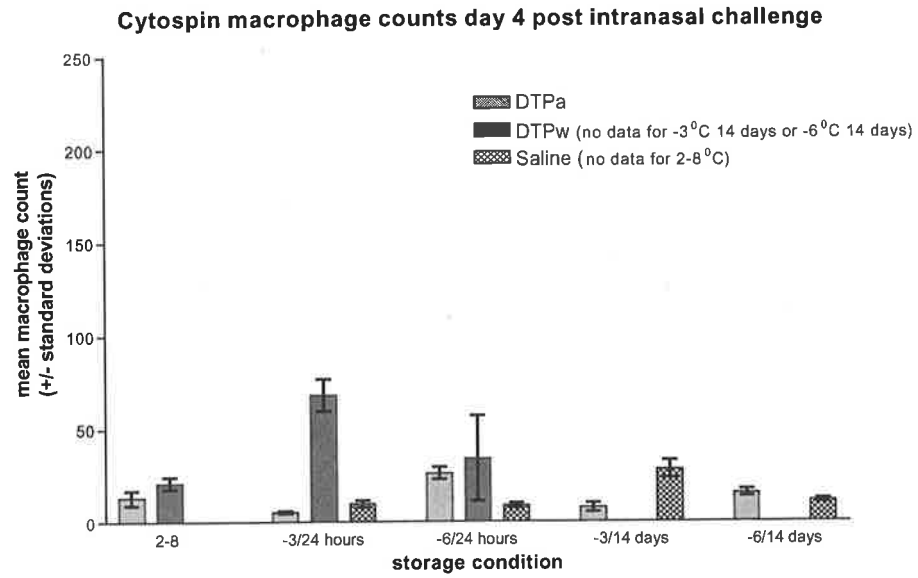
## Figures 7.6a, 7.6b, 7.6c Cytospin cell counts



## Figures 7.7a, 7.7b, 7.7c Cytospin cell counts: individual cell types



## Figures 7.8a, 7.8b, 7.8c BAL cytokine concentrations

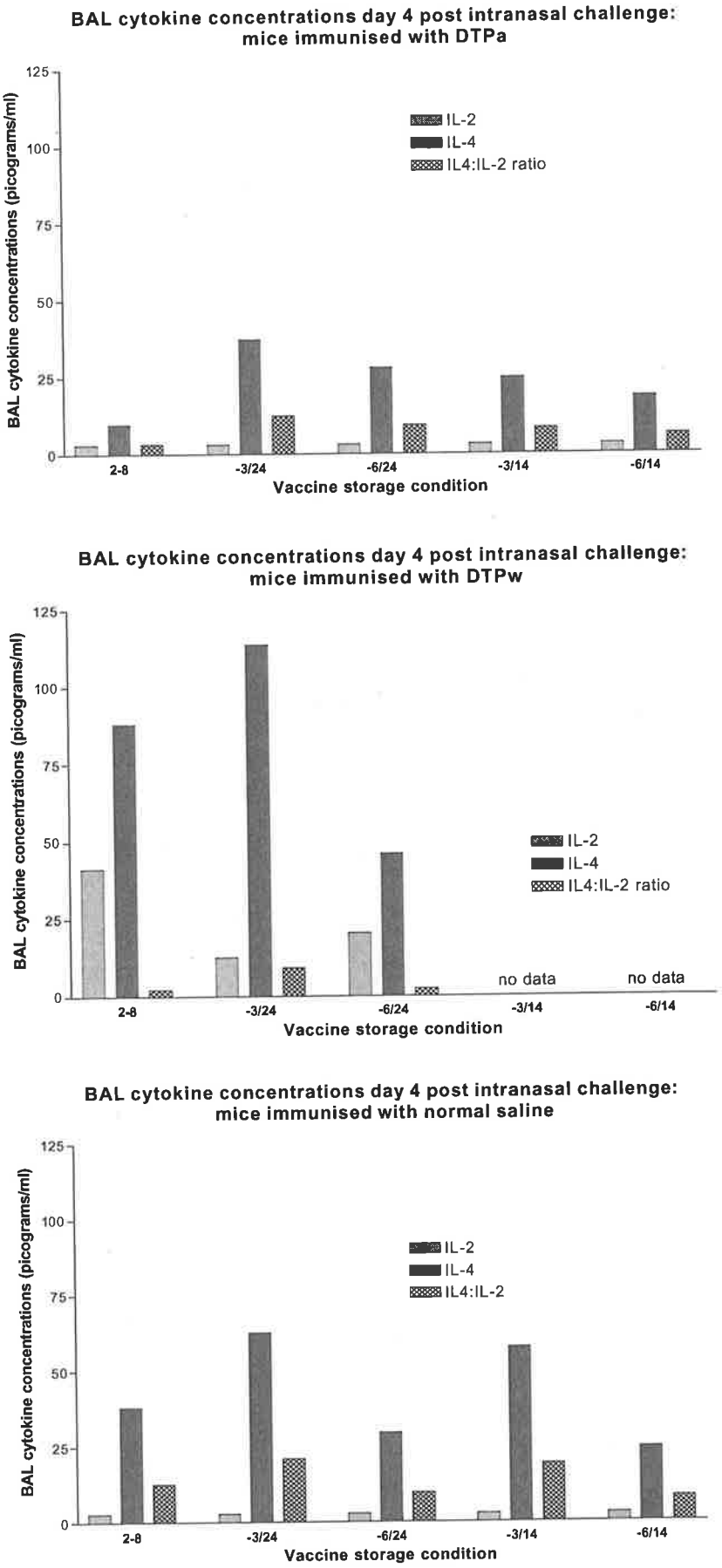
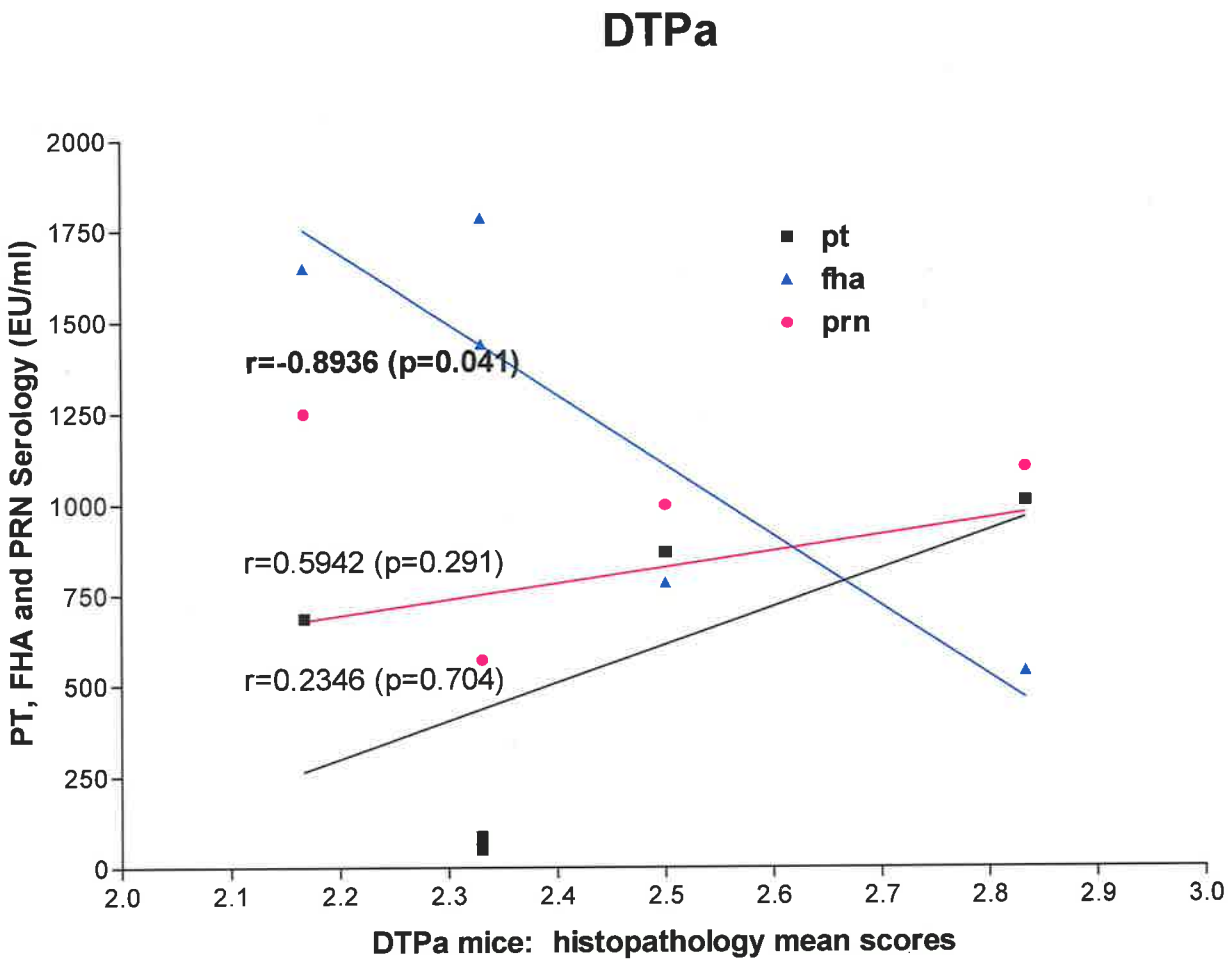
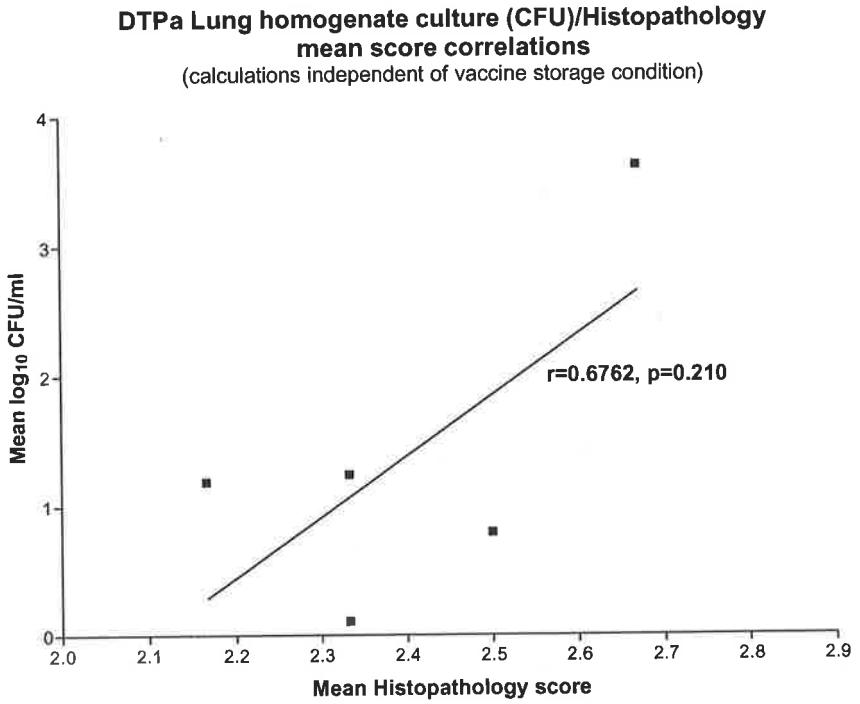
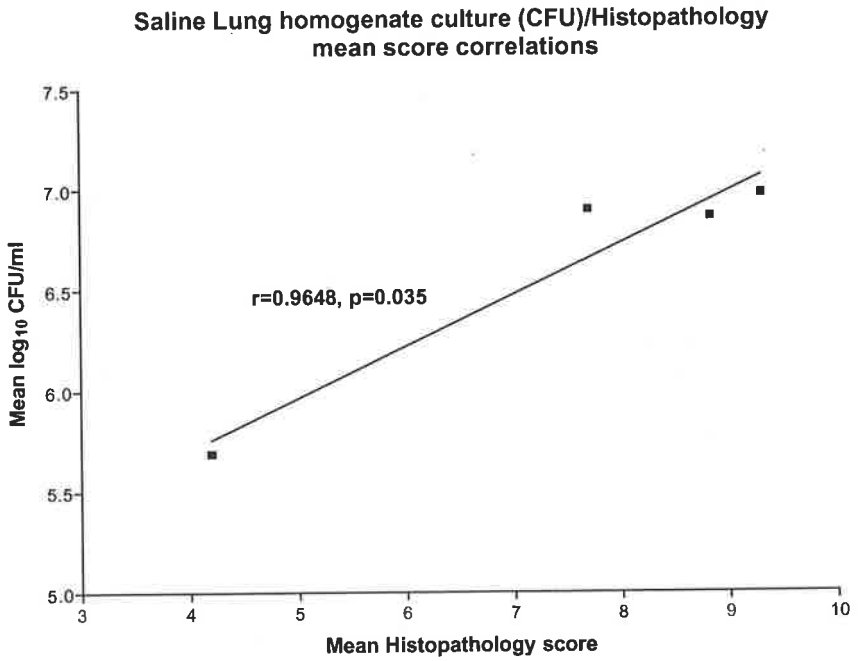


**Figure 7.9 Correlations between DTPa serology and histopathology mean scores**



**Figures 7.10a and b Correlations between lung homogenate cultures (mean CFU/ml) and mean histopathology scores by vaccine**



# Chapter 8 Antibody responses to routine immunisations in premature and term infants

## 8.1 Introduction

Two studies were undertaken to investigate antibody responses to routine childhood immunisations in premature and term infants. The first was a longitudinal comparative cohort study in which premature and term infants were enrolled at 2 months of age and followed until 19 months of age. Blood samples were collected at 2, 7, 18 and 19 months of age for comparison of antibody responses to antigenic components of the triple antigen and *Haemophilus influenzae* type b vaccines. A second, separate study was undertaken to compare the antibody responses to vaccines in ex-premature and term infants immunised with an acellular triple antigen vaccine (Infanrix® DTPa), or the CSL whole cell triple antigen vaccine (DTPw) for the primary series of immunisations (at 2, 4 and 6 months of age) and with Infanrix® DTPa for the 18-month booster immunisation. Infants immunised with DTPw in the primary series had been enrolled in another vaccine trial conducted by the Vaccine Research Unit in the University of Adelaide Department of Paediatrics at the Women's and Children's Hospital, South Australia and had serum samples collected and 2, 8, 18 and 19 months of age•. Newly recruited ex-premature infants in the second study had blood collected for seroanalysis before and one month after the 18-month booster immunisations and received immunisations as outlined in the 1997 NH&MRC Australian Immunisation Handbook (6th edition, (117) and in table 8.1. Comparisons between term and premature infants antibody responses is the second study were made in both a longitudinal and cross sectional manner. All studies were approved by the Women's and

• The parents/guardians of these infants had given permission for collected serum samples to be used in other studies approved by the Women's and Children's Hospital Research Ethics Committee, provided Research Ethics Committee approval of the supplementary study was obtained.

Children's Hospital Research Ethics Committee. Details of numbers of infants in individual cohorts and timing of blood collection for each cohort are detailed in table 8.2.

## 8.2 Methods

### 8.2.1 Recruitment

#### 8.2.1.1 Term infants, first study

Healthy term infants (gestation ≥ 37 weeks: cohort 1b) born at the Women's and Children's Hospital delivered between March and June 1999 were recruited from the postnatal wards of the Women's and Children's Hospital. Mothers who had delivered in the preceding 24 to 72 hours were invited to enrol their infants in the study after discussion with ward nursing staff to determine that mothers and their infants were well enough to be approached. Parent information sheets were distributed at the time of this first contact. These mothers were subsequently contacted by telephone when their infants were 4-6 weeks of age to arrange the first immunisation at eight weeks of age. Mothers who were discharged from hospital before the study investigator could interview them were initially contacted by mail and subsequently by telephone when their infants were 6 weeks of age. If these mothers agreed to participate in the study, an appointment was arranged at the time of telephone contact. Written, informed consent was obtained at the time of the first appointment.

#### 8.2.1.2 Premature infants

##### *8.2.1.2.1 First study*

Premature infants (gestation <37 weeks) born between March and July 1999 at the Women's and Children's Hospital (WCH) and admitted to the neonatal unit (cohort 1a) were recruited from one month prior to their first immunisations. Slower recruitment of premature infants necessitated an extra month of recruitment. Once a week during the recruitment period, a ward round was conducted with the neonatologist involved in the

study to determine which infants met the selection criteria for recruitment (section 8.2.2). Neonatal research nurses then determined an appropriate time for the study investigator to interview the parents of these infants in the neonatal unit. Informed consent was obtained, usually at a subsequent interview, after the infants' parents/guardians had read the parent information sheet and agreed to participate in the study.

#### *8.2.1.2.2 Second study*

Ex-premature infants approaching 18 months of chronological age between March 1999 and October 2000 were identified from a database of admissions to the neonatal unit at the WCH (cohort 2). The parents/guardians of a subset of infants selected by the study neonatologist who fulfilled recruitment criteria (refer to table 8.3) were contacted by post and then by telephone to determine their interest in the study. Written, informed consent was obtained at the first appointment.

### 8.2.2 Recruitment criteria

Table 8.3 outlines the selection criteria for premature and term infants for recruitment into the studies. Both socio-demographic and medical factors were taken into account during the selection process. Only parents who could understand English were approached. In addition, it was a requirement that parents could be contacted by telephone and lived at a fixed address to ensure that immunisation and blood sample collection occurred at appropriate times. Infants with a known chromosomal abnormality, a major congenital anomaly, or a serious chronic illness related to extreme prematurity were excluded from the study. Infants with bronchopulmonary dysplasia, a chronic lung disease related to extreme prematurity and prolonged mechanical ventilation, were not excluded from the study due to the relative frequency with which this condition occurs. Ante- or postnatal corticosteroid administration was not an exclusion criterion. However, the first

immunisation was not administered until 14 days after cessation of postnatal steroid therapy to minimise the effect on antibody responses in these infants (227). Transfusion of blood or blood products did not exclude infants from participation unless intravenous immunoglobulin had been administered in the pre-immunisation period.

### 8.2.3 Informed consent

Written, informed consent from each infant's parent(s) or guardian(s) was a mandatory requirement prior to entry into the study and was obtained by the principal investigator. Please refer to appendix 1 to view the consent form.

### 8.2.4 Appointment schedule and data collection

Tables 8.4 and 8.5 outline the appointment schedules for each study. Each infant had data recorded on a study report form at each visit (appendix 2). This included the results of physical examination by the principal investigator, the infant's health status between appointments, whether an adverse reaction to immunisation had occurred and any medication prescribed in the intervening period. Data were also collected from the infant's casenotes and included the infant's date of birth, gender, birth APGAR scores and percentiles, the number of blood transfusions received, the age at the first blood transfusion, the duration of hospital admission and the amount of time spent in the neonatal unit, and the duration of mechanical ventilation and oxygen therapy, if required. The mother's gravidity, parity, level of education, country of birth and residential postcode were also recorded (appendix 2).

### 8.2.5 Data management

Data items recorded on the study report forms were transcribed into a database created using Microsoft Access '97®. These items were cross-referenced against the hospital casenotes at the time of data entry to ensure accuracy. Different tables in the database were linked using the participant's hospital unit record number. Independent validation of all data entry was conducted by a laboratory technical officer. Access to the computer database was protected by a password known only by the principal investigator and validating laboratory technical officer.

### 8.2.6 Blood sampling procedures

Blood samples were collected from each infant immediately prior to and one month after completion of the primary schedule (the first three immunisations at 2, 4 and 6 months of age) in the first study and immediately prior to and one month after the 18-month booster immunisations in both studies. A minimum of 1.5 ml of blood was required to provide enough serum for analysis of antibody response to all bacterial vaccine antigens by enzyme immunoassay (EIA). Blood was collected by venepuncture using a 25-gauge needle by the principal investigator or by heel or fingerprick if venepuncture was unsuccessful. To prevent pain associated with venepuncture, infants over 6 months of age had EMLA® cream (a eutectic mixture of local anaesthetics containing lignocaine and prilocaine in 5% concentrations, Astra Pharmaceuticals, Australia) applied to the skin of the dorsum of the hand or the antecubital fossa one hour before the appointment. Systemic absorption of topical anaesthetic may occur if used in infants less than six months of age.

### 8.2.7 Separation of serum, labelling and storage of samples

Blood samples were left to clot at room temperature for one hour before being spun at 3000 rpm for 10 minutes. Serum was then stored at $-20^0$C for 48 hours and then at $-70^0$C until assays were performed. Sample tubes were labelled with the specimen number, the infant's name, study enrolment number and the date of sample collection prior to storage.

### 8.2.8 Immunisation procedures and vaccine details

#### 8.2.8.1 Australian Immunisation Schedule

All newly recruited infants received immunisations as outlined by the 1997 NH&MRC Australian Immunisation Handbook 6th Edition (table 8.1). The Handbook recommends three consecutive injections of DTPa and Hib immunisations with oral polio vaccine administered at eight-week intervals from eight weeks of age. This is followed by an injection of MMR vaccine at 12 months of age and booster doses of DTP and Hib immunisations at 18 months of age. All injectable vaccines were administered by the deep subcutaneous or intramuscular route.

Immunisations were administered by the principal investigator to infants in rooms especially designated for the University of Adelaide Department of Paediatrics Vaccine Research Unit at the Women's and Children's Hospital. Premature infants who had not been discharged from hospital at the time of their first immunisation were immunised in the neonatal unit. In accordance with hospital policy, the first dose of oral polio vaccine was not administered to inpatient premature infants until the day of discharge to prevent possible infection of other neonates with vaccine strain virus. Where possible, immunisation of study participants occurred at the nominated chronological age (i.e.

uncorrected for gestation) as stipulated by the NH&MRC immunisation guidelines. For a description of the individual vaccines administered to study participants refer to table 8.6.

#### *8.2.8.2.1 Management of infants unresponsive to the Hib vaccine after the primary course*

As in previous studies, infants not seroconverting to the Hib vaccine after the primary course of immunisation (as determined by antibody concentration of <0.15µg/ml at age 7m determined by EIA) were offered an extra dose of Hib vaccine at 12 months, at the same time as the Measles-Mumps-Rubella vaccine, but in a separate limb. With parental consent, extra blood samples were obtained immediately prior to and one month after this booster dose, to determine serological response.

#### *8.2.8.2.2 Management of adverse events following immunisation*

An adverse event is defined as any serious, uncommon or unexpected event following immunisation (123). This includes events such as anaphylaxis, abscess formation at the injection site, hypotonic-hyporesponsive episodes, sepsis or severe local reaction (for example). All immunisation rooms were equipped with adrenaline, suction and basic resuscitation equipment (suction and oxygen outlets, suction and, oxygen tubing, bag and mask apparatus) to manage any acute severe reactions which may have occurred. Parents were advised during the consent procedure that in the event of any adverse event following immunisation, their infant would be referred to the WCH Immunisation Adverse Events Clinic and a formal report would be made to the South Australian Immunisation Coordination Unit prior to subsequent immunisations.

### 8.2.9 Sample size and power calculations

Although serological correlates of protection against infection have been determined for *C. diphtheriae, Cl. tetani* and *H. influenzae* type b, a similar correlate has not yet been determined for *B. pertussis*. In addition, available data regarding premature and term infant responses to acellular pertussis vaccines at the commencement of the study were from a study using a combined DTPa-HBV vaccine administered to infants at 3, 5 and 11 months of age (244). The NH&MRC guidelines for routine childhood immunisation in Australia recommend DTPa given separately at 2, 4, and 6 months of age for the primary series. Therefore, the estimation of the required sample size to detect a significant difference in IgG antibodies to the DTPa and Hib vaccine antigens was based on the hypothesis that there would be a 10% difference in antibody response between premature and term infants to all vaccine antigens. To achieve a power of 80% using this hypothesis, the minimum number of infants required in each group was determined to be 32. Because of anticipated loss to follow-up of study participants by 18 months of age, a target of 50 infants in each group was set.

### 8.2.10 Materials and Methods of seroanalysis

Immunoglobulin G (IgG) concentrations directed against all antigens in the acellular triple antigen vaccine (Infanrix®) and the capsular polysaccharide in the Hib vaccine (HibTiter®) used in this study were measured using sandwich EIAs specific for IgG.

#### 8.2.10.1 Pertussis assays

In-house EIAs were developed to measure IgG antibodies to three pertussis antigens: pertussis toxin (PT), filamentous haemagglutinin (FHA) and pertactin (PRN). from methods supplied Mr A Lawrence, Senior Research Scientist, Department of

Microbiology, Women's and Children's Hospital, South Australia. A summary of these assays is presented in table 8.7.

The plates used for these assays were either Nunc Maxisorp 96-well microtitre plates (PT and PRN assays) or Nunc Polysorp 16-well strips (FHA). Plates were coated at 0.5µg/ml with purified PT and FHA (List Biologicals catalogue numbers 181 and 170 respectively) or at 1.0µg/ml purified PRN (supplied by SmithKline Beecham Biologicals, Rixensart, Belgium) diluted in sterile carbonate/bicarbonate buffer pH 9.6, 100µl per well. Plates were incubated either for 60 minutes (PT and FHA) or 90 minutes (PRN) at $37^0$C in a humid chamber and then washed four times with sterile phosphate-buffered saline pH 7.2-0.5% tween 20 (PBST) using a NUNC® immunowash-12 manual plate washer.

Plates were then blocked with 100µl per well of a 1% solution of non-fat milk (NFM)-PBS and were incubated for 60 minutes at $37^0$C in a humid chamber and washed four times as before. Patient sera and standards were diluted in 1% NFM-PBST. One hundred microlitre aliquots of each serum sample were then placed in wells in triplicate at the appropriate dilutions. Standard curves were created using the appropriate serum plated in duplicate in eight doubling dilutions in the first two columns on each plate (see section 8.2.10.1.1). High and low quality controls (QCs) were included at appropriate dilutions on each plate. Three serum blanks were included on each plate to measure background assay activity. Serum samples were incubated at $37^0$C for 60 minutes in a humid chamber for all three assays.

The plates were then washed five times as previously and 100µl of a 1:1000 concentration of Silenus® horseradish peroxidase conjugated sheep anti-human IgG (catalogue number 982033020) was added to each well. The plates were incubated at $37^0$C for 45 minutes

(PT assay) or 30 minutes (FHA and PRN assays) in a humid chamber. After washing five times as before, 100µl of 2.2.-azino-di-3-ethylbenzthiazoline sulphonate (ABTS) and 33% w/v hydrogen peroxide ($H_2O_2$) was added to all wells.

Quantitation of the antibody-antigen reaction was by the development of colour read on a Dynex Opsys MR® plate reader at dual wavelengths of 410 and 540nm. Plates were read when the most concentrated dilution of reference serum achieved an optical density of 2.0 units. The development time for all assays was approximately 10-20 minutes.

#### *8.2.10.1.1 Reference sera*

The reference serum used in the PT assay was the USFDA lot 3 pertussis reference serum and that for the PRN assay the FDA lot 4 pertussis reference serum. The reference serum for the FHA assay comprised serum samples from eight study participants collected at their 19-month visit (pooled infant serum). This was calibrated against the USFDA lot 3 pertussis reference serum to determine the FHA antibody concentration.

#### *8.2.10.1.2 Quality Controls (QCs)*

The USFDA lot 3 was also used as the high QC in the FHA assay. The pooled infant serum (referred to in section 8.2.10.1.1) was the high QC in the PT and PRN assays. The low QC in all three assays was a pooled adult serum created from the serum of five adult volunteers working in the University of Adelaide Department of Paediatrics. In addition, the NIBSC *B. pertussis* human antiserum 89/530 was used as a second external QC for each assay and was included on each plate.

This NIBSC *B pertussis* human antiserum does not have assigned values for the antibody concentrations against different pertussis antigens, but is currently being calibrated against the USFDA lots 3 and 4 human anti-pertussis reference sera (Dr D Sesardic, NIBSC, personal communication, 2000). Therefore, both the internal reference sera and quality controls were calibrated against the USFDA lots 3 and 4. Results for all samples were expressed as the concentration of antibodies in ELISA Units (EU)/ml of serum.

#### *8.2.10.1.3 Standardisation of the immunoassays*

An immunoassay data management programme, Multicalc, (Pharmacia: Uppsala, Sweden) was used to standardise the immunoassays and to quantitate antibody concentrations of the study participants' samples. This programme analysed the differences between the means and the variances of the real and the determined values. The comparison of these results was then viewed through a precision profile, which was of high precision if there was no bias from the true value among repeated observations and if random errors were small. Only samples for which the deviation of the true value from the expected value had a coefficient of variation of less than 18% (the limit set by the computer programme) were included in the analyses.

All assays were developed such that each dilution of the standard curve had two replicates from which the mean values were used to construct the curve. To fit the curve, a method of least squares was used. Replicates lying outside a specified range were rejected and the curve was then refitted.

Each quality control sample was assayed several times by the Mulitcalc® programme to determine a target value for each of the controls. Every time an assay was performed, the measured response of the quality controls was accepted only if it was with in +/- 2 standard

deviations of the determined target value. Hence, inter- and intra-assay variation could be assessed by calculation of the coefficient of variation of the results of the quality controls.

#### 8.2.10.2 Anti-Diphtheria and anti-Tetanus toxoid IgG EIAs

In-house EIAs were developed to measure antibodies to diphtheria and tetanus toxoids in patient sera from methods provided by Dr H. MacDonald and Mr A. Lawrence in the Department of Microbiology and Infectious Diseases and the WCH, South Australia (table 8.8).

NUNC Polysorp strips were coated with 100µl/well of a 1:1000 concentration of CSL Tetanus toxoid fractionated concentrate (2155 LF/ml, batch # 552602501) or CSL Diphtheria toxoid fractionated concentrate (3600 LF/ml, batch # 082033301) and incubated for 60 minutes at $37^0$C in a humid chamber. After washing four times with a NUNC immunowash-12 manual plate washer using sterile PBS (pH 7.2) Tween (0.5%) {PBST}, 100µl aliquots of participants' serum samples, quality controls and dilutions of the appropriate reference sera were added and incubated for 45 minutes at $37^0$C in a humid chamber.

For both assays the internal reference serum was the pooled adult serum collected from five volunteers working in the laboratories at the WCH. This was calibrated against the NIBSC Diphtheria Antitoxin, human (cat # 91/534) or the NIBSC Tetanus antitoxin, human (cat # 76/589) and plated in duplicate in serial doubling dilutions from an initial 1:50 concentration in the first two columns of each plate.

A pooled human serum, comprising samples from 5 infants aged 9-18 months who had participated in another vaccine trial conducted by the University of Adelaide Department

of Paediatrics Vaccine Research Group was used as the high quality control in the diphtheria assay and the low quality control in the tetanus assay (appropriately diluted). The parents/guardians of these children had previously given consent for these sera samples to be used in other research projects approved by the WCH Research Ethics Committee. The high QC for the tetanus assay and the low QC for the diphtheria assay were the two pooled adult sera supplied by Mr M. Hanlon (pool 1 and pool 2 respectively). Both of these QCs were calibrated against the appropriate NIBSC international reference serum. The same NIBSC reference serum was also run as an external quality control in each assay.

The plates were washed four times as before and 100µl of a 1:1000 concentration of DAKO® rabbit anti-human, horseradish peroxidase-conjugated IgG (PO214, lot 128{4a}) was added to each well. After a final incubation of 30 minutes at $37^0$C in a humid chamber the plates were washed again, four times and 100µl of ABTS with 33% $H_2O_2$ was added to each well.

Once again, when the most concentrated samples of the standard curve reached an optical density of 2.0 OD units, the plates were read and data values transferred into the Multicalc® software programme to evaluate and standardise the results of the known and unknown samples. Antibody concentrations were expressed as IU/ml of serum.

#### 8.2.10.3 Anti-PRP IgG antibody EIA

The EIA used to measure anti-PRP IgG in this study had been previously developed in the University of Adelaide Department of Paediatrics laboratory by Ms L Dinan (table 8.8).

To enhance adsorption to polystyrene microtitre plates for the coating step in the assay, the immunogen, PRP (Aventis-Pasteur, France), was conjugated to tyramine (Sigma Chemical Co., Missouri, US) using cyanogen bromide (Lab supply, Sydney, Australia) according to the method of Insel and Anderson (Insel R, Aderson P. *Haemophilus influenzae* type b: assays for the capsular polysaccharide and for anti-polysaccharide antibodies (259).

Fifty millilitres (ml) of a 150 mg/ml solution of cyanogen bromide in distilled water were added to 1mg of PRP in 1ml 0.1 Molar (M) sodium hydrogen carbonate ($NaHCO_3$), pH 7.2. The pH of the solution was maintained for six minutes using 0.1 Normal (N) sodium hydroxide (NaOH), and 0.4 mg tyramine in 0.1M $NaHCO_3$ was then added before the solution was mixed in a water bath at $20^0$C for 10 minutes. After adjusting the pH to 8.6 with 0.1N NaOH the solution was then dialysed using three changes of phosphate buffered saline (PBS) and 0.01% methiolate. The antigen preparation was stored at $4^0$C for short-term storage (<48 hours) and at $-70^0$C for long-term storage.

To determine optimal coating concentrations of PRP-tyramine to be used in the anti-PRP IgG ELISAs, checkerboard titrations of differing concentrations of the antigen were performed. The USFDA lot 1983 human pooled serum standard, a known sample containing a high concentration of antibody to PRP, and a known anti-PRP negative sample were all used to standardise the checkerboard assay. The PRP-tyramine was determined to be at optimal concentration when the highest concentration of the USFDA standard reached an optical density of 2.0 units at a wavelength of 410nm in 30-40 minutes.

Wells of Costar® polystyrene microtitre plates (Jomar Diagnostics, Adelaide, Australia) were coated with 100µl of an optimum concentration of PRP-tyramine diluted in 0.01M

carbonate/bicarbonate buffer pH 9.6, incubated at $37^0$C for 90 minutes and then left overnight at $4^0$C in a humid chamber. After washing with PBS-0.01% methiolate-Tween using a Denley 5® automated plate washer, unbound sites were blocked with 1% casein sodium (Ajax Chemicals, Sydney, Australia) diluted in wash buffer and the plates were incubated for two hours at $37^0$C in a humid chamber. The plates were washed as before and 100µl of appropriately diluted serum samples, quality controls and reference serum were added to the wells. Plates were left at room temperature for four hours and washed again as previously described. The addition of 100µl/well of a 1:1000 dilution of Silenus sheep anti-human IgG (gamma chain specific) peroxidase conjugate in 1% casein sodium was followed by incubation at $37^0$C for one hour in a humid chamber. After washing again, 100µl of ABTS with 33% $H_20_2$ was added to all wells. The plates were read using a Dynex MR Opsys® plate reader at a wavelength of 410 nm when the highest concentration of the USFDA reference serum reached an optical density of 2.0 units.

The reference serum used to construct the standard curve was the international reference standard, USFDA human anti-Hib capsular polysaccharide serum, lot 1983, assigned a value of 60.9 µg/ml of anti-PRP IgG antibody by the FDA. Short-term storage of this human reference serum was at $-20^0$C and long-term storage was at $-70^0$C.

Quality control samples were used to validate results within and between assays. High quality control wells contained a serum sample taken 14 days post immunisation with HibTiter® vaccine from an adult volunteer working in the University of Adelaide Department of Paediatrics Laboratory. The low quality control sample was the pooled adult serum collected from five adult volunteers in the University of Adelaide Department of Paediatrics.

Results were expressed as μg/ml of serum in relation to the concentration of anti-PRP antibody assigned to the USFDA reference. The same data management programme referred to in section 8.2.10.1.3 was used to quantitate the concentration of anti-PRP IgG antibody in the patient samples.

### 8.2.11 Statistical analysis

Statistical analysis was performed using Systat 10®. One-way ANOVA was performed to compare term infant responses with those of premature infants by gestational age subgroup and to compare responses between premature infant subgroups. These subgroups were defined as premature (33-36 weeks), very premature (28-32 weeks) and extremely premature (27 weeks or less). Comparisons between any two groups of data were performed using the Student's two groups t-test. P values less than or equal to 0.05 were deemed to be statistically significant. All data were $\log_{10}$ transformed prior to statistical analysis.

Demographic and medical characteristics associated with significant differences in antibody titres (responses to pertussis antigens) or protective levels of antibody titres (results of diphtheria, tetanus and Hib serology) were explored using multiple regression and were kindly performed by Mr Craig Hirte of the Public Health Research Unit associated with the WCH..

## 8.3 Results

### 8.3.1 First study

#### 8.3.1.1 Numbers of infants recruited

Forty-two premature and 54 term infants were recruited to participate in the study. Of the premature infants, 11 were born at 27 weeks gestation or less (extremely premature), 13 between 28 and 32 weeks (very premature) and 18 between 33 and 36 weeks gestation (premature).

#### 8.3.1.2 Numbers of infants completing the study

Forty premature (95.2%) and 44 term infants (81.5%) completed the 19 month study providing an overall completion rate of 87.5%.

Of the premature infants not completing the study, one withdrew after the first appointment at the birth age of 2 months (gestational age 36 weeks), but no reason was given by the parents for withdrawal. The second infant (gestational age 27 weeks) was withdrawn after the 18 month visit, as the family moved interstate and the new local medical officer was unable to collect the 19 month blood sample.

Three term infants were withdrawn after the first appointment, at 2 months of age, as their parents did not wish further blood samples to be collected. Five term infants withdrew after the 7 month visit: two were lost to follow-up, the families of two participants moved interstate and decided not to complete the study and the family of one participant could not attend the WCH for appointments due to transportation difficulties. Two further term infants were withdrawn after the 18 month visit as their parents did not wish further blood samples to be collected.

#### 8.3.1.3 Compliance with immunisation schedule

The aim for the timing of immunisations was to comply with the NH&MRC guidelines, that is to commence immunisations at 2 months (approximately 60 days from birth) and to administer the subsequent primary immunisations (four and six months) at intervals of 7-11 weeks or 49-77 days thereafter. For the 18 month booster immunisations, administration between 17 and 19 months of age was considered an acceptable range.

##### *8.3.1.3.1 Premature infants*

Premature infants received the two month immunisation an average of 62 days after birth (range 49-87 days, standard deviation {s.d} 7.67 days). One infant in the extremely premature infant group was first immunised on day 87 due to a prolonged oral corticosteroid requirement. At four and six months the average number of days between immunisation was 57 in both instances (2-4 months range 49-71, s.d. 3.89, 4-6 months range 49-69 days, s.d. 3.64 days). All premature infants received the 18 month immunisations between 17 and 19 months of age.

##### *8.3.1.3.2 Term infants*

Term infants received the two month immunisation an average of 61 days post partum (range 49-77 days, s.d. 6.52 days). Good compliance with the schedule was maintained at four and six months of age with average durations between immunisations of 58 and 56 days respectively (2-4 months range 52-79, s.d. 5.43 , 4-6 months range 49-62, s.d. 2.19). All term infants received the 18 month immunisation between 17 and 19 months of age.

#### 8.3.1.4 Numbers of blood samples collected

In total, 345 blood samples were collected (out of a possible 353 after study withdrawals). Three samples were not collected as parents of these infants inadvertently attended another immunisation provider prior to attending a study appointment (one term infant at two and 18 months of age and one premature infant at 2 months of age). The remaining five samples were not collected due to technical difficulties with venous sampling. Three of these missed samples occurred in premature infants (one each at 2, 7 and 18 months of age) and two of these in term infants (one at 2 and one at 7 months of age). These parents did not wish finger- or heel-prick sampling to occur.

#### 8.3.1.5 Summary of demographic data

These data are summarised in tables 8.8 and 8.9

##### *8.3.1.5.1 Sex distribution and birth weight*

Female infants comprised 44.4% of the term infants, 53% of the premature infants and 54.5%, 46.1% and 55.6% of the extremely premature, very premature and premature infant subgroups respectively.

The average birth weight of the term infants was 3470 gm, and that of the premature infants was 1638 gm. Extremely premature, very premature and premature infants had average birth weights of 774, 1276 and 2426 gms respectively. Other birth parameters are presented in tables 8.9 and 8.10.

#### *8.3.1.5.2 Ante- and postnatal corticosteroid medication*

No term infant received ante- or postnatal corticosteroid medication whereas 45.5% of extremely premature, 69.2% of very premature and 11.1% of premature infants received antenatal corticosteroids, and 81.8% of extremely premature and 38.5% of very premature infants received postnatal corticosteroids. The mean duration of postnatal steroid administration was 12 days for extremely premature infants (range 0-33 days) and 4.8 days for very premature infants (range 0-10 days) None of the premature infants born at 33-36 weeks gestation received postnatal corticosteroid medication.

#### *8.3.1.5.3 Duration of breast-feeding*

There were 83.3% of term infants and 81.0% of premature infants who were breast fed, although most premature infants received a combination of expressed breast milk and formula feeds whilst in hospital. Mothers of term infants continued exclusive breast-feeding for an average of 4.8m and mothers of premature infants for 3.7 months. There was little difference in the percentage of infants breast fed and the average duration of breast-feeding between the premature infant subgroups.

#### *8.3.1.5.4 Blood transfusions prior to immunisation*

None of the term infants received blood transfusions, whereas 45.2% of premature infants (91.0%, 61.5% and 5.6% of premature infant subgroups) received blood transfusions with washed packed red cells prior to immunisation. The average number of transfusions received was six with a range of 0-16.

#### *8.3.1.5.56 Time in hospital, in the Neonatal Intensive Care Unit (NICU) and Special Care Baby Unit (SCBU)*

Term and premature infants spent an average of 4.7 (range 1-22) and 51 (range 3-113) postnatal days in hospital respectively. None of the term infants required neonatal intensive care, but 16.7% of term infants spent an average of 31 hours in the SCBU (range 0-192) for management of subacute problems requiring more specialised medical and nursing care than could be provided on a general postnatal ward (e.g. oxygen requirement not severe or prolonged enough for mechanical ventilation, hypothermia, hypoglycaemia, slow weight gain or persistent feeding difficulties). The 72% of premature infants requiring NICU care spent an average of 455 hours in the NICU (range 0-1998 hours) and 761 hours in the SCBU (range 24-1448 hours). As expected, extremely premature infants had a longer requirement for intensive and special care facilities than very premature or premature infants. All extremely premature infants were admitted to the NICU and to the SCBU (NICU average time 1171 hours, range 288-1992 hours; SCBU average time 1121 hours, range 720-1430 hours). All very premature infants were admitted to both the NICU and the SCBU (NICU average time 522 hours, range 17-1176 hours; SCBU 826 hours, range 0-1416 hours). Fifty percent of premature infants spent an average of 28 hours (range 0-130 hours) in the NICU and 100% of premature infants spent an average of 522 hours in the SCBU (range 24-864 hours).

#### *8.3.1.5.6 Duration of oxygen therapy*

None of the term infants had an oxygen requirement for more than 4 hours, whereas the average oxygen requirement for premature infants was 63.5 days. The range of oxygen requirement was 0-267 days. As expected, 100% of extremely premature infants required oxygen therapy, with an average requirement of 165 days (range 75-249 days). Fifty four percent of very premature infants (average 62 days, range 0-267 days) and 33% of infants

33-36 weeks gestation at birth (average 2 days, range 0-18 days) had a prolonged oxygen requirement.

#### *8.3.1.5.7 Health insurance*

Private health insurance was held by 11.1% of parents of term infants and 38.1% of premature infants. This may reflect the method of recruitment of premature infants whose parents may have been more likely to participate in the study if their infants were cared for by the study neonatologist.

#### *8.3.1.5.8 Adverse events following immunisation*

Two premature infants (one 29 and one 35 weeks gestation at birth) had a severe local reaction to the 18 month booster dose of DTPa. These reactions were initially noted 24 hours after immunisation and consisted of red, warm, indurated swelling around the injection site extending halfway down the arm and up to the shoulder. Reactions were not associated with fever, extreme irritability or inability to move the arm and responded well to non-steroidal anti-inflammatory analgesia, resolving in a further 24-48 hours. Both reactions were reported to the South Australian Immunisation Coordination Unit and both infants have been referred to the WCH Immunisation Adverse Events Clinic for review regarding appropriate management of subsequent immunisations. This type of adverse event is becoming increasingly recognised at 18 months and is thought to be due to the diphtheria component of the DTPa vaccine (260).

### 8.3.2 Results of IgG antibody determination

#### 8.3.2.1 Pertussis serology

Single antigen pertussis EIAs are still used mainly for research purposes rather than as diagnostic assays, as several countries still immunise children routinely with whole cell pertussis vaccines, and there is no universally accepted serological correlate of protection against infection with *B. pertussis*. Infants in this study were immunised with an acellular DTP vaccine. Therefore development of assays to measure IgG antibodies to individual pertussis antigens contained in the vaccine was considered to be an essential part of the study.

The single antigen assays used in this study were modified from methods to detect IgG antibodies to diphtheria and tetanus in human sera. However, the USFDA lots 2 and 3 were used either to generate the standard curve (PT, PRN) or as external quality control samples (FHA). The NIBSC pertussis human antiserum (89/530) was also used as a second external quality control in all assays. The coefficients of inter- and intra-assay variation were <6% for FHA and PRN and <12% for PT. The lower limits of detection for these assays were 0.38 IU/ml for PT and PRN and 0.78 IU/ml for FHA.

##### *8.3.2.1.1 Comparison of term and premature infant antibody concentrations*

The results are presented in figures 8.1-8.3. IgG antibody results are represented as GMCs with 95% confidence intervals. P values for each comparison are included.

For all three pertussis antigens (PT, FHA and PRN) term infant IgG antibody concentrations were significantly greater than those of premature infants at all time points

(2, 7, 18 and 19 months) except for PRN at 19 months of age (p=0.507: refer to figures 8.1a, 8.2a and 8.3a).

#### *8.3.2.1.2 Comparison between term and premature infant subgroups*

Term infant antibody responses to all three antigens were significantly greater than those in all premature infant subgroups except to PT at 7 months (p=0.076) and PRN at 19 months (p=0.576), when analysed using ANOVA for combined group comparisons.

P values for subanalyses comparing term infant responses to those of individual premature infant subgroups are presented in table 8.11

##### 8.3.2.1.2.1 Comparison between term and extremely premature infants (gestation = 27 weeks)

IgG PT antibody concentrations were significantly greater in term infants than extremely premature infants at all study times. Concentrations of IgG FHA antibodies were significantly higher in term infants at all study times except 2 months (p=0.118). Term infant IgG PRN antibody concentrations were significantly greater than those of extremely premature infants only at the 7 and 18 month study times (2m p=0.401, 19m p=0.723).

##### 8.3.2.1.2.2 Comparison between term and very premature infants (gestation 28-32 weeks)

IgG PT, FHA and PRN antibody concentrations were significantly higher in term infants than very premature infants at the 2 and 18 month study times. Antibody concentrations at the 7 month study time were significantly higher in term infants in response to FHA (p=0.034) and PRN (p=0.003) but not to PT (p=0.340). Antibody concentrations at the 19

month study time were only significantly higher in term infants in response to PT (p=0.046).

8.3.2.1.2.3 Comparison between term and premature infants (gestation 33-36 weeks)

IgG PT, FHA and PRN antibody concentrations were significantly higher in term infants than premature infants at the 2 month study time, but not significantly higher at any other study time except for PT at the 18 month study time (p=0.006).

### *8.3.2.1.3 Comparisons between premature infant subgroups*

The only statistically significant difference on overall comparison between premature infant subgroups (one way ANOVA) was to PRN at 18 months (p=0.041). However, comparisons for FHA at 7 months tended towards significance (p=0.057). When the individual gestational agegroups were compared using all combinations, significant differences were found in comparison between premature and extremely premature infants only. These were demonstrated for the comparison of IgG FHA concentrations at the 7, 18 and 19 month study times (p=0.046, p=0.044 and p=0.043 respectively) and for the comparison of IgG PRN concentrations at the 18 month study time (p=0.025). Refer to table 8.12 for these data.

### *8.3.2.1.4 Summary of pertussis serology results*

In general, responses to pertussis antigens were significantly greater in term infants than in premature infants. The exceptions were the comparisons of term with premature infants to PRN at 19 months and term infants with the three premature infant subgroups to PT at 7 months and PRN at 19 months. In contrast, comparisons of responses between premature

infant subgroups were, in general, not significantly different except between extremely premature and premature infants at 7 months to FHA and at 18 months to PRN. Nevertheless, it can be seen from the figures that in most cases the magnitude of antibody response varied with gestational age at birth and was lowest in extremely premature infants and greatest in term infants at all four time points.

#### 8.3.2.2 Diphtheria and Tetanus serology

Enzyme immunoassays to measure premature and term infant IgG responses to diphtheria and tetanus antigens were developed from protocols used in the NATA accredited laboratory of the Department of Microbiology at the WCH. The relevant NIBSC international reference serum was used as the high quality control and was used to calibrate the pooled reference serum in both assays. Coefficients of inter- and intra-assay variation were <5% for the diphtheria assay and <12% for the tetanus assay.

Serological correlates of protection against infection have been determined for both diphtheria and tetanus. For diphtheria antibody responses, intermediate levels of protection are provided by antibody concentrations of 0.01-0.1 IU/ml and full protection by levels =0.1 IU/ml on EIA. Protective concentrations of tetanus toxoid antibodies are defined as those =0.1 IU/ml on EIA. The lower limit of detection for both assays was 0.0001 IU/ml.

##### *8.3.2.2.1 Comparison between term and premature infant responses*

Significant differences between the IgG antibody concentrations of term and premature infants to were demonstrated for diphtheria toxoid at 2 months (p=0.033) and tetanus

toxoid at 18 months (p=0.011). No other comparisons were statistically significant. Refer to figure 8.4a and 8.5a.

#### *8.3.2.2.2 Comparison between term and premature infant subgroup responses*

Significant differences were found between term infants and premature infant subgroups only in response to diphtheria and tetanus toxoids at 18 months (p=0.033 and p=0.040 respectively). Refer to figures 8.4b and c, 8.5b and c.

##### 8.3.2.2.2.1 Comparison between term and extremely premature infants

At 2 months of age, IgG diphtheria and tetanus toxoid antibody concentrations were significantly higher in term than in extremely premature infants (p=0.002 and p=0.027 respectively). At 18 months, IgG tetanus toxoid antibody concentrations tended to be higher in term infants (p=0.050). No other comparisons were statistically significant.

##### 8.3.2.2.2.2 Comparison between term and very premature infants

No comparisons between term and very premature infants to either toxoid were statistically significant.

##### 8.3.2.2.2.3 Comparison between term and premature infants (33-36 weeks gestation)

None of the comparisons between these groups differed significantly at the study times of 2, 7, 18 or 19 months of age.

#### *8.3.2.2.3 Comparison between premature infant subgroups*

The only statistically significant comparison between premature infant subgroups by one-way ANOVA was in response to tetanus toxoid at 2 months of age (p=0.037). Further

analysis demonstrated a significant difference in antibody responses between premature and extremely premature infants at 2 months (p=0.011). At 2 months a tendency to statistical significance existed between responses of premature and extremely premature infants to diphtheria toxoid (p=0.050). Refer to tables 8.13 and 8.14 for a summary of these data.

#### *8.3.2.2.4 Proportion of infants in each age group achieving protective antibody concentrations*

##### 8.3.2.2.4.1 Diphtheria antibodies

At 2 months, 41.2% of term infants and 60% of premature infants had diphtheria antibody concentrations <0.1IU/ml ($\chi^2$ =7.22, p=0.0072). One premature infant had a concentration of <0.01IU/ml. At 18 months 9.8% of term infants and 28.2% of premature infants had antibody concentrations <0.1 IU/ml but >0.01IU/ml ($\chi^2$ =10.53, p=0.0118). At 7 and 19 months, all term and premature infants achieved protective antibody concentrations. Of the premature infants with antibody concentrations between 0.01IU/ml and 0.1IU/ml at 2 months, 38.5% came from the extremely premature infant group, 34.6% from the very premature and 26.9% from the premature infant group. At 18 months 40%, 60% and 20% of premature infants with antibody concentrations between 0.01IU/ml and 0.1IU/ml came from the extremely premature, very premature and premature infant groups respectively.

##### 8.3.2.2.4.2 Tetanus antibodies

At 7 and 19 months all term and premature infants achieved "protective" antibody concentrations to tetanus toxoid, although at 7 months one term infant had an antibody concentration of exactly 0.1IU/ml. At 2 months 94.1% of term infants and 92.5% of premature infants had protective antibody concentrations (Fisher's exact test, p=0.5794) and at 18 months 97.6% of term and 74.4% of premature infants had antibody

concentrations >0.1IU/ml (Fisher's exact test p<0.0001). Of the premature infants without protective antibody concentrations at 2months, one third came from each premature infant subgroup and at 18 months 33.3% were from the extremely premature infants, 44.4% from the very premature and 22.3% from the premature infant subgroup.

#### *8.3.2.2.5 Summary of diphtheria and tetanus serology results*

Although there were, in general, no significant differences in the antibody concentrations to tetanus and diphtheria toxoids between premature and term infants, premature infants clearly had a reduced magnitude of response at all time points. In addition, the same gradation of magnitude of response as seen with pertussis antigens was demonstrated between the premature infant subgroups, with extremely premature infants having the lowest and premature infants having the highest antibody concentrations. However, after the primary and 18 month booster immunisations, all premature infants achieved protective concentrations of antibodies to both diphtheria and tetanus toxoids.

### 8.3.2.3 Hib-PRP serology

The University of Adelaide Department of Paediatrics Laboratory is the only laboratory in South Australia able to perform EIAs measuring antibody levels to the Hib-PRP antigen. The USFDA lot 1983 human anti-PRP serum has been used to generate the standard curve since inception of the assay. Inter- and intra-assay coefficients of variation were <12%. The lower limit of detection of this assay was 0.02 µg/ml.

#### *8.3.2.3.1 Comparison between term and premature infant responses*

Term infants had significantly greater concentrations of anti-PRP IgG than did premature infants at 7 months (p=0.023) and tended to demonstrate greater antibody concentrations at

2 months (p=0.056) and at 18 months (p=0.057) than premature infants. Refer to figure 8.6a for graphical representation of these data.

#### *8.3.2.3.2 Comparison between term and premature infant subgroup responses*

On one way ANOVA, anti-PRP antibody levels were significantly greater in term infants at 7 months, but not at 2, 18 or 19 months of age (2m p=0.055, 7m p=0.006, 18m p=0.269, 19m p=0.151: figures 8.6b and 8.6c).

##### 8.3.2.3.2.1 Comparison between term and extremely premature infants

Term infants achieved significantly greater antibody concentrations at 2 and 18 months ($p<0.001$ and p=0.029 respectively), with a trend towards significance at 7 months (p=0.054) and19 months (p=0.055).

##### 8.3.2.3.2.2 Comparison between term and very premature infants

Comparisons between these groups differed at 7 months only, with term infants having greater antibody concentrations of IgG anti-PRP (p=0.008).

##### 8.3.2.3.2.3 Comparison between term and premature infants

There were no significant differences in PRP antibody concentrations at any time point between these two groups of infants.

#### *8.3.2.3.3 Comparison between premature infant subgroups*

Significant differences were not found between the antibody responses of the premature infant subgroups to PRP on one-way ANOVA (table 8.16). However, on further analysis, the comparison between extremely premature and premature infants was significant at 2

months (p=0.004) and the comparison between very premature and premature infants was significant at 7 months (p=0.016).

#### *8.3.2.3.4 Proportion of infants in each age group achieving protective antibody concentrations*

Previous investigators have categorised anti-PRP IgG antibody concentrations according to levels of protection. Concentrations ≥0.15μg/ml are considered to indicate short-term protection against infection and those ≥1.0 μg/ml are consistent with long-term protection (261).

##### 8.3.2.3.4.1 Short-term protection

At 2 months, 75.5% of term infants and 60% of premature infants had PRP antibody concentrations consistent with short-term protection ($\chi^2$ =5.88, p=0.01529). Of the premature infants who did not have protective antibody concentrations, 43.8% were in the extremely premature infant group, 31.2% in the very premature and 25% in the premature infant group. These results reflect the relationship between the degree of prematurity and the amount of trans-placental transfer of IgG.

At 7 months, one premature and one term infant did not achieve short-term protection, giving a seroconversion rate of 98% for term infants and 97.5% for premature infants (the premature infant was 29 weeks gestation at birth, i.e. in the very premature infant group). Both infants received a booster dose of Hib at 12 months and both had an appropriate serological response to this extra dose of vaccine.

At 18 months 100% of term and 95% of premature infants had antibody concentrations ≥0.15 μg/ml (Fisher's exact test p=0.0594). Of the two premature infants with non-

protective antibody concentrations, one was from the very premature and the other from the premature infant group.

At 19 months, all term and premature infants had antibody concentrations consistent with short-term protection.

8.3.2.3.4.2 Long-term protection (determined at 7 and 19 months)

At 7 months, 98% of term and 77.5% of premature infants had antibody concentrations consistent with long-term protection (i.e.>1.0 μg/ml: Fisher's exact test $p<0.0001$). Again, most of the less-responsive infants were in the extremely premature infant group (50%), with 37.5% in the very premature and 12.5% in the premature infant group.

At 19 months 100% of term and 94.7% of premature infants had antibody concentrations ≧1.0 μg/ml. Both of the two less responsive premature infants were in the extremely premature infant group.

### *8.3.2.3.5 Summary of PRP serology results*

Term infants had significantly higher responses to PRP than premature infants only at 7 months, with a trend towards higher antibody concentrations at 2 and 18 months. Comparison of term infants and premature infant subgroups revealed that there were no statistical differences in responses of term infants and premature infants born between 33-36 weeks gestation. Infants in the very and extremely premature infant groups differed significantly from term infants in their PRP antibody concentrations at 2 months only. It was only at 2 and 7 months that significant differences in antibody concentrations were found between the premature infant subgroups. The proportion of premature infants achieving protective antibody concentrations was less than term infants at all study times,

but differed most significantly with respect to the ability to achieve long-term protective antibody concentrations, particularly at the 7 month study time.

#### 8.3.2.4 Multiple regression analysis

All medical and demographic variables were analysed initially to determine which caused the significant differences in magnitude of antibody responses to vaccine antigens between term and premature infants. Out of all parameters nine were determined to have the most influence. These were: gender, gestational age at delivery, private heath insurance status, oxygen therapy, breast-feeding, antenatal steroid administration, postnatal steroid administration, maternal education, and maternal ethnicity. Of these nine parameters private heath insurance, antenatal steroid administration, postnatal steroid administration gestational age at delivery and oxygen therapy most frequently influenced the differences in antibody responses between term and premature infants. However, these variables were all related to prematurity, as none of the term infants required oxygen therapy for more than four hours, none received ante- or postnatal steroids and more premature infants had parents with private heath insurance than the parents of term infants.

Using backwards regression, for each antigen and each study time these parameters were analysed further to determine the proportion of the variability between term and premature infant antibody responses which could be accounted for by each parameter. Because of the relatively large number of vaccine antigens and study times few analyses revealed parameters which could account for more than 25% of the variability ($R^2$ ) in antibody responses between premature and term infants.

For example, premature infant gestational age group adjusted for breast feeding accounted for 23.8% of the variability in responses to PT at 2m of age and for 25% of the variability

in responses to PRN at 7 months of age in term and premature infants. Premature gestational age group alone accounted for 28.4% of the variability in antibody responses to PT at 18 months of age. Private heath insurance adjusted for maternal ethnicity, antenatal corticosteroid administration and maternal age at delivery accounted for 29.1% of the variability in responses to tetanus toxoid at 18 months of age.

Because of the high proportion of parents of premature infants with private health insurance, this variable was removed from the model. However, the $R^2$ values describing the proportion of the total variability in antibody responses explained by each factor were reduced after this adjustment

Thus it would appear that prematurity may be the single most important factor in the determination of antibody concentrations after immunisation in these studies.

### 8.3.2 Second study

Serum samples from 11 premature (cohort C) and 11 term infants (cohort D) previously enrolled in a study to investigate premature and term infant antibody responses to the Hib vaccine were used for further comparisons. These infants had been immunised with DTPw in the primary series and with DTPa at the 18 month booster immunisation. Serum samples were collected from these infants at 2, 8, 18 and 19 months of age. These samples were selected on the basis of the amount of serum available at each study time, the requirement for serum from all four study times for each infant, and consent from the parent(s)/guardian(s) for the use of these samples in other studies. Longitudinal comparisons were made between these infants and those in the first study (cohorts 1a and 1b, who had all been immunised with DTPa from 2 months of age) to investigate antibody responses to DTPa and DTPw in the primary series and how this impacted on 18 month

booster responses to DTPa. Separate, cross-sectional comparisons with newly recruited ex-premature infants at 18 months of age (cohort 2) and cohort C were made to confirm 18 month booster responses to DTPa in premature infants with different primary immunisation schedules. Finally, cohorts 1a and 2 were combined to provide a larger cohort of newly recruited premature infants and therefore larger premature infant subgroup cohorts for further cross-sectional comparisons with term infants (cohort 1b) at the 18 and 19 month study times. Table 8.2 outlines these cohorts and the numbers of infants in each cohort.

#### 8.3.2.1 Numbers of infants recruited in Cohort 2 and demographic characteristics

Thirty-nine ex-premature infants were recruited at eighteen months of age with gestational ages at birth ranging from 23 weeks (two infants) to 36 weeks (three infants). There were 11 infants in the extremely premature infant group, 12 in the very premature and 16 in the premature infant group. Twelve infants (30.8%) were female. Forty-one percent had parents with private health insurance. Six pairs of twins were enrolled: four pairs were single sex (three male and one female) and two were male:female pairs. All infants completed the study and had immunisations within the ages of 17-19 months with post-immunisation blood samples collected 4-6 weeks later. One infant did not have a blood sample collected at 18 months, as he was inadvertently immunised by his local medical officer after his parents had agreed to participate in the study. All other blood samples were collected without difficulty by the principal investigator.

All serum samples were assayed to determine IgG antibody responses to PT, FHA, PRN and diphtheria and tetanus toxoids. Responses to Hib are not reported for cohorts C and D as these were performed in the previous study by Dinan. Antibody concentrations are once

again represented as GMCs with 95% confidence intervals on all figures. P values are also represented on each figure. Figures representing comparisons between term infants and premature infant subgroups display the degree of significance in sub-comparisons represented by the symbols * for $p \leq 0.05$ and ** for $p \leq 0.005$.

### 8.3.2.2 Longitudinal comparisons of IgG antibody responses between premature infants immunised with DTPa (cohort 1a) or DTPw in the primary series and DTPa at 18 months (cohort C)

#### *8.3.2.2.1 IgG antibody responses to pertussis antigens*

No significant differences in IgG antibody concentrations to PT, FHA or PRN were demonstrated between the two groups at 2 months, reflecting similar concentrations of transplacentally-acquired maternal antibody.

Antibody concentrations to FHA and PRN, however, were significantly higher in premature infants immunised with DTPa for the primary series (cohort 1a) at 7 months/8 months, 18 and 19 months of age (p=0.005 in all cases). Concentrations of anti-PT IgG were only higher in infants from cohort 1a at 7 months (p=0.011). Refer to figures 8.7a-c for graphical representation of these data.

#### *8.3.2.2.2 IgG antibody responses to diphtheria and tetanus toxoids*

No differences in concentrations of diphtheria or tetanus toxoid antibodies were demonstrated at 2 months of age between the two groups of infants (figures 8.8a and 8.8b). At 18 and 19 months, however, infants immunised with DTPw in the primary series demonstrated significantly higher antibody responses to both toxoids than those immunised with DTPa from 2 months of age (diphtheria 18 months p=0.030, 19 months p=0.040;

tetanus 18 and 19 months p<0.001). Antibody responses to tetanus toxoid were also higher in infants immunised with DTPw after primary immunisation (comparison at 7m/8m of age: p<0.001).

### 8.3.2.3 Longitudinal comparisons of IgG antibody responses between term infants immunised with DTPa (cohort 1b) or DTPw in the primary series and DTPa at 18 months (cohort D)

#### *8.3.2.3.1 IgG antibody responses to pertussis antigens*

A similar pattern of antibody responses was demonstrated for term infants. Concentrations of PT, FHA and PRN antibodies did not differ between the two groups of term infants at 2 months of age (figures 8.9a-c). Infants immunised with DTPa in the primary series demonstrated significantly higher PT, FHA and PRN antibody concentrations at 7 months/8 months and 18 months of age ($p \leq 0.001$ in all cases). However, only PRN antibody concentrations were significantly higher in infants in cohort 1b at 19 months (PT p=0.335, FHA p=0.090, PRN p=0.002).

#### *8.3.2.3.2 IgG antibody responses to diphtheria and tetanus toxoids*

Term infants immunised with DTPw in the primary series demonstrated significantly higher concentrations of antibodies to tetanus toxoid at 7 months/8 months, 18 and 19 months of age (p<0.001 in all cases: figures 8.10a and 8.10b), with no differences demonstrated between responses at 2 months of age (p=0.900). However, term infants immunised with DTPa in the primary series demonstrated significantly higher antibody concentrations of anti-diphtheria toxoid IgG at 7 months/8 months (p=0.001) and 18 months of age (p=0.012). No significant differences were demonstrated at 2 months (p=0.829) or 19 months (p=0.061), however.

### 8.3.2.4 Cross-sectional studies

The first studies (section 8.3.2.4.1) were performed in order to determine if the results from sections 8.3.2.1.1 and 8.3.2.1.2 were comparable if another cohort of separately recruited premature infants (born approximately 18 months earlier but immunised with DTPa for all immunisations: cohort 2) were used to compare with the same cohort of premature infants receiving primary immunisations with DTPw and 18 month booster immunisation with DTPa (cohort C).

The second study, (section 8.3.2.4.3), by combining the two newly recruited premature infant cohorts (cohorts 1a and 2), provided premature infant subgroup numbers of 21 extremely premature infants (≤27 weeks gestation), 25 very premature infants (28-32 weeks gestation) and 33 premature infants (33-36 weeks gestation). These second cross-sectional comparisons were made in order to assess whether the relatively small numbers of infants in each premature infant subgroup in cohort 1a affected statistical comparisons with term infant antibody concentrations as reported in the first study at 18 and 19 months.

#### *8.3.2.4.1 Cross-sectional comparisons of IgG antibody 18 month booster responses between premature infants immunised with DTPa (cohort 2) or DTPw in the primary series and DTPa at 18 months (cohort C)*

Antibody responses to all three pertussis antigens at 18 months were greater in infants immunised with DTPa in the primary series (cohort 2: PT $p=0.030$, FHA and PRN $p<0.001$), whereas only responses to FHA and PRN were significantly greater in cohort 2 at 19 months (PT $p=0.420$, FHA $p<0.001$, PRN $p=0.024$). These results were very similar to those documented previously between cohort C and cohort 1a, although PT antibody concentrations at 18 months did not differ significantly between the two groups in the second study.

Similar to the previous comparisons between cohorts 1a and C, antibody responses to diphtheria and tetanus toxoid at 18 and 19 months were greater in infants immunised with DTPw in the primary series (diphtheria 18 months p=0.018, 19 months p=0.014; tetanus p<0.001 in both cases). Refer to figures 8.11a-c and 8.12a and 8.12b for graphical representation of these data.

#### *8.3.2.4.2 Proportion of infants with protective antibody concentrations of diphtheria and tetanus antibodies in cohorts 2, C and D.*

All infants in cohorts C and D demonstrated protective antibody concentrations of diphtheria and tetanus antibodies at 8, 18 and 19 months of age. Similarly, 100% of infants in cohort 2 had diphtheria and tetanus antibody concentrations >0.1 IU/ml at 18 and 19 months of age.

#### *8.3.2.4.3 Cross-sectional comparisons of IgG antibody responses between all newly recruited premature infants (cohorts 1a and 2 combined) and term infants (cohort 1b) at 18 and 19 months of age*

##### 8.3.2.4.3.1 IgG antibody responses to pertussis antigens

8.3.2.4.3.1.1 Comparisons between term and premature infants

At both the 18 and 19 month study times, term infants had significantly greater antibody concentrations of IgG anti-PT, -FHA and –PRN antibodies than term infants using the Student's t-test (18 months: PT p<0.001, FHA p=0.002, PRN p=<0.001; 19 months: PT p=0.001, FHA p=0.007, PRN p=0.36). These results are represented in figures 8.13a, 8.14a and 8.15a respectively.

8.3.2.4.3.1.2 Comparisons between term infants and premature infant subgroups

Using one-way ANOVA, term infant IgG PT, and FHA antibody concentrations were significantly greater at both the 18 and 19 month study times (PT 18m $p<0.001$, 19m $p=0.001$; FHA 8m $p=0.009$, 19m $p=0.012$). Antibody concentrations of IgG anti-PRN were only significantly greater in term infants at the 18 month study time (PRN 18m $p=0.005$, 19m $p=0.170$). Refer to figures 8.13b, 8.14b, 8.15b.

In all cases, comparisons between term and extremely premature infants were significant at 18 months (PT, $p=0.001$, FHA $p=0.007$, PRN $p=0.006$). In addition, term infants had significantly greater IgG anti-PT antibody concentrations than premature infants (33-36 weeks gestation) at 18 months ($p=0.007$). At 19 months, term infants had significantly greater PT and FHA antibody concentrations than extremely premature infants at 19 months (PT $p=0.001$, FHA $p=0.007$). However, no similar significant differences were demonstrated for PRN at the 19 month study time ($p=0.170$).

8.3.2.4.3.1.3 Comparisons between premature infant subgroups

No significant differences in pertussis antibody concentrations were demonstrated between premature infant subgroups at either 18 or 19 months of age using one-way ANOVA.

8.3.2.4.3.1.4 Comparison of pertussis antibody results with first study

Term infants had significantly greater PT, FHA and PRN antibody concentrations than premature infants at both the 18 and 19 month study times. This differs from the results of the first study only with respect to the 19 month concentrations of PRN antibodies, which did not differ significantly between the two cohorts ($p=0.427$, fig 8.3a).

Comparisons between term and premature infant subgroups demonstrated greater PT and FHA antibody concentrations in term than extremely premature infants at 18 and 19 months, greater term infant PRN antibody concentrations at 18 months and greater tern than premature infant (33-36 weeks gestation) PT antibody concentrations at 18 months. These mirrored the results of the first study. However, in addition, term infants also demonstrated significantly higher PT, FHA and PRN antibody concentrations at 18 months and greater PT antibody concentrations at 19 months than very premature infants in the first study.

8.3.2.4.3.2 IgG antibody responses to diphtheria and tetanus toxoids

8.3.2.4.3.2.1 Comparisons between term and premature infants

At the 18 month study time, term infant concentrations of IgG anti-diphtheria and-tetanus antibodies were significantly greater than those of premature infants (diphtheria p=0.039, tetanus p=0.008). At 19 months, only concentrations of tetanus antibodies were significantly greater in term infants (diphtheria p=0.673, tetanus p=0.014). Refer to figures 8.16a and 8.17a.

8.3.2.4.3.2.2 Comparisons between term infants and premature infant subgroups

Using one-way ANOVA, term infants had significantly greater antibody concentrations than premature infant subgroups at 19 months for tetanus only (p=0.025). None of the other comparisons reached statistical significance. (diphtheria 18m, p=0.119, 19m p=0.911: tetanus 18m p=0.064). Refer to figures 8.16b and 8.17b.

At the 19 month study time, term infants had significantly greater tetanus antibody concentrations than extremely premature infants (p=0.030). None of the other sub-comparisons reached or approached statistical significance.

8.3.2.4.3.2.3 Comparisons between premature infant subgroups

No significant differences in diphtheria or tetanus antibody concentrations were demonstrated between premature infant subgroups at either 18 or 19 months of age using one-way ANOVA.

8.3.2.4.3.2.4 Comparison of diphtheria and tetanus antibody results with first study

Term infant antibody diphtheria and tetanus concentrations were greater than those of premature infants at 18 months, but only tetanus antibody concentrations were significantly greater than those of premature infants at 19 months. There results differed from those of the first study in which differences between term and premature diphtheria antibody concentrations at 18 months and tetanus antibody concentrations at 19 months were not statistically significant (diphtheria 18m p=0.252, fig 8.4a, tetanus 19m p=0.057, fig 8.5a).

There were no significant differences demonstrated between term and premature infant subgroup responses to either diphtheria or tetanus toxoid at 18 months in direct contrast to the first study. In addition, significant differences between term and extremely premature infant tetanus antibody concentrations were demonstrated at 19 months in the second study, but not in the first.

8.3.2.4.3.3 IgG antibody responses to Hib PRP

8.3.2.4.3.3.1 Comparisons between term and premature infants

Neither at 18 nor at 19 months were significant differences demonstrated between term and premature infant PRP antibody concentrations (18m p=0.113, 19m p=0.186).

8.3.2.4.3.3.2 Comparisons between term infants and premature infant subgroups

Using one-way ANOVA, No significant differences between term and premature infant subgroup concentrations of PRP antibodies were demonstrated t either 18 or 19 months (18m p=0.470, 19m p=0.374).

8.3.2.4.3.3.3 Comparisons between premature infant subgroups

No significant differences in PRP antibody concentrations were demonstrated between premature infant subgroups at either 18 or 19 months of age using one-way ANOVA.

8.3.2.4.3.3.4 Comparison of PRP antibody results with first study

Term infant PRP antibody concentrations did not differ from premature infants or from those of the premature infant subgroups at either 18 or 19 months of age. These results differed from those of the first study only with respect to the comparison between term and extremely premature infant PRP antibody concentrations at 18 months which were significant in the first study (p=0.29).

## 8.4 Conclusions

### 8.4.1 First study

#### 8.4.1.1 General trends in antibody concentrations

Term infants in this study demonstrated significantly higher concentrations of maternal antibody measured at two months of age to all pertussis vaccine antigens and to diphtheria toxoid than premature infants. Concentrations of antibodies to tetanus toxoid and Hib PRP were not significantly higher in term infants at 2 months, but only approached significance for Hib PRP ($p=0.056$). Both term and premature infants demonstrated good booster responses to vaccine antigens at 7 and 19 months of age. In addition all infants demonstrated protective diphtheria and tetanus antibody concentrations at 7 and 19 months of age. All but one term and one premature infant demonstrated protective antibody concentrations of IgG anti-PRP at 7 months, and all infants demonstrated protective PRP antibody concentrations at 19 months of age.

#### 8.4.1.2 Pertussis antibodies

Term infants also demonstrated significantly greater concentrations of PT and FHA antibodies at all study times and significantly greater concentrations of PRN antibodies at the study times of 2, 7 and 18 months. These significant differences were also mirrored in the comparisons between term infant and premature infant subgroup pertussis antibody concentrations. In general, extremely premature infants (gestation $\leq$ 27 weeks) demonstrated significantly lower pertussis antibody concentrations than term infants at all study times. Very premature infants (gestation 28-32 weeks) often demonstrated significantly lower pertussis antibody concentrations than term infants. Pertussis antibody concentrations in premature infants (33-36 weeks gestation) did not differ from those of term infants for PT, FHA or PRN antibodies at any of the study times. Comparisons

between premature infant subgroup pertussis antibody concentrations were only significant between extremely premature and premature infants at 7 months in response to FHA and at 19 months in response PRN.

#### 8.4.1.3 Diphtheria and tetanus antibodies

Diphtheria and tetanus toxoids appeared to be more immunogenic in premature infants than pertussis antigens, as comparisons between term and premature infants antibody concentrations were significant at 2 months for diphtheria antibodies and at 18 months for tetanus antibodies only. At 19 months term infants also had greater tetanus antibody concentrations but this comparison approached statistical significance only (p=0.057). Comparison between term infant and premature infant subgroup antibody concentrations were significant at 18 months for both diphtheria and tetanus antibodies. However, comparisons of term infant with individual premature infant subgroup antibody concentrations were significant for tetanus antibodies only; extremely premature infants had significantly lower antibody concentrations at both 2 and 18 months. Comparisons between premature infant subgroup antibody concentrations did not revel any statistically significant differences.

#### 8.4.1.5 Hib PRP antibodies

Although only the 7 month comparison between term and premature infant responses to Hib PRP were statistically significant, comparisons at both 2 and 18 months approached statistical significance (2m p=0.056, 18 m p=0.057). In addition, at both the 2 and 18 month study times, extremely premature infants had significantly lower PRP antibody concentrations. At 7 months, the one-way ANOVA comparing term and premature infant subgroup PRP antibody concentrations was not significant, however, term infants had

significantly higher antibody concentrations than very premature infants. Comparisons between premature infant subgroups demonstrated significantly higher PRP antibody concentrations in premature infants than extremely premature infants at 2 months and significantly higher PRP antibody concentrations in premature than very premature infants at 7 months.

Previous studies investigating the responses of premature and term infants to DTP vaccines are summarised in table 8.16. Two of these studies used DTPa preparations. The study of Schloessor et al (262) demonstrated significantly higher PT and FHA and Diphtheria antibody concentrations in term infants at 7 months of age after three doses of vaccine. The study of Faldella (244) demonstrated no significant differences between term and premature infants in PT or FHA antibody concentrations, but demonstrated significantly higher pertactin antibody concentrations in term infants. Diphtheria antibody concentrations were not compared in this study. The two studies differed from the study reported here and eachother both in the vaccine formulation used and/or the immunisation schedule as well as using different EIA methods. Therefore, these factors may have influenced the magnitude of antibody responses in premature and term infants.

A previous study performed in the University of Adelaide Department of Paediatrics (L Dinan, MPH thesis) investigated PRP antibody responses in premature and term infants before, during and after primary immunisation. That study demonstrated that term infants had significantly higher PRP antibody concentrations than premature infants at 2 and 4 months of age (before and after the first immunisation), but responses did not differ significantly between the two groups after the second or third immunisations (at 6 and 8 months of age). This was similar to the findings of Washburn et al (263) who demonstrated significantly lower PRP antibody concentrations in premature infants after

one and two doses of vaccine in comparison with those in term infants. The infants in the study of Washburn et al were not followed beyond four months of age, however. D'Angio et al (264 ) also demonstrated no significant differences between term and premature infant responses to Hib PRP after three doses of vaccine (albeit using a different schedule). These results differ from those in the present study, where PRP antibody responses were significantly different between term infants and premature infants (and premature infant subgroups) only at 7 months of age (figure 8.6a). These differences may be explained in terms of sample size (in Dinan's study total infant numbers were more than twice that of the present study, whereas D'Angio et all investigated Hib PRP antibody responses in 16 premature infants, less than half the numbers of premature infants enrolled in the present study), and differing times of post-primary immunisation blood sampling (7 months on the present study vs. 8 months in Dinan's study and 12 months in the study of D'Angio et al).

A more recent study demonstrated significantly lower PRP antibody concentrations in premature infants receiving dexamethasone for chronic lung disease in comparison with infants of the same gestation who were not treated with steroid medication (227). Premature infants who had received steroid medication in the present study were immunised two weeks after cessation of therapy, to prevent this from being a confounding factor.

The division of premature infants into gestational age subgroups in the present study demonstrated that antibody responses to vaccine antigens varied with gestational age at birth, with extremely premature infants demonstrating the lowest concentrations and term infants the highest antibody concentrations. This pattern of response was consistent for all antigens at all time points. In addition, where statistically significant differences in antibody responses were demonstrated between term infants and premature infant

subgroups (most commonly at 2 and 18 months of age), significance was usually generated by the difference in responses between term and extremely premature infants.

Two of the previous studies documented in table 8.15 separated premature infants on the basis of gestational age at birth (244, 265). Faldella et al compared premature infants born at ≤ 31 weeks gestation with those born between 31 and 36 weeks gestation. After two doses of DTPa-Hepatitis B vaccine administered at 3 and 5 months of age, infants born at ≤31 weeks demonstrated significantly lower antibody responses to PT, FHA and PRN than premature infants born higher gestational ages. Responses to diphtheria toxoid did not differ between the two groups. However, after the third dose administered at 11 months of age, antibody responses to PRN were significantly higher in infants born between 31 and 36 weeks gestation. Pullen and Hull divided premature infants into three groups, those born at 26-27 weeks, 28-31 weeks or 32-36 weeks. This study demonstrated that all infants developed "satisfactory" antibody responses to DTP immunisation regardless of gestational age at birth. Infants born at 26-27 weeks demonstrated lower tetanus and pertussis antibody concentrations than other premature infant groups and term infants. However, all infants were immunised with a DTPw vaccine, pertussis antibody responses were assessed by whole cell EIA and formal statistical analyses were not performed on the data. Therefore, whilst neither study is directly comparable to the present study in terms of vaccine administered and/or immunisation schedule, both demonstrated similar differences in antibody concentrations between infants of lower gestational age at birth and premature infants born later in gestation and term infants. All other studies reported in table 8.16 used DTPw preparations. None reported significant differences between term and premature infant antibody responses to vaccine antigens (264, 266, 267).

Dinan also separated premature infants into three groups on the basis of gestational age at birth (similar to those in the present study) and found that PRP antibody responses were lower in premature infant subgroups than in term infants at 2 and 4 months of age, but not at 8 months of age. Extremely premature infants and premature infants demonstrated significantly lower PRP antibody concentrations than term infants at 2 months, but by 8 months no significant differences between term infant and individual premature infant subgroup PRP antibody concentrations. These results also differ from those in the present study as only extremely premature infants demonstrated significantly lower PRP antibody concentrations than term infants at 2 months and very premature infants also demonstrated lower PRP antibody concentrations at 7months of age than term infants. Once again, however, the smaller number of infants in the present study may explain the difference in results.

In general, premature infant subgroup antibody responses to all vaccine antigens did not differ significantly in the present study. Where significant differences were demonstrated between the subgroups, however, they were generated in all but one case by comparisons between the antibody responses of premature infants (gestation 33-36 weeks) and extremely premature infants (gestation $\leq$27 weeks). In addition, the antibody responses of premature infants born between 33-36 weeks gestation did not differ from term infants for any comparison.

It is interesting to note that of all vaccine antigens, responses to PRN demonstrated the greatest fold rises both in terms of pre and post primary antibody responses (approximately 50-fold for term infants and 25-fold for premature infants) and comparison between 7 and 19 month antibody responses (approximately 7-fold for term infants and 10-fold for premature infants). None of the previous studies assessed fold rises in PRN antibodies.

However, Schloessor et al reported a 14-fold rise in PT titres after primary immunisation in premature infants and a 20-fold rise in term infants (262). This differs from the present study in which term infants demonstrated a 13-fold rise and premature infants a 25-fold rise in PT antibody concentrations after primary immunisation. A 14-fold rise in FHA antibody concentrations was demonstrated in term and premature infants before and after primary immunisation in the present study. Comparisons between 7 and 19 month fold-rises in PT and FHA antibody concentrations were similar for term and premature infants: PT antibody responses rose two-fold and FHA responses six-fold. Some investigators have used a four-fold rise in pertussis antibody concentrations as a marker of pertussis vaccine protective efficacy (262). However, this method requires the assumptions that all assay results lie on the linear portion of an EIA standard curve and that the magnitude of antibody response correlates with vaccine protective efficacy. A serological correlate of protection against pertussis infection has not yet been found, and it would appear that cell mediated immunity has a large role in the protection aginst infection with *B. pertussis* (162, 184). Therefore, fold-rises in antibody concentrations should be interpreted with caution.

### 8.4.2 Second study

#### 8.4.2.1 Comparison of immunisation responses in infants immunised with DTPw or DTPa in the primary series and DTPa at 18 months

##### *8.4.2.1.2 Longitudinal comparisons*

Premature infants immunised with DTPw in the primary series and DTPa at 18 months demonstrated significantly lower FHA and PRN antibody concentrations at 7/8months, 18 and 19 months of age, significantly lower PT antibody responses at 7 months, but paradoxically higher 7 month responses to tetanus toxoid and 18 and 19 month responses to diphtheria and tetanus toxoids than infants immunised with DTPa from 2 months of age.

Comparisons in term infants were also similar. However, at 19 months infants immunised with DTPw in the primary series did not differ in response to FHA or diphtheria toxoid. In addition, responses to PT at 18 months and diphtheria toxoid after primary immunisation were significantly higher in infants immunised with DTPa from 2 months of age.

All infants demonstrated protective concentrations of diphtheria and tetanus antibodies at 2 and 19 months of age.

##### *8.4.2.2.1 Cross sectional comparisons*

Premature infants in cohort 2 (recruited from 18 months of age) who were also immunised with DTPa from 2 months of age demonstrated a similar pattern of responses as did premature infants in cohort 1a in comparison with cohort C.

Concentrations of FHA and PRN antibodies were significantly higher (with similar degrees of significance) than those of infants in cohort C who had been immunised with DTPw for the primary series at both 18 and 19 months. However, in contrast to the previous study, responses to PT were also significantly greater in infants in cohort 2.

Similar results and degrees of significance were also demonstrated in the comparisons of diphtheria and tetanus antibody concentrations between infants in cohorts 2 and C as between infants in cohorts1a and C. That is, primary immunisation with DTPw conferred significantly higher diphtheria and tetanus antibody concentrations at both 18 and 19 months than in infants immunised with DTPa in the primary series. However, the number of infants achieving protective diphtheria and tetanus antibody concentrations at 19 months was unaffected.

Several previous studies have investigated comparative antibody responses in infants and children to DTPa and DTPw vaccines (91, 92, 239, 240, 242, 243, 268-275). In general, these studies also demonstrated higher antibody responses in DTPa recipients to PT, FHA and PRN (although not all DTPa vaccines were tricomponent and some contained agglutinogens) after primary, 18 month and 4-7 year booster immunisations whether previous vaccinations had been with DTPa or DTPw. Antibody responses to the diphtheria and tetanus components of these vaccines were not investigated in all studies, but were either the same in DTPa and DTPw recipients or greater in DTPw recipients.

There are also similarities of the results of the present study with the murine IgG antibody results reported in chapter six. Mice immunised with DTPa demonstrated higher antibody concentrations of pertussis antibodies, but lower concentrations of diphtheria and tetanus IgG antibodies after immunisation and infection than mice immunised with DTPw.

Therefore in both mice and humans, killed whole pertussis organisms are likely to be an important adjuvant for diphtheria and tetanus toxoids in the DTPw vaccine. Alternatively, there is a relatively higher dose of both toxoids administered with the DTPw vaccine which in turn stimulates greater antibody concentrations. However, CSL Ltd reports diphtheria and tetanus toxoid concentrations in DTPw in International Units (IU), whereas the SB Biologicals reports the amount of toxoid in flocculation units (Lf) a measure of opacity and functional activity. These units are not convertible and therefore the effect of dose cannot be assessed accurately.

Human infants demonstrated antibodies to PT after immunisation regardless of vaccine preparation and gestational age, whereas mice immunised with DTPw appeared to develop PT antibodies only after infection, emphasising the need for caution when comparing murine studies to the human situation.

#### 8.4.2.2.1.1 Cross-sectional comparison of antibody responses in term and premature infants at 18 and 19 months using a combined cohort of premature infants

In general, statistical comparisons between term infants and premature infant PT, FHA and PRN and PRP antibody concentrations were unaffected by the addition of a second cohort of premature infants. However, comparisons between term infant and very premature infant PT, FHA and PRN antibody concentrations at 18 months and PT antibody concentrations at 19 months no longer differed significantly in the second study. However, as comparisons between term and extremely premature infant pertussis antibody concentrations remained statistically significant, it would appear that extremely premature infants may be most at risk of pertussis infection on the basis of antibody concentrations alone, and in the absence of a serological correlate of protection.

Diphtheria antibody concentrations at 18 months and tetanus antibody concentrations at 19 months were also significantly greater in term infants in the second study, but not in the first study (although comparison of tetanus antibody concentrations at 19 months approached statistical significance in the first study: p=0.057). In addition, term infant tetanus antibody concentrations became significantly higher than those of the premature infant subgroups at both 18 and 19 months in the second study. In contrast, diphtheria antibody concentrations were significantly higher in term infants than those of the premature infant subgroups at 18 months in the first study, but not in the second. As both the term infant/premature infant and term infant/premature infant subgroup comparisons were affected for both diphtheria and tetanus antibody concentrations, it would appear that there is more inherent variability in 18 and 19 month IgG antibody responses to both of these toxoids in premature infants. However, as the published data regarding premature infant antibody responses to diphtheria and tetanus toxoids are limited (table 8.16) further studies are required to determine the veracity of this statement.

Statistical comparisons between term infants and premature infant PRP antibody concentrations were also unaffected by the addition of a second cohort of premature infants. At 18 months, extremely premature infant PRP antibody responses were no longer significantly different from those of term infants, indicating either that increasing the number of infants in these subgroups increased the statistical power of the comparisons, or that the two premature infant subgroups were, in fact, from two immunologically distinct populations.

In summary, these studies have demonstrated that premature infants demonstrate lower IgG antibody concentrations to vaccine antigens after primary and 18 month booster immunisations with DTPa and Hib vaccines and that extremely premature infants

demonstrated the lowest antibody responses of all groups. Premature infants born between 33 and 36 weeks gestation demonstrated no differences in IgG antibody response to any vaccine antigen in comparison with term infants. However, all infants demonstrated protective IgG antibody concentrations for diphtheria and tetanus antibodies at 7 months and diphtheria, tetanus and PRP antibodies at 19 months regardless of gestational age. Premature and term infants all demonstrated high fold-rises in pertussis antibody titres after primary and 18 months booster immunisations, but the biological significance of these results is uncertain.

Immunisation with DTPw in the primary series of immunisations conferred higher tetanus antibody concentrations after primary and 18 month booster immunisation in premature and term infants, and greater 18 month booster immunisation responses to diphtheria toxoid. However, these infants had lesser PT, FHA and PRN antibody concentrations after primary immunisation, and lesser FHA and PRN antibody concentrations after 18 month booster immunisation than infants immunised with DTPa from 2 months of age. These results are important, as DTPw is still used in childhood immunisation schedules in many parts of the world.

Future studies should be directed towards further delineation of premature infant immune function, particularly in relation to pertussis infection and immunisation. In addition, studies of antibody avidity may reveal qualitative differences in antibodies, even when the magnitude of antibody response does not differ between groups.

## Table 8.1: NH&MRC Australian childhood immunisation schedule

From the Australian Immunisation Handbook, 6th Edition, 1997

| AGE | VACCINES ADMINISTERED |
|---|---|
| 2months | Acellular pertussis triple antigen (DTPa)<br>Hib conjugate vaccine<br>Oral polio |
| 4 months | Acellular pertussis triple antigen (DTPa)<br>Hib conjugate vaccine<br>Oral polio |
| 6months | Acellular pertussis triple antigen (DTPa)<br>Hib conjugate vaccine<br>Oral polio |
| 12 months | Measles- mumps- rubella |
| 18 months | Acellular pertussis triple antigen (DTPa)<br>Hib conjugate vaccine |

## Table 8.2 Description of different cohorts of infants recruited for human studies of antibody responses to childhood immunisations

| Cohort | Premature /Term | Numbers | DTP formulation administered | Serum sample collection times |
|---|---|---|---|---|
| **1a*** | Premature | 42 | DTPa at 2, 4, 6,and 18m | 2, 7, 18 and 19m |
| **1b*** | Term | 54 | DTPa at 2, 4, 6,and 18m | 2, 7, 18 and 19m |
| **2*** | Premature | 39 | DTPa at 2, 4, 6,and 18m | 18 and 19m |
| **C†** | Premature | 11 | DTPw at 2, 4,and 6m, DTPa at 18 m | 2, 8, 18 and 19m |
| **D†** | Term | 11 | DTPw at 2, 4,and 6m, DTPa at 18 m | 2, 8, 18 and 19m |

*Infants newly recruited by the principal investigator

†Infants who participated in a previous study performed in the University of Adelaide Department of Paediatrics: consent obtained from parent(s)/guardian(s) for use of serum samples in other studies

## Table 8.3: Recruitment criteria

| Medical exclusion criteria | |
|---|---|
| 1. | a chromosomal abnormality |
| 2. | a congenital or acquired immunodeficiency syndrome |
| 3. | a major congenital abnormality [1] |
| 4. | serious chronic illness[2] |
| 5. | a history of convulsions or epilepsy[3] |
| 6. | a history of pertussis, diphtheria, tetanus, polio, or *Haemophilus influenzae* type b infection or immunisation prior to recruitment |
| 7. | a history of intravenous gamma globulin therapy or immunosuppressive therapy prior to recruitment[4] |

| Socio-demographic exclusion criteria | |
|---|---|
| 1. | Parents unable to understand English |
| 2. | Parents not accessible by telephone |
| 3. | Parents unable to attend the Women's and Children's Hospital for appointments |
| 4. | Families with no fixed address, or a history of use of drugs of dependence, domestic violence, or child abuse |

[1]One infant with surgically corrected congenital heart disease (a double aortic arch, an atrial septal defect and a ventricular septal defect) was recruited at the request of his parents.

[2]Infants with bronchopulmonary dysplasia were not excluded from participation in the study.

[3]The NH&MRC Immunisation handbook (6th edition) states that infants with active or progressive neurological disease should not receive pertussis-containing vaccines

[4]Infants receiving corticosteroid therapy for the treatment of bronchopulmonary dysplasia were immunised 14 days after the cessation of steroid therapy.

**Table 8.4: Outline of study procedures for first study**

| Contact | VISIT 1 | VISIT 2 | VISIT 3 | VISIT 4* | VISIT 5 | VISIT 6 |
|---|---|---|---|---|---|---|
| Age | 7-11 weeks | 15-19 weeks | 23-27 weeks | 27-31 weeks | 17-19 months | 19-21 months |
| Informed Consent | ● | | | | | |
| Check selection criteria | ● | ● | ● | ● | ● | ● |
| Medical History | ● | ● | ● | ● | ● | ● |
| Physical examination | ● | ● | ● | ● | ● | ● |
| Blood sampling | ● | | | ● | ● | ● |
| Recording of medication usage | ● | ● | ● | ● | ● | ● |
| Vaccination (DTPa, Hib conjugate, oral polio†) | ● | ● | ● | ● | No polio vaccination ● | |
| Recording of medication usage | ● | ● | ● | ● | ● | ● |

*telephone contact between visits 4 and 5 to ensure that MMR vaccination is given; offer of extra Hib vaccination at 12 months and 2 extra blood samples at 12 and 13 months of age if Hib anti-PRP IgG levels low.

† Oral polio vaccine was administered only if child was not an inpatient at the time of immunisation

**Table 8.5: Outline of study procedures for second study**

| | VISIT 1 | VISIT 2 |
|---|---|---|
| **Age** | 17-19 months | 19-21 months |
| Informed Consent | ● | |
| Check selection criteria | ● | ● |
| Medical History | ● | ● |
| Physical examination | ● | ● |
| Blood sampling | ● | ● |
| Recording of medication usage | ● | ● |
| Vaccination (DTPa, Hib conjugate) | ● | |
| Recording of medication usage | ● | ● |

## Table 8.6: Details of vaccines administered to study participants

| VACCINE | COMPOSITION |
|---|---|
| **DTPa**<br>Infanrix® (SmithKline Beecham) | -Triple antigen vaccine with acellular pertussis component<br>-Not less than 25Lf of Diphtheria toxoid, 10Lf of Tetanus toxoid, 25 µg Pertussis Toxoid, 25µg Filamentous Haemagglutinin, 8 µg Pertactin,<br>-Sodium phenoxyethanol 2.5 mg preservative, -Aluminium hydroxide adjuvant<br>-Sodium chloride 4.5mg and water for injections to 0.5 ml. |
| **Hib**<br>Hib Titer® (Lederle Laboratories) | -10 µg Eagen *Haemophilus influenzae* type b strain polysaccharide (PRP)conjugated with a mutant diphtheria toxin ($CRM_{197}$) in 0.5 ml |
| **Oral Polio (Sabin)**<br>(SmithKline Beecham) | -Live, attenuated viral strains 1, 2 and 3<br>-Neomycin B sulphate 5µg per 2 drop dose |
| **MMR (measles, mumps rubella)**<br>Priorix® (SmithKline Beecham) | -Lyophilised, live attenuated measles virus (Schwartz strain), RIT 4385 mumps virus strain (derived from the Jeryl Lynn strain), Wistar RA 27/3 rubella virus strain<br>-Lactose, neomycin sulphate, albumin<br>-Sorbitol and mannitol as stabilisers<br>-Reconstituted with 0.5 ml sterile water for injection |

## Table 8.7: Pertussis enzyme immunoassays: summary

| | Pertussis Toxin | Filamentous Haemagglutinin | Pertactin |
|---|---|---|---|
| **COATING** | | | |
| Plates | Maxisorp | Polysorp | Maxisorp |
| Antigen concentration | 0.5µg/ml (1:100) | 0.5µg/ml (1:100) | 1.0µg/ml (1:20) |
| Incubation time/temp | 60 minutes at 37$^0$C | 60 minutes at 37$^0$C | 90 minutes at 37$^0$C |
| **BLOCKING** | | | |
| Reagent | 1%NFM[?]-PBS[?] | 1%NFM-PBS | 1%NFM-PBS |
| Incubation time/temp | 60 minutes at 37$^0$C | 60 minutes at 37$^0$C | 60 minutes at 37$^0$C |
| **SERA SAMPLES** | | | |
| Diluent | 1%NFM-PBST[¦] | 1%NFM-PBST | 1%NFM-PBST |
| **Reference sera** | | | |
| assigned concentration | USFDA[?] lot 3<br>200U | Pooled infant serum<br>200U | USFDA lot 4<br>90U |
| initial dilution factor | 1:50 | 1:200 | 1:50 |
| External QC<br>assigned concentration | NIBSC89/530<br>200 U/ml | NIBSC 89/530<br>200 U/ml | NIBSC 89/530<br>90 U/ml |
| High QC<br>assigned concentration | Pooled infant serum*<br>93.7U | USFDA lot 3<br>200U | Pooled infant serum*<br>478U |
| Low QC<br>assigned concentration | Pooled adult serum†<br>11.2U | Pooled adult serum<br>77.3U | Pooled adult serum<br>207.8U |
| Incubation time/temp | 60 minutes at 37$^0$C | 60 minutes at 37$^0$C | 60 minutes at 37$^0$C |
| **HRP-LABELLED CONJUGATE** | | | |
| Type | Silenus | Silenus | Silenus |
| Concentration | 1:1000 | 1:1000 | 1:1000 |
| Incubation time/temp | 45 minutes at 37$^0$C | 30 minutes at 37$^0$C | 30 minutes at 37$^0$C |

*The pooled infant serum comprised sera from eight study participants collected at 19 months

†The pooled adult serum comprised sera from five adult volunteers in the Department of Paediatrics laboratory

NIBSC *B. pertussis* human antiserum (catalogue number 89/530)

[?] United States Federal Drug Authority

[?] Non fat milk

[?] Phosphate buffered saline

[¦] Phosphate buffered saline-20% Tween

## Table 8.8 Diphtheria, Tetanus and Hib PRP ELISAs: summary

| | Diphtheria | Tetanus | Hib PRP |
|---|---|---|---|
| **COATING** | | | |
| Plates | Polysorp | Polysorp | Costar |
| Antigen concentration | 3.6 Lf/ml (1:1000) | 2.155 Lf/ml 1:1000) | 1µg/ml |
| Incubation time/temp | 60 minutes at 37$^0$C | 60 minutes at 37$^0$C | 90 minutes at 37$^0$C and overnight at 4$^0$C |
| **BLOCKING** | | | |
| Reagent | no blocking step | no blocking step | 1% Casein/PBST |
| Incubation time/temp | - | - | 120 minutes at 37$^0$C |
| SERA SAMPLES | | | |
| Diluent | PBST pH 7.2 | PBST pH 7.2 | 1% Casein/PBST |
| **Reference Sera** | | | |
| assigned concentration<br>initial dilution factor | Pooled adult serum*<br>6.3IU/ml<br>1:50 | Pooled adult serum<br>2.5IU/ml<br>1:50 | USFDA lot 1983†<br>60.9µg/ml<br>1:100 |
| External QC assigned concentration | NIBSC<br>1IU/ml | NIBSC<br>1IU/ml | - |
| High QC<br>assigned concentration | Pooled human serum♠<br>2.05IU | Pooled adult serum from NCH #1‡<br>5.03IU | Adult volunteer (post immunisation sample)<br>4µg/ml |
| Low QC<br>assigned concentration | Pooled adult serum from NCH #2<br>0.85IU | Pooled human serum<br>0.92IU | Pooled adult serum*<br>0.7µg/ml |
| Incubation time/temp | 60 minutes at 37$^0$C | 60 minutes at 37$^0$C | Four hours at room temperature |
| **HRP-LABELLED CONJUGATE** | | | |
| Type | Silenus | Silenus | Silenus |
| Concentration | 1:1000 (PBST) | 1:1000 (PBST) | 1:1000 1%Casein/PBST |
| Incubation time/temp | 45 minutes at 37$^0$C | 30 minutes at 37$^0$C | 60 minutes at 37$^0$C |

* The pooled adult serum comprised samples from five adult volunteers in the WCH laboratories
†USFDA human anti-Hib capsular polysaccharide serum lot 1983
♠The pooled human serum comprised samples from infants 8-19 months of age enrolled in a previous trial conducted in the University of Adelaide department of Paediatrics
‡ One of two pooled adult sera supplied by Mr M Hanlon, in the Department of Immunology and Infectious Diseases, New Children's Hospital, New South Wales, Australia

## Table 8.9 Demographic description of term and premature infant study populations

| Parameter | Term infants n=54 | Premature infants n=42 |
|---|---|---|
| **Male:Female ratio** | 1.25 | 0.91 |
| **Average birth weight (grams)** (range) | 3470 (2000-4640) | 1638 (620-3260) |
| **Average birth length (cm)** (range) | 50.2 (43-55) | 39.6 (30-48) |
| **Average birth head circumference (cm)** (range) | 34.8 (31-43) | 28.3 (20-35) |
| **Breast feeding (%)** | 83.3 | 81.0 |
| **Average duration of breast feeding (months)** (range) | 4.8 (0-14) | 3.7 (0-11) |
| **Number of twin births** (sex) | 2 (f/f, m/f) | 4 (f/f, f/f, f/f, m/f) |
| **Intrauterine growth restriction (%)** | 5.5 | 14.3 |
| **Antenatal steroids (%)** | 0 | 38.1 |
| **Postnatal steroids (%)** **Average duration (days)** (range) | 0 0 (0-0) | 33.3 9.2 (0-33) |
| **Blood transfusions prior to immunisation** (% infants) **Average number transfusions** (range) | 0 0 (0-0) | 45.2 5.9 (0-16) |
| **Average stay in hospital (days)** (range) | 4.7 (1-22) | 51* (3-113)* |
| **Average time in NICU (hours)** (range) | 0 (0-0) | 455 (0-1998) |
| **Average time in SCBU (hours)** (range) | 31 (0-144) | 761 (24-1448) |
| **Private health insurance (%)** | 11.1 | 38.1 |
| **Average maternal age at delivery (years)** (range) | 30.7 (18-41) | 30.5 (21-41) |
| **Level of maternal education (%)** **-tertiary** **-secondary** **-<secondary** | 29.6 22.2 48.2 | 45.2 26.2 28.6 |
| **Maternal Country of Origin (%)** **Australia-indigenous** **-non-indigenous** **Other countries** | 1.9 83.3 14.8 | 4.8 88.1 7.1 |

* Total time in hospital does not include time after transfer from the WCH to a SCBU in another hospital.

## Table 8.10 Demographic description of premature infant subgroup study populations

| Parameter | Extremely premature n=11 | Very premature n=13 | Premature n=18 |
|---|---|---|---|
| **Male:Female ratio** | 0.83 | 1.17 | 0.80 |
| **Average birth weight (grams)** (range) | 774 (620-1000) | 1276 (800-2060) | 2426 (1620-2880) |
| **Average birth length (cm)** (range) | 32.6 (30-36.5) | 37.7 (32.5-43) | 45.4 (41.5-50) |
| **Average birth head circumference (cm)** (range) | 23.7 (21.5-26) | 27 (20-31.5) | 32 (29.5-34.5) |
| **Breast feeding (%)** | 72.7 | 84.6 | 83.3 |
| **Average duration of breast feeding (months)** (range) | 3.5 (0-11) | 3.2 (0-6) | 3.3 (0-10.5) |
| **Number of twin births** (sex) | 1 (f/f) | 2 (f/f, m/f) | 1 (f/f) |
| **Intrauterine growth restriction (%)** | 18.2 | 15.4 | 11.8 |
| **Antenatal steroids (%)** | 45.5 | 69.2 | 11.1 |
| **Postnatal steroids (%)**<br>**Average duration (days)** (range) | 81.8<br>12 (0-33) | 38.5<br>4.8 (0-10) | 0<br>0 (0-0) |
| **Blood transfusions prior to immunisation** (% infants)<br>**Average number transfusions** (range) | 91.0<br>7.3 (0-16) | 61.5<br>2.7 (0-7) | 5.6 (1 infant)<br>(1 transfusion) |
| **Average stay in hospital (days)** (range) | 94.8 (63-113) | 61.6 (41-86) | 16.8 (3-42) |
| **Average time in NICU (hours)** (range) | 1065 (0-1992) | 522 (17-1176) | 36 (0-168) |
| **Average time in SCBU (hours)** (range) | 1194 (720-2112) | 1420 (576-2064) | 399 (24-1008) |
| **Private health insurance (%)** | 45.5 | 46.2 | 27.8 |
| **Average maternal age at delivery (years)** (range) | 29.4 (22-39) | 31.5 (21-40) | 30.5 (23-38) |
| **Level of maternal education (%)**<br>**-tertiary**<br>**-secondary**<br>**-<secondary** | <br>45.5<br>27.3<br>27.2 | <br>38.4<br>30.8<br>30.8 | <br>50.0<br>22.2<br>27.8 |
| **Maternal Country of Origin (%)**<br>**Australia-indigenous**<br>**-non-indigenous**<br>**Other countries** | <br>0<br>91<br>9 | <br>0<br>92.3<br>7.7 | <br>5.6<br>88.8<br>5.6 |

## Table 8.11 Comparison between term infant and premature infant subgroup antibody responses to pertussis antigens

Highlighted numbers are those for which comparisons not significant ($p<0.05$, Student's independent t-test)

| | PT | | | FHA | | | PRN | | |
|---|---|---|---|---|---|---|---|---|---|
| | **extremely premature** | **very premature** | **premature** | **extremely premature** | **very premature** | **premature** | **extremely premature** | **very premature** | **premature** |
| **2m** | 0.002 | 0.003 | 0.010 | 0.118 | <0.001 | 0.007 | **0.401** | 0.011 | 0.001 |
| **7m** | 0.021 | **0.340** | **0.459** | <0.001 | 0.034 | **0.083** | 0.003 | 0.003 | **0.089** |
| **18m** | 0.001 | <0.001 | 0.006 | <0.001 | 0.022 | **0.195** | 0.007 | 0.015 | **0.426** |
| **19m** | 0.005 | 0.046 | **0.098** | <0.001 | **0.182** | **0.338** | <0.001 | **0.182** | **0.828** |

## Table 8.12 Comparison of IgG PT, FHA and PRN antibody levels between premature infant subgroups

| | 2 months | | | 7 months | | | 18 months | | | 19 months | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **PERTUSSIS TOXIN (PT)** | **Ext prem** | **Very prem** | **Prem** | **Ext prem** | **Very prem** | **Prem** | **Ext prem** | **Very prem** | **Prem** | **Ext prem** | **Very prem** | **Prem** |
| **Sample size** | 11 | 13 | 18 | 11 | 13 | 17 | 11 | 13 | 16 | 11 | 13 | 16 |
| **Geometric Mean Concentration (GMC)** | 1.09 | 1.02 | 1.33 | 17.94 | 26.99 | 29.70 | 2.14 | 2.68 | 3.48 | 38.39 | 52.02 | 46.93 |
| **95% confidence intervals for GMC** | 0.74-1.60 | 0.62-1.69 | 0.86-2.05 | 11.28-28.53 | 16.54-44.03 | 20.00-44.09 | 1.31-3.51 | 0.60-0.77 | 2.37-5.11 | 36.70-55.18 | 38.87-69.62 | 27.74-79.40 |
| **ANOVA p value** | **p=0.679** | | | **p=0.285** | | | **p=0.325** | | | **p=0.635** | | |
| | **2 months** | | | **7 months** | | | **18 months** | | | **19 months** | | |
| **FILAMENTOUS HAEMAGGLUTININ (FHA)** | **Ext prem** | **Very prem** | **Prem** | **Ext prem** | **Very prem** | **Prem** | **Ext prem** | **Very prem** | **Prem** | **Ext prem** | **Very prem** | **Prem** |
| **Sample size** | 11 | 13 | 18 | 11 | 13 | 17 | 11 | 13 | 16 | 11 | 13 | 16 |
| **Geometric Mean Concentration (GMC)** | 2.90 | 1.87 | 2.44 | 19.61 | 37.54 | 46.44 | 3.33 | 4.71 | 7.69 | 128.32 | 216.82 | 290.95 |
| **95% confidence intervals for GMC** | 11.60-5.24 | 1.28-2.73 | 1.59-3.74 | 14.57-26.40 | 21.51-65.51 | 28.34-76.10 | 2.27-4.88 | 2.30-9.62 | 4.14-14.28 | 83.44-197.33 | 93.85-500.91 | 158.05-535.61 |
| **ANOVA p value** | **p=0.464** | | | **p=0.057*** | | | **p=0.187*** | | | **p=0.278*** | | |
| | **2 months** | | | **7 months** | | | **18 months** | | | **19 months** | | |
| **PERTACTIN (PRN)** | **Ext prem** | **Very prem** | **Prem** | **Ext prem** | **Very prem** | **Prem** | **Ext prem** | **Very prem** | **Prem** | **Ext prem** | **Very prem** | **Prem** |
| **Sample size** | 11 | 13 | 18 | 11 | 13 | 17 | 11 | 13 | 16 | 11 | 13 | 16 |
| **Geometric Mean Concentration (GMC)** | 1.48 | 0.93 | 0.90 | 39.27 | 46.2 | 72.18 | 4.56 | 6.66 | 16.51 | 585.66 | 434.53 | 734.21 |
| **95% confidence intervals for GMC** | 0.62-3.52 | 0.54-1.57 | 0.62-1.30 | 23.76-64.90 | 24.72-16.10 | 48.42-107.60 | 1.91-10.91 | 3.19-13.89 | 10.20-26.71 | 272.93-1256.74 | 229.43-822.97 | 415.44-1297.56 |
| **ANOVA p value** | **p=0.430** | | | **p=0.135** | | | **p=0.041*** | | | **p=0.503** | | |

* Comparisons for which statistically significant differences existed between premature infant subgroups. These were found for comparisons between premature and extremely premature infant serological responses only, $p<0.05$ (Student's t-test: independent groups)

**Table 8.13 Comparison of IgG diphtheria antibody levels between premature infant subgroups**

| | 2 months | | | 7 months | | | 18 months | | | 19 months | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **DIPHTHERIA TOXOID** | **Ext prem** | **Very prem** | **Prem** | **Ext prem** | **Very prem** | **Prem** | **Ext prem** | **Very prem** | **Prem** | **Ext prem** | **Very prem** | **Prem** |
| **Sample size** | 11 | 13 | 18 | 11 | 13 | 17 | 11 | 13 | 16 | 11 | 13 | 16 |
| **Geometric Mean Concentration (GMC)** | 0.04 | 0.07 | 0.09 | 1.46 | 1.51 | 1.80 | 0.15 | 0.14 | 0.39 | 4.55 | 5.36 | 10.03 |
| **95% confidence intervals for GMC** | 0.03-0.06 | 0.05-0.11. | 0.05-0.15 | 0.85-2.53 | 0.93-2.44 | 1.15-2.83 | 0.06-0.41 | 0.05-0.39 | 0.24-0.63 | 1.97-10.49 | 2.37-12.14 | 5.60-17.80 |
| **ANOVA p value** | **p=0.141*** | | | **p=0.804** | | | **p=0.130** | | | **p=0.288** | | |

**Table 8.14 Comparison of IgG tetanus antibody levels between premature infant subgroups**

| | 2 months | | | 7 months | | | 18 months | | | 19 months | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **TETANUS TOXOID** | **Ext prem** | **Very prem** | **Prem** | **Ext prem** | **Very prem** | **Prem** | **Ext prem** | **Very prem** | **Prem** | **Ext prem** | **Very prem** | **Prem** |
| **Sample size** | 11 | 13 | 18 | 11 | 13 | 17 | 11 | 13 | 16 | 11 | 13 | 16 |
| **Geometric Mean Concentration (GMC)** | 0.20 | 0.22 | 0.47 | 0.74 | 1.01 | 1.19 | 0.16 | 0.15 | 0.24 | 3.41 | 3.62 | 5.23 |
| **95% confidence intervals for GMC** | 0.14-0.29 | 0.13-0.39 | 0.27-0.77 | 0.50-1.11 | 0.60-1.70 | 0.93-1.53 | 0.08-0.30 | 0.07-0.33 | 0.15-0.38 | 1.73-6.72 | 2.08-6.29 | 2.98-9.29 |
| **ANOVA p value** | **p=0.037**** | | | **p=0.238** | | | **p=0.490** | | | **p=0.565** | | |

*At 2 months a tendency to statistical significance existed between responses of premature and extremely premature infants (p=0.050, Student's t-test)

** At 2 months statistically significant differences existed between responses of premature and extremely premature infants (p=0.011, Student's t-test)

**Table 8.15 Comparison of IgG Hib PRP antibody levels between premature infant subgroups**

| | 2 months | | | 7 months | | | 18 months | | | 19 months | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **HIB PRP** | **Ext prem** | **Very prem** | **Prem** | **Ext prem** | **Very prem** | **Prem** | **Ext prem** | **Very prem** | **Prem** | **Ext prem** | **Very prem** | **Prem** |
| **Sample size** | 11 | 13 | 18 | 11 | 13 | 17 | 11 | 13 | 16 | 11 | 13 | 16 |
| **Geometric Mean Concentration (GMC)** | 0.19 | 0.36 | 0.48 | 1.67 | 1.59 | 6.74 | 0.62 | 0.77 | 0.75 | 8.59 | 26.80 | 26.57 |
| **95% confidence intervals for GMC** | 0.15-0.24 | 0.15-0.88 | 0.29-0.80 | 0.50-5.65 | 0.68-3.71 | 3.42-13.27 | 0.42-0.90 | 0.42-1.43 | 0.42-1.43 | 3.05-24.17 | 10.90-65,84 | 12.62-55.92 |
| **ANOVA p value** | **0.134*** | | | **0.035**** | | | **0.847** | | | **0.156** | | |

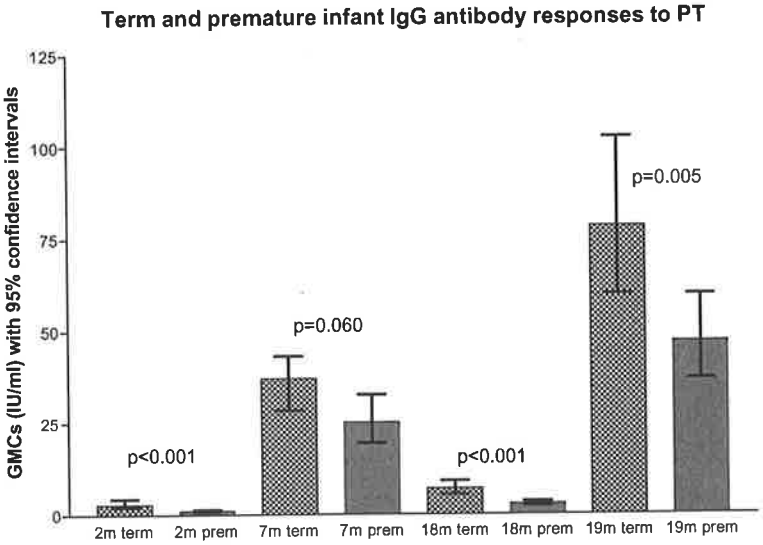
* At 2 months statistically significant differences were found between premature and extremely premature infants (p=0.004, Student's t-test)

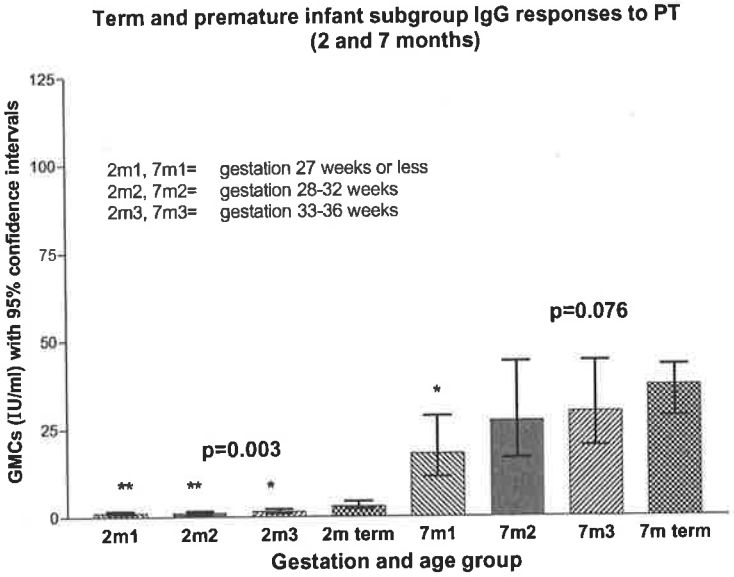
** At 7 months statistically significant differences were found between premature and very premature infants (p=0.016, Student's t-test)

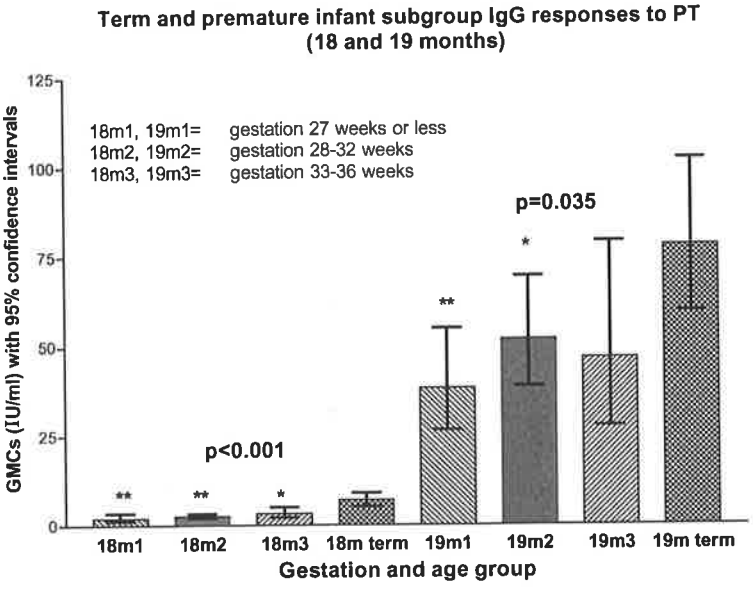
**Table 8.16 Summary of previous studies investigating premature and term infant responses to DTP immunisation**

| Author | Study numbers | Vaccine/Schedule | Comparative serology (premature vs term infants) after three doses | | | | |
|---|---|---|---|---|---|---|---|
| | | | PT | FHA | PRN | Diphtheria | Tetanus |
| **Schloessor et al Pediatrics, 1999** | 50 prems<br>46 terms | Bivalent DTPa at 2, 3, 4m or 2, 4, 6m (PT and FHA) | p<0.003 (term infant responses greater: EIA) | p<0.0001 (term infant responses greater: EIA) | not tested | p=0.0004 (term infant responses greater: EIA) | not tested |
| **Faldella et al Vaccine, 1998** | 34 prems<br>28 terms | Trivalent DTPa-HBV at 3,5,11m (PT, FHA and PRN) | p=0.15 | p=0.26 | p=0.0002 (term infant responses greater: EIA) | not tested | not tested |
| **D'Angio et al Pediatrics 1995** | 16 prems (< 29 weeks)<br>46 terms | DTPw at 2,4, 6m | not tested | not tested | not tested | not tested | p=0.630 |
| **Pullan and Hull Arch Dis Child 1989** | 50 prems<br>21 terms | DTPw at 3, 5, 9-12m | Prem infants separated by gestational age<br>26-27 weeks n=9, 28-31 weeks n=19, 32-36 weeks n=22<br>Pertussis: whole cell EIA, satisfactory responses in all infants: no formal statistical analyses<br>Diphtheria and tetanus: EIAs, satisfactory responses in all infants: no formal statistical analyses | | | | |
| **Koblin et al Pediatr Inf Dis J 1988** | 110 prems<br>146 terms | DTPw at 2, 4, 6m | *B. pertussis* agglutination assay and CHO cell assay results not significantly different between prems and terms | | | no significant differences, both antigens (agglutination) | |
| **Bernbaum et al J Pediatrics 1985** | 25 prems<br>38 terms | DTPw at 2, 4, 6m | *B. pertussis* agglutination assay results not significantly different between prems and terms | | | no significant differences, both antigens (toxin neutralisation) | |

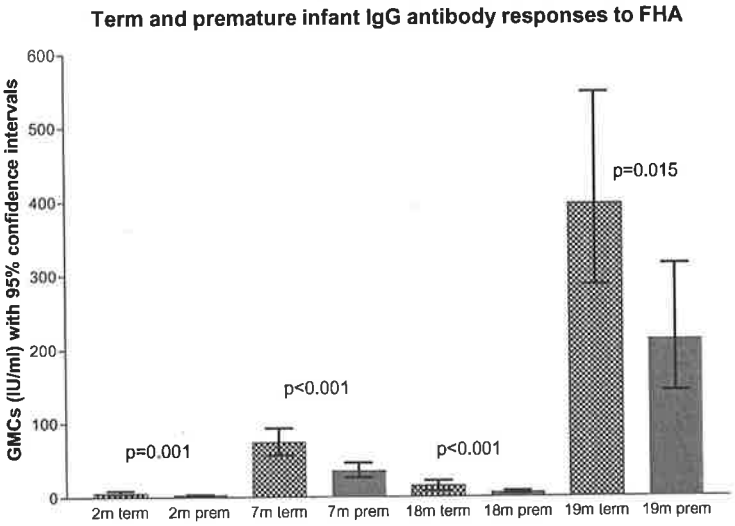
**Figures 8.1a, 8.1b. 8.1c** **Comparison of term and premature infant responses to PT**

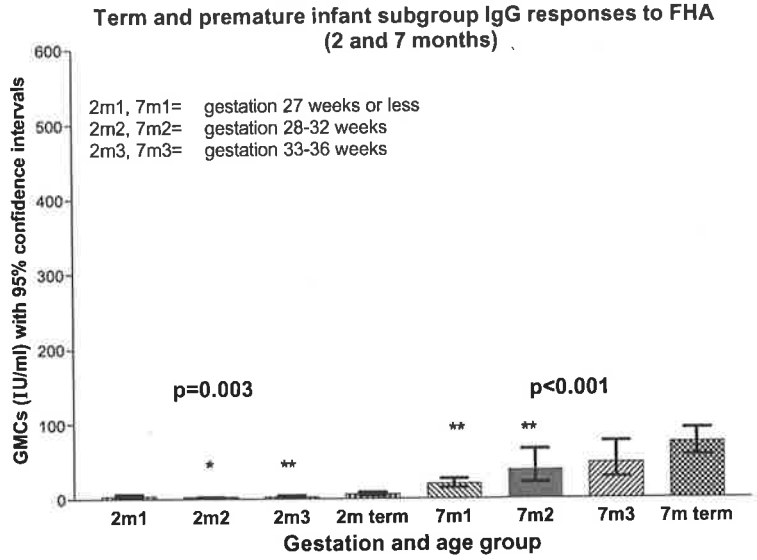


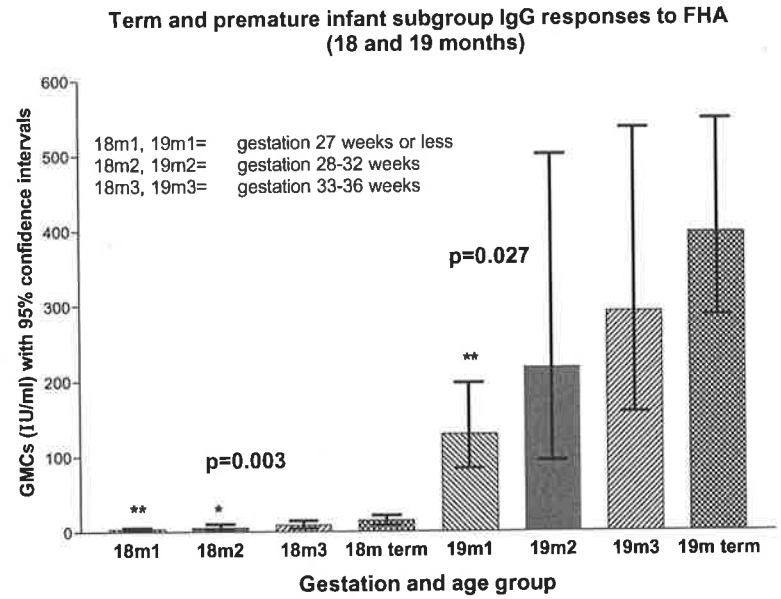



## Figures 8.2a, 8.2b. 8.2c Comparison of term and premature infant responses to FHA

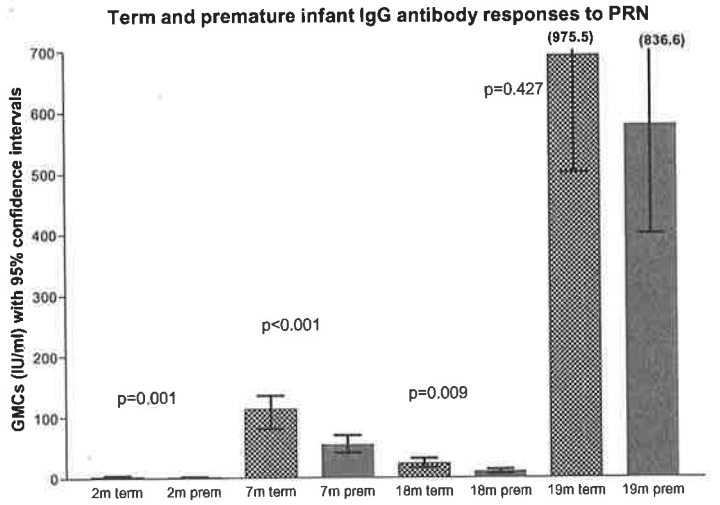


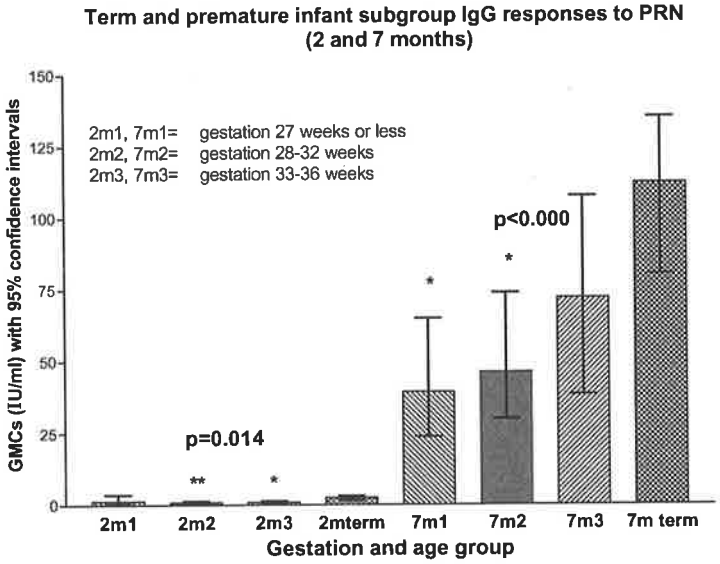



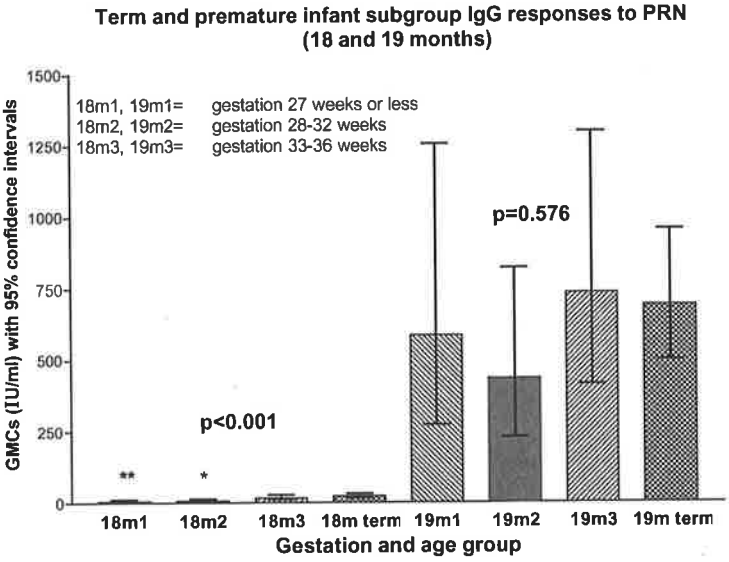



**Figures 8.3a, 8.3b, 8.3c** **Comparison of term and premature infant responses to PRN**

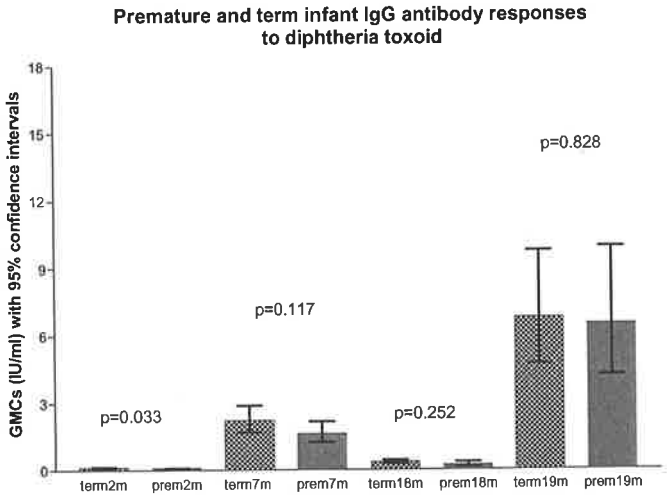
(note differences in x-axis scales)

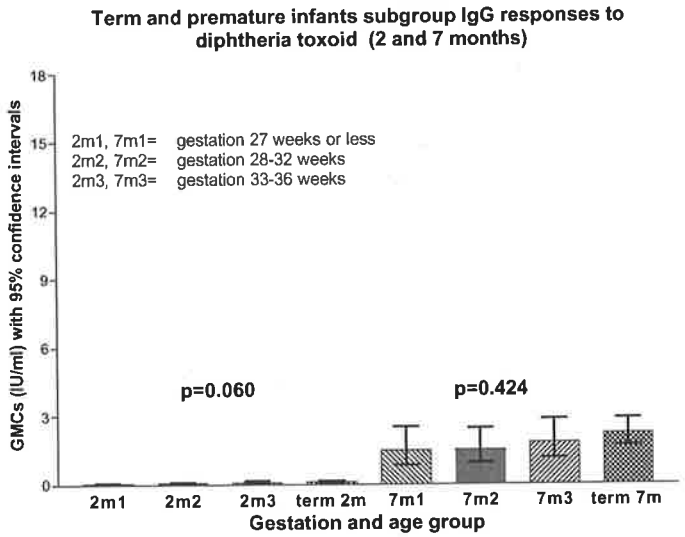


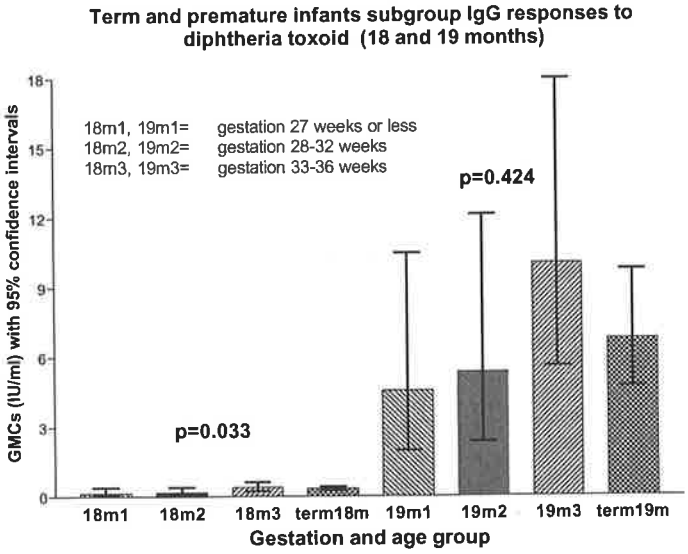



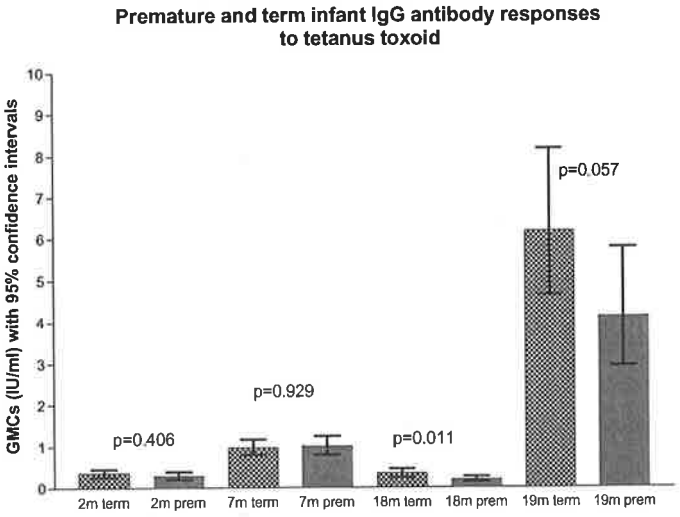
**Figures 8.4a, 8.4b, 8.4c** **Comparison of term and premature infant responses to diphtheria toxoid**

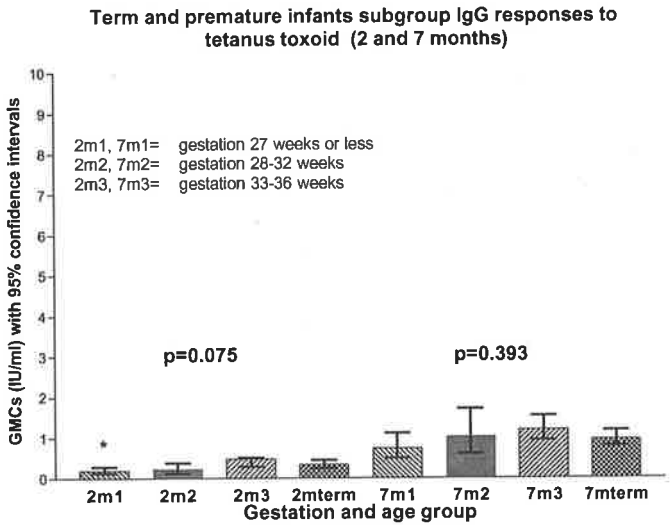


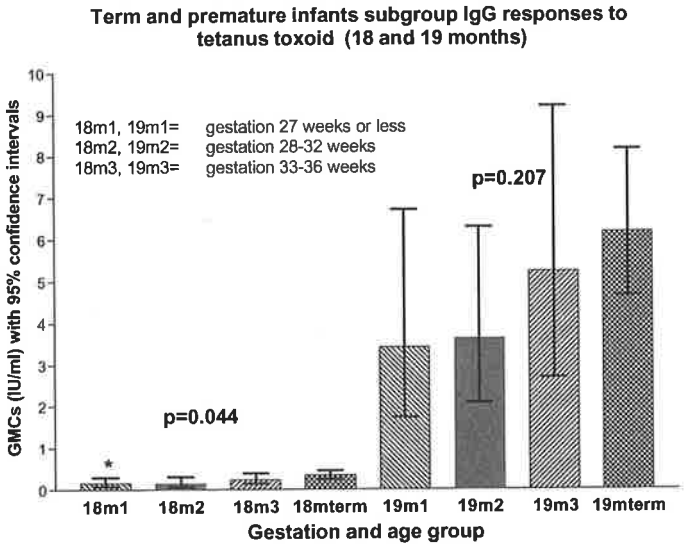



**Figures 8.5a, 8.5b, 8.5c** **Comparison of term and premature infant responses to tetanus toxoid**

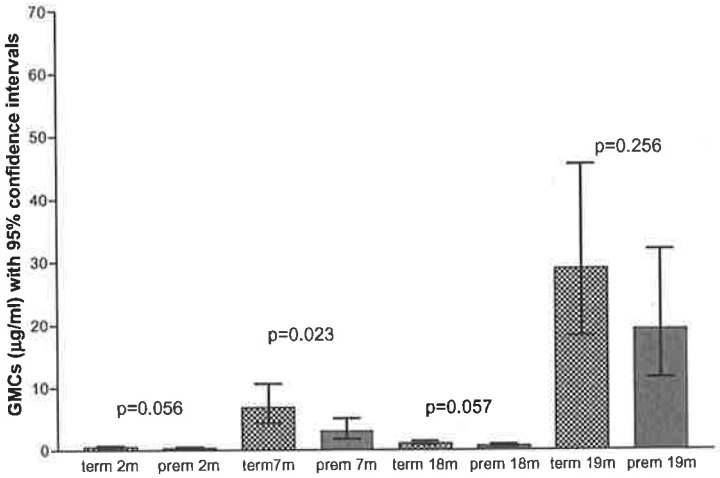


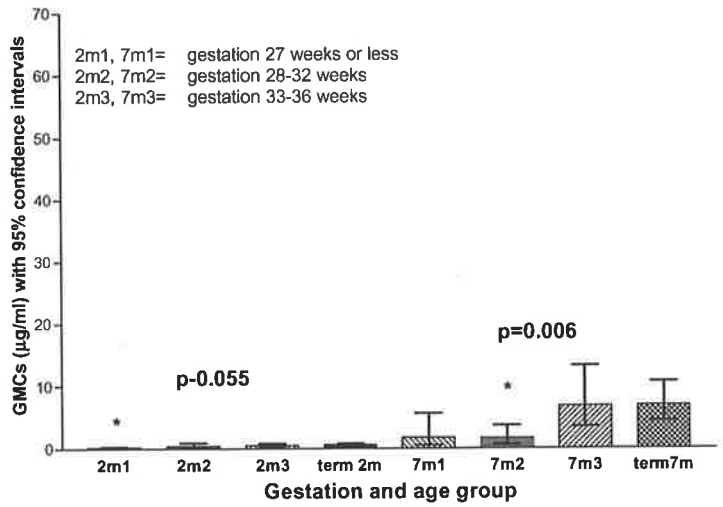



## Figures 8.6a 8.6b, 8.6c Comparison of term and premature infant responses to Hib PRP

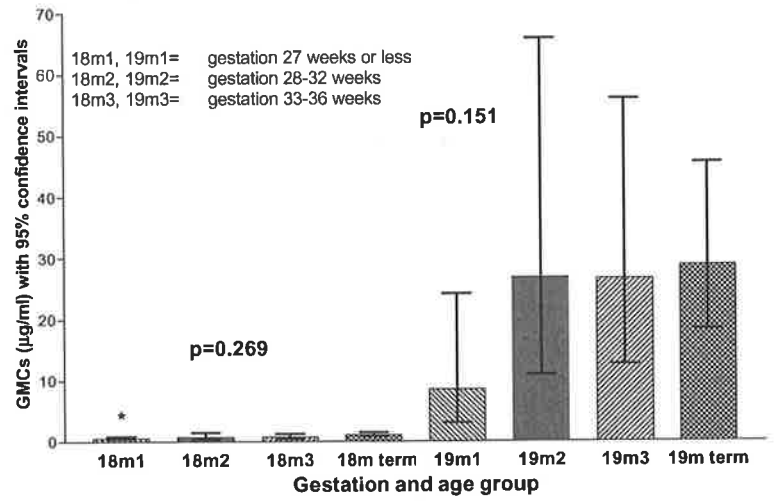
**Premature and term IgG antibody responses to Hib PRP**



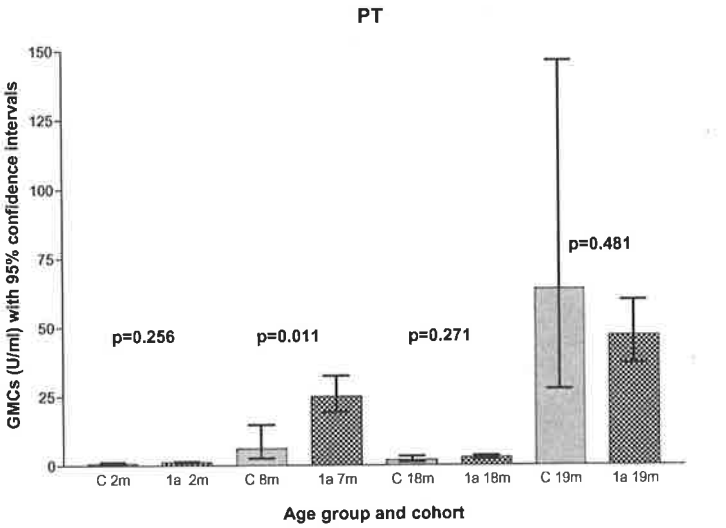

**Term and premature infant IgG responses to Hib PRP (2 and 7 months)**

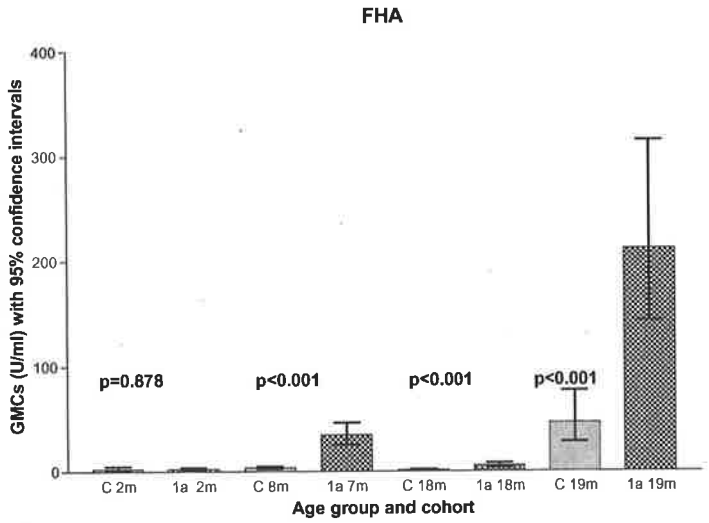


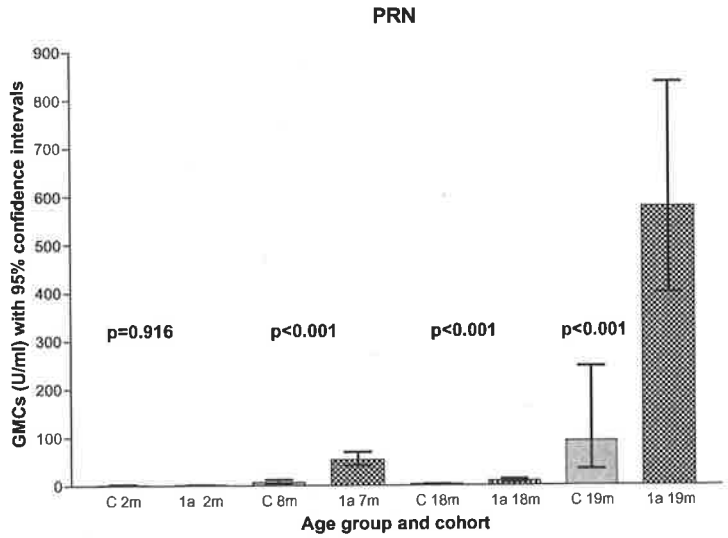

**Term and premature infant subgroup IgG responses to Hib PRP (18 and 19 months)**



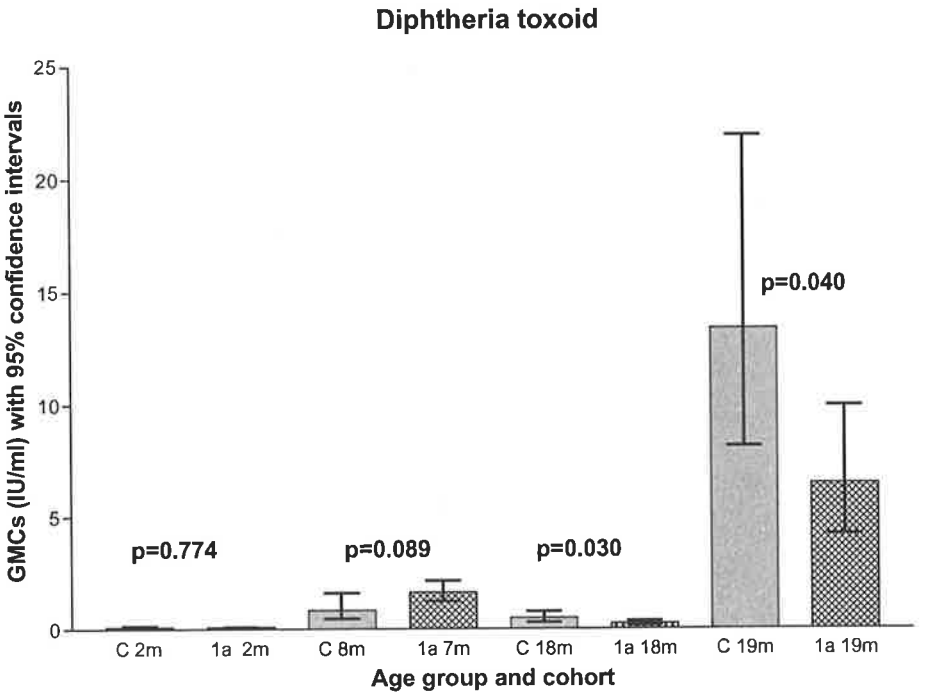
**Figures 8.7a-c Comparison of pertussis IgG antibody concentrations between cohorts premature infants in cohorts 1a (first study) and C**

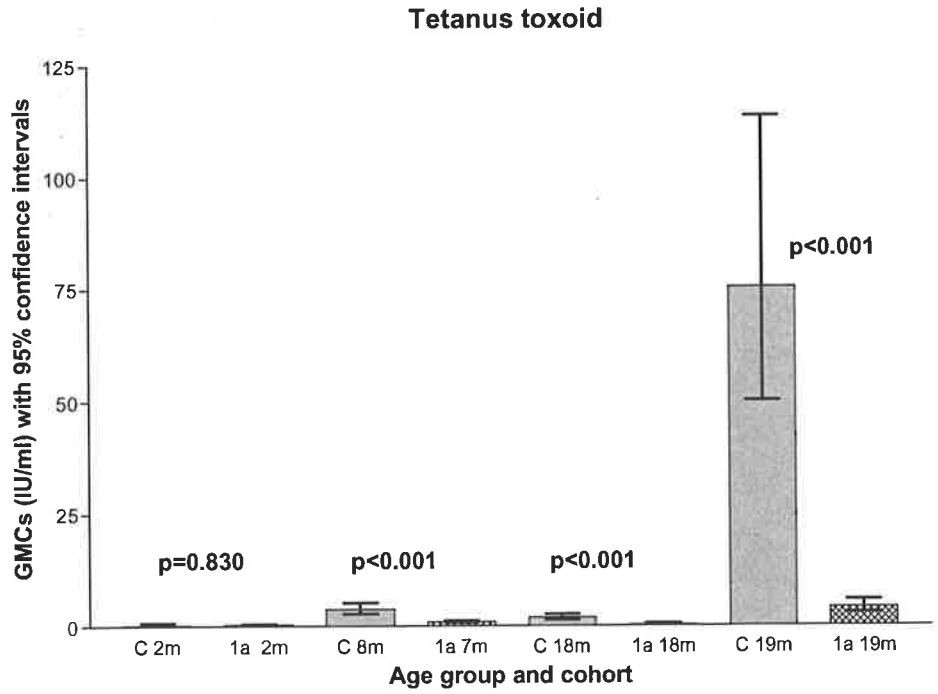


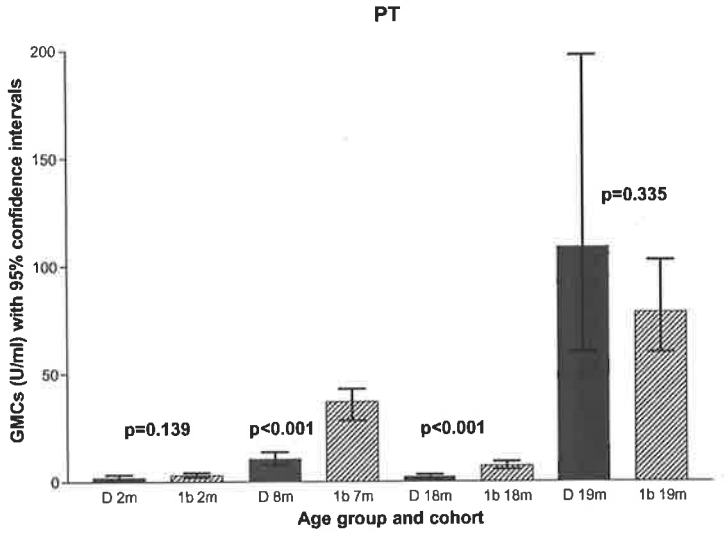



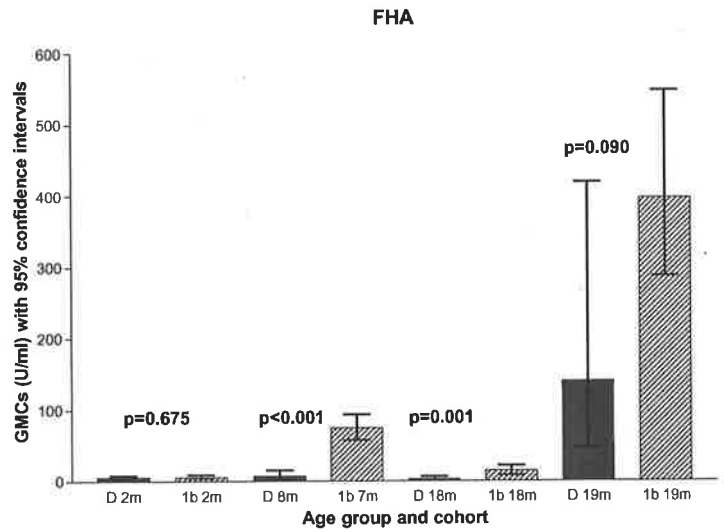
**Figures 8.8a and b** **Comparison of diphtheria and tetanus IgG antibody concentrations between premature infants in cohorts 1a (first study) and C**

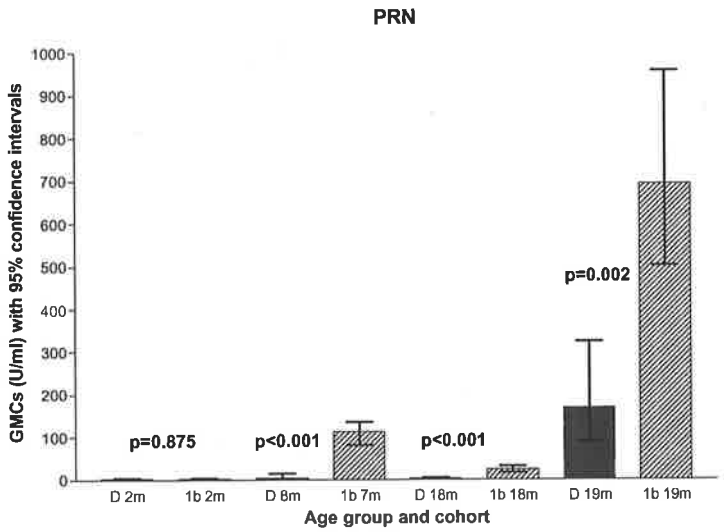


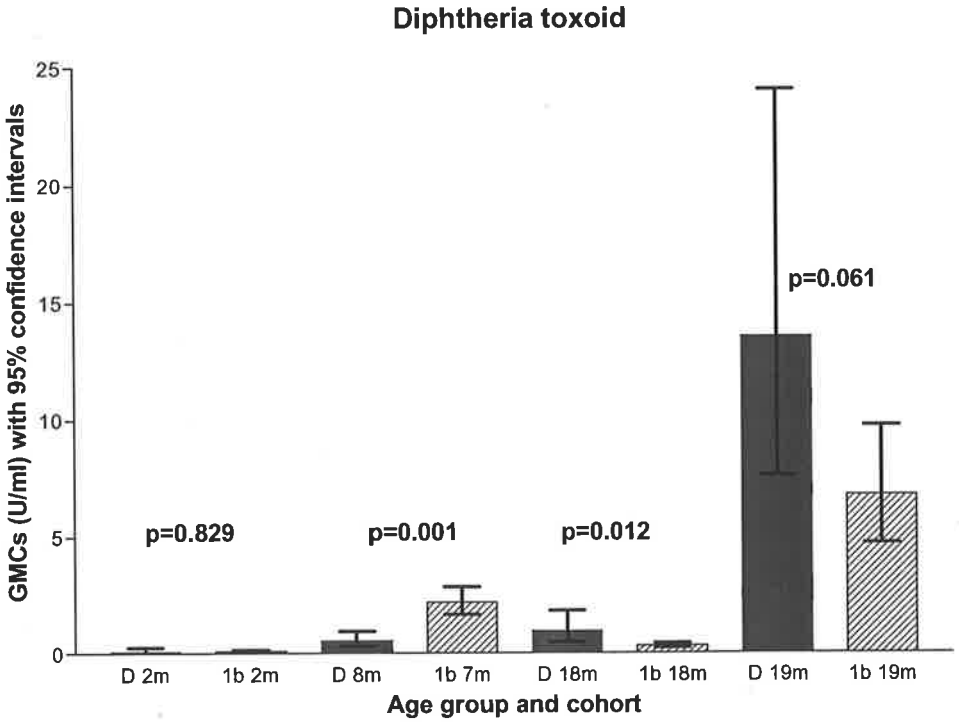
**Figures 8.9a-c Comparison of pertussis IgG antibody concentrations between term infants in cohorts 1b (first study) and D**

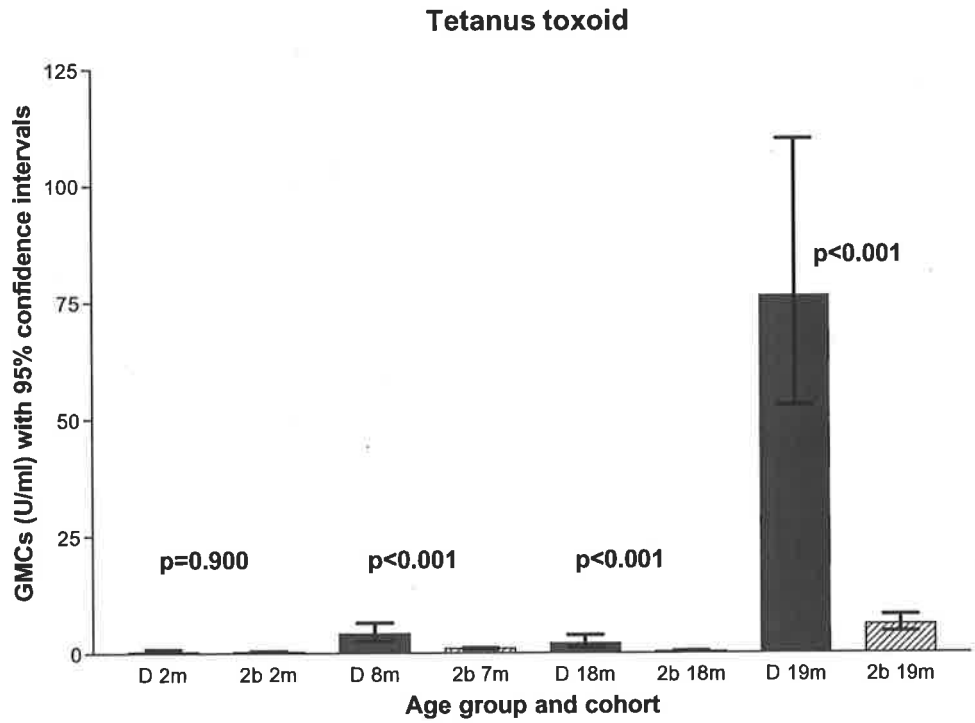


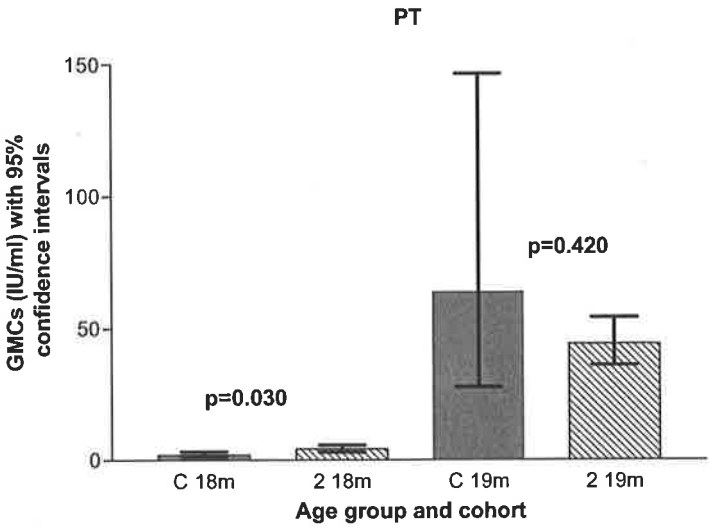



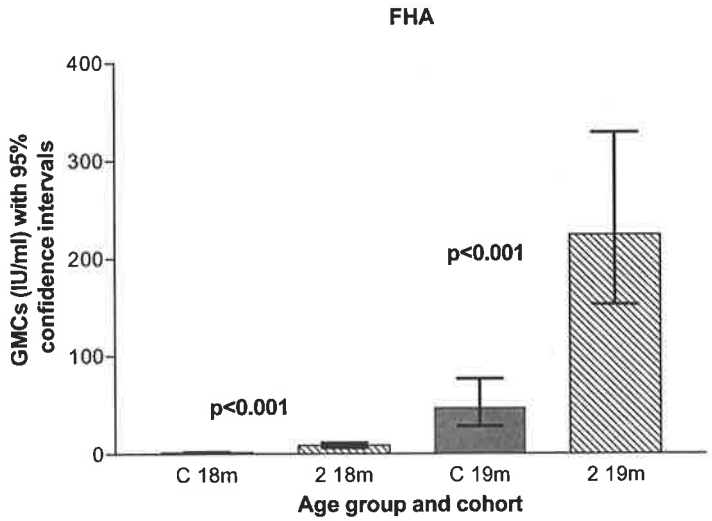
**Figures 8.10a and b Comparison of diphtheria and tetanus IgG antibody concentrations between term infants in cohorts 1b (first study) and D**

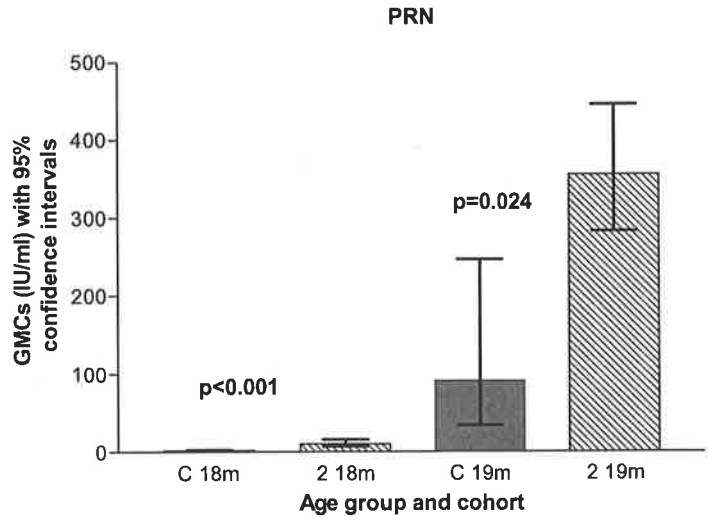


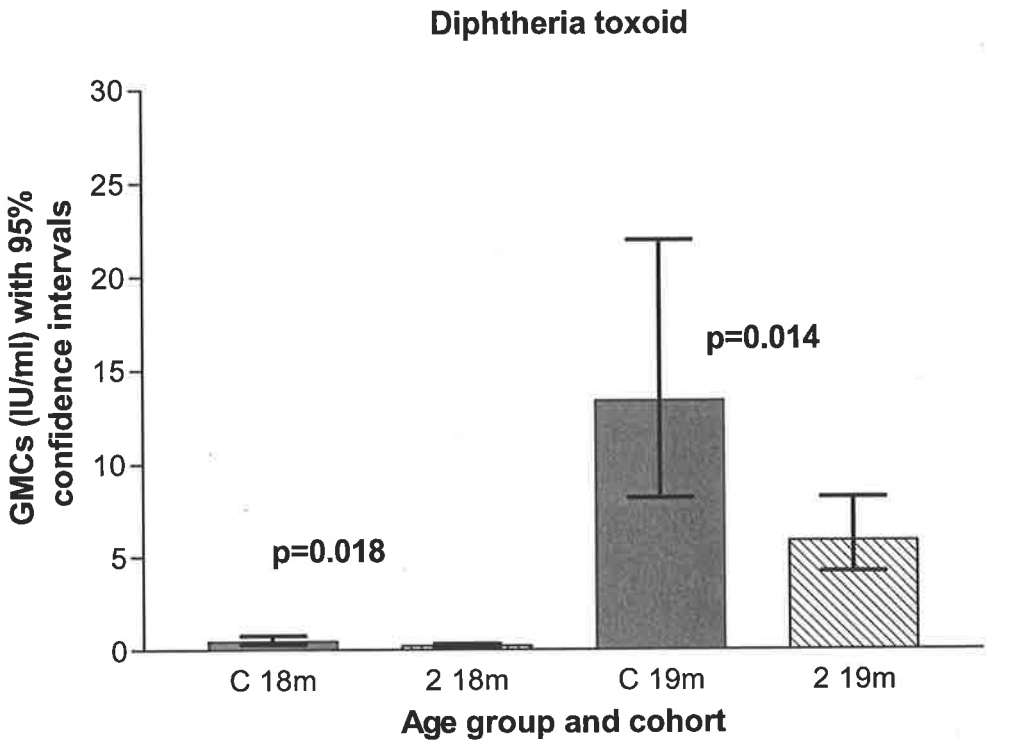
**Figures 8.11a-c Comparison of pertussis IgG antibody concentrations between cohorts 2 (second study) and C**

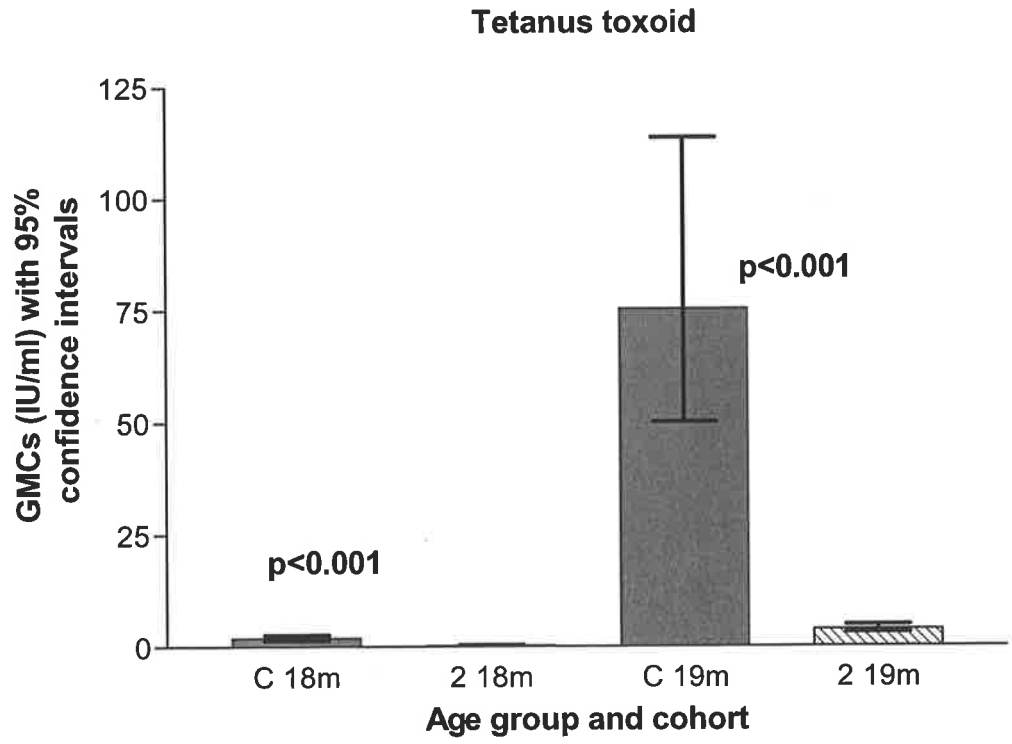


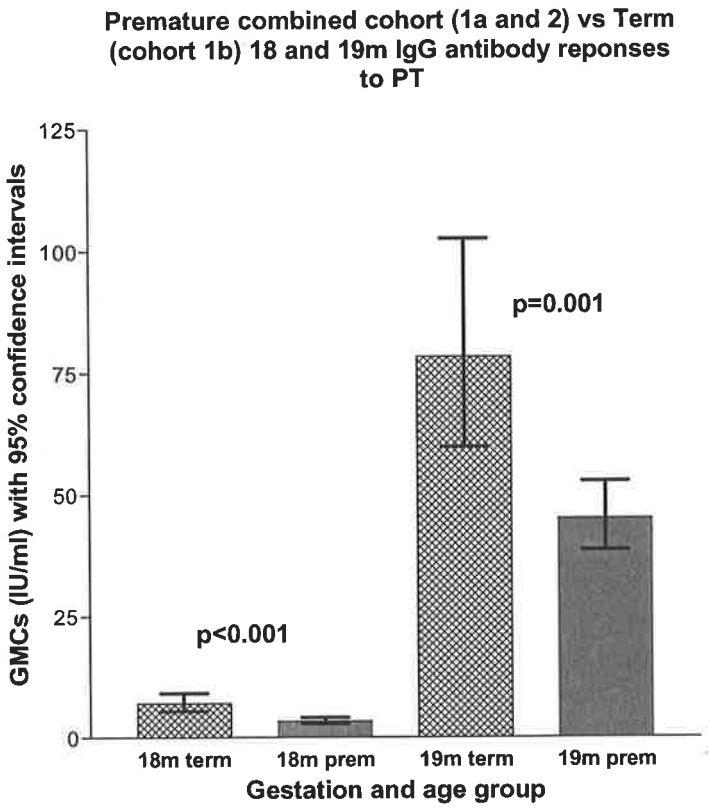



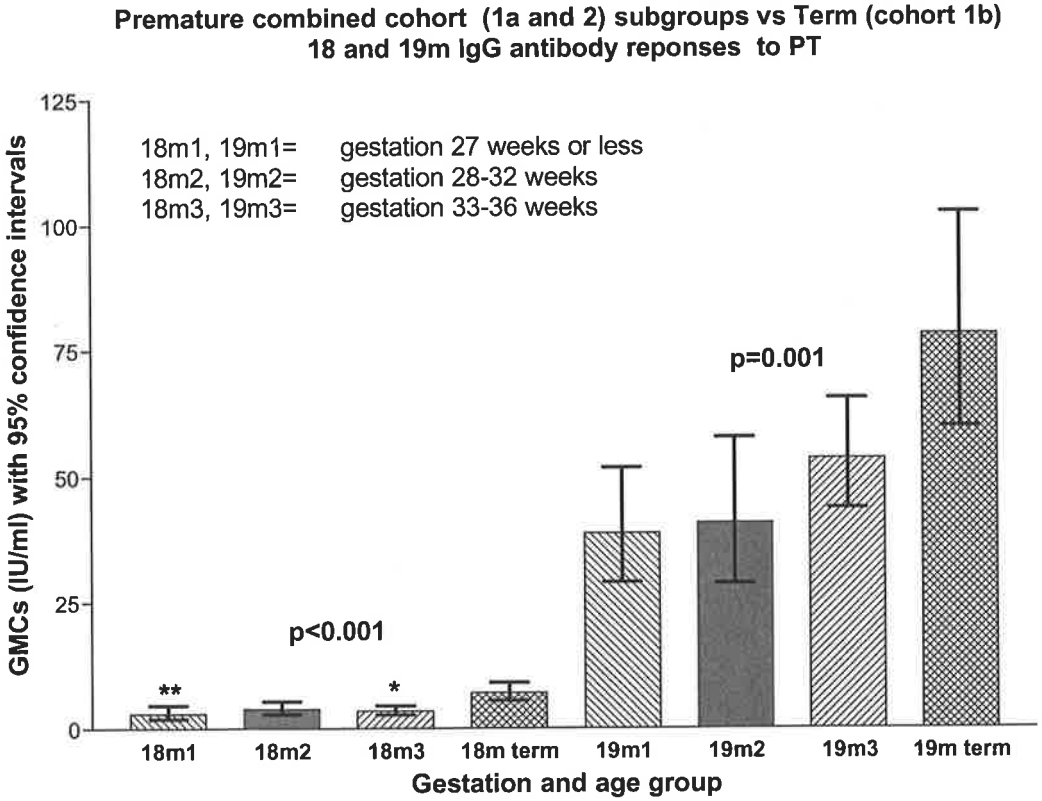
**Figures 8.12 a and b** **Comparison of diphtheria and tetanus IgG antibody concentrations between cohorts 2 (second study) and C**



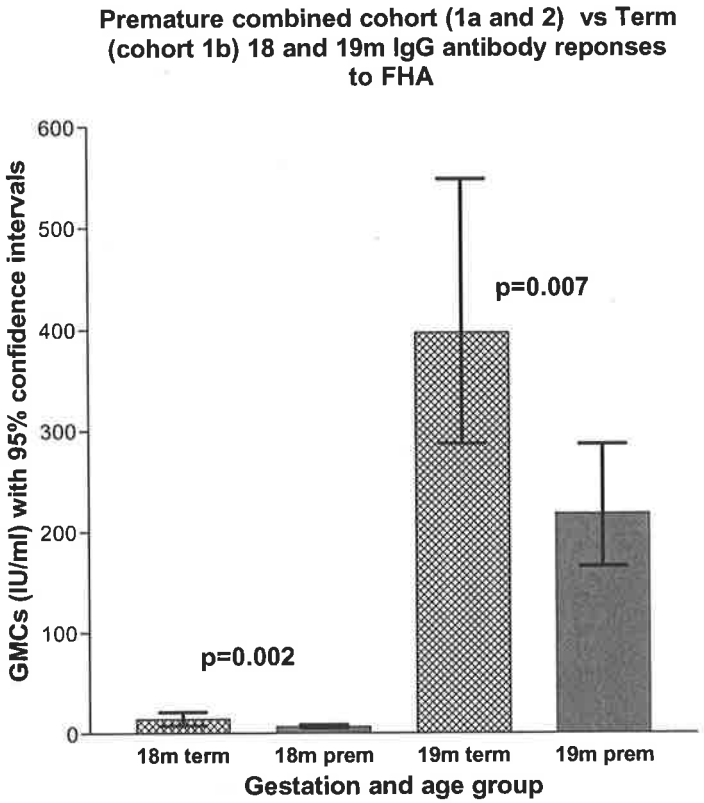



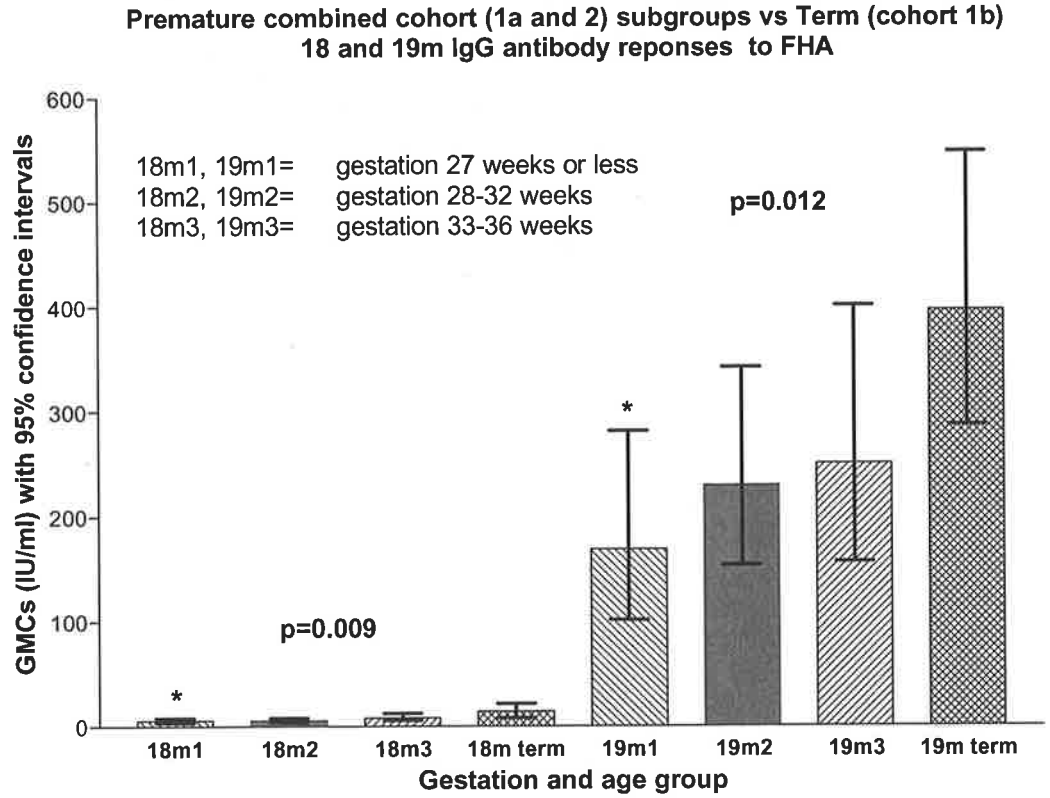
**Figures 8.13a and b** **Comparison between term and combined premature infant cohort (1a and 2) PT antibody concentrations**



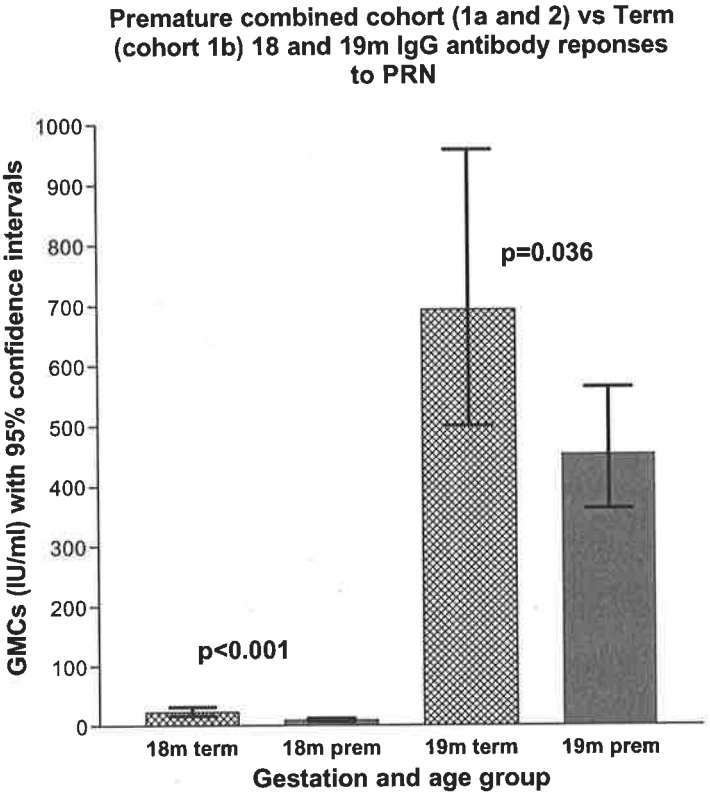



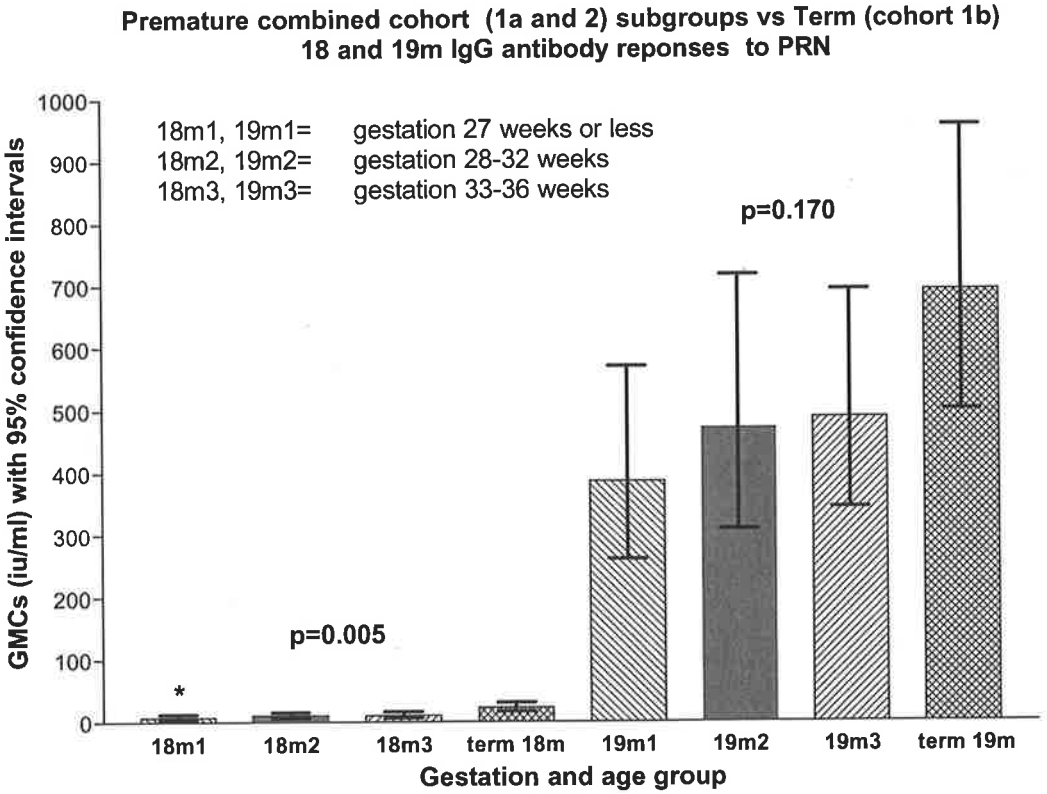
**Figures 8.14a and b Comparison between term and combined premature infant cohort (1a and 2) FHA antibody concentrations**



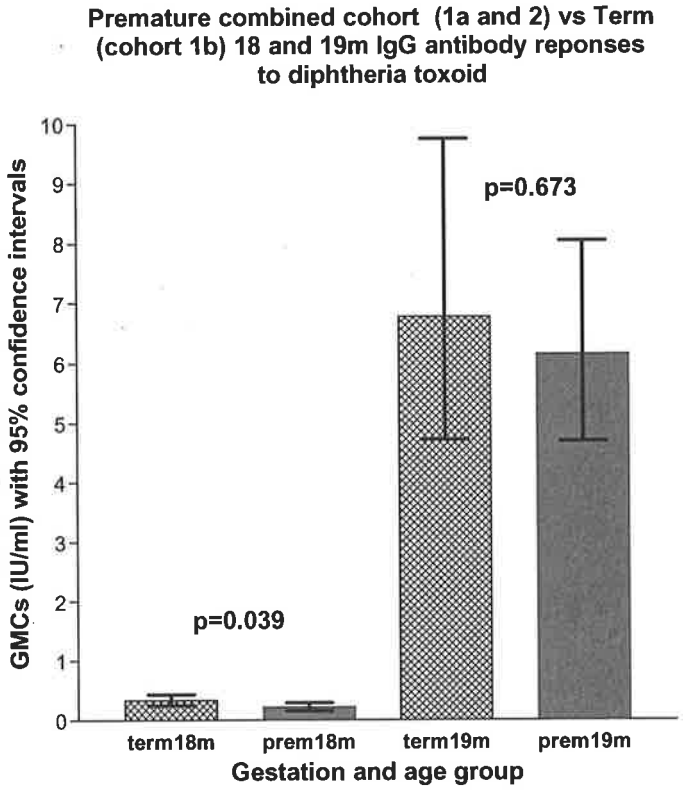



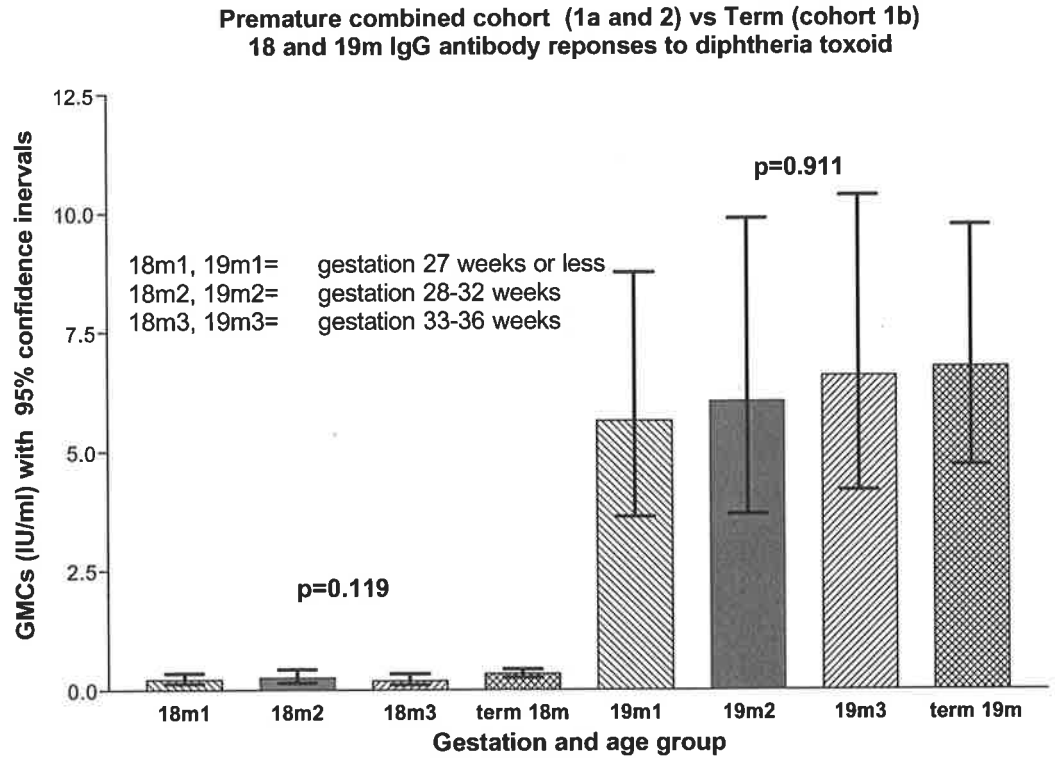
**Figures 8.15a and b Comparison between term and combined premature infant cohort (1a and 2) PRN antibody concentrations**



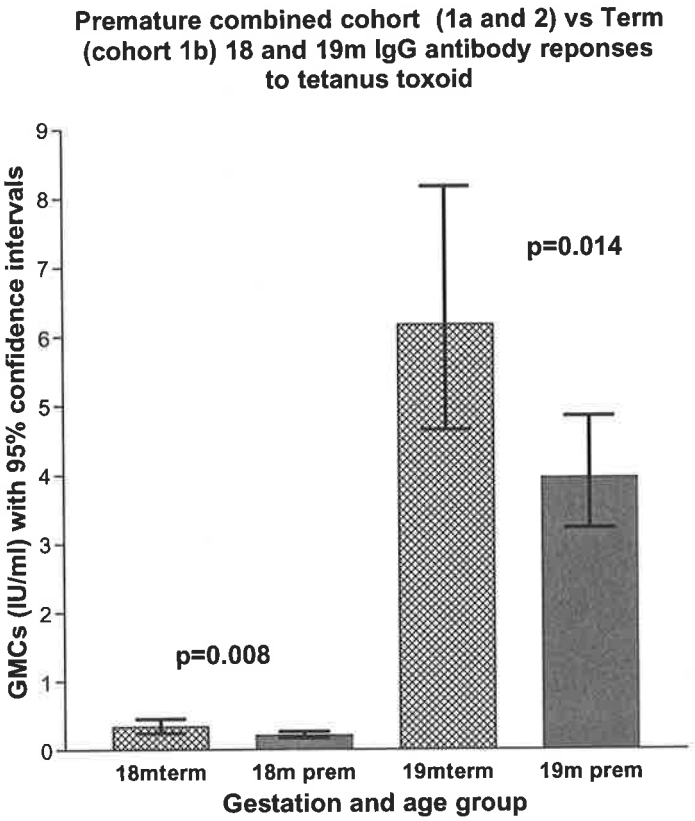



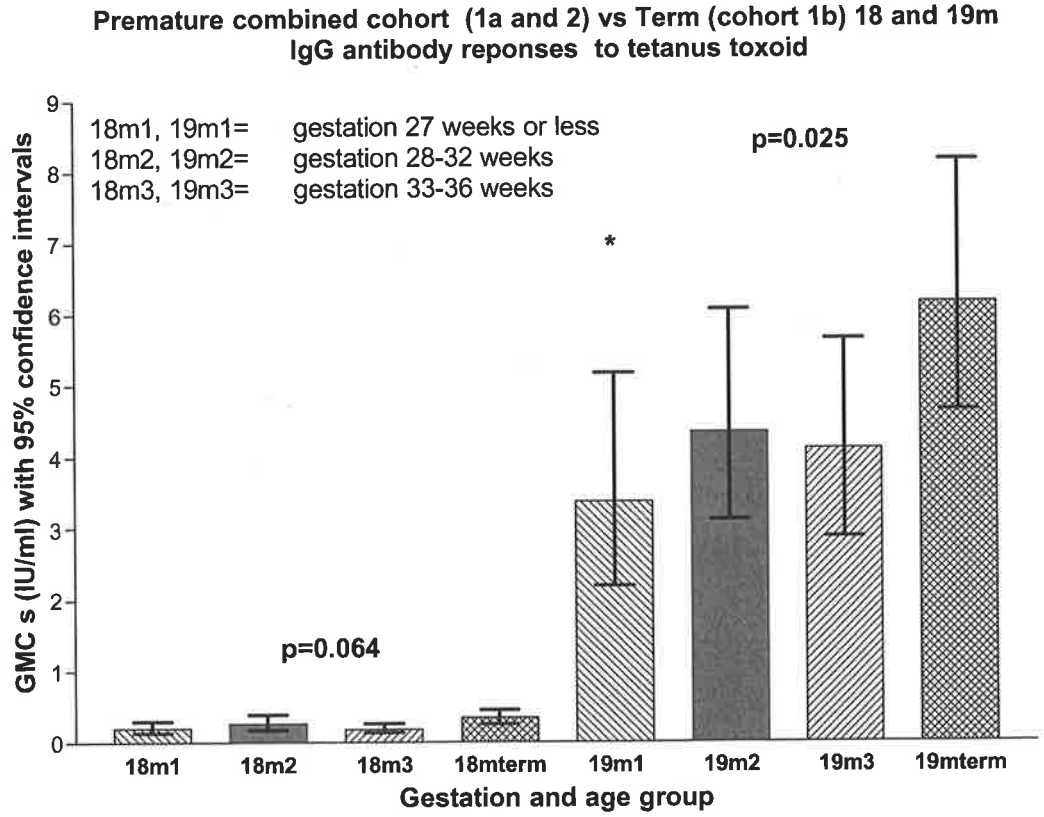
**Figures 8.16a and b Comparison between term and combined premature infant cohort (1a and 2) diphtheria antibody concentrations**



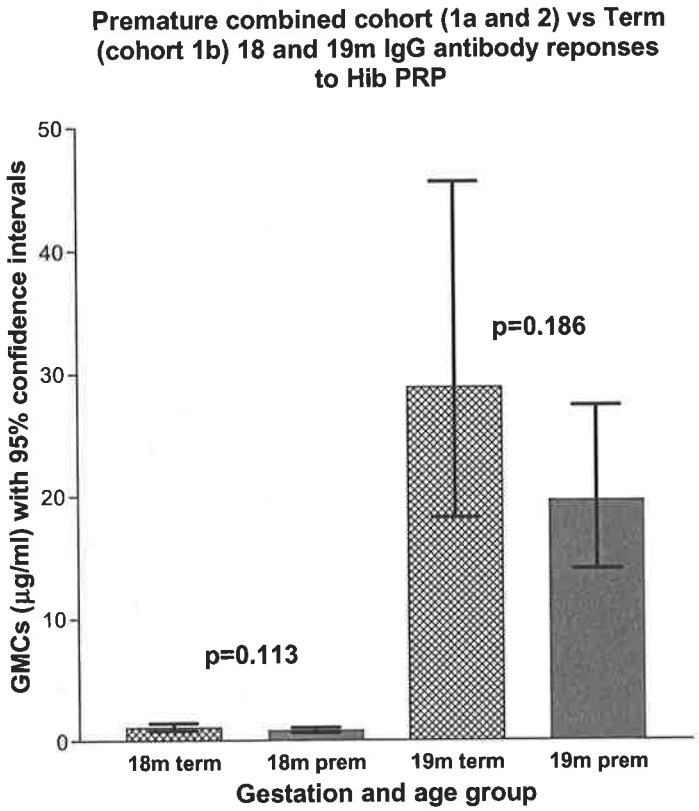



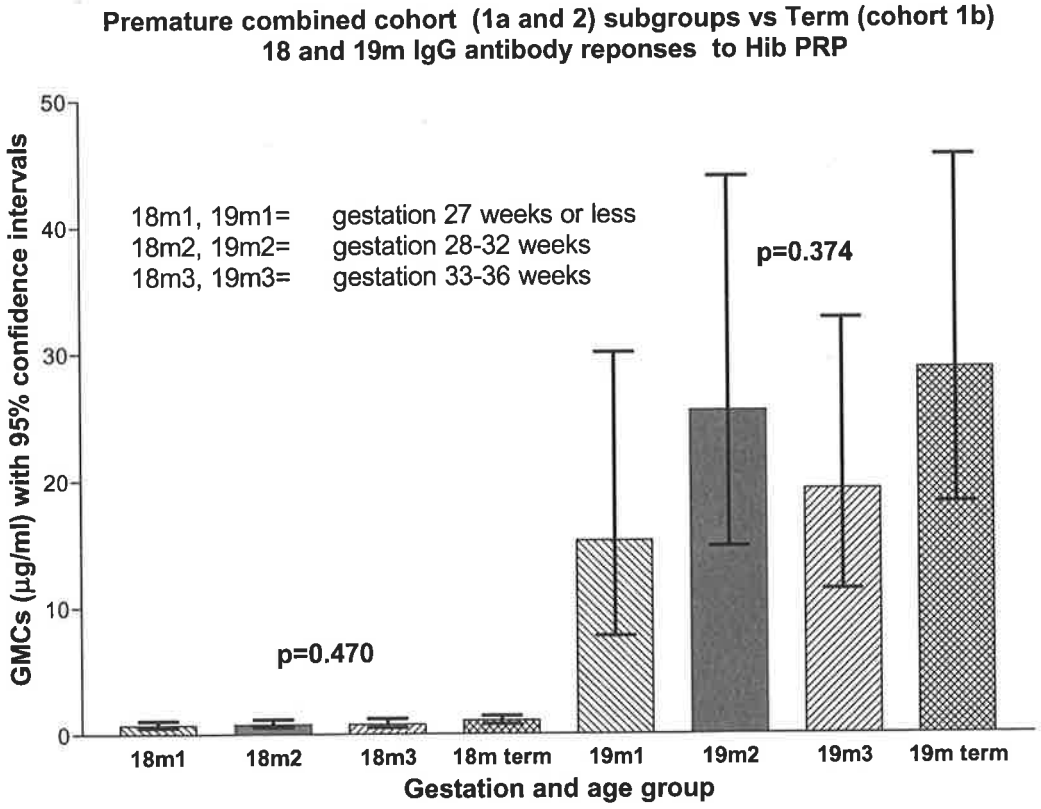
**Figures 8.17a and b Comparison between term and combined premature infant cohort (1a and 2) tetanus antibody concentrations**



**Figures 8.18a and b Comparison between term and combined premature infant cohort (1a and 2) Hib PRP antibody concentrations**



# Chapter 9 Discussion

## 9.1. Murine Immunogenicity Studies

These studies were based on the hypothesis that adverse storage of pertussis vaccines below $0^0$C, which occurs frequently at vaccine provider sites may affect their immunogenicity (128, 131, 133, 134, 136, 138, 139, 276). The principal aim of these studies was to assess the effect of such adverse storage of pertussis vaccines on their immunogenicity.

A murine model was developed successfully and single antigen pertussis assays were performed to compare the immunogenicity of correctly and adversely stored vaccines. The adverse vaccine storage conditions chosen for investigation were $-3^0$C for 24 hours or 14 days and $-6^0$C for 24 hours prior to immunisation.

Studies in Swiss Outbred mice clearly demonstrated that longer periods of adverse storage below $0^0$C enhanced vaccine immunogenicity in these mice relative to ideal storage and shorter periods of adverse storage. In addition, responses to DTPa were of a greater magnitude than those to DTPw for all storage conditions. However, the IgG antibody GMCs demonstrated very wide confidence intervals.

Therefore, further similar studies were performed in Balb/c mice, to determine how much of the variability in results in experiments in outbred mice could be accounted for by mouse strain. Much narrower confidence intervals were demonstrated in the GMCs of IgG pertussis antibody responses in Balb/c mice. In general, antibody responses to pertussis antigens in DTPa or DTPw were unaffected by adverse storage with the exception PRN antibody responses in mice immunised with DTPa. An unexpected finding was the lack of antibody responses to PT in mice immunised with DTPw. It was also demonstrated that

Swiss outbred mice had antibody responses to pertussis antigens in both vaccines which were greater in magnitude than those of Balb/c mice.

These studies have provided a reproducible model to investigate the immunogenicity of correctly and adversely stored pertussis vaccines in mice. They have extended the collective body of knowledge in this area, as no previous investigators have examined the immunogenicity of acellular pertussis vaccines after adverse storage, and none has compared the immunogenicity of the pertussis component of DTP preparations in outbred and inbred mice.

The first question these studies raise relates to the mechanism(s) by which the immunogenicity of DTP vaccines is altered under conditions of adverse storage. Such mechanisms could include physico-chemical changes to the antigens themselves or to the adjuvant.

Secondly, the lower antibody responses to the pertussis component of DTPw in both strains of mice requires further exploration. Investigation of the relative doses of antigen administered is relevant, as it was not possible to calculate exact amount of antigen delivery in mice immunised with DTPw.

In addition, further investigation of the differences in immunogenetics in the different mouse strains may be of importance both in relation to the lower magnitude of responses to DTPw in general, and to the lower magnitude of antibody responses in Balb/c mice.

## 9.2 Murine Protective efficacy studies

Because IgG pertussis antibody concentrations in isolation do not reflect the degree of protection afforded by immunisation, there was a need to find a method to investigate the protective efficacy of pertussis vaccines under similar conditions of adverse storage.
These studies were based on the hypothesis that adverse vaccine storage below $0^0$C would affect the protective efficacy of acellular and whole cell pertussis triple antigen vaccines.

The aims of these studies were to develop and use a reproducible murine model to investigate the the following: the protective efficacy of correctly and adversely stored pertussis vaccines by assessing lung homogenate culture colony counts after infection, the development of single antigen pertussis EIAs and diphtheria and tetanus toxoid EIAs to measure the serological responses to immunisation and infection in these mice, and the assessment of the degree of lung inflammation using histopathological analysis of lung tissue, and performing cytokine analysis and cytospin cell counts on the BAL fluid of infected mice.

Based on the methods of previous investigators (115, 186, 252) a murine intranasal challenge model was developed successfully. EIAs were developed successfully in our laboratory to assess antibody responses to PT, FHA, PRN, diphtheria and tetanus toxoids. The conditions of adverse vaccine storage were the same as in the immunogenicity studies with the addition of experiments utilising storage at $-6^0$C for 14 days and $+13^0$C for 48 hours prior to immunisation. The primary outcome measure was the determination of lung homogenate culture results four days after intranasal challenge, although some experiments included sacrifice of mice for lung homogenisation at additional time points after challenge.

In general, the protective efficacy of DTPa and DTPw stored above and below the recommended temperature range of $2^0$C-$8^0$C were unaffected, with the exception of storage of vaccines at $-3^0$C for 24 hours which paradoxically decreased lung clearance of organisms. Although this result remains unexplained, it was found for both vaccines, making technical error a less likely cause. Overall, DTPa appeared to provide better protective efficacy than DTPw.

In initial experiments examining serial time points of sacrifice post intranasal challenge (storage of vaccine between $2^0$C and $8^0$C and at $+13^0$C) mice immunised with DTPa and DTPw demonstrated a steady increase in PT, PRN and diphtheria antibody concentrations after each immunisation. A further increase in PT and PRN antibody concentrations was demonstrated in mice immunised with either vaccine on day 14 post challenge. However, only mice immunised with DTPa demonstrated a continued rise in antibody titre 14 days after intranasal challenge.

In general, pertussis, diphtheria and tetanus antibody responses to DTPa and DTPw were unaffected by adverse storage below $0^0$C or at $+13^0$C. In some cases greater pertussis, diphtheria or tetanus antibody concentrations were demonstrated in mice immunised with adversely stored vaccine, and these were mostly found in mice immunised with DTPw. Mice immunised with DTPa demonstrated greater pertussis antibody concentrations after immunisation and mice immunised with DTPw demonstrated greater diphtheria and tetanus antibody concentrations. As in the immunogenicity studies and in contrast to the work of other investigators, mice immunised with DTPw did not produce PT antibodies until 14 days after challenge (185, 231).

Mice immunised with normal saline demonstrated earlier antibody responses to intranasal challenge than previous investigators with similar study design. However, these other studies utilised aerosol rather than intranasal challenge to infect mice (184, 185).

No significant correlations were found between lung clearance and pertussis antibody concentrations in these studies.

Histopathological analysis of mouse lungs from these experiments demonstrated significantly less inflammation in mice immunised with DTPa than with DTPw or normal saline and similar degrees of lung inflammation in mice immunised with DTPw or normal saline. Lung inflammation was essentially unaffected by storage condition for both vaccines, although some variability between experiments was demonstrated in mice immunised with normal saline. The degree of acute inflammation was the most significant of the four parameters assessed in determining the total lung inflammatory score.

Correlations between lung histopathology inflammation scores and pertussis serology or lung homogenate culture results were in general, not statistically significant. However, the numbers of mice for each comparison were small, thus reducing the power of these analyses.

Cytospin examination of BAL fluid demonstrated significantly fewer inflammatory cells in the BAL of mice immunised with DTPa than mice immunised with DTPw or normal saline. Macrophages were the predominant cell type in the BAL of mice immunised with DTPa and neutrophils predominated in the BAL cells of DTPw-immunised mice. Differences between vaccine storage conditions could not be analysed due to the small numbers of mice with BAL fluid collected.

Cytokine analysis of BAL fluid demonstrated higher IL4:IL-2 ratios in mice immunised with DTPa and normal saline than in DTPw-immunised mice regardless of storage condition: mice immunised with DTPw had relatively high concentrations of both cytokines in BAL fluid. These results suggest a Th-2 –type immune responses in mice immunised with DTPa or normal saline and a Th-1-type immune response in mice immunised with DTPw, although mouse numbers were small and data were incomplete for DTPw-immunised mice.

These experiments have demonstrated the development and use of a reproducible model of assessment of protective efficacy of pertussis vaccines. In addition, new data have been provided regarding the protective efficacy of adversely stored pertussis vaccines, which has not been assessed previously for DTPa or using a respiratory challenge model of infection.

The first question arising as a result of these studies is whether lung clearance is an appropriate model with which to assess pertussis vaccine protective efficacy. These studies have demonstrated very clear information regarding bacterial clearance in unimmunised mice and mice immunised with correctly and adversely stored DTPa and DTPw. However, no serological correlate of protection was identified. Most other investigators have also found this to be the case, although most have used similar (relatively small) numbers of mice as in the present studies, thus reducing the power of such correlations (115, 162, 183, 252). There also appears to be a significant body of evidence from murine and human studies to suggest that the contributions of humoral and cell-mediated immunity cannot easily be separated when assessing protection against pertussis infection (162, 172, 182, 184, 185, 188, 190, 277, 278).

Secondly, there is the question of how these results may relate to protection in human infants and children. A significant amount of our knowledge of the composition and function of the human immune system has been based on murine studies. However, because of the inability to replicate the protective efficacy studies presented in this dissertation in humans, the murine model remains the best existing method with long-term studies of the rates of pertussis infection in immunised children as the alternative.

Future studies which delineate lung clearance prior to four days after challenge and which have large enough numbers to assess the relationships between lung clearance, serological responses to pertussis infection and immunisation, and markers of lung inflammation will help to address some of these questions.

## 9.3 Human immunogenicity studies

The aims of these studies were to determine the difference in antibody responses to routine childhood immunisations (DTP and Hib vaccines) between premature and term infants followed longitudinally from two to 19 months of age and to compare the effect of immunisation with DTPa or DTPw in the primary series on primary immunisation antibody responses and upon subsequent 18 month booster antibody responses to DTPa. The hypothesis upon which these studies were based was that premature infants would demonstrate lesser antibody responses than term infants to routine childhood immunisations. Infants were recruited at 2months of age for longitudinal comparisons of antibody responses and from 18 months of age for cross-sectional studies.

Enzyme immunoassays were developed in the University of Adelaide Department of Paediatrics Laboratory to measure IgG antibody responses to PT, FHA, PRN, diphtheria and tetanus toxoids. Pre-existing EIA methods to measure IgG Hib-PRP were already

established. Antibody concentrations were assessed at 2, 7 18 and 19 months of age in longitudinal studies and at 18 and 19 months of age in cross-sectional studies.

In the first longitudinal study, premature infants demonstrated significantly lower pertussis IgG antibody concentrations to all three antigens at 2, 7, and 18 months of age and to PT and FHA at 19 months of age. When premature infant antibody responses were analysed according to gestational age at birth, the significance was generated by the comparison of term and extremely premature infant (gestational age at birth < 28 weeks) antibody responses in most cases. Premature infant antibody responses to diphtheria and tetanus toxoids and Hib PRP were also of lesser magnitude but not significantly different in most cases. In addition, all infants achieved protective concentrations of antibody to diphtheria and tetanus toxoids at the 7 and 19 month study times. All but one term and one premature infant achieved protective concentrations of PRP antibodies at 7months and all achieved protective levels at 19 months.

In addition, subdivision of premature infants on the basis of gestational age at birth (extremely premature: <28 weeks, very premature 28-32 weeks, premature 33-36 weeks) revealed that premature infants born at 33-36 weeks gestation responded to immunisation almost identically to term infants for all vaccine antigens.

Immunisation with DTPw in the primary series and DTPa at the 18 month booster immunisation conferred lower pertussis and greater diphtheria and tetanus antibody concentrations in premature and term infants than in infants immunised with DTPa for all immunisations. These findings were confirmed in cross-sectional studies which analysed serum samples from a separate cohort of premature infants recruited at 18 months of age. These results are similar to those of the murine studies reported here, in which mice

immunised with DTPw demonstrated greater diphtheria and tetanus antibody concentrations and lesser pertussis antibody concentrations that mice immunised with DTPa.

Cross-sectional comparisons between term infants and a second cohort of premature infants recruited at 18 months of age demonstrated similar results when pertussis antibody concentrations were compared. Term/premature infant and term/premature infant subgroup comparisons for diphtheria and tetanus antibody responses were not similar and did not follow a particular pattern of differences, indicating variability between the two cohorts of premature infants in this regard, and possibly also in premature infants in general.

Although several previous studies investigated the comparative responses of term and premature infants to childhood immunisations (table 8.16), none has reported responses to all bacterial antigens in the same study. In addition, none has examined antibody responses past the age of 12 months, and none has compared responses to DTPa and DTPw in premature and term infants. Therefore these studies have provided valuable new information regarding longitudinal and cross-sectional comparisons between term and premature infant responses to routine immunisations and regarding the responses of premature infants in relation to gestational age.

The questions raised by the studies reported here relate principally to premature infants. After division of premature infant cohorts on the basis of gestational age at birth, the number of infants in each subgroup was relatively small, thus potentially reducing the power of term/premature and term/premature infant subgroup analyses. Analyses of larger cohorts of premature infants is of great importance, as this information could potentially

influence decisions regarding timing and formulation of immunisation in premature infants and in particular extremely premature infants in neonatal intensive care units.

These studies did not examine and compare the cell-mediated immune responses of recruited infants to immunisation. This is particularly important in relation to pertussis infection, where it would appear that humoral and cell-mediated immunity play equal roles in protection against infection. Although neonatal immune function in term infants is becoming increasingly understood (203, 206, 211, 213, 214), there is little information regarding the immune function of premature infants and how differences may affect responses to immunisation. There is clearly a need for further investigation in this area, as the introduction of new vaccines or the alteration of existing immunisation schedules will require direct and indirect measures of efficacy which can be applied to premature as well as term infants in carefully designed, prospective, controlled cohort studies with large numbers of participants.

In summary, these studies have provided better understanding of the immunogenicity and protective efficacy of adversely stored pertussis vaccines and of the longitudinal and cross-sectional differences between premature and term infant antibody responses to immunisation. The directions which further research in these areas should take have been outlined. This information will enable the future provision of safe, stable, immunogenic and protective vaccines to all infants regardless of gestational age at birth.

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**Appendix 1**
**Parent Information Sheet and Consent Form**

**Parent Information Sheet**

**PRINCIPAL INVESTIGATORS:**
*Dr Tina Boros (page 4146, extn 6228), Dr A McPhee, Professor Don Roberton, Dr. Mike Gold*

**STUDY TITLE:**
**The effect of gestational age on the immunogenicity of childhood immunisations: Cohorts 2a and 2b**

## Background and description of the study

Your child has been invited to participate in a vaccine study to determine the immunogenicity (the body's ability to produce protective antibody) in premature infants to routine triple antigen (DTPa) and Hib (meningitis) immunisation. Premature infants may have weaker immune systems than term infants. However, the immune system matures as the child grows older. In previous studies, premature infants were shown to have lower antibody responses to the Hib immunisation after the first three doses than term infants. This diminished response appeared to be related to prematurity. However, after the booster dose at 18 months, their immune responses to this vaccination were similar to those of term infants. We are particularly interested in the way premature infants respond to the triple antigen and Hib immunisation compared to term infants.

Whooping cough, diphtheria, tetanus and Hib bacteria can all cause potentially serious infections, especially in young infants whose immune systems are not yet fully developed, but are preventable by the use of triple antigen vaccines. Premature infants may be at higher risk of these infections, particularly if their responses to immunisation are low. However, there have been few previous studies designed to investigate this possibility. Information provided from this study will help us determine if we are providing appropriate immunisation schedules for premature and term infants.

Before agreeing to have your child participate in this study, it is important that you read and understand the following explanation of the steps involved in the study. Please ask the study doctor or staff to explain any words or information that you do not understand.

The study is being carried out in the Department of Paediatrics, at the Women's and Children's Hospital.

### Purpose of this Study

The purpose of this study is to evaluate the immune response to the acellular, or DTPa triple antigen and Hib vaccines in healthy premature and term infants such as your own and to compare their responses.

The DTPa vaccine to be used was introduced into the South Australian Childhood Immunisation Schedule in February 1997. It is used to protect children against whooping cough, tetanus and diphtheria infections and replaces "whole-cell" (DTPw) triple antigen vaccines which are associated with more reactions to immunisation.

The Hib vaccine to be used is part of the normal Childhood Immunisation Schedule and is therefore the same as that given to any other child. Both vaccines are known to be safe and effective in infants.

Your infant will be among approximately 100 term and preterm participants who will be enrolled from the Women's and Children's Hospital in Adelaide from March 1999. For each child the study will last 19 months from commencement.

**Study Processes**

To participate in this vaccine study your infant should be due for their first immunisations at 2 months of age, in good health and be available to participate in the study for 19 months. You (parent or guardian) should be available to be contacted by telephone.

To be in this study, your infant should have:

- no known or suspected weak immune system or be receiving medications which weaken the immune system (such as steroids)
- no birth defect or serious chronic disorder
- no chronic cardiac or respiratory illness
- no previous serious allergic response to any vaccine
- not received any previous vaccination except to Hepatitis B
- no previous whooping cough, diphtheria , tetanus or Hib infection

In addition, your child should not live in a house with individuals with weak immune systems.

If, at the time of immunisation, your child has:

- a current illness or fever (temperature greater than 37.5°C), or
- is taking antibiotics for an illness, or
- has received other vaccines within the last 2 weeks (if received the Hepatitis B vaccine)

they will be excluded from the study until these conditions have passed.

Children enrolled in this study will have a total of 4 blood samples taken over the 19 months in the study. A small amount of blood (approximately 1-2 teaspoons) will be obtained from a vein in your child's arm at each of these times to measure the response to immunisation. EMLA cream (an anaesthetic cream) will be applied to your child's arm before the blood sample is collected to numb the skin. Children whose immune responses to Hib immunisation are low after three doses (at 2, 4 and 6 months of age) will be offered a booster dose at 12 months of age.

**Your visits to us at the Women's and Children's Hospital will involve:**

**Visit 1:at 2 months of age**

- a physical examination with our medical officer
- a blood sample to be collected from a vein in the hand or arm.
- immunisation with DTPa, Hib and polio vaccinations
- enquiry about the use of any medications by your child

**Visits 2 and 3: at 4 and 6 months of age**

- enquiry about the use of any medications by your child
- another physical examination with our medical officer
- immunisation with DTPa Hib and polio vaccinations
- These visits may occur at your child's local doctor's surgery if you wish

**Visit 4: at 8 months of age**

- enquiry about the use of any medications by your child
- a physical examination with our medical officer/ research nurse
- a blood sample to be collected from a vein in the hand or arm.

If your child has a low response to the Hib vaccination after this visit, you will be offered the chance to give them an extra dose of this vaccination at 12 months of age.

**Visit 5: at 18 months of age**

- review any symptoms, medical problems or medications used
- a physical examination with our medical officer/ research nurse
- a blood sample to be collected from a vein in the hand or arm
- immunisation with DTPa and Hib vaccinations

**Visit 6: at 19 months of age**

- enquiry about the use of any medications by your child
- a physical examination with our medical officer/ research nurse
- a blood sample to be collected from a vein in the hand or arm

The immunisations, physical examinations and blood tests will be performed by the research study staff of the Department of Paediatrics, at the Women's and Children's Hospital

The protocol has been reviewed and accepted by an independent Ethics Review Committee.

## Possible Benefits From This Study:

Your child and other children in the future may benefit from this and other medical research studies.
In this case, consideration of such benefits include:

- close follow-up of your child by a medical officer/research study staff.
- the knowledge that your child will have had all the correct immunisations given in the Childhood Immunisation Schedule by the end of the study
- the opportunity to have a booster dose of Hib vaccination if required
- the satisfaction of having participated in research that will help us understand more about the responses of premature and term infants to immunisation.

## Risks Associated with this Study:

Risks associated with participation in this study include, but are not limited to, mild pain and swelling at the vaccination site, mild to moderate fever and possible irritability of your child in the 24 hours after vaccination. These are the same risks that any child not participating in the trial would have when receiving DTPa or Hib vaccination.

The risks of taking blood samples may include discomfort (we will provide a topical anaesthetic cream to numb the skin), redness and swelling and/or bruising at the needle site.

## Confidentiality:

All documentation containing personal details of your child's identity will remain confidential. However, any results generated by this study may be published in medical journals. No information that could identify a particular individual will be made public. Your child's medical records will also be kept confidential.

If you have any questions about your child's participation in this study, you may contact the study coordinators who are:

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|---|---|
| Dr Tina Boros<br>Department of Paediatrics,<br>Women's and Children's Hospital<br>**Tel:** (08) 8204 6228. | Ms Leonie Dinan<br>Department of Paediatrics<br>Women's and Children's Hospital<br>**Tel:** (08) 8204 6328 |

**Right to Ask Questions or Withdraw from the Study:**

You have the right to ask any questions concerning this study or the potential risks related to it at any time. You will be informed of any significant information pertaining to your child's safety.

Your child's participation in this study is voluntary. You will receive a signed copy of the consent form. You may refuse to participate or ask to withdraw your child from the study at any time without prejudice to his/her future treatment.

# WOMEN'S & CHILDREN'S HOSPITAL RESEARCH ETHICS COMMITTEE

## CONSENT FORM

I _____

**hereby consent to my child's involvement in the research project entitled:**

The effect of Gestational Age on the Immunogenicity of Acelllular Pertussis vaccine.

1. The nature and purpose of the research project described on the attached Information Sheet has been explained to me. I understand it, and agree to my child taking part.

2. I understand that my child may not directly benefit by taking part in this study.

3. I acknowledge that the possible risks and/or side effects, discomforts and inconveniences, as outlined in the Information Sheet, have been explained to me.

4. I understand that while information gained in the study may be published, my child will not be identified and information will be confidential.

5. I understand that I can withdraw my child from the study at any stage and that this will not affect medical care or any other aspects of my child's relationship with this hospital.

6. I understand that there will be no payment to my child for taking part in this study

7. I have had the opportunity to discuss taking part in this research project with a family member or friend and/or have had the opportunity to have a family member or friend present whilst the research project was being explained by the researcher.

8. I am aware that I should retain a copy of the Consent Form, when completed, and the Information Sheet.

9 a) I consent to two specimens of blood being taken from my child and being used in the above project.

b) I do / do not consent to the blood samples being used in any other research project, provided the project has the approval of the Women's & Children's Hospital Research Ethics Committee.

10. I understand that I am free to stop my chlid from donating blood samples at any stage, without giving any reason, and that my action of donating/not donating a sample will not affect (i) my prospects in any position; (ii) any academic prospects; or (iii) any other conceivable situation.

Signed .....

Relationship to Patient: .....

Full name of patient: .....

Dated:.....

I certify that I have explained the study to the parent and consider that he/she understands what is involved
.Signed: .....

Title: .....

Dated: .....

**Appendix 2**
**Clinical report Form**

# CLINICAL REPORT FORM
# TERM/PRETERM STUDY

## COHORT 1A / COHORT 1B

**Subject Initials:** ☐☐☐

**Subject Identification:** _____

Full Name: _____

D.O.B: _____

Sex: M/F

Race: _____

Parent/Guardian Name: _____

_____

Address: _____

_____

Telephone No: _____hm/ wk

_____hm/ wk

Grandparent Name: _____

Telephone No. _____hm/ wk

Medical History: _____

_____

_____

Birth weight: _____g

Birth length: _____cm

Birth HC: _____cm

Medications: _____

(identify start and stop dates) _____

_____

| Vaccination History: | Vaccine(s) | Date Administered |
|---|---|---|
| | _____ | _____ |
| | _____ | _____ |
| | _____ | _____ |

- Informed consent given, copied (2x), & copy given to parent Y/N

**Subject Initials:** ☐☐☐

**Subject Identification:** _____

**Visit 1** (age 8wks ± 1wk) Date: __ / __ / __

## Medical Examination

Temperature (axillary) ____°C

Abnormal findings Y/N

_____
_____
_____
_____

| | | |
|---|---|---|
| Ears | -L____ | R_____ |
| Nose | -Congestion | Y/N |
| | -Discharge | Y/N |
| Throat | -Inflammation | Y/N |
| | -Exudate | Y/N |
| Lymph Nodes | -Tender | Y/N |
| Chest | -_____ | |
| CVS | -_____ | |
| Abdo | -_____ | |
| Hips | -_____ | |
| Length | -_____cm | |
| Weight | -_____g | |
| HC | -_____cm | |

- Pre-immunisation blood sample obtained (4ml) Y/N
  Where was this sample obtained from, eg cubital/dorsal? _____

## Vaccine Administration

| | | |
|---|---|---|
| DTPa left thigh | Y/N | batch #: _____ |
| DTPa left arm | Y/N | |
| Hib right thigh | Y/N | batch #: _____ |
| Hib right arm | Y/N | |
| Oral polio | Y/N | batch #: _____ |
| Observation (15 min.) | Y/N | |

Comments : _____
_____
_____

- CYH record completed Y/N

**Subject Initials:** ☐☐☐

**Subject Identification:** _____

**Visit 2** (Visit 1 + 8wks) Date: __/__/__

♦ Hospitalisation post Visit 2 Y/N

*AE's: _____

_____

♦Medications: Y/N

-identify start & stop dates & reason

_____

_____

## Medical Examination

Temperature (axillary) ____°C

Abnormal findings Y/N

_____

_____

_____

_____

| | | |
|---|---|---|
| Ears | -L____ | R_____ |
| Nose | -Congestion | Y/N |
| | -Discharge | Y/N |
| Throat | -Inflammation | Y/N |
| | -Exudate | Y/N |
| Lymph Nodes | -Tender | Y/N |
| Chest | -_____ | |
| CVS | -_____ | |
| Abdo | -_____ | |
| Hips | -_____ | |
| Length | -_____cm | |
| Weight | -_____g | |
| HC | -_____cm | |

## Vaccine Administration

DTPa left thigh Y/N batch #: _____

DTPa left arm Y/N

Hib right thigh Y/N batch #: _____

Hib right arm Y/N

Oral polio Y/N batch #: _____

Observation (15 min.) Y/N

Comments : _____

_____

_____

♦ CYH record completed Y/N

**Subject Initials:** ☐☐☐

**Subject Identification:** _____

**Visit 3** (Visit 2 + 8wks) Date: __/__/__

♦ Hospitalisation post Visit 2 Y/N

*AE's: _____

_____

♦ Medications: Y/N

-identify start & stop dates & reason

_____

_____

## Medical Examination

Temperature (axillary) ____°C

Abnormal findings Y/N

_____

_____

_____

_____

| | | |
|---|---|---|
| Ears | -L____ | R_____ |
| Nose | -Congestion | Y/N |
| | -Discharge | Y/N |
| Throat | -Inflammation | Y/N |
| | -Exudate | Y/N |
| Lymph Nodes | -Tender | Y/N |
| Chest | -_____ | |
| CVS | -_____ | |
| Abdo | -_____ | |
| Hips | -_____ | |
| Length | -_____ | cm |
| Weight | -_____ | g |
| HC | -_____ | cm |

## Vaccine Administration

DTPa left thigh Y/N batch #: _____

DTPa left arm Y/N

Hib right thigh Y/N batch #: _____

Hib right arm Y/N

Oral polio Y/N batch #: _____

Observation (15 min.) Y/N

Comments : _____

_____

_____

♦ CYH record completed Y/N

**Subject Initials:** ☐ ☐ ☐

**Subject Identification:** _____

**Visit 4** (Visit 3 + 8 wks)Date: __ / __ / __

♦ Hospitalisation post Visit 3 Y/N

*AE's: _____

_____

♦ Medications: Y/N

-identify start & stop dates & reason

_____

_____

## Medical Examination

Temperature (axillary) ____°C

Abnormal findings Y/N

_____

_____

_____

_____

| | | |
|---|---|---|
| Ears | -L____ | R_____ |
| Nose | -Congestion | Y/N |
| | -Discharge | Y/N |
| Throat | -Inflammation | Y/N |
| | -Exudate | Y/N |
| Lymph Nodes | -Tender | Y/N |
| Chest | -_____ | |
| CVS | -_____ | |
| Abdo | -_____ | |
| Hips | -_____ | |
| Length | -_____cm | |
| Weight | -_____g | |

♦ Post-immunisation blood sample obtained (4ml) Y/N
Where was this sample obtained from, eg cubital/dorsal? _____

♦ CYH record completed Y/N

**Subject Initials:** ☐☐☐

**Subject Identification:** _____

**Visit 5** (18mths ± 1mth) Date: __/__/__

♦ Hospitalisation post Visit 4 Y/N

*AE's: _____

_____

♦ Medications: Y/N

-identify start & stop dates & reason

_____

_____

## Medical Examination

Temperature (axillary) ____°C

Abnormal findings Y/N

_____

_____

_____

_____

| | | |
|---|---|---|
| Ears | -L____ | R_____ |
| Nose | -Congestion | Y/N |
| | -Discharge | Y/N |
| Throat | -Inflammation | Y/N |
| | -Exudate | Y/N |
| Lymph Nodes | -Tender | Y/N |
| Chest | -_____ | |
| CVS | -_____ | |
| Abdo | -_____ | |
| Hips | -_____ | |
| Length | -_____cm | |
| Weight | -_____g | |
| HC | -_____cm | |

♦ Pre-18 month booster immunisation blood sample obtained (4ml) Y/N
Where was this sample obtained from, eg cubital/dorsal? _____

## Vaccine Administration

DTPa left thigh Y/N batch #: _____
DTPa left arm Y/N

Hib right thigh Y/N batch #: _____
Hib right arm Y/N

Observation (15 min.) Y/N
Comments : _____
_____
_____

MMR administration @12 months? Y/N*
*details_____
_

♦ CYH record completed Y/N

**Subject Initials:** ☐ ☐ ☐

**Subject Identification:** _____

**Visit 6** (Visit 5 +1mth) Date: __/__/__

♦ Hospitalisation post Visit 5 Y/N

*AE's: _____

_____

♦ Medications: Y/N

-identify start & stop dates & reason

_____

_____

_____

## Medical Examination

Temperature (axillary) ____°C

Abnormal findings Y/N

_____

_____

_____

_____

| | | |
|---|---|---|
| Ears | -L____ | R____ |
| Nose | -Congestion | Y/N |
| | -Discharge | Y/N |
| Throat | -Inflammation | Y/N |
| | -Exudate | Y/N |
| Lymph Nodes | -Tender | Y/N |
| Chest | -_____ | |
| CVS | -_____ | |
| Abdo | -_____ | |
| Hips | -_____ | |

♦ Post-18 month booster immunisation blood sample obtained (4ml) Y/N
Where was this sample obtained from, eg cubital/dorsal? _____