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The protective and immunomodulatory properties of *Bordetella pertussis* adenylate cyclase toxin

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Abstract

Bordetella pertussis is the causative agent of the disease whooping cough in man, and although there is an effective whole-cell vaccine (WCV), recent efforts have focused on the development of the acellular vaccines (ACVs) to avoid the problems of reactogenicity associated with WCVs.

The cell-invasive toxin, adenylate cyclase toxin (CyaA), is an important virulence factor of *B. pertussis* and, when used as a vaccine, is able to protect mice against intranasal challenge with *B. pertussis*. The project was designed to investigate the mouse immune responses to two forms of the toxin, an enzymically-active, acylated form (CyaA) and an enzymically-inactive, acylated form (CyaA*). These were expressed as recombinant forms in *E. coli*, purified and their enzymic, haemolytic and cytotoxic properties characterised. The adenylate cyclase (AC) and cytotoxic activities of CyaA* were low compared to CyaA, but the haemolytic activities of the two toxins were similar. This indicated that the toxic activity of CyaA is dependent on AC enzymic activity, but that haemolytic activity is independent of AC activity.

Mouse responses to intraperitoneal immunisation with CyaA or CyaA* were examined, initially when each toxin was administered alone and later when administered in combination with protective antigens commonly used in ACV preparations (pertussis toxin (PT), filamentous haemagglutinin (FHA), and pertactin (P.69)). Both forms of the toxin when administered alone produced a strong serum IgG response which was boosted by a second vaccination. Anti-PT, -FHA and –P.69 IgG levels, in response to immunisation with PT, FHA and P.69 mixture in the presence of CyaA* were all raised compared to mice immunised with just the antigen mixture alone when measured at five

weeks post-immunisation, however only anti-P.69 levels were raised by co-administration with CyaA. When detoxified PT (dPT) was co-administered alone with CyaA*, the anti-PTd levels decreased but if FHA or P.69 were also included in the antigen mixture, the anti-PTd levels increased in comparison to mice immunised with PTd alone. When either FHA or P.69 antigen was co-administered with CyaA*, both anti-FHA and anti-P.69 levels respectively were raised in comparison to those immunised with FHA or P.69 alone, again when measured at five weeks post-immunisation.

Other work had shown that induction of cell-mediated immunity (CMI) was necessary for effective clearance of *B. pertussis* in the mouse. In this study, nitric oxide production by peritoneal macrophages was measured as a marker of macrophage activation. Macrophages collected from mice vaccinated with PT, FHA and P.69 together with CyaA or CyaA* responded more to stimulation with *B. pertussis* heat-killed cells or CyaA* than those taken from mice immunised with just the antigen mixture alone. Increased protection following aerosol challenge with *B. pertussis* was also observed when mice received a vaccination of CyaA* co-administered with PT, FHA and P.69 mixture.

Taken together these results indicate that CyaA is a candidate antigen for inclusion in acellular vaccines, even in the detoxified CyaA* form. As well as being a protective antigen in its own right, it may also afford improved CMI responses than the current ACV formulations.

Abbreviations

aa = amino acid
AC = adenylate cyclase enzymic activity
ACV = acellular vaccine
AFC = antibody forming cell
Amp = ampicillin
ATP = adenosine 5'-triphosphate
BAL = bronchoalveolar lavage
BAM = bovine alveolar macrophage
BBMC = bone marrow-derived mast cell
Bp = base pair
BG = Bordet Gengou
BSA = bovine serum albumin
bvg = *Bordetella* virulence gene
CAS = casamino acid solution
°C = degrees Celsius
CFU = colony forming units
Chlor = chloramphenicol
Ci/mCi = curie/millicurie
CMC = critical micelle concentration
CMI = cell-mediated immunity
CTL = cytotoxic T lymphocyte
CyaA = adenylate cyclase toxin
Da/kDa = daltons/kilodaltons
DC = dendritic cell
DEAE = diethylaminoethyl
DNA = deoxyribonucleic acid
DNT = dermonecrotic toxin
dNTP = deoxynucleoside triphosphate
dPT = detoxified pertussis toxin
DTPa = diphtheria tetanus pertussis acellular vaccine
EDTA = ethylenediamineteraacetic acid
ELISA = enzyme-linked immunoabsorbent assay
FCS = foetal calf serum
FFBP = formalin-fixed *Bordetella pertussis*
FHA = filamentous haemagglutinin
Fim = fimbriae
g/mg/μg/ng = grams/ milligrams/ micrograms/ nanograms
GFP = green fluorescent protein
h = hours
HBSS = Hank's balanced salt solution
kb = kilobase
KDO = 2-keto-3-deoxyoctonate
IFNγ = interferon gamma

IgG = Immunoglobulin G
i.m = intramuscular
i.p = intraperitoneal
IPTG = Isopropyl-1-thio-β-$_D$-galactoside
l/ ml/ μl = litres/ millilitres/ microlitres
LA = Luria-Bertani agar
LAL = *Limulus amoebocyte* lysate
LB = Luria-Bertani broth
LMCV = lymphocytic choriomeningitis virus
LPS = lipopolysaccharide
M/ mM/ μM/ nM = molar/ millimolar/ micromolar/ nanomolar
mA = milliamps
MFP = membrane fusion protein
MHC = major histocompatibility complex
min= minutes
mRNA = messenger RNA
MTT = 3-(4,5-di-methylthiazol-2-3yl)-2,5-diphenol tetrazolium bromide
MWT = molecular weight
NO = nitric oxide
OA = ovalbumin
$O.D._{xxnm}$ = optical density at wavelength XXnm
OMP = outer membrane protein
ON = overnight
P.69 = pertactin
PAGE = poly-acrylamide gel electrophoresis
PBS = phosphate buffered saline
PCR = polymerase chain reaction
PFGE = pulse-field gel electrophoresis
PIF = polymorphonuclear leukocyte inhibitory factor
PMN = polymorphonuclear leukocyte
PRP = *Haemophilus influenzae* type b capsular polysaccharide
PRP-T = PRP tetanus conjugate
PT = pertussis toxin
RNA = ribonucleic acid
RNAse = ribonuclease
rpm = revolutions per minute
RPMI = Roswell park memorial institute
rRNA = ribosomal RNA
RT = room temperature
RTX = repeat in toxin
s = seconds
SCID = severe combined immunodeficiency
SDS = sodium dodecyl sulphate
Sec =
SHD = single human dose
TAE = Tris/ Acetic acid/ EDTA (buffer)

TBE = Tris/ Boric acid/ EDTA (buffer)
TCF = tracheal colonisation factor
TCR = T cell receptor
TCT = tracheal cytotoxin
TE = Tris/ EDTA (buffer)
Tet = tetracycline
TBS = Tris buffered saline
TTBS = TBS + Tween
Tris = tris(hydroxylmethyl)aminoethane
U = units
UV = ultraviolet
$^{v}/_{v}$ = volume by volume
vag = virulence activated gene
vrg = virulence repressed gene
$^{w}/_{v}$ = weight by volume
WCV = whole-cell vaccine

List of Contents

List of Figures

List of Tables

CHAPTER 1

INTRODUCTION

1.1 CHARACTERISTICS OF THE GENUS BORDETELLA

1.1.1 Taxonomy and association with disease

Bordetella spp, are minute, Gram-negative, aerobic, non-acid fast, non-sporing coccobacilli (0.2-0.5) x (0.5-2)μm, arranged singly or in pairs with an optimum growth temperature of 35-37°C. Currently there are 8 named species, of which *B. pertussis* is the type species. *B. pertussis* was first described by Bordet and Gengou (1906) as the cause of pertussis or whooping cough, a highly contagious acute bacterial disease involving the respiratory tract, (Per, meaning intensive or pernicious, and tussis, meaning cough). It was initially named *Haemophilus pertussis* and remained as such until 1952 when Moreno-Lopez created the new genus *Bordetella* in honour of the work carried out by Jules Bordet (Moreno-Lopez, 1990). *B. bronchispetica*, first obtained by Ferry (1910) from the respiratory tract of dogs, and *B. parapertussis*, isolated by Bradford and Slavin (1937) from mild cases of whooping cough, were also included in the genus *Haemophilus* before the introduction of the genus *Bordetella*.

B. pertussis and *B. parapertussis* are the aetiological agents of whooping cough in humans. *B. pertussis* is an obligate pathogen for humans but *B. parapertussis* strains are also found in sheep where they can cause chronic pneumonia (Cullinane *et al.*, 1987). *B. bronchiseptica* causes respiratory disease in various mammalian species including dogs, rodents and horses, but occasionally affects man (Woolfrey and Moody (1991). *B. avium* is a pathogen for birds causing severe respiratory problems

in poultry, especially turkeys (Kersters *et al.*, 1984) and *B. hinzii* is mainly found as a commensal of the respiratory tracts of fowl and recently has been reported as the causative agent of fatal human septicaemia (Katter *et al.*, 2000). *B. holmesii* was included in the genus *Bordetella* on the basis of genetic and chemotaxonomic analyses and has been isolated repeatedly from blood of young adults and occasionally from sputum (Weyant *et al.*, 1995). *B. trematum,* which was isolated from wounds and ear infections in humans, was the most recently described member of this family (Vandamme *et al.*, 1996) until *B. petrii* was isolated from a mixed anaerobic, dechlorinating culture. Comparative 16S rDNA sequence analysis, DNA base composition and DNA-DNA hybridisation experiments suggested that *B. petrii* should be assigned to the genus *Bordetella* as a novel species (von Wintzingerode *et al.*, 2001) and have also led to a new description of the genus *Bordetella*: Gram-negative, catalase-positive, asaccharolytic coccobacilli with a DNA G+C content of 60-69 mol%. Growth occurs strictly aerobically although *B. petrii* is able to grow under anaerobic conditions favouring respiratory nitrate and selenate reduction. All species assimilate citrate under aerobic conditions and one species, *B. petrii*, assimilates D-gluconate. Based on comparative 16S rRNA gene sequence analysis, the *Bordetella* genus forms a distinct cluster separated from members of the genera *Achromobacter* and *Alcaligenes* within the *β-Proteobacteria* (von Wintzingerode *et al.*, 2001).

1.2 The Disease Pertussis

Whooping cough is transmitted from patients (usually young children) to non-immune individuals by means of the secretions in the mouth and nose but largely by droplet infection (Long *et al.*, 1990). After inhalation of these droplets, the first step

of infection involves the bacterium attaching to the ciliated epithelial cells of the bronchial tree. In the second step, the bacteria proliferate and colonise the mucosa of the respiratory tract. The ciliated cells will ultimately be damaged as a direct result of bacterial colonisation.

Typical pertussis disease can be divided into three symptomatic stages, 1) catarrhal, 2) paroxysmal and 3) convalescent. After infection with *B. pertussis*, there is an incubation period of 6 to 20 days. The catarrhal stage lasts 1-2 weeks and symptoms include only a mild cough, sneezing and occasionally conjunctivitis. During the paroxysmal stage (1-20 weeks) the cough is more forceful and more frequent (Cherry *et al.*, 1988). In approximately 50% of patients, coughing ends with the characteristic inspiratory 'whoop', sometimes with vomiting and apnoea and also cyanotic attacks which is the most frequent cause of pertussis hospital admissions (Cherry *et al.*, 1988). The convalescent stage is characterised by a reduction in the number and frequency of paroxysms, which may still occur sporadically for up to 6 months post-infection, and the disappearance of viable *B. pertussis* organisms from the respiratory tract.

Worldwide, *B. pertussis* causes some 20-40 million cases of pertussis, 90% of which occur in developing countries, and an estimated 200-300 000 fatalities each year (WHO, 1999). Although pertussis may occur at any age, most cases of serious disease and the majority of fatalities are observed in early infancy. Major complications are pneumonia, encephalitis, and malnutrition, due to repeated vomiting (Edwards *et al.*, 1999).

1.2.1 Diagnosis

The major problem in diagnosing pertussis is the resemblance between the first stage of pertussis infection and a common cold, meaning that diagnosis may not be possible until the paroxysmal stage. Diagnosis during the catarrhal stage involves culture of the bacteria from nasopharyngeal aspirates or swabs (Onorato *et al.*, 1987). The specimen from the patient is usually cultured on freshly prepared Bordet Gengou (BG) agar and incubated at 37°C in a moist atmosphere for 5 to 7 days. The biochemical reactions of *B. pertussis* including a positive oxidase test, negative urease test or negative motility can be used for differentiation from other species of the *Bordetella* genus. Serological identification tests such as agglutination, complement fixation and ELISA are also available for use in identification of *B. pertussis* (Friedman, 1988). More recently a rapid, real-time multiplex PCR assay for detecting and differentiating *B. pertussis* and *B. parapertussis* in nasopharyngeal swabs was developed (Sloan *et al.*, 2002), thereby significantly improving *B. pertussis* detection by decreasing the length of time taken to generate a result.

1.2.2 Epidemiology

Before the worldwide introduction of pertussis vaccine into the routine childhood vaccination programmes, pertussis was of considerable public health concern in developed as well as in developing countries. Due to the highly contagious nature of this disease, there is always a large number of secondary cases among non-immune contacts. There are several antibiotics, including erythromycin, chloramphenicol,

and tetracycline, which are effective in vivo against pertussis but erythromycin is generally the drug of choice for treatment of pertussis patients (Hoppe, 1992).

Although prophylactic antibiotic treatment in the early incubation period may prevent disease, the difficulty of early diagnosis and the costs involved, as well as the inherent ecological concerns related to induction of drug resistance, limit prophylactic treatment to selected individual cases.

1.3 Regulation of virulence

Bordetella pertussis possesses a genetic locus that encodes a biological 'switch' enabling it to change between different phenotypic states depending on the environment. This has also been shown to be the case in *B. parapertussis*, *B. bronchiseptica* and *B. avium* (Arico *et al.*, 1991; Gentry-Weeks *et al.*, 1991). In the human respiratory tract, a subset of *B. pertussis* genes is expressed which allow the bacterium to colonise the host. In the laboratory, the virulence control switch can be manipulated to bring *Bordetella* species into an avirulent state by reducing the culture temperature from 37°C to 25°C or by the addition of sulphate ions or nicotinic acid to the culture medium (Melton and Weiss, 1993). Virulent and avirulent phases are now referred to as Bvg^+ and Bvg^- respectively.

Weiss *et al.* (1983) used the transposon Tn*5* as a mutagen in order to produce a series of strains that were deficient in a single virulence-associated determinant, and from this, were able to identify a virulence regulatory locus. Weiss and Falkow (1984) proposed that modulating conditions were able to influence the expression of the regulatory locus, which they designated *vir*, that in turn was able to alter expression of virulence determinants. The *vir* locus was later termed the *Bordetella* virulence

gene (*bvg*). This genetic locus encodes two proteins, which enable the bacterium to sense the prevailing environmental conditions, and then act accordingly by controlling the expression of specific genes. The sensor protein is termed BvgS and the activator is known as BvgA. BvgS is a 135-kDa membrane-bound protein with an N-terminal periplasmic region flanked by two transmembrane sequences that hold the protein in position within the cytoplasmic membrane (Stibitz and Yang, 1991). BvgA is a 23-kDa cytoplasmic protein. The 2-domain structure of BvgA, consisting of a receiver at the N-terminus and a C-terminal helix-turn-helix (HTH) motif, is characteristic of response regulators (Arico *et al.*, 1989; Stibitz and Yang, 1991), and it is this HTH module that facilitates specific DNA sequence recognition and binding at the promoters of Bvg-activated genes (Boucher *et al.*, 1994). The heptameric consensus sequence of the BvgA binding site, TTTCCTA, first proposed by Roy and Falkow (1991), is present as either direct or inverted repeats upstream of various Bvg-regulated genes. The presence of these repeats in a number of virulence-activated promoters has been confirmed by DNase I protection studies (Marques and Carbonetti, 1997; Boucher and Stibitz, 1995).

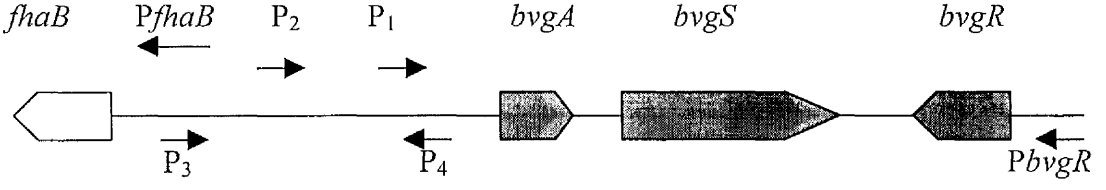
1.3.1 BvgAS signal transduction

Together BvgA and BvgS belong to the two-component regulatory family of signal-transducing proteins that control bacterial gene expression via phosphorylation/de-phosphorylation of component proteins (Arico *et al.*, 1989; Stibitz and Yang, 1991; Parkinson and Kofoid, 1992). The *bvgA* and *bvgS* genes are transcriptionally linked, whilst a third gene, *bvgR*, is transcribed separately on the opposing DNA strand. Transcription of *bvgAS* is controlled by 3 self-regulated promoters; P_1, P_3 and P_4, and one Bvg-independent promoter, P_2 (Roy *et al.*, 1990) (see Figure 1.1). Transcription

from P2 is constitutive, thereby maintaining low-level production of intracellular BvgAS.

BvgAS uses a complex phosphotransfer system to transmit signals between histidine and aspartate residues (His-Asp-His-Asp). When active, BvgS autophosphorylates at the histidine residue position 729 in the transmitter domain, followed by the transfer of the phosphate group to the aspartic acid residue position 1023 in the receiver domain, then on to the histidine residue position 1172 in the histidine phospho-transfer output domain (HPT) before a final phosphotransfer to BvgA (Uhl and Miller, 1994; Uhl and Miller, 1996). The receiver and HPT domains of BvgS are essential for signal transduction to BvgA, thus the histidine kinase transmitter portion of BvgS is unable to pass the phosphate group to BvgA and only the phosphohistidine of the HPT domain can complete the transfer to BvgA (Uhl and Miller, 1994).

Figure 1.1 Schematic representation of the genetic region of *B. pertussis* covering the *bvg* operon and *fhaB* gene.



The Bvg^+ phase is characterised by the expression of all Bvg-activated adhesins and toxins and lack of expression of all Bvg-repressed phenotypes. The Bvg^- phase is characterised by the absence of all Bvg-activated adhesins and toxins and the presence of all Bvg-repressed phenotypes. A third phase, Bvg intermediate (Bvg^i) has been assigned to wild-type *B. bronchiseptica* able to grow in submodulating concentrations of magnesium sulphate or nicotinic acid. This phase is characterised by the absence of Bvg-repressed phenotypes and the presence of some but not all Bvg-activated virulence factors (Cotter and Miller, 1997; Cotter and DiRita, 2000). BvgAS is thought to control four classes of genes in response to environmental conditions 1) those that are expressed only in the Bvg^+ phase, 2) those that are expressed in both the Bvg^+ and Bvg^i phase, 3) those that are expressed only in the Bvg^i phase and 4) those that are expressed only in the Bvg^- phase (Cotter and DiRita, 2000). Examples of the different classes include, *ptx-ptl* operon and *cyaABDE* operon, that belong to class 1 and encode pertussis toxin and adenylate cyclase toxin respectively, *fhaB* which encodes filamentous haemagglutinin and belongs to class 2, *bipA* which belongs to class 3 and is predicted to encode a large outer membrane protein with a region of homology to the intimin protein of enteropathogenic *E. coli,* and the *frlAB* operon belonging to class 4 and encodes flagellin genes in *B. bronchiseptica.* Dnase I protection studies indicate that in the presence of BvgA~P sufficient to activate transcription *in vitro*, the *cyaA* and *ptx* promoter regions bound to BvgA~P extend from position 139 for cyaA and –168 for ptxA to the –35 regions, suggesting that binding of multiple BvgA~P dimers to multiple but relatively weak binding sites is important if not essential for transcriptional activation at the *ptxA* and *cyaA* promoters (Karimova *et al.*, 1996; Zu *et al.*, 1996). Class 1 genes can be

distinguished from class 2 genes on the basis that a significantly higher concentration of BvgA~P is required to activate transcription.

1.3.2 BvgR and RisAS

While BvgA exercises direct control over the expression of *Bordetella* virulence-activated genes (*vag's*) by binding to promoter sequences, control over virulence-repressed genes (*vrg's*) is indirect, implicating a Vag repressor protein. A gene that controls repression of the *vrg's*, has been identified in *B. pertussis* and termed BvgR (Merkel and Stibitz, 1995).

It has been shown that *B. pertussis* mutants lacking BvgR are less efficient colonisers than wild-type strains in a mouse aerosol challenge model, demonstrating that BvgR regulation of the *vrg's* makes a significant contribution to infection in mice (Merkel *et al.*, 1998). The recent discovery in *B. bronchispetica* of a second two-component regulatory system distinct from *bvg* has furthered our knowledge of genetic regulation in *Bordetella* spp. Like *bvg*, this system, designated the *ris* locus (regulator of intracellular response) also consists of genes encoding a response regulator (RisA) and a sensor kinase (RisS) (Jungitz *et al.*, 1998). The *ris* locus is essential for bacterial resistance to oxidative stress and the production of acid phosphatase, as well as *in vivo* persistence. However the function of *ris* in *B. pertussis* has not been determined.

1.4 Virulence factors of *B. pertussis*

The sets of genes expressed by *B. pertussis*, which allow it to invade and persist inside the host, are termed, amongst others, virulence factors or determinants. Falkow and Moxon divided virulence into three main areas; 1) virulence consists of bacterial factors required for host and tissue tropism, 2) virulence includes factors required for multiplication within the host and 3) virulence includes factors involved in aspects of host toxicity (ASM news). The gene products include adhesins, which facilitate attachment to target host cells, and toxins, which enable the bacterium to evade the host immune system. At present the consensus with regard to the strategy for developing whooping cough vaccines seems to be to include a few purified immunogens in an acellular, multicomponent vaccine. Many of the virulence factors of *B. pertussis* have been considered for this purpose. Candidate antigens include filamentous haemagglutinin, serotype-specific fimbriae, pertactin, pertussis toxin and adenylate cyclase toxin. The properties of some *B. pertussis* virulence factors are described below and represented schematically in Figure 1.2, and their role in pathogenesis is summarised in Table 1.1.

1.4.1 Filamentous haemagglutinin (FHA)

The critical step in *B. pertussis* infection is attachment of the pathogen to the ciliated epithelium of the respiratory tract of host cells (Tuomanen and Weiss, 1985), and FHA is consistently referred to as the major adhesin of this bacterium. It is synthesised as a 367 kDa precursor, FhaB, which possesses an N-terminal signal sequence and undergoes extensive C-terminal proteolytic maturation in the course of FHA secretion to form the mature 220 kDa FHA protein (Jacob-Dubuisson *et al.*,

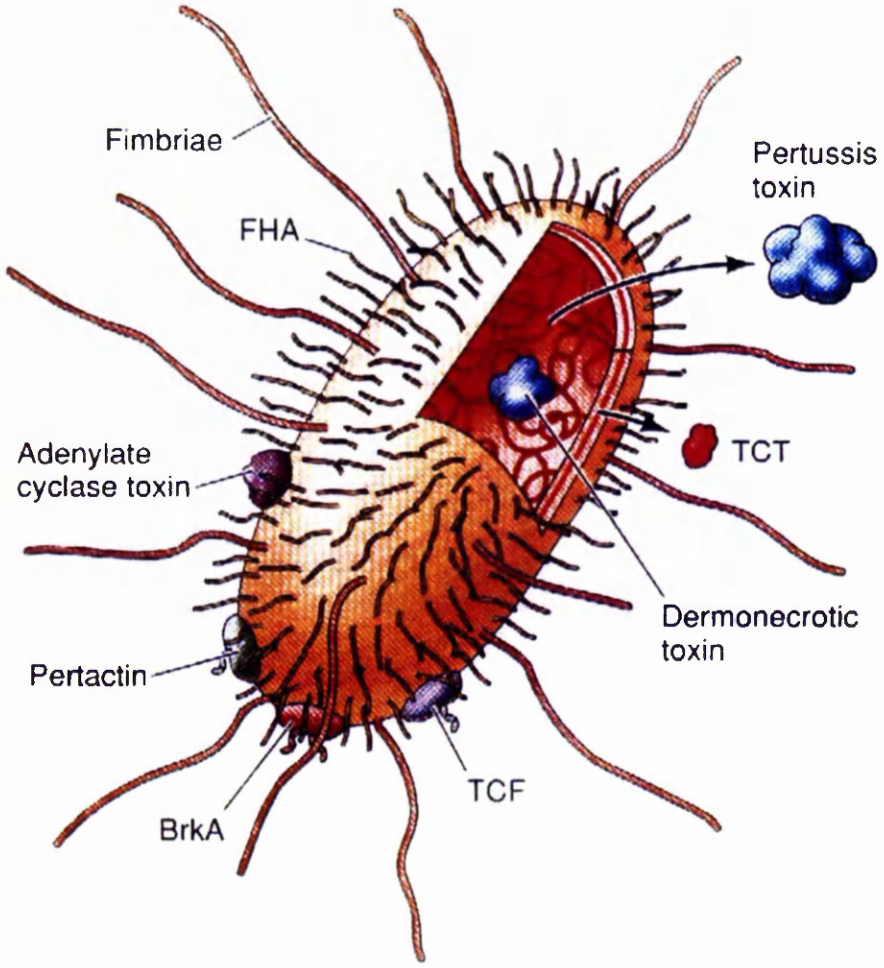
1996; Renauld-Mongenie *et al.*, 1996). FHA has a filamentous structure, supported by electron microscopy studies, giving the dimensions of the molecule as 2 nm wide and 45-50 nm long (Arai and Sato, 1976; Makhov *et al.*, 1994).

Makhov and co-workers (1994) proposed that FHA consists of a polypeptide chain folded into a monomeric hairpin, comprising head, shaft and tail regions. The model predicts that whilst the head contains the terminal domains, and the tail the important RGD (arginine-glycine-aspartic acid) sequence for adhesion, the shaft is composed of tandem 19-amino acid residue repeat regions R1 (38 cycles) and R2 (13 cycles) which maintain the structural integrity of the molecule. The carbohydrate-binding domain of FHA, which has a unique affinity for glycolipids, mediates attachment to cells in the respiratory tract region. This domain has been further narrowed down to amino acid residues 1141-1279 using monoclonal antiobodies (Prasad *et al.*, 1993). Animal experiments, involving infection experiments with a *B. pertussis* mutant unable to produce FHA, have shown that the colonisation in the lung and persistence in the upper respiratory tract were significantly reduced (Weiss and Goodwin, 1989). However, Alonso *et al.*, (2001) demonstrated that FHA-deficient mutants colonised the mouse respiratory tract nearly as efficiently as the parent strain. When *B. pertussis* is in suitable conditions for colonisation, FHA is one of the first proteins produced and is detected in a matter of minutes (Scarlato *et al.*, 1991).

Table 1.1 Role of virulence factors associated with the pathogenesis of *B. pertussis*

Virulence factor	Role in pathogenesis	Reference
Filamentous haemagglutinin	Contains sites that mimic ligands recognised by the integrin complement receptor 3 (CR3) that leads to phagocytosis via CR3 thereby enabling bacteria to avoid triggering an oxidative burst. Also a domain that mediates attachment to cells in the respiratory tract	Relman *et al.*, 1990; Prasad *et al.*, 1993; Liu *et al.*, 1997
Fimbriae	Involved in attachment and required for effective colonisation in the respiratory tract.	Willems *et al.*, 1990; Mooi *et al.*, 1992
Pertactin	Acts as an adhesin for *B. pertussis*, the cell attachment site is an RGD motif which may bind integrin	Charles *et al.*, 1989; Charles *et al.*, 1994.
Pertussis toxin	Mediates attachment to ciliated respiratory epithelial cells and macrophages. Upregulates macrophage integrin CR3 that acts as a receptor for FHA. ADP-ribosylation of G proteins.	Katada and Ui, 1982; Relman *et al.*, 1990; Van't *et al.*, 1992
Adenylate Cyclase Toxin	Activated by eukaryotic calmodulin to catalyse the conversion of ATP to cAMP, resulting in impairment of phagocytic cells and also apoptosis.	Wolff *et al.*, 1980; Hanski and Farfel, 1985; Gueirard *et al.*, 1998
Dermonecrotic Toxin	Belongs to a family of dermonecrosis-inducing toxins and has been shown to have a role in disease although mutants deficient in DNT do not exhibit any differences in virulence in mouse studies.	Walker and Weiss, 1994; Horiguchi *et al.*, 1994
Tracheal Cytotoxin	Causes ciliostasis and impairs neutrophil functions thereby contributing to *B.pertussis* survival *in vivo*.	Goldman, 1988; Cundell *et al.*, 1994
Lipopolysaccharide	Adjuvant and pyrogenic properties.	Allen *et al.*, 1998
Bordetella resistance to killing	Important in virulence. Mutant strains of *B. pertussis* deficient in the expression of the *brk* locus are less virulent than the wild-type and are more susceptible to complement-activated killing.	Weiss and Goodwin, 1989; Fernandez and Weiss, 1994
Tracheal colonisation factor	*bvg*-regulated protein, shown to be important in bacterial colonisation in the mouse model.	Finn and Stevens, 1995

Figure 1.2 Virulence factors of *Bordetella pertussis*. Taken from Weiss (1997)



FHA, filamentous haemagglutinin

BrkA, *Bordetella* resistance to *k*illing

TCF, tracheal colonisation factor

TCT, tracheal cytotoxin

The tail region of FHA includes a binding moiety specific for complement receptor 3 (CR3) integrins present on the surface of macrophages. The RGD sequence in the tail region facilitates binding to CR3 and in turn leads to phagocytosis via CR3, which enables the bacteria to avoid triggering an oxidative burst and thereby permitting them to survive intracellularly whilst evading the immune system (Relman *et al.*, 1990). FHA is also known to bind the human serum protein C4BP. This protein is known as a regulator of complement activation and acts to inhibit the classical complement pathway, ceasing the formation of the membrane attack complex (Berggard *et al.*, 1997). How *B. pertussis* is able to make full use of this binding capacity is still unclear, but demonstrates the extent to which *B. pertussis* interacts with the immune system. A recent report by McGuirk and Mills (2000) showed purified FHA was able to stimulate the release of IL-10 and IL-6 and to suppress IL-10-mediated IL-12 secretion in J774 murine macrophages. This in turn suggests that FHA may delay the development of CMI, thereby facilitating the persistence of *B. pertussis* in the respiratory tract. Further work by Abramson *et al.* (2001) confirmed these findings by showing that FHA was able to stimulate secretion of TNFα and may induce apoptosis in human monocyte-like cells, macrophages and bronchial epithelial cells. Thus, as well as the important role FHA plays in adhesion, it may also modify the development of the host immune response to *B. pertussis* by contributing to proinflammatory and proapoptotic responses, which could in turn favour the bacteria by providing a more favourable location for their adherence.

1.4.2 Serotype-specific fimbriae

Fimbriae, also known as pili or agglutinogens, are long filamentous protrusions, which extend from the bacterial cell surface and facilitate a variety of binding capacities. Both major and minor sub-units are incorporated in the fimbriae. The major sub-units form the fimbrial strand, being grouped into pentameric repeat units, each 13 nm in length and comprising two full helical turns (Steven *et al.*, 1986). The major fimbrial subunits that form the two main serotypes Fim 2 and Fim 3 are encoded by chromosomal loci *fim2* and *fim3*, respectively (Livey *et al.*, 1987; Mooi *et al.*, 1987). A third locus, *fimX*, is expressed at very low levels (Riboli *et al.*, 1991) and a fourth locus, *fimN*, has been identified in *B. bronchiseptica* (Kania *et al.*, 2000). The most noticeable aspect of the fimbrial promoters is the C stretch, a long run of cytosine residues that have been implicated in fimbrial phase variation. Insertion or deletion of extra cytosine residues in this C stretch is the source of fimbrial phase variation by slip-strand mispairing with the cytosine residues located between the –10 and –35 elements of *fim2, fim3, fimX* and *fimN* promoters (Willems *et al.*, 1990). All fimbrial serotypes share a common minor fimbrial subunit, FimD, which forms the tip adhesin and is located within the fimbrial biogenesis operon, downstream of *fimB* and *fimC*, genes that are thought to function as a periplasmic chaperone and outer membrane usher protein, necessary for fimbrial biogenesis (Locht *et al.*, 1992; Willems *et al.*, 1992). As with other virulence factors employed by *B. pertussis* to attach to host cells, the expression of fimbriae is controlled by the *bvg* locus.

Purified fimbrial proteins have been shown to protect mice against respiratory infection with different serotypes of *B. pertussis* (Robinson *et al.*, 1989) and *in vivo*

studies have shown that Fim⁻ *B. pertussis* strains are defective in their ability to multiply in the nasopharynx and trachea of mice (Geuijen *et al.*, 1997). More recently, Mattoo *et al.* (2000), have shown that fimbriae are absolutely required for persistence in the trachea in both rat and mouse models of *B. bronchiseptica* infection.

1.4.3 Pertactin

Pertactin is known as P.69 or 69-kDa OMP because of its apparent MWT determined by SDS-PAGE (Montaraz *et al.*, 1985, Makoff *et al.*, 1990). It is actually a 60 kDa OMP involved in bacterial adherence (Makoff *et al.*, 1990; Leininger *et al.*, 1991). Other membranes of the *Bordetella* genus produce similar molecules, for example, P.70 in *B. parapertussis* and P.68 in *B. bronchiseptica* (Montaraz *et al.*, 1985; Li *et al.*, 1991).

The *prn* gene encodes a 95.5 kDa polypeptide comprising 910 amino acids. This precursor, termed p.93, later undergoes the removal of a 34 amino acid N-terminal signal peptide and cleavage of a 30 kDa polypeptide (p.30) from the C-terminus (Charles *et al.*, 1989). Emsley *et al.* (1996) determined the X-ray crystal structure of P.69 and showed that the protein folded to consist of a 16-stranded parallel β-helix, the largest beta-helix known to date. The structure is described as a helix with several protruding loops that contain sequence motifs concerned with the biological activity of the protein. In particular, there is an RGD motif that is also found in attachment sites of fibronectin, vitronectin and fibrinogen, thereby indicating that pertactin may also bind integrins (Hynes, 1987). No accessory proteins are thought to be involved in the transport of pertactin across the outer membrane (Charles *et al.*, 1994). It is assumed that there are three domains within the autotransporter protein:

the leader sequence, the amino-terminal passenger domain and the carboxy-terminal β-barrel domain. It is proposed that the signal-cleaved amino-terminal domain is translocated through a β-barrel pore made by the C-terminal 30 kDa region to the cell surface, where either the protein remains intact with a carboxy-terminal membrane-bound domain and an amino-terminal domain extending into the environment, or the protein becomes autoproteolytic and cleaves itself, or is cleaved by an outer membrane protease (Henderson *et al.*, 1998). Many other proteins, such as VacA of *Helicobacter pylori*, IgA1 protease of *Haemophilus influenzae, Neisseria gonorrhoeae* and *N. meningitidis*, have all been shown to use a similar mechanism in order to cross the membrane, and these have been collectively termed members of the autotransporter family (Henderson *et al.*, 1998). Other *Bordetella* proteins with predicted autotransporter ability include TcfA (Finn *et al.*, (1995), BrkA (Fernandez and Weiss, 1994) and Vag8 (Finn and Amsbaugh, 1998), all of which show significant amino acid sequence similarity in their C-termini and contain one or more RGD motifs within the N- or C-termini. In a comparison of the *prn* gene sequences of *B. pertussis, B. parapertussis and B. bronchiseptica*, the precursors of pertactin were found to be extremely homologous, with the C-termini displaying the most conserved regions, suggesting the functional relevance of P.30 to these organisms (Li *et al.*, 1992). Mutants deficient in pertactin have been shown to be 30-40 % less efficient in adherence to Chinese Hamster Ovary (CHO) cells and HeLa cells (Leininger *et al.*, 1991; Leininger *et al.*, 1992). However, recent work involving site-directed mutagenesis (aspartic acid to glutamic acid) to obtain a pertactin molecule with an RGE instead of RGD site showed no role for the pertactin RGD sequence as a mediator of eukaryote cell adhesion. Strains expressing the mutation displayed no

difference in their ability to promote adhesion to HEp-2 or CHO cells (Everest *et al.*, 1996).

Pertactin has been shown to be an immunoprotective antigen, being used in subunit vaccines to protect mice from respiratory challenge with virulent *B. pertussis* (Novotny *et al.*, 1991; Romanos *et al.*, 1991; Roberts *et al.*, 1993) and is now included in some commonly available acellular pertussis vaccines (See Section 1.5.2).

1.4.4 Pertussis toxin

Pertussis toxin (PT) was initially termed histamine-sensitising factor or mouse protective antigen according to its activities (Levine and Pieroni, 1966). In 1979 it was re-named pertussis toxin by Pittman (Pittman, 1979). PT has a wide range of biological activities both *in vitro* and *in vivo*. Activities of PT demonstrated *in vitro* include; ADP-ribosylation of G proteins, inhibition of chemotaxis and oxidative responses in neutrophils and macrophages, and adhesion and invasion of ciliated respiratory epithelial cells (Bockoch and Gilman, 1984; Meade *et al.*, 1984; Okajima and Ui, 1984; Lad *et al.*, 1985; Tuomanen and Weiss, 1985). Activities of PT demonstrated *in vivo* include; histamine and endotoxin sensitisation, enhancement of insulin secretion, adjuvanticity and acute toxicity (Pittman, 1984; Munoz, 1988; Kaslow and Burns, 1992). Of the *Bordetella* species, only *B. pertussis* synthesises and secretes PT. A cryptic PT operon is present in *B. parapertussis* and *B. bronchiseptica*, but a group of mutations in the promoter region leaves these operons transcriptionally silent (Arico *et al.*, 1987). PT is a complex 106 kDa, hexameric protein comprising 5 distinct subunits, S1 – S5 in the ratio 1:1:1:2:1. Each subunit is synthesised with an N-terminal signal sequence, which may suggest that transport of

PT subunits into the periplasmic domain occurs via a general export pathway. The PT subunits are assembled in the periplasm into an A-B architecture, similar to cholera toxin, except that the binding subunit of PT is more complex. The A protomer, consisting of the S1 enzymically-active subunit, sits above the ring-like B oligomer comprising the remaining subunits S2-S5. The B region of the protein facilitates attachment of toxin to the host cells and increases the efficiency with which the S1 subunit gains entry into host cells (Tamura *et al.*, 1982). The genes encoding the PT subunits are clustered together in an operon and genetic analysis has revealed that each subunit is translated separately with an amino-terminal signal sequence, which is cleaved during transport to the periplasm where the holotoxin is then assembled (Locht and Keith, 1986). Secretion across the outer membrane involves a specialised transport mechanism composed of nine Ptl (pertussis toxin liberation) proteins (Covacci and Rappuoli, 1993; Weiss *et al.*, 1993; Farizo *et al.*, 1996). The *ptl* locus located downstream of the *ptx* operon is related to the *Agrobacterium tumefaciens virB* operon, which encodes a type IV secretion system to export the tumour-inducing T-DNA complex (Das, 1988; Ward *et al.*, 1990; Kaldau *et al.*, 1990). It has been suggested that the two systems may function by a common mechanism, as both are involved in the transport of large protein complexes.

When in its reduced form, the S1 subunit is able to catalyse the transfer of ADP-ribose from NAD to the alpha subunit of guanine nucleotide binding proteins (G proteins) in eukaryotic cells (Tamura *et al.*, 1982; Katada *et al.*, 1983). G proteins are molecular switches involved in many cell functions such as cell proliferation, tissue differentiation, signal transduction and protein synthesis. G proteins that PT has been shown to inactivate by ADP ribosylation are G_i (inhibitory G protein), G_t

(transducin) and G_o. When active, G_i inhibits adenylate cyclase and activates potassium channels, G_t activates cyclic GMP phosphodiesterase and G_o activates potassium channels, inactivates calcium channels and activates phospholipase C-beta (Ui, 1990). The disruption of these signalling pathways *in vivo* leads to effects such as histamine sensitisation and enhancement of insulin secretion in response to regulatory signals (Pittman, 1984; Munoz, 1988).

The vast diversity of the biological activities of PT can be accounted for by its enzymatic ADP-ribosyltransferase activity. However, some biological activities of PT including its mitogenicity and its ability to agglutinate red blood cells, are independent of the enzymatic activity. Based on the knowledge of the molecular mechanisms of PT action, genetically-detoxified (enzymically inactive) derivatives were developed as vaccine candidates, in that they induced high levels of protection without causing harmful side effects (Pizza *et al.*, 1989). Overexpression and secretion of genetically detoxified PT has since been acheived in *B. pertussis* and made available for use as an acellular pertussis vaccine component (Zealey *et al.* 1992).

1.4.5 Adenylate cyclase toxin

The adenylate cyclase of *B. pertussis* is the main subject of this thesis and will be discussed separately (Section 1.7).

1.4.6 Dermonecrotic toxin

Dermonecrotic toxin (DNT) was one of the first *B. pertussis* toxins described and originally named endotoxin (Bordet and Gengou, 1906). It was subsequently termed

heat-labile toxin because it is completely inactivated by heating at 56°C for 10 min. The term DNT is due to the characteristic necrotic skin lesions caused by the toxin when it is injected subcutaneously into mice (Livey and Wardlaw, 1984) but the toxin also causes other side effects such as spleen atrophy, reduced weight gain and is considered to be a virulence factor for the production of turbinate atrophy in porcine atrophic rhinitis (Wardlaw and Parton, 1983; Roop *et al.*, 1987). DNT is also lethal for mice at low doses after intravenous inoculation (Iida and Okonogi, 1971). The genes encoding the DNT's of *B. pertussis* and *B. bronchiseptica* have been cloned and sequenced (Walker and Weiss, 1994; Pullinger *et al.*, 1996; Kashimoto *et al.*, 1999) and the open reading frame of DNT shown to consist of 4395bp coding for 1464 amino acids. Kashimoto *et al.* (1999) identified Cys 1305 as an essential amino acid for the enzymic activity of DNT, as the introduction of a mutation at Cys 1305 eliminated the activity. DNT belongs to a family of bacterial toxins that include CNF1 and CNF2 of *E. coli* (cytotoxic necrotising factor 1 and 2) (Falbo *et al.*, 1993; Oswald *et al.*, 1994) and the *Pasteurella multocida* toxin (PMT) (Rozengurt *et al.*, 1990). The toxins serve as regulators of cell growth or division. There is limited homology among the group members at the amino acid level. PMT and CNF sequences and CNF and DNT sequences show regions of significant amino acid homology at their N and C-termini respectively (Lax *et al.*, 1990; Falbo *et al.*, 1993; Oswald *et al.*, 1994; Walker and Weiss, 1994), but there is no significant homology between the sequences of DNT and PMT. It has been suggested that DNT targets and modifies Rho proteins (Oswald *et al.*, 1994; Horiguchi *et al.*, 1995), a family of GTP-binding proteins involved in the regulation of cell functions such as the formation of actin stress fibres and focal adhesins (Horiguchi *et al.*, 1995). DNT deaminates Gln residue 63 of Rho and the corresponding residues of the Rho family

proteins, Rac and Cdc42 (Horiguchi *et al.*, 1997). This results in the reduction of its capacity to hydrolyse GTP to GDP and thus renders the Rho family proteins constitutively active. The exact role of DNT in the pathogenesis of *B. pertussis* is still unclear, because although DNT is termed a virulence factor, mutants deficient in DNT do not exhibit any differences in virulence in mouse studies (Weiss and Goodwin, 1989).

1.4.7 Tracheal cytotoxin

Tracheal cytotoxin (TCT) is a low-molecular-mass glycopeptide, whose primary structure has been determined by mass spectrometry as a 921 Da, N-acetylglucosaminyl-1,6-anhydro-N-acetylmuramylalanyl-γ-glutamyl-diaminopimelylalanine, identical to the *Neisseria gonorrhoeae* ciliostatic anhydropeptidoglycan (Cookson *et al.*, 1989b). The destruction of cilia and ciliated epithelial cells by TCT results in ciliostasis and may be responsible for relentless coughing of the infected individual in order to remove accumulating mucus. It has been demonstrated in hamster trachea epithelial cells, that the lactyl tetrapeptide part of the molecule is responsible for full toxic activity (Luker *et al.*, 1995). The toxicity conferred by TCT is indirect, being caused by the induction of host cells to produce IL-1. This activates host cell nitric oxide synthase leading to high levels of nitric oxide radicals. The nitric oxide acts by destroying iron-dependent enzymes, eventually inhibiting mitochondrial function and DNA synthesis in nearby host cells (Heiss *et al.*, 1994). TCT also has a toxic effect on other cells, impairing neutrophil function at low concentrations and conferring toxic activity in larger quantities (Cundell *et al.*, 1994). Such effects could explain the cause of some of the pathological events in pertussis infection such as coughing, excess mucus production

in the airways and the predisposition to secondary bacterial infections (Wardlaw and Parton, 1988). TCT could just be termed a breakdown by-product of peptidoglycan manufacture, however it is considered an integral component in the pathogenesis of *B. pertussis* infection.

1.4.8 Lipopolysaccharide

Lipopolysaccharide (LPS) is present in most Gram-negative bacteria and generally comprises a lipophilic portion termed lipid A, the core oligosaccharide and a long polysaccharide O-antigen (O-specific chain). The structure of *B. pertussis* LPS is different to *B. parapertussis* and *B. bronchiseptica*, in that it lacks the O-antigen and because of this is sometimes referred to as lipooligosaccharide (Martin *et al.*, 1992; Preston *et al.*, 1996). *B. pertussis* produces two types of LPS, designated LPS-A and LPS-B, which resolve as two distinct bands on silver-stained SDS-PAGE gels (Peppler, 1984). LPS-A has a standard lipid A portion like the LPS of *E. coli*, whereas LPS-B has a distinctive lipid portion named Lipid X (2,3-diacylglucosamine 1-phosphate). The two types of LPS also have different polysaccharide regions. LPS-A has a polysaccharide region containing an oligosaccharide core with 2-keto-3-octulosinic acid and LPS-B contains an oligosaccharide core with phosphorylated ketodeoxyoctulosonic acid (KDO). Although all *Bordetella* species produce LPS, their structures vary somewhat between the different species, which may reflect the differences in some of the biological activities among the *Bordetella* species. *B. parapertussis* strains isolated from humans and sheep display distinct LPS profiles, adding to the host-specific feature of this molecule (Porter *et al.*, 1995; van den Akker, 1998). The LPS produced by *B. bronchiseptica* is similar to that of *B. parapertussis*, as they both express O-antigen in a temperature-dependent manner

(van den Akker, 1998). Recently, Allen and Maskell identified, cloned and sequenced the genetic loci required for LPS biosynthesis in *B. pertussis, B. bronchiseptica* and human *B. parapertussis,* and went on to construct strains with mutant LPS phenotypes (Allen and Maskell, 1996; Allen *et al.*, 1998). When compared with their wild type parental strains, *B. pertussis B. bronchiseptica* and human *B. parapertussis* strains that synthesised only LPS-B, due to a deletion of the *wbl* locus that is required for the expression of LPS-A, were found to be less efficient at colonisation of the trachea in a mouse model of respiratory infection (Harvill *et al.*, 2000). The three mutants all displayed a different degree of defectiveness in their colonisation. The *B. pertussis wbl* mutant was less able to colonise the nasal cavity than the wild type and the *B. bronchiseptica* mutant colonised the lungs like the wild-type initially but was cleared by the second week post-infection. Both *B. parapertussis* and *B. bronchiseptica* mutants were sensitive to killing by normal rabbit serum, whereas the parent strains were not, but both the *B. pertussis* mutant and wild-type were killed. These results indicate a difference in the host-pathogen interaction between the different *Bordetella* species. The data also conflict with the proposed role of BrkA of *B. pertussis* in resistance to complement-activated killing (see section 1.4.10).

1.4.9 Tracheal colonisation factor

Tracheal colonisation factor (TCF) is a *bvg*-regulated protein, exclusively produced among the *Bordetellae* by *B. pertussis*. The gene encoding TCF, *tcfA*, has been cloned and sequenced, and the derived amino acid sequence predicts a 68.6 kDa precursor, the first 39 amino acids of which comprise a likely signal peptide, leaving a 64.4 kDa protein (Finn and Stevens, 1995). The precursors of the pertactins of

Bordetellae species possess a C-terminus with significant homology to that of TCF, as does the serum resistance factor, BrkA (Section 1.4.10), (Fernandez and Weiss, 1994). The exact role that TCF plays in the pathogenesis of pertussis is yet to be fully understood, although experiments involving infection of mice with a strain of *B. pertussis* defective in TCF, demonstrated that the number of bacteria isolated from the trachea was decreased ten-fold in comparison to the wild type parent strain, indicating the importance that this protein has in colonisation (Finn and Stevens, 1995).

1.4.10 Serum resistance locus

The *brk* locus, which has homologues in *B. parapertussis* and *B. bronchiseptica*, consists of two genes transcribed in opposite directions, *brkA* and *brkB* (Fernandez and Weiss, 1994; Rambow *et al.*, 1998). The proteins predicted by these genes, BrkA and BrkB; are both required for serum resistance. BrkA, translated as a precursor molecule, is proteolytically cleaved to form 73 kDa and 30 kDa products. This cleavage pattern is very similar to that of pertactin, the protein to which BrkA is most closely related, and Tcf (Finn and Stevens, 1995). Mutant strains of *B. pertussis* deficient in the expression of the serum resistance (*brk*) locus are significantly less virulent in mice than the wild-type strains (Weiss and Goodwin, 1989). These strains have also been shown to be more susceptible to complement-activated killing in the presence of human serum (Fernandez and Weiss, 1994). A recent study has demonstrated that *B. pertussis* clinical isolates were serum-resistant, suggesting that circulating strains gain an advantage *in vivo* by evading complement-activated killing (Fernandez and Weiss, 1998).

1.4.11 Invasion

It is well known that different *Bordetella* species are able to invade and survive within a range of cell types in particular; epithelial cells (Ewanowich *et al.*, 1989; Savelkoul *et al.*, 1993), dendritic cells (Guzman *et al.*, 1994) and macrophages (Bromberg *et al.*, 1992; Friedman *et al.*, 1992; Forde *et al.*, 1998). This capacity of persistence within a cellular environment that *Bordetella* species possess, may allow them to escape any host clearance mechansims that they would otherwise be susceptible to, and also provide them with a habitat rich in nutrients and devoid of competing microorganisms (Finlay and Falkow, 1989). *B. bronchiseptica* strains have been shown to differ to other *Bordetella* species, as both wild type and Bvg^- strains are able to invade and survive within eukaryotic cells (Guzman *et al.*, 1994; Schipper *et al.*, 1994), whereas intracellular survival in other *Bordetella* species is dependent on *bvg*-activated products (Banemann and Gross, 1997; Friedman *et al.*, 1992). Jungitz *et al.* (1998) generated bacterial mutants in order to identify genes encoding proteins invovled in the intracellular survival process, which subsequently led to the identification of a novel 2-component regulatory system (Ris) (Section 1.3.2). The sensitivity of *B. bronchispetica* to superoxide dismutase (SOD) and hydrogen peroxide (H_2O_2) was analysed using the compound paraquat. Paraquat generates intracellular free-oxygen radicals, which by further reduction, can accumulate as toxic hydroxy-radicals, whereas H_2O_2 is an important compound of the respiratory burst and can also generate hydroxy-radicals. Jungitz and co-workers demonstrated that the *ris* mutants were susceptible to both compounds thereby proposing that *ris* regulates a protein required to survive the accumulation of such hydroxy radicals and necessary for intracellular survival.

1.5 Vaccination against pertussis

Given that antibiotics are not very effective for the prevention or control of pertussis due to the fact that diagnosis usually occurs after the bacteria have already damaged the respiratory tract and released the toxins responsible for severe symptoms, the best current alternative is vaccination, with either WCV or the newer ACV's.

1.5.1 Whole-cell vaccine

Whole-cell vaccines (WCV) have been available for many years and are manufactured in several countries. An effective vaccine against whooping cough was developed in the 1940's and although the basic preparation procedures are similar between different vaccines, the vaccines frequently elicit markedly different immune responses to various *B. pertussis* antigens. In general, *B. pertussis* is grown in bulk culture, harvested, concentrated by centrifugation, and suspended in a buffered saline solution (Cherry *et al.*, 1988). Concentrated bacteria are killed and partially detoxified by heat or a chemical agent or by a combination of these methods. Currently available WCVs are usually given in a combined preparation that contains diphtheria and tetanus toxoids and a suspension of killed *B. pertussis* cells, which are adsorbed onto an aluminium adjuvant to enhance the immunogenicity and efficacy of the vaccine. All whole-cell pertussis vaccines contain both endotoxin and other active toxins, so it is not surprising that reactions occur.

Immunisation of infants following approved schedules, with WCV vaccines, has shown an efficacy of 80% or more. An accelerated immunisation schedule at two,

three and four months of age, was introduced in the UK in 1990 in an attempt to increase the protection given to very young children in whom pertussis is most severe (DOH, 1990). In countries with good vaccination coverage, morbidity and mortality from pertussis have gradually been reduced to low levels. However, in recent years an increase in the incidence of pertussis has been reported from a number of countries, including the US, Australia, Canada, and several countries in Europe. The reasons for this increase are largely unknown, but sub-optimal quality of the involved WCV and cyclical variation in disease patterns may have added to the effect of waning vaccine-derived immunity in certain countries. It has also been suggested that the increase could be due to antigenic divergence between vaccine strains and circulating strains (Willems and Mooi, 1996). Mooi *et al.* (1999) conducted an analysis of *B. pertussis* strains from Finland and reported that different alleles of pertactin (*prn*-1, *prn*-2, *prn*-3 and *prn*-4) were present in isolated strains. The results from the study suggested the possibility that, pertussis vaccines, which used a strain with a *prn*-1 genotype, protected less well against *prn*-3, which predominate in some countries such as The Netherlands, but are less common in Finland. A more recent report indicates an antigenic divergence between clinical isolates and vaccine strains in The Netherlands, particularly with respect to pertactin and pertussis toxin, therefore suggesting that *B. pertussis* has been able to adapt and remain endemic and thereby contribute to the re-emergence of pertussis in The Netherlands (Mooi *et al.*, 2001).

In the UK, after a 1974 report ascribing neurological reactions to WCV (Kulenkampff *et al.*, 1974), public confidence in the vaccine fell and a sharp reduction in coverage followed. Although the health authorities resisted pressure to withdraw the vaccine, the decrease in confidence resulted in pertussis epidemics

(incidence at approx. 150 per 100,000). Confidence was eventually restored and the disease incidence declined to approx. 1 in 100,000 and has since been low (Gangrosa *et al.*, 1998). Following the pertussis epidemic in The Netherlands in 1996, and the demonstration by Mooi and co-workers (1998, 1999) of a clear shift in the genetic composition of *prn* and *ptx* genes of *B. pertussis* in circulating *B. pertussis* strains, a similar study was undertaken to investigate whether such variation had occurred in the UK (Fry *et al.*, 2001). Isolates of *B. pertussis* in the UK were examined both before and after the introduction of the UK WCV. The precise year of emergence in the UK of *prn* types distinct from those in the UK WCV cannot be determined, however *prn*-2 and *prn*-3 were found in strains from 1982 onwards. Data from The Netherlands and Finland show that by the 1990's, approximately 90% of isolates were of a non-vaccine *prn* type (Mooi *et al.*, 1998; Mooi *et al.*,1999), whereas data from the UK showed that although non-vaccine *prn* types had increased, almost half of all circulating strains remained of the vaccine *prn* type. These results have led to a possible hypothesis that a WCV containing *prn-1* may protect better against *B. pertussis* with *prn-2*, than against *B. pertussis* strains with *prn-3*, thereby suggesting why there is a continued low level of pertussis in the UK compared to The Netherlands (Fry *et al.*, 2001). A further study was carried out by Weber *et al.* (2001) using genotyping with pulse-field gel electrophoresis (PFGE) to investigate the influence of temporal and geographic factors on the French population of *B. pertussis* isolates circulating over different periods of time. Chromosomal DNA from isolates collected from different parts of France was analysed by PFGE and distinguished into groups by clustering anlaysis. These groups correlated well with the P.69 type expressed by the isolates and one group conatining isolates that expressed *prn-2* could be further divided; a group composed of isolates circulating

between 1993 and 1996 and a second group composed of isolates circulating since 1997. These results are consistent with a shift in the circulating *B. pertussis* population every 3 years, which may correspond to periodic pertussis incidences observed in different countries (Weber *et al.*, 2001).

A recent study on antigen expression of vaccinal strains in the Pasteur Merieux (Aventis Pasteur) french WCV from multiple lots stored since 1984 showed that all lots of the vaccinal strains expressed the major adhesins such as FHA and P.69, together with CyaA and PT, indicating that, despite lyophilisation being performed on the strains for over 30 years, they still continued to express the toxins and adhesins expressed by currently circulating isolates (Njamkepo *et al.*, 2002). Since neither the vaccine quality nor the coverage has changed in France, the most likely cause of a pertussis resurgence is a waning immunity in the absence of booster vaccination. France therefore decided to introduce a booster dose of acellular pertussis vaccine (see below) at 13 years of age, which, at this age, has been shown to protect adolescents against *B. pertussis* infection (Anonymous, 1998).

1.5.2 Acellular pertussis vaccine

Vaccination in Japan against pertussis began in 1947. By 1974, there were few cases and no deaths, however during a national debate about the adverse events resulting from smallpox vaccine, news reports of neurological reactions after DTP vaccination, gave rise to Japan's movement against whole-cell pertussis vaccines, resulting in claims that vaccination would no longer be needed because there was practically no more pertussis in the community (Kimura *et al.*, 1990, Kanai., 1980). In response to this, the Okayama Perfectural Medical Association switched from

DTP to DT only vaccine. Pertussis vaccination coverage for infants fell from nearly 80% in 1974 to 10% in 1976 (Kimura *et al.*, 1990). A pertussis epidemic occurred in 1979 with more than 13,000 cases and 41 deaths.

Following adverse publicity concerning the side effects associated with WCV, in particular the severe effects such as brain damage and death, the World Health Organisation (WHO) recommended continuing vaccination with WCV but with a greater emphasis on the development of new vaccines devoid of side effects. In order to develop a new vaccine, the composition of the WCV was studied. It is composed of many different molecules, including lipopolysaccharide, nucleic acids, lipids and proteins, some of these being important for the virulence of *B. pertussis*. Therefore the first step in the vaccine development was to identify and isolate the antigens that conferred protection. Sato and co-workers were the first to identify and separate PT and FHA from the culture supernatant of *B. pertussis*, before moving on to detoxify them by formaldehyde treatment. This led to the development of the first ACV containing PT and FHA (Sato and Sato, 1984). Japan began replacing whole-cell with ACV in 1981, and a striking fall in pertussis incidence followed (incidence was approx. 1 per 100,000). To evaluate the efficacy of this vaccine (Takeda vaccine) and another Japanese preparation containing only purified PT (Biken vaccine), a field test was organised in Sweden in 1986. The results of this trial showed that both vaccines were able to protect children with an efficacy similar to that of the WCV (Ad Hoc group, 1988; Olin and Storsaeter, 1989). One other result of the trial was the ability of the formaldehyde-inactivated PT to revert to the active toxic form (Storsaeter *et al.*, 1990). In order to reduce the risks associated with detoxification, better methods were required and Rappuoli and co-workers approached this problem using recombinant-DNA technology (Rappuoli *et al.*,

1991). They developed genetically detoxified pertussis toxin molecules containing amino acid substitutions that were able to abolish the enzymatic activity of the S1 sub-unit, the best Arg9 to Lys and Glu129 to Gly. This was then used as a component of new acellular vaccines, one containing only 15μg of the mutant PT-9K/129G and the other containing 7.5μg of the mutant PT-9K/129G, 10μg of FHA and 10μg of 69K. Results from a trial involving over 1000 infants receiving three doses of the vaccines alone or combined with diphtheria and tetanus toxoids, showed both vaccines to be safe and highly immunogenic (Rappuoli *et al.*, 1992).

As a result of these trials, plans were made for further trials of different ACV's. One of these took place in Sweden and compared a two-component vaccine with a five-component vaccine and a conventional WCV used in the USA (Gustafsson *et al.*, 1996). The two-component vaccine (SmithKline Beecham (SKB)) consisted of 25μg PT detoxified by glutaraldehyde and formalin treatment, plus 25μg of formalin-treated FHA and the five-component vaccine (Connaught) consisted of 10μg glutaraldehyde-inactivated PT, 5μg FHA, 5μg Fim 2 and 3 combined and 3μg of P.69. Infants received 3 injections, the first at two months and the second and third at eight-week intervals. The results of the trial showed both ACV to be highly immunogenic with regard to each antigen studied, although overall the five-component vaccine was more efficacious against mild disease. They concluded that the multi-component vaccine was more protective against both typical pertussis and mild disease than the one and two-component vaccines, results that were also confirmed in earlier trials (Edwards *et al.*, 1995; Greco *et al.*, 1996). A further study by Olin and co-workers investigated the effects of a two, three or five-component vaccine and a UK whole-cell DTP vaccine (Olin *et al.*, 1997). The two-component vaccine was from SKB as before, the three-component vaccine (Chiron) consisted of

5μg of the genetically detoxified PT (9K/129G), 2.5μg of FHA and 2.5μg of P.69, and the five-component vaccine (Pasteur-Merieux-Connaught) consisted of 20μg glutaraldehyde-inactivated PT, 20μg of formalin-treated FHA, 3μg P.69 and 5μg of fimbriae 2 and 3 combined. The two and five-component vaccines contained phenoxyethanol as a preservative, whereas the three-component and WCV contained thiomersal. The DTP vaccines were given in a series of three intra-muscular injections at 3 months, 5 months and 12 months, according to previous Swedish vaccination schedules for DT. Overall the main finding was that the WCV and the five-component vaccine had similar efficacy against culture-confirmed typical pertussis, whereas the three-component vaccine was less effective than the five-component or WCV vaccine, again suggesting that the multi-component vaccines are more protective and efficacious. However a more recent report contradicted this and suggested that a mono-component acellular vaccine containing pertussis toxoid was sufficient (Taranger *et al.*, 2001). The trial was a double-blinded, placebo-controlled one with pertussis toxoid compounded with diphtheria and tetanus toxoids. The results indicate that the level of efficacy is as good as the multi-component acellular vaccines, with an average efficacy of 71%, although this value is usually between 90 and 95% for WCV, and that the correlation between the levels of pertussis toxoid-induced antibodies and protection against pertussis were statistically significant. This result possibly highlights the different serological criteria set by various organisations when diagnosing pertussis and suggests that a standard set of criteria should be set. Contradictory results from the various acellular vaccine trials further emphasise the necessity to identify the most important compounds and to try and assign a clear method of correlating protection.

At present, all of the acellular vaccines used in the trials described above have been licensed for use in various countries. Booster doses of the multi-component pertussis vaccines given to adolescents or adults could possibly have an epidemiological impact through reducing the prevalence of mild, clinically unrecognised infection in the adult population (parents), thereby reducing the risk of spreading disease to young children or babies. However, the role of adults in transmission of the disease to susceptible individuals is unclear. Whilst pertussis has traditionally been thought of as an infant or childhood illness, recent attention has shifted more to the occurrence of respiratory illness caused by *B. pertussis* in adolescents and adults. The advent of acellular pertussis vaccines has now provided an opportunity to give a late booster dose to stimulate antibody levels in adolescents and young adults. The best ways of providing this booster will vary in different settings. The less reactogenic acellular pertussis vaccine, may, therefore, be used as a booster dose to maintain immunity against pertussis among older children, adolescents and adults in the future. Campins-Marti *et al.* (2002) put forward some strategies for reducing the burden of pertussis, the ideal one being a booster vaccination programme for adults and adolescents throughout life. However, as this is unrealistic, a more achievable programme would be one targetted at adolescents still at school and adults likely to come into contact with very young children. A consensus group consisting of international experts from different countries recommended that the decision of who to vaccinate be made on an individual country basis and that the combined diphtheria, tetanus, pertussis acellular (DTPa) vaccine be used, or when tetanus and/or diphtheria immunity is not indicated, a monovalent pertussis vaccine may be made available. The U.K Joint Committee on Vaccination and Immunisation (JCVI) also considered the introduction of a booster dose of pertussis to redue the levels of

morbidity and mortality, and as of November 2001 the DTPa vaccines were determined safe to use as a routine booster for school children in England (DOH, 2001).

1.6 Repeat in Toxin (RTX) toxins

Bacterial pore forming toxins represent a heterogeneous group of exotoxins. Those belonging to the Gram-negative bacteria are commonly synthesised as inactive protoxins, which are subsequently converted into the active form by either modification or proteolytic processing. Examples of Gram-positive and Gram-negative pore-forming toxins are listed in Table 1.2. The Repeat in Toxin (RTX) toxins represent the largest family of bacterial pore forming cytolysins (Welch, 1991; Coote, 1992; Ludwig and Goebel, 1999). Examples of toxins belonging to the RTX family are listed in Table 1.3.

Table 1.2. Pore-forming toxins

Bacterium	Toxin	Reference
Streptococcus pyogenes	Streptolysin O	Alouf and Geoffroy,1988
S. pneumoniae	Pneumolysin	Saunders *et al.*, 1989
Bacillus alvei	Alveolysin	Geoffroy and Alouf, 1988
Clostridium tetani	Tetanolysin	Blumenthal and Habig, 1984
C. perfringens	Perfringolysin O	Tweten *et al.*, 1991
Listeria monocytogenes	Listeriolysin O	Mengaud *et al.*, 1988
Aeromonas hydrophila	Aerolysin	Buckley, 1991
Staphylococcus aureus	Alpha toxin	Bhakdi and Tranum-Jensen, 1985

Table 1.3 Example of some toxins belonging to the RTX family

Bacterium	Toxin	Reference
E. coli	HlyA	Welch *et al.*, 1981
Proteus vulgaris	PvxA	Strathdee and Lo, 1987
Morganella morganii	MmxA	Koronakis *et al.*, 1987
Actinobacillus actinomycetemcomitans	AaltA	Kraig *et al.*, 1990
Pasteurella haemolytica	LktA	Strathdee and Lo, 1987
B. pertussis	CyaA	Glaser *et al.*, 1988
V. cholerae	RtxA	Nagamune *et al.*, 1996

1.6.1 Characteristics of RTX toxins

RTX toxins are synthesised by Gram negative bacteria as inactive proteins with molecular masses typically around 100 to 120 kDa, with the exception of adenylate cyclase toxin from *B. pertussis* and RtxA from *Vibrio cholerae*, which have predicted sizes of 177 kDa and 500 kDa respectively. The C-terminal half of the RTX toxin proteins includes a tandem array of glycine and aspartate-rich nonameric repeats with the consensus sequence UXGGXG(N/D)DX, where U is a large hydrophobic amino acid and X is an arbitrary amino acid. The designation 'RTX toxins' refers to these characteristic repeats. The number of repeats varies among the RTX toxins between about 10 and 40 (Welch, 1991; Coote, 1992).

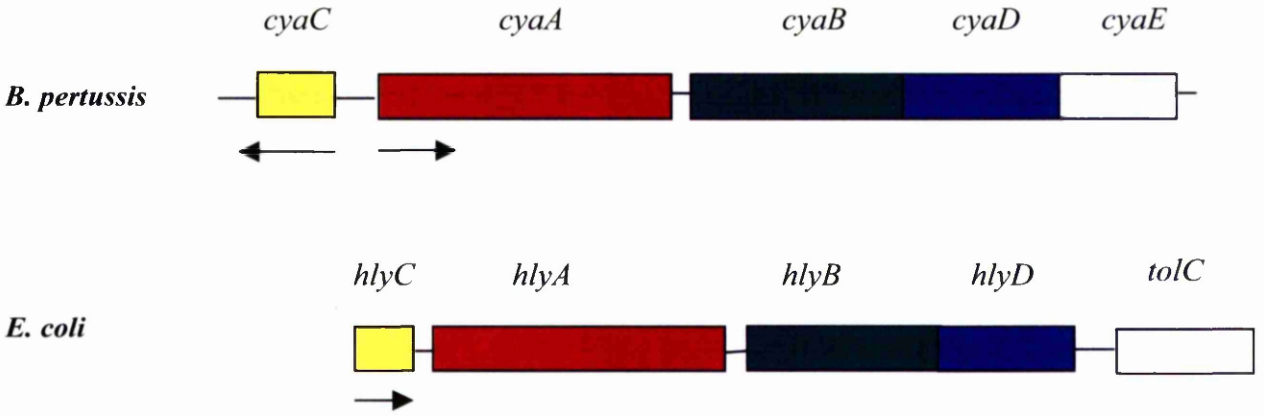
The RTX toxins lack a cleavable N-terminal signal peptide and their secretion is not sec-dependent; therefore they are not exported via the general secretory pathway. The extracellular secretion of the RTX toxins rather proceeds via the type-1 secretion pathway, which allows direct translocation of the toxins across both the inner and the outer membrane in one step, without any detectable periplasmic intermediate. Secretion of RTX toxins therefore depends upon specific, highly conserved export systems, composed of three envelope proteins. One is an inner membrane ATPase belonging to a family of ATP-binding cassette (ABC) transporters. The ABC protein provides energy for the secretory process through hydrolysis of ATP. The second protein is also anchored in the inner membrane and belongs to a family of membrane fusion proteins (MFP). These are transport accessory proteins found mostly in Gram-negative bacteria, where they function in conjunction with inner membrane transporters such as the ABC proteins. The third component of the exporter is an outer membrane protein with a typical N-terminal signal sequence. The entire secretion apparatus comprising the three proteins is usually designated as an ABC exporter. The genes specifically required for synthesis, activation and secretion of the RTX toxins are clustered either on the bacterial chromosome or on a plasmid and usually represent a single operon. This operon typically contains four genes in the order C-A-B-D. Gene A is the structural gene of the toxin protein, Gene C encodes the activator protein and the genes B and D encode the ABC protein and the MFP component, respectively, of the ABC exporter. The gene encoding the outer membrane component of the secretion apparatus is, in most cases, not linked to this gene cluster but located elsewhere on the chromosome (Ludwig and Goebel, 1990).

1.6.2 *E. coli* α-haemolysin

E. coli α-haemolysin (HlyA) is one of the best-characterised members of the RTX toxin family (Welch *et al.*, 1981) and was the first RTX toxin to be cloned and characterised. It is frequently produced by *E. coli* strains causing urinary tract and other extra-intestinal infections and contributes significantly to the virulence of these strains. The synthesis and secretion of *E. coli* HlyA are determined by the *hlyCABD* operon (Issartel *et al.*, 1991) (Figure 1.3). The HlyA secretory apparatus comprises HlyB and HlyD (an inner membrane traffic ATPase and inner membrane protein) and TolC (an outer membrane protein) (Wandersman and Delepelaire, 1990). In *E. coli* and most other pathogens TolC, or its equivalent protein, is encoded by a gene separated from the operon, but in *B. pertussis,* the operon includes a *tolC* equivalent (*cyaE*) (Section 1.7.1). HlyA is synthesised as a non-toxic pro-haemolysin (pro-HlyA) that is activated intracellularly by a mechanism dependent on the co-synthesised HlyC. HlyC alone cannot activate proHlyA, but requires a cytosolic activating factor, namely the acyl carrier protein (ACP). Activation to the mature toxin is achieved by the transfer of a fatty acid acyl group, from the acyl carrier protein to pro-HlyA (Issartel *et al.*, 1991).

E. coli α-haemolysin was the first protein shown to be exported via a type-I secretion system, a sec-independent mechanism (Wagner *et al.*, 1983; Blight and Holland, 1994). α-Haemolysin lyses erythrocytes from many species, but also exhibits strong cytotoxic and cytolytic activity against a variety of nucleated cells. In particular, it causes lysis of immune cells involved in first-line defence mechanisms, including polymorphonuclear leukocytes (PMN) and monocytes (Cavalieri and Snyder, 1992). The lytic action of α-haemolysin on red blood cells may promote bacterial growth in

Figure 1.3 **Comparison of the *B. pertussis cya* and the *E. coli hly* loci (Mock and Ullmann, 1993).**



Arrows indicate the direction of transcription and homologous genes are shaded in the same colour.

the host as it further increases the concentration of the available iron. Complete loss of pore formation is observed when HlyA is mutated in the hydrophobic domain between amino acids 299 and 327, suggesting an essential function in pore formation for this part of HlyA (Ludwig *et al.*,1996).

1.6.3 Leukotoxins

The leukotoxins of *Pasteurella haemolytica* (LktA) and *Actinobacillus actinomycetemcomitans* (AaltA) share high sequence identity to *E. coli* HlyA (Strathdee and Lo, 1997; Kraig *et al.*, 1990). Both of these toxins have little erythrolytic activity but possess potent cytotoxic activity towards phagocytic cells of particular animals, for example LktA lyses leukocytes from cattle, sheep and other ruminants (Shewen and Wilkie, 1982) whereas AaltA lyses human and primate polymorphonuclear lymphocytes only (Zambon *et al.*, 1983). As in *E. coli,* the *P. haemolytica* and *A. actinomycetemcomitans* toxins are encoded by a cluster of four genes in which the A gene encodes the toxin and the products of the B, C, and D genes are involved in post-translational modification of the toxin and its membrane insertion and secretion. The *A. actinomycetemcomitans* leukotoxin is not secreted but remains associated with the bacterial membrane, possibly through a hydrophobic domain at the carboxyl terminus, which distinguishes it from the *E. coli* and *P. haemolytica* toxins (Lally *et al.*, 1989).

When bovine alveolar macrophages (BAM) are exposed to low concentrations of LktA, an increase in cytokine gene expression and intracellular calcium elevation is observed (Hsuan *et al.*, 1999). Increased calcium levels contribute to the release of

leukotriene B_4 (LTB_4), an important chemotactic agent responsible for the influx of neutrophils into infected lungs (Henricks *et al.*, 1992, Cudd *et al.*, 1999). These non-lytic effects seen at low toxin concentrations, may be relevant to the *in vivo* situation where toxin produced at the site of infection will lyse phagoytic cells, but dissemination of toxin through the host cell will allow signalling effects on mammalian cells.

1.7 Adenylate cyclase toxin

Utsumi *et al.* (1978) noticed that a urea extract of *B. pertussis* contained a new factor that inhibited phagocytosis to opsonised targets and chemotaxis of PMN's and named it polymorphonuclear leukocyte-inhibitory factor (PIF) of *B. pertussis*. The partially-purified extract showed little histamine sensitising and cytotoxic activities. Confer and Eaton (1982) demonstrated that this toxic activity was due to an adenylate cyclase, which was able to enter human phagocytes and catalyse the unregulated formation of cyclic 3'-5'AMP, an intracellular mediator of hormone action and a signalling molecule.

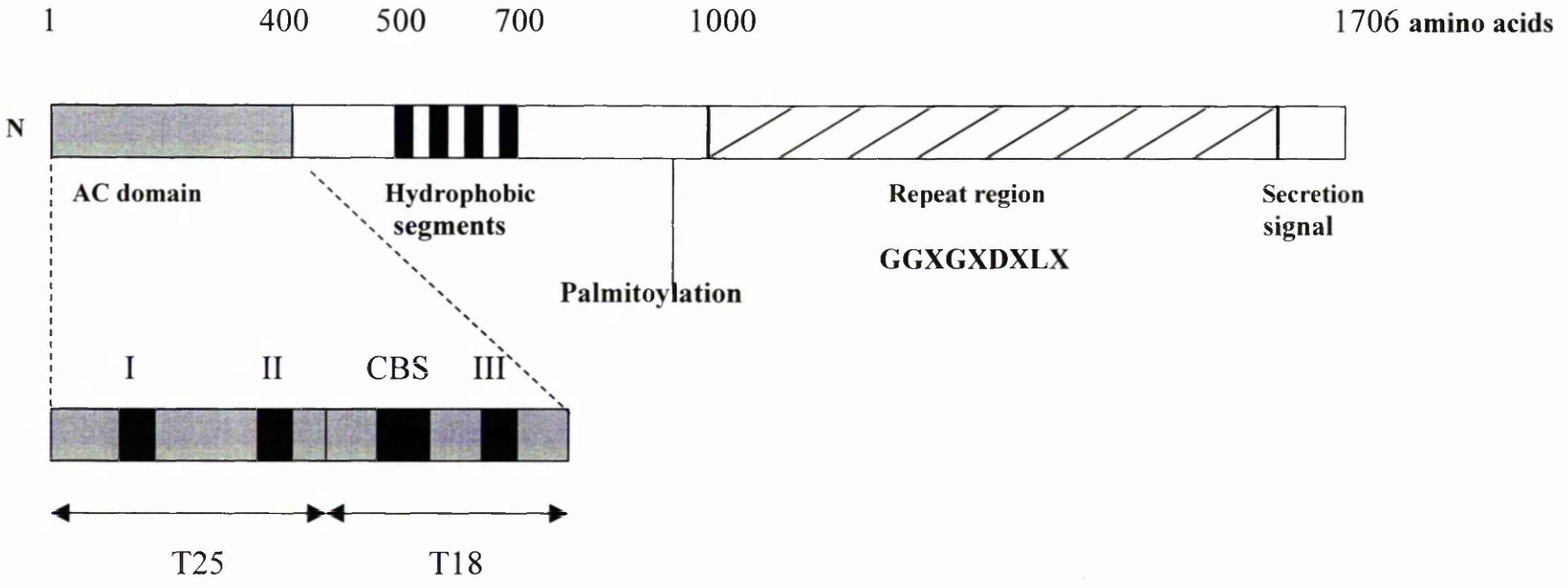
1.7.1 Structure and synthesis of CyaA

Native CyaA from *B. pertussis* has been isolated in multiple forms with reported molecular masses ranging from 43 to greater than 200 kDa. The toxic form of *B. pertussis* CyaA has an apparent molecular mass of 220 kDa (Hewlett *et al.*, 1989; Rogel *et al.*, 1989); and is able to release a catalytically-active 43 kDa form of

adenylate cyclase following proteolytic digestion (Bellalou *et al.*, 1990). Glaser *et al.* (1988a) and Brownlie *et al.* (1988) both reported the cloning and expression of CyaA in *E. coli*. The nucleotide sequence of the *cyaA* gene showed CyaA to be synthesised as a large molecule of 1706 amino acids, with the calmodulin-stimulated adenylate cyclase activity residing in the amino-terminal 400aa and the haemolytic and invasive activities located in the 1306 carboxy terminus (Glasser *et al.*, 1988a) (Figure 1.4). The catalytically-active region of CyaA is organised into two domains, the N-terminal domain of 25 kDa which carries the catalytic site and the 18 kDa C-terminal domain which carries the calmodulin-binding site. Ladant *et al.* (1989) characterised the calmodulin binding and catalytic domains of CyaA by cleavage of the 43 kDa N-terminal portion into tryptic fragments (T25 and T18 peptides). They concluded that: (1) the catalytically-active 43 kDa form of *B. pertussis* CyaA lies within the first 400 residues of the protein encoded by the *cya* gene, (2) T18, which is the main calmodulin binding domain, occupies the C-terminal segment of the 43 kDa (residues 236/238-399) and is devoid of catalytic properties and (3) the complementary peptides, T25 and T18, re-associate only in the presence of calmodulin, leading to significant recovery of the original activity. Figure 1.4 shows the structural organisation of the CyaA toxin. Glaser *et al.* (1988a) showed that the 1250 C-terminal aa of the precursor showed 25% similarity with the *E. coli* alpha-haemolysin (HlyA) and 22% similarity with *Pasteurella haemolytica* leukotoxin.

Three open reading frames were identified downstream from the *cyaA* gene, *cyaB*, *cyaD* and *cyaE* (Figure 1.3) (Glaser *et al.*, 1988b). As for *E. coli* HlyA, secretion of *B. pertussis* CyaA requires the expression of additional genes. The genes *cyaB* and *cyaD*, similar to *hlyB/D*, are necessary for the transport of CyaA across the cell envelope and for its release into the external medium.

Figure 1.4 Structural organisation of the *B. pertussis* CyaA toxin.



The catalytic domain (AC) is enlarged to show the subdomains T25 and T18. CBS corresponds to the main calmodulin binding site and boxes I, II and III represent the regions involved in catalysis (Ladant and Ullmann, 1999).

Complementation and functional studies indicated that *B. pertussis* CyaA is secreted by a similar mechanism to that of HlyA, requiring the presence of a third gene product. In *E. coli*, TolC is the outer membrane product encoded by *tolC*, which is separated from the *hlyCABD* locus however, in *B. pertussis*, *tolC* (*cyaE*) is included on the toxin locus (Glaser *et al.*, 1988b). Rogel *et al.* (1989) showed that expression of the *cyaA* gene in *E. coli* led to the production of a catalytically-active, 200 kDa CyaA but devoid of invasive and haemolytic activities. They described how post-translational modification confers the toxic properties upon CyaA. Further work showed that the *cyaC* gene in *B. pertussis*, homologous to the *hlyC* gene in *E. coli*, was required for the haemolytic and toxic activities of the *cyaA* gene product (Barry *et al.*, 1991). Sebo *et al.* (1991) reconstructed in *E. coli*, an expression system consisting of *cyaA* and *cyaC*, which enabled production of active CyaA toxin, endowed with catalytic, haemolytic and invasive activities.

1.7.2 Interaction of CyaA with target cells

Rogel *et al.* (1989) showed that extracellular calcium was absolutely essential *for B. pertussis* adenylate cyclase entry into target cells, and the penetration was blocked by neutralising anti-*B. pertussis* adenylate cyclase antibodies. They also demonstrated that calmodulin-mediated inhibition of enzyme penetration was dose-dependent and inversely proportional to the CaM-dependent stimulation of the enzymatic activity of the toxic form.

The haemolytic features of CyaA were characterised by purifying the 200 kDa protein. It was demonstrated that full haemolytic activity required the structural integrity of the CyaA protein, whether the protein was cell-associated or secreted into

the culture medium. The haemolytic activity of CyaA was calcium-dependent but very weak in comparison to previously characterised haemolysins. Bellalou *et al.* (1990) constructed in-phase deletion mutants within the *cyaA* gene. These mutants had reduced haemolytic activities and their toxin activity was abolished; however these proteins did not lose their capacities to be transported without processing across the bacterial cell envelope, suggesting that the structural integrity of the CyaA protein is necessary for toxin activity and that different structural determinants are required for secretion and pore-forming functions (Bellalou *et al.*, 1990). Hewlett *et al.* (1991) demonstrated that the CyaA molecule exhibits a conformational change when free calcium concentrations exceed 100 μM, as demonstrated by a shift in intrinsic tryptophan fluorescence. This conformational change was associated with an alteration in binding of an anti-CyaA monoclonal antibody and a structural modification that could be observed by electron microscopy. They proposed that an increase in the ambient calcium concentration to a critical point and the ensuing interaction of the toxin with the calcium induced a conformational change that is necessary for its insertion into the target cell and for the delivery of its catalytic domain to the cell interior. The process of CyaA toxin penetration into sheep erythrocytes was further shown by Rogel and Hanski (1992) to be carried out in three stages; insertion, translocation and intracellular cleavage. Insertion into the membrane occurs over a wide temperature range (4°C to 36°C) whereas translocation across the membrane was highly temperature-dependent and occurred only above 20°C. The N-terminal fragment of the toxin was cleaved in the cell, releasing the active 45-kDa portion of CyaA into the cytosol. Intoxication is a rapid process and an increase in cAMP levels in target cells can be detected within seconds of adding the CyaA toxin, evidence which supports the theory of direct translocation,

as entry of toxins through a trafficking mechanism or endocytosis would require a much longer exposure time (Rogel and Hanski, 1992). Work by Gray *et al.* (1999; 2001) demonstrated that a particular monoclonal antibody Mab 3D1, directed against an epitope within amino acids 373 to 399, was able to both prevent delivery of the catalytic domain to the target cell interior when bound to CyaA, and enhance the haemolytic activity of CyaA thereby suggesting that the catalytic domain be defined as ending at amino acid 399.

1.7.3 Activation of CyaA by CyaC

Using the technique of mass spectrometry, Hackett *et al.* (1994) demonstrated that wild type *B. pertussis* CyaA toxin was modified by amide linked palmitoylation on the epsilon-amino group of lysine 983. A synthetic palmitoylated peptide corresponding to the tryptic fragment (glutamine 972 to arginine 984) that contained the acylation, blocked CyaA toxin-induced accumulation of adenosine 3'-5'-monophosphate in target cells, whereas the non-acylated peptide had no effect. These data suggest that palmitoylation at this site represents the modification that is required for the toxicity and haemolytic activity of CyaA. Heveker *et al.* (1994) demonstrated that undirected transfer of lauric, myristic, or palmitic acid chains to the CyaA pro-toxin *in vitro*, conferred both haemolytic and toxic activities to CyaA. However, both activities were shown to be low in comparison to CyaA modified *in vivo*, suggesting that *in vitro* fatty acylation of the pro-toxin may involve random modification of nucleophilic residues present in the toxin. Westrop *et al.* (1996) observed similar differences in the *in vitro* activated CyaA. Full AC enzymic activity was obtained but the level of haemolytic activity was less than 10% of that

expected from its invasive enzymic activity. A possibility suggested for this observation was the use of a non-specicifc S100 extract as a asource of acyl-ACP in an *in vitro* system. In contrast to CyaA, the HlyA from *E. coli* was found to be acylated at two sites, Lys 564 and Lys 690 (Stanley *et al.*, 1994). The CyaC-activated recombinant ACT produced in *E. coli* is palmitoylated at Lys 860 as well as acylation of Lys 983. Basar *et al.* (1999) demonstrated, by replacing Lys 860 with either arginine, leucine or cysteine residues, that Lys 860 and Lys 983 from *E. coli* CyaA, are acylated independently *in vivo* and that mutations of Lys 860 did not affect the quantitative acylation of Lys 983 by palmitic and palmitoleic fatty-acyl groups, nor the invasive nor haemolytic activity of CyaA.

1.7.4 CyaA as a virulence factor

CyaA has been shown to be an important virulence factor in animal models of pertussis. Weiss *et al.* (1984) demonstated how *B. pertussis* mutants deficient in either, PT, CyaA, or all toxins, had a reduced ability to cause lethal infection in infant mice. Further work by Weiss and co-workers examined the proliferation of *B. pertussis* in the lungs of infant mice challenged by the intranasal route. They demonstrated how an adenylate cyclase mutant (BP348) was rapidly cleared from the lungs with no viable bacteria remaining 10 days post challenge, suggesting that CyaA is critical for colonisation and initiation of infection (Weiss and Goodman, 1989; Goodwin and Weiss, 1990). This data was also confirmed by Brownlie *et al.* (1988) following mouse intranasal challenge with *B. pertussis* BP348, a Tn5-insertion mutant deficient in adenylate cyclase and haemolytic activities. The mutant was considerably less virulent than the wild-type strains of *B. pertussis* but virulence

could be restored when the recombinant plasmid, pRMB1, containing both these activities was expressed. Khelef *et al.* (1992) showed that mutants deficient in CyaA were unable to adhere to and multiply in the lungs, suggesting that CyaA is required to initiate infection. They subsequently showed that there was no overall cellular influx into the brochoalveolar lavage (BAL) fluids of infected mice, in particular PMNs, when compared with parental virulent strains, indicating an important role that CyaA plays during *B. pertussis* infection (Khelef *et al.*, 1994).

1.7.5 Immune response to infection

In dealing with extracellular pathogens, the immune system aims to destroy the pathogen itself and neutralise its products. In response to intracellular pathogens, there are two main options; either the cytotoxic T cells can destroy the infected cell or the T cells can activate the infected cell to deal with the pathogen itself. Because many pathogens have both intra and extracellular phases of infection, different mechanisms are usually effective at different times. In vertebrates, immunity against pathogens or against other foreign substances is divided into two categories; innate or non-specific immunity (natural) or acquired immunity. Innate immunity is present from birth and includes such functions as physiologic barriers, cellular defenses and inflammation; whereas acquired immunity develops as a consequence of an encounter with a foreign substance. B and T lymphocytes are responsible for the specificity exhibited by the acquired immune response. All lymphocytes are derived from the bone-marrow stem cells, but T lymphocytes then develop in the thymus, while B lymphocytes develop in the bone marrow. B lymphocytes (B cells) combat extracellular pathogens and their products by synthesising and secreting into the bloodstream antibodies, which, specifically recognise and bind to particular target

molecules known as the antigen. This type of immunity is termed humoral immunity. T lymphocytes (T cells) perform various effector functions when antigen presenting cells (APC) bring antigens into the secondary lymphoid organs. T cells also interact with B cells and help the latter make antibodies; they activate macrophages and have a central role in the development and regulation of acquired immunity. Acquired immunity mediated by T cells is termed cellular or cell-mediated immunity (CMI). CMI was originally used to describe localised reactions to intracellular pathogens mediated by lymphocytes and phagocytes, rather than humoral immunity which is mediated by antibodies. However it is now no longer reasonable to consider cell-mediated and antibody-mediated responses separately, as no cell-mediated response is likely to occur in the total absence of antibodies.

The interaction between B and T cells is a two-way process, in that B cells present antigen to T cells and also receive signals from T cells for division and differentiation. B and T cells both recognise antigens in different ways. Antibodies recognise antigen in solution or on cell surfaces, but always in their native confirmation; whereas T cell receptors only recognise the unique combination of MHC plus the antigenic peptide when presented by APCs.

Immunoglobulins are a group of glycoproteins present in the serum and tissue fluids of all mammals. Some are carried on the surface of B cells where they act as receptors for specific antigens. Others (antibodies) are free in the lymph or blood. Contact between B cells and antigen is needed to cause the B cells to develop into antibody forming cells (AFC). The basic structure of all immunoglobulin molecules is a unit consisting of two identical heavy and light polypeptide chains linked by disulphide bonds. The heavy-chain type determines the class and subclass of an immunoglobulin molecule. The primary function of an antibody is to bind antigen.

In order for an antigenic determinant (epitope) and an antibody-combining site (paratope) to combine, the shape of the combining site must fit the epitope (Roitt *et al.*, 2001). T cells can be distinguished by their different T-cell antigen receptors (TCR), of which there are two types. One contains two disulphide-linked polypeptides (α and β), the other contains γ and δ polypeptides. Between 90 and 95% of T cells in the blood are αβ, the remaining 5-10% are γδ T cells. The αβ TCR cells can be sub-divided into two distict populations; a subset that carries the CD4 marker (T-helper cells) and a subset that carries the CD8 marker (cytotoxic T cells). $CD4^+$ T cells recognise their specific antigens in association with MHC Class II molecules, whereas $CD8^+$ T cells recognise antigens in association with MHC Class I molecules. Two groups of $CD4^+$ T cell clones have been identified namely, Th1 and Th2. $CD4^+$ T-helper cells provide help for B cells in proliferation and antibody production, and secrete a range of cytokines that are involved in a variety of immunoregulatory functions. Th1 cells secrete IL-2, IFN-γ and TNF-β and are involved in delayed type hypersensitivity and inflammatory responses, and display cytotoxic T-lymphocyte activity *in vitro*. A key function of this population in the immunological defence mechanism *in vivo* is the activation of macrophages, which are stimulated to take up and kill invading microorganisms. IFNγ is a particularly important cytokine during the early phase of infection with intracellular pathogens and when in combination with TNFα, is able to activate macrophages, which results in them being better able to control the growth and/or kill intracellular organisms. Th2 cells secrete IL-4, IL-5, IL-6 and IL-10 and these secreted cytokines play an important role in immunoglobulin class switching and B cell differentiation. IL-10 inhibits the production of IFNγ, antigen presentation and macrophage production of IL-1, IL-6 and TNFα. The production of IFN-γ without IL-4 or IL-5 is considered to

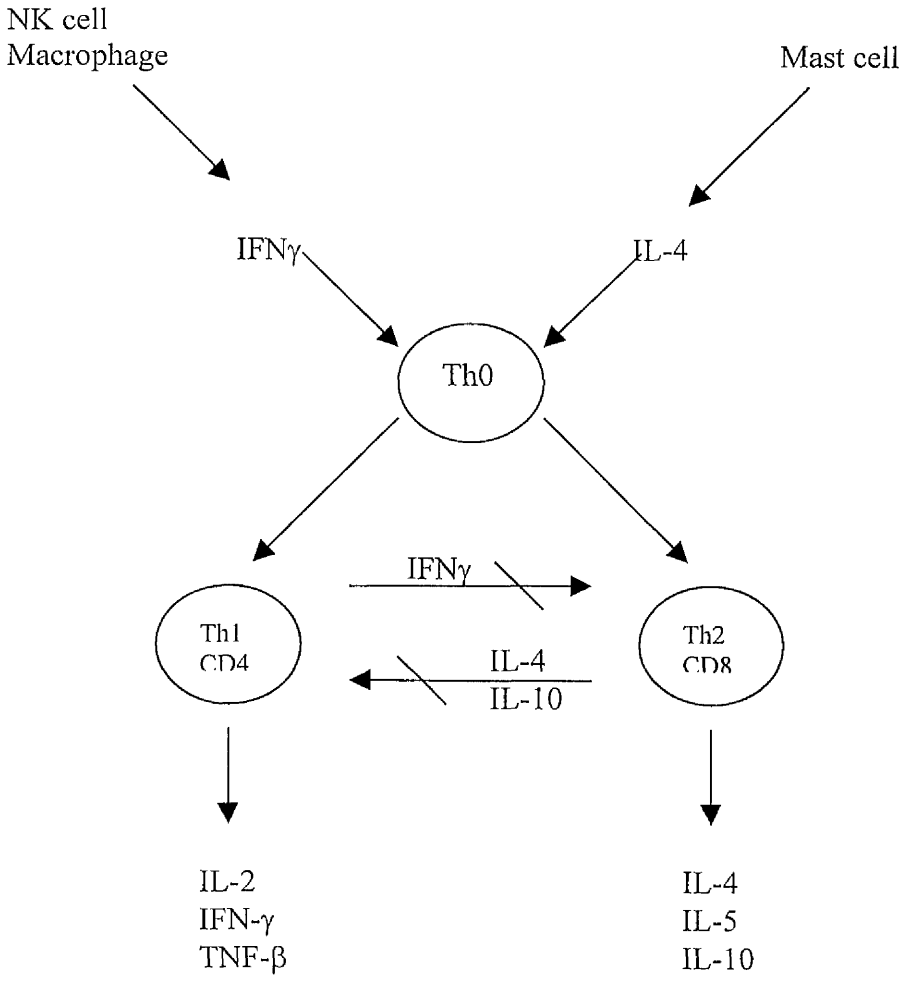
be the most reliable indicator of a Th1 response and conversely IL-4 or IL-5 production without IFN-γ is indicative of a Th2 response (Swain *et al.*, 1988). T cells expressing cytokines of both Th1 and Th2 cells are termed Th0 cells (Mosmann *et al.*, 1989). Figure 1.5 illustrates the cytokine control of the Th1 and Th2 subsets.

$CD8^+$ cytotoxic T lymphocytes (CTL) are important immune effectors arising in response to intracellular pathogens such as viruses and intracellular bacteria (Lin and Askonas, 1981). $CD8^+$ T cells can be divided into specific subsets, one of these expresses CD28 molecules and produces IL-12 in response to activation signals. Another subset responds to IL-12 and expresses the CD11b/CD18 cell receptor molecule. CD11b/CD18 or CR3 is a member of the β-integrin family and is expressed on monocytes, PMNs and some B and T cells. CR3 (alpha M beta 2, CD11b/CD18) has been shown to mediate macrophage binding and uptake of infectious agents including *B. pertussis* (Relman *et al.* (1990). $CD8^+$ T cells mediate their effector function through the production of cytokines such as IFNγ and TNFα. T cell activation involves the transduction of signals from both the TCR and CD28. Clustering of surface receptors upon ligand binding leads to activation of associated tyrosine kinases. These kinases become activated by dephosphorylation and are then able to phosphorylate γ chains of CD3 which permits association with other kinases. The end result of these complex pathways is the induction of gene synthesis by the activation of transcription factors (Roitt *et al.*, 2001).

Natural killer (NK) cells also play an essential role in killing pathogens through cytotoxic attack, mediated through the release of perforin or through the induction of receptor-mediated apoptosis, or activation of macrophages via IFNγ production.

Figure 1.5 Cytokine control of Th1 and Th2 $CD4^+$ subsets

Adapted from Benjamini *et al.* (2000)



Experiments *in vitro* implicate that the dose of antigen administered can dictate the development of a Th1- or Th2-like phenotype. As the dose of peptide was increased, the development of Th1-like cells producing IFNγ was observed. However, at very high doses of peptide (greater than 10μM) or very low doses (less than 0.05μM) the development was switched to Th2-like cells and the production of increasing amounts of IL-4 but less IFNγ. Affecting the balance of cytokine production, brought about by altering the antigen dose, may determine the class and strength of the immune response to foreign antigens (Hosken *et al.*, 1995).

1.7.5.1 Immunity in pertussis

B. pertussis has the ability to invade and survive intracellularly within murine and human macrophages (Cheers *et al.*, 1969; Masure, 1993; Friedman *et al.*, 1992) suggesting that the intracellular localisation may be an important mechanism in the disease process. Because of these different habitats it is likely that the bacterium employs a variety of both humoral and cell-mediated immune evasion strategies to prolong survival in the host.

Antibodies may function either by neutralising bacterial toxins, inhibiting extracellular bacteria from binding to cells in the mucosal tracts or by enabling bacterial uptake and destruction by macrophages and neutrophils. Evidence of a role for antibodies has been shown in the murine model, particularly for PT and FHA. Munoz *et al.* (1981) compared the protective activities of FHA and PT and found, through protection studies, that antisera known to contain antibodies to PT was able to protect mice against intracerebral challenge, whereas sera lacking anti-PT

antibodies but containing anti-FHA antibodies was unable to protect mice. Sato and Sato, (1984) correlated the titres of specific IgG antibodies against PT and FHA following intracerebral and aerosol challenge with *B. pertussis*. They demonstrated that in the intracerebral system FHA did not elicit a protective response but was able to increase the response generated by immunisation with PT. Further evidence for the role of antibodies was provided by the use of Ig defective mice. Mahon *et al.* (1997) demonstrated that B cell-deficient mice were unable to clear bacteria after aerosol infection when compared with the control mice. The Ig $^{-/-}$ mice, which lack mature B cells, failed to mount an IgG antibody response whereas the wild-type mice developed *B. pertussis* specific serum antibody by day 24 after aerosol challenge, indicating a requirement for complete bacterial elimination from the lungs. The nature of the protective immunity to pertussis was investigated further by Leef *et al.* (2000). They found that mice given subcutaneous or intranasal WCV did have pertussis-specific antibodies in both serum and lung-lavage samples that exceeded the levels found in naturally infected mice. Nonetheless, passive transfer of immune serum obtained after immunisation intranasally with formalin-fixed *B. pertussis* (FFBP) had minimal effects on the bacterial burden. However, repeated immunisation of B cell knockout mice resulted in partial protection and transfer of pertussis-immune serum achieved complete protection, thereby indicating the importance of B cells in protective immunity. Canthaboo *et al.* (2001) investigated both the cellular and humoral responses to immunisation with WCV and ACV. It was demonstrated that mice immunised with WCV were found to have lower antibody titres to PT, FHA and P.69 compared to those immunised with the ACV. However, immunisation with the WCV had a strong effect on macrophage activation and was associated with a fast clearance of bacteria from the lungs from a very early

stage. In contrast, mice immunised with ACV generated humoral responses, which were associated with inhibition of bacterial growth in the first stage but delayed bacterial clearance following aerosol challenge. These results infer that antibody responses may have a greater role in immunity generated by ACV vaccines and that IgG may confer protection by inhibition of bacterial adherence and neutralisation of toxins.

The first direct evidence for the importance of T cells in immunity to *B. pertussis* was provided by the demonstration that severe combined immunodeficient (SCID) mice devoid of T and B cells, failed to clear the bacterium from the respiratory tract, whereas immunocompetent BALB/c mice cleared the infection after about 5 weeks, demonstrating that T and/or B cells contribute to bacterial elimination (Mills *et al.*, 1993). Mills and co-workers also demonstrated that *B. pertussis*-specific CD4+ T cells from mice primed by infection were capable of conferring protection to athymic mice. Redhead *et al.* (1993) used a mouse respiratory challenge model to examine the induction of cellular and humoral immune responses and their role in protection against *B. pertussis* following immunisation or previous infection. They found that spleen cells taken from mice convalescing from a previous infection, secreted high levels of IL-2 and IFNγ but not IL-4 or IL-5, characteristic of a $CD4^+$ Th1 cytokine profile. In contrast, mice immunised with an acellular vaccine had high levels of spleen cells secreting IL-5 but not IFNγ, a profile characteristic of $CD4^+$ Th2 cells. They also found that after exposure to a respiratory challenge with *B. pertussis*, the convalescent mice and those immunised with the WCV eliminated the bacteria faster than those immunised with the ACV. Peppoloni *et al.* (1991) demonstrated that human $CD4^+$ T cell clones specific for pertussis toxin and other *B. pertussis* antigens produced mostly IL-2 and IFNγ. Further evidence for a role of $CD4^+$ T cells was

provided by the demonstration that $CD4^+$ knockout mice could not be protected by intra-nasal challenge with inactivated bacteria, whereas in contrast, $CD8^+$-depleted mice did not differ from wild-type mice in their ability to control aerosol pertussis challenge (Leef *et al.*, 2000).

B. pertussis has been shown to be capable of down-regulating local cell-mediated immunity as a means of prolonging its survival in the respiratory tract (McGuirk *et al.*, 1998). The majority of T cells from the lungs of naïve mice express the co-stimulatory molecule CD28, which interacts with B7 on APCs, as an essential second signal for T cell activation. However during murine infection with *B. pertussis,* McGuirk *et al.* demonstrated a significant decrease in the number of T cells in the lungs expressing CD28, especially at the peak of the bacterial load.

Respiratory infection or immunisation of mice with WCV, selectively primes Th1 cells (Mills *et al.*, 1993; Redhead *et al.*, 1993), whereas immunisation of mice with ACV generates T cells that secrete IL-4 an IL-5, plus low levels of IFNγ (Barnard *et al.*, 1996). *B. pertussis*-specific T-cell response in mice primed with ACV, shifted to a mixed Th1/Th2 profile after respiratory infection with *B. pertussis* (Mahon *et al.*, 2000) and addition of IL-12 to an ACV formulation resulted in the induction of IFNγ secreting T cells (Mahon *et al.*, 1996). McGuirk and Mills (2000) examined the protection mediated in mice following immunisation with Th1 and Th2-inducing pertussis vaccines. They found that the Th1-inducing WCV resulted in a significant neutrophil influx after aerosol bacterial challenge whereas in contrast the inflammatory response was suppressed following immunisation with the Th2-inducing 3 component acellular vaccine. They suggested that Th2 cells provide help for IgG_1, IgG and IgA production, whereas Th1 cells activate the antimicrobial

activity of phagocytic cells and also stimulate B cells to produce opsonising and complement-fixing antibodies of the IgG_{2a} subclass.

IFNγ has been shown to enhance the ability of macrophage to kill *B. pertussis in vitro* (Torre *et al.*, 1994; Mahon *et al.*, 1999). It has also been demonstrated that NO is produced *in vitro* by peritoneal or alveolar macrophages stimulated with *B. pertussis* and *in vitro* by spleen cells and peritoneal macrophages following immunisation with WCV, or by alveolar macrophages following immunisation with ACV (Xing *et al.*, 2000; Torre *et al.*, 1993; Torre *et al.*, 1996). NO and reactive nitrogen intermediates are associated with the antimicrobial functions of macrophages (Ding *et al.*, 1988) and macrophage activation, as measured by NO synthesis, correlates closely with protection *in vivo* against *B. pertussis* challenge. By using NO production as a marker of macrophage activation it is possible to identify the relevant components of any vaccine that are responsible for activation of macrophages (Xing *et al.*, 2000).

McGuirk and Mills (2000) demonstrated that FHA is capable of suppressing IL-12 production by macrophages and that this inhibition was dependent on an IL-10 mechanism. Agglutinogens 2 and 3 and PT did not suppress IL-12 production, suggesting that *B. pertussis* is able to increase its survival *in vivo* by stimulating the production of inhibitory cytokines which, in turn, subvert the protective cellular immune responses. McGuirk *et al.* (2002) investigated the hypothesis that FHA may contribute to the suppression of Th1 responses during acute infection with *B. pertussis* by the induction of T cells with regulatory activity, as a result of its interactions with cells of the innate immune system. They demonstrated that FHA interacts directly with dendritic cells to induce IL-10 and inhibit LPS-induced IL-12 production. The DCs generated after interaction with FHA selectively stimulates the

induction of T regulatory (Tr) cells from naïve T cells. Results of this study demonstrate that activation of DCs that secrete IL-10 but not IL-12, can direct naïve T cells to a Tr1 subtype and that FHA can provide the stimulus.

Mielcarek *et al.* (2001) studied the interaction of *B. pertussis* with mast cells using the technique of transmission electron microscopy. They were able to demonstrate that bone marrow-derived mast cells (BMMCs), following *in vitro* exposure to *B. pertussis,* were able to release increased levels of TNFα and IL-6 compared with BMMCs incubated with medium alone. These pro-inflammatory cytokines are known to be released by mast cells and are thought to play a critical role during infection (Gordon and Galli, 1990; Van Snick, 1995; Malaviya *et al.*, 1996) therefore mast cells may participate in the immunity against *B. pertussis* in the lungs via the production of cytokines, in particular TNFα and IL-6, possibly by enhancing phagocytosis.

1.7.6 Delivery of multiple epitopes by CyaA

Recombinant CyaA toxoids with disrupted catalytic activity have been shown to be capable of delivering vaccinal $CD8^+$ T cell epitopes into the cytosol of MHC class I APCs and have been successfully used for antigen delivery and induction of protective antiviral and therapeutic antitumour $CD8^+$ CTLs in mice (Sebo *et al.*, 1995; Saron *et al.*, 1997; Fayolle *et al.*, 1999, Guermonprez *et al.*, 1999). Dadaglio *et al.* (2000) investigated the potency of recombinant CyaAs carrying between one and four copies of the MHC class I and class II T-cell epitopes from the nucleoprotein of the lymphocytic choriomeningitis virus (LCMV), to induce T cell

responses characterised by IL-2 and IFNγ production and a lack of detectable levels of IL-4 and IL-5, indicative of a Th1-like cytokine profile. From this it was found that CyaA molecules bearing $CD8^+$ and $CD4^+$ T cell epitopes were able to induce both CTL and Th1-like responses. Th1 responses play an important role in protection against intracellular pathogens (Section 1.7.5.1), therefore this is very useful observation in view of development of CyaA as an antigen delivery system. They also showed that peptides of more than 60 amino acids inserted into the catalytic domain, did not affect the efficiency of CTL induction. This is useful, in that insertion of multiple peptides into the same site may lead to CTL responses with broad MHC class restriction. Further work by Fayolle *et al.* (2001) showed that CyaA was able to deliver simultaneously three immunodominant CTL epitopes from LCMV, HIV type 1 and chicken ovalbumin and induce epitope-specific cytotoxic responses *in vivo* against each epitope.

1.7.7 Protection by CyaA

Antibody-mediated protection against intracellular pathogens is important in vaccine design and there is conclusive evidence that antibodies can protect against a variety of intracellular pathogens for which CMI has also been shown to play a crucial role in host defence. Antibodies may function by neutralising bacterial toxins or by enabling bacterial uptake and destruction by macrophages and neutrophils. Confer and Eaton (1982) demonstrated the ability of CyaA to inhibit the functions of neutrophils, namely phagocytosis and killing of microorganisms. The development of a method to quantify phagocytosis using *B. pertussis* labelled with green fluorescent protein (GFP) and differential staining, led to the discovery that 98% of

phagocytosed *B. pertussis* bacteria by human neutrophils were killed and few, if any, *B. pertussis* cells were capable of surviving within these neutrophils (Lenz *et al.*, 2000). Further work by Weingart and Weiss (2000) examined the role that *B. pertussis* virulence factors and the presence or absence of opsonising antibodies played in phagocytosis by human neutrophils. They showed that *B. pertussis* mutants lacking FHA were unable to attach to neutrophils and were not phagocytosed, thereby demonstrating a major role for FHA in promoting attachment to neutrophils and therefore antagonising phagocytosis by these neutrophils. They also showed that CyaA was able to block phagocytosis, as proven by the efficient phagocytosis of mutants that failed to express CyaA. More recently Weingart *et al.* (2000) demonstrated that antibodies which neutralised CyaA enhanced phagocytosis, but only when combined with human opsonising antibodies. *B. pertussis* is an unusual organism, in that opsonisation does not appear to promote phagocytosis of wild-type *B. pertussis*, and the bacteria remain extracellular. However when CyaA toxin activity is absent or diminshed by the presence of neutralising antibodies, inceased phagocytosis occurs, suggesting that the increase in cAMP production inhibits the signalling that normally occurs following the interaction of the antibody and Fc receptor. These results suggest that neutralising antibodies to CyaA in conjunction with opsonising antibodies could increase the immune response generated by enhancing the phagocytic defence mechanisms.

Guiso *et al.* (1989) showed that active immunisation with 3μg of purified *B. pertussis* CyaA by the subcutaneous route, three times, at one-week intervals, protected mice against lethal challenge by the intranasal route with 10^8 *B. pertussis* 18.323 bacteria. In a subsequent study using the mouse respiratory challenge model, they demonstrated that two subcutaneous immunisations with 4μg of purified CyaA

holotoxin at two-week intervals, induced protection against colonisation with *B. pertussis* (Guiso *et al.*, 1991). Betsou *et al.* (1993) showed that recombinant CyaA expressed in the presence of the CyaC product was able to protect mice after an intranasal challenge with *B. pertussis*, whereas the recombinant CyaA expressed in the absence of the CyaC product had no protective ability. They further showed, using a set of truncated recombinat CyaA proteins, that the palmitoylated Lysine 983 residue was an essential component for protective activity, haemolytic and cytotoxic activities; as these functions were absent when either acylation did not occur or the last amino acids were removed (Betsou *et al.*, 1995). Hormozi *et al.* (1999) showed that mice exhibited protective activity against intranasal challenge with a sublethal dose of *B. pertussis* 18.323 after receiving two immunisations subcutaneously with 15μg of purified active (acylated) CyaA, either the native active form from *B. pertussis* or the recombinant active form. This was shown by low lung to body weight ratio and low bacterial numbers in the lungs compared to mice vaccinated with purified inactive (non-acylated) CyaA. These results confirm that CyaA modification by CyaC is critical for the protective ability of CyaA. The group also investigated the adjuvanticity of purified active or inactive recombinant CyaA on the co-administered antigen, ovalbumin (OA). They found that anti-OA IgG levels were enhanced several fold when active CyaA was co-administered with OA, whereas the enhancement was less marked when the inactive recombinant CyaA was co-administered. This suggested that the invasive activity of CyaA, via increases in the levels of cAMP, may be responsible for the enhancement of specific antibody responses to OA.

The ultimate goal of a vaccine is to deliver long-lived immunological protection by an enhancement of memory responses. In some situations protective immunity can

be mediated by just antibodies or $CD8^+$ T cells, but for optimal control both the humoral and cellular immune responses need to be activated (Casadevall., 1998; Bromberg *et al.*, 1991).

Aims and Objectives

B. pertussis has been shown to invade and survive within intracellular cells therefore whilst humoral immunity has traditionally been considered to play the central role in immunity it may not be totally effective and instead the induction CMI may be necessary for complete bacterial clearance. Adenylate cyclase toxin (CyaA) invades target cells where host-cell calmodulin activates the adenylate cyclase enzymic moiety to produce high levels of cAMP. High levels of cAMP impairs functions such as chemotaxis and phagocytosis, therefore assists in the survival of, and colonisation of, this bacterium in the respiratory tract. Directing an immune response toward CyaA may therefore be an important anti-colonisation factor. Following vaccination this project aimed to define the immune response to CyaA and compare it with other protective pertussis antigens, thereby furthering the understanding of the nature of immunity to pertussis.

The main objectives were to firstly produce and purify two forms of CyaA, an enzymically active and an enzymically inactive form. Following vaccination of the two different forms the relevance and importance of the enzymic moiety could be determined with respect to 1) protection against *B. pertussis* or *B. parapertussis* challenge; 2) any potential adjuvant effect to stimulate the immune response to other antigens present in the ACV and 3) direction of the immune response towards cellular immunity and a Th1 type of response.

CHAPTER 2

MATERIALS AND METHODS

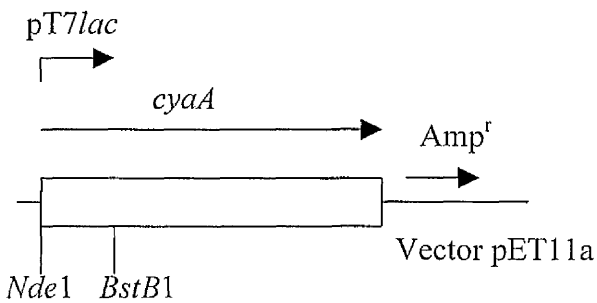
2.1 BACTERIAL STRAINS AND PLASMIDS

The *E. coli* host strain used for expression vectors was BL21/DE3. This strain contains chromosomally-located λDNA expressing the gene encoding T7 RNA polymerase under the control of the inducible lacUV5 promoter. Addition of isopropyl-1-thio-β-D-galactoside (IPTG) to a growing culture induces the polymerase which, in turn, transcribes the target DNA in the plasmid via a T7 promoter.

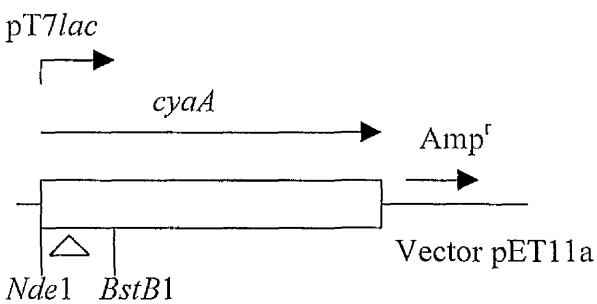
The CyaA pro-toxin, which has adenylate cyclase activity, and the CyaC protein required for post-translational activation of cytotoxic activity, were expressed from separate compatible plasmids, pGW44 and pGW54 respectively, initially from pET11a (Westrop *et al.*, 1996, 1997) (Figure 2.1). The plasmid encoding CyaA pro-toxin lacking adenylate cyclase activity, pGW44/188, was constructed (S, Bailie and J.G Coote, unpublished) by replacing a 1.1 kb *Nde1/BstB*1 fragment at the 5' end of the *cyaA* gene in pGW44 with the equivalent fragment from pACM188 (Ladant *et al.*, 1992). This creates a pro-toxin that is catalytically inactive due to insertion of the dipeptide Leu-Gln between codons 188 and 189 within the ATP-binding site (Ladant *et al.*, 1989). Co-expression of pGW44/188 with pGW54 led to the production of the invasive toxin devoid of catalytic activity. The stock of bacterial cells and competent bacterial cells for transformation of plasmids were stored at –70°C in 50% (v/v) glycerol.

Figure 2.1 Schematic representation of plasmids used for expression of different forms of CyaA in *E. coli*

pGW44

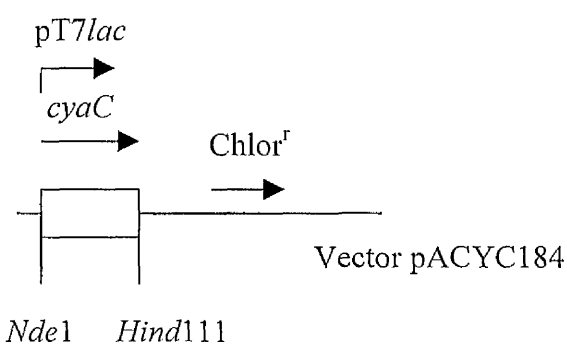


pGW44/188



△ di-peptide amino acid insertion

pGW54



2.1.1 Growth of *E. coli*

E. coli strains were routinely grown overnight at 37°C on Luria Bertani (LB) agar (Appendix A.1). Where necessary, 500 ml LB broth (Appendix A.1) in a 2 L flask was inoculated from such plates and incubated overnight at 37°C with shaking at between 150 and 200 rpm. Ampicillin (Amp) at a final concentration of 75μg/ml was included in all cultures inoculated with *E. coli* containing pGW44 or pGW44/188, and chloramphenicol (Chlor), at a final concentration of 12.5μg/ml, was added to *E. coli* cultures inoculated with cells containing pGW54.

2.1.2 Growth of *B. pertussis* and *B. parapertussis*

B. pertussis and *B. parapertussis* (strains 18.323 (NCTC 107399) and NCTC 5952 respectively) were grown on Charcoal agar base (Oxoid Ltd, UK) containing 10 % (v/v) defibrinated horse blood (E+O, UK). Plates were incubated in a humidified atmosphere at 37°C for 2 to 3 days.

2.2 DNA EXTRACTION

2.2.1 Plasmid purification

Plasmid DNA was extracted using the QIAprep® Miniprep purification system (Qiagen, UK) according to the manufacturer's instructions. Briefly, 5 ml of overnight culture of *E. coli* was centrifuged at 10,000 x g for 10 min (Biofuge, Heraeus). The resultant bacterial pellet was re-suspended in 250 μl of Buffer P1 and lysed using 250 μl of Buffer P2 (alkaline detergent solution) for 5 min at room temperature. The lysate was subsequently neutralised and adjusted to high salt

binding conditions by the addition of 350 µl of Buffer N3, followed by centrifugation at 10,000 x g for 10 min. The supernatant was added to a QIAprep® spin column and centrifuged at 10,000 x g for 1 min. The flow-through was discarded and the final wash was performed with 750 µl of Buffer PE, with a further centrifugation as described previously, to ensure efficient removal of salts. Finally, the DNA was eluted by centrifugation after the addition of 50 µl of sterile distilled water heated to 65°C.

2.3 AGAROSE GEL ELECTROPHORESIS

2.3.1 Sample preparation

The sample DNA (5-20 µl) was mixed with 6x DNA loading buffer (Appendix B.2) in a ratio of 5:1 prior to loading into the wells. Molecular weight markers (1 Kb ladder, Gibco BRL, UK) were utilised according to manufacturer's instructions.

2.3.2 Gel preparation

Agarose Type II-A (Sigma) was suspended in 1x Tris acetate EDTA electrophoresis (TAE) buffer (Appendix B.1) to give a 1 % (w/v) solution and heated until the agarose was completely dissolved. The solution was allowed to cool, ethidium bromide (BioRad) added to a final concentration of 1.0 µg/ml, then poured into an appropriate gel tray. Upon setting, the gel was immersed in 1x TAE in a horizontal gel electrophoresis tank (Bethesda Research Labs, USA)

2.3.3 Electrophoresis

A powerpack (DeltaElektronika) was used to provide a current of 100 mA through the agarose gel. Electrophoresis was carried out until the marker dye migrated an appropriate distance to enable visualisation of the DNA.

2.3.4 Visualisation of DNA

A high performance ultraviolet transilluminator (Ultra Violet Products, Cambridge, UK) coupled to an image acquisition and analysis software package (LabWorks) was used to visualise and store electronically, images of the ethidium bromide-stained DNA. Images were printed using a video graphic printer (model UP-860, Sony) and edited using Adobe Photoshop Version 4.

2.4 STANDARD TRANSFORMATION

2.4.1 Preparation of electroporation-competent cells

A culture of *E. coli* grown overnight in LB at 37°C was diluted 1 in 100 in 500ml of LB in a 2 L dimpled flask. The flask was shaken at 200 rpm at 37°C until an OD_{600nm} of between 0.6 and 1.0 was obtained. The flask was chilled on ice and cells harvested at 5,000 x g for 15 min in a Sorvall superspeed (rotor GS-3). The resultant bacterial pellet was suspended in 500 ml ice-cold sterile distilled water and centrifuged as previously described. The cell pellet was then suspended in 250ml ice-cold sterile distilled water, centrifuged as before and resuspended in 10 ml cold sterile glycerol 10 % (v/v) and 100 µl aliquots were snap frozen on dry ice and used immediately or stored at -70°C.

2.4.2 Electroporation procedure

Prior to electroporation, a 0.2cm electroporation cuvette (Flowgen, UK) was chilled at -20°C. A mixture of 1.5 μl of plasmid DNA and 40 μl of competent cells were added to the cold cuvette and shaken to the bottom of the cuvette. Cells were pulsed once in a Bio-Rad Gene Pulser (model 1652078, Bio-Rad laboratories, UK) set at 2.5 kV, 25μFD and connected to a Bio-Rad pulse controller (model 1652098) set to 200Ω. Immediately following electroporation, 1 ml of pre-warmed SOC (Appendix A.2) medium was added and the cells were incubated at 37°C for 1 h. After incubation of the transformed cells in SOC, 100 μl was spread onto LB agar plates containing appropriate antibiotics and the plates were incubated at 37°C overnight. Resultant colonies were cultured and plasmid DNA was extracted (Section 2.2.1).

2.4.3 Expression of recombinant proteins

An overnight culture of *E. coli* BL21/DE3 containing the relevant plasmid DNA was diluted 1 in 100 in 1 L of LB containing appropriate antibiotics. The culture was shaken at 200 rpm at 37°C until an OD_{600nm} of between 0.5 and 0.8 was obtained. IPTG was then added to a concentration of 1 mM and the culture shaken at 37°C for a further 4 h. Cells were harvested at 5,000 x g for 15 min in a Sorvall superspeed centrifuge (rotor GS-3).

2.4.4 Urea extraction of inclusion bodies

After expression of recombinant proteins, the resultant cell pellet was re-suspended in 20 ml ice-cold Buffer A (Appendix C.1). The cell suspension was disrupted using

a One-Shot sonic cell disrupter (Constant Systems Ltd, UK) for 15 sec and repeated between three and five times until all the suspension had passed through the disrupter. The resultant lysate was centrifuged at 17,000 x g in a Sorvall superspeed centrifuge (rotor SS34) for 30 min to allow inclusion bodies to be pelleted. Pellets were re-suspended in 10 ml of Buffer B (Appendix C.2) and inclusion bodies solubilised with rotation at room temperature until the suspension cleared, approximately 3 hours. A final centrifugation step of 100,000 x g for 90 min at 4°C was performed and the supernatant, containing urea-soluble protein, was retained and stored at -20°C.

2.5 PURIFICATION OF CyaA

2.5.1 DEAE Sepharose Chromatography

A 10 ml volume of DEAE Sepharose Fast Flow (Sigma) was applied to a glass Econo-Column (1.5 cm x 20 cm) (BioRad) and washed with 30 ml water to remove excess ethanol. The column was equilibrated at room temperature by washing with 20, 50 and 30 ml volumes of Buffer 1, 2 and 3 (Appendix C.3.1, C.3.2, C.3.3) respectively. The urea extract was adjusted to give a final concentration of 50 mM NaCl and then applied directly to the column. The column was then washed with 25 ml of Buffer 3 adjusted to 130 mM NaCl and then eluted in aliquots with Buffer 3, gradually increasing the NaCl concentration from 250 mM up to 2 M (Appendix C.3.4). The eluted extracts were then stored at -20°C until aliquots could be analysed by SDS-PAGE for the presence of CyaA.

2.5.2 Phenyl Sepharose Chromatography

Eluted fractions from the DEAE Sepharose chromatography were analysed by SDS-PAGE and the relevant fractions containing CyaA were pooled and diluted 1:4 in Solution A (Appendix C.4.1). A clean Econo-column (1.5 cm x 20 cm) was filled with 2 ml Phenyl Sepharose CL-4B (Sigma) and washed with 30 ml water to remove excess ethanol, followed by an equilibration with 20 ml Solution A. The diluted fractions were applied to the column in 20 ml aliquots and washed with Wash buffer (Appendix C.4.2). Elution was performed with Solutions B1 to B3 (Appendix C.4.3), gradually decreasing the NaCl content from 0.5 M to zero and increasing the urea content from 2 M to 8 M. Fractions were analysed by SDS-PAGE.

2.5.3 Concentration of CyaA samples

Centricon (Millipore) centrifugal concentrators with a molecular weight cut-off value of 100 kDa were used to concentrate CyaA samples. A 2ml volume of CyaA was applied to the centricon sample reservoir (Millipore) and centrifuged at 1000 x g for 30 min at 4°C as per the manufacturers' instructions. The filtrate was left in the filtrate vial and the retentate transferred to a microfuge tube, and both were subjected to SDS-PAGE analysis.

2.6 ENDOTOXIN REMOVAL

2.6.1 Polymyxin B

A polypropylene disposable column (0.8 cm x 9.0 cm) (Bio-Rad) was packed with 2 ml Affi-Prep Polymyxin Matrix (BioRad) and washed with 3 column volumes of 0.1

M NaOH, followed by ten to fifteen bed volumes of pyrogen-free water (Baxter). The column was then equilibrated with pyrogen-free PBS at room temperature until the eluate was at neutral pH. Purified CyaA was loaded onto the column in 2 X 2.5 ml volumes and the eluate recovered. A sample of the recovered solution was sent for endotoxin content analysis (Division of Endocrinology, NIBSC), the remainder stored at -20°C.

2.6.2 Triton X-114

Purified ACT sample was diluted in a ratio of 1:1 in 50mM Tris, pH 8.0. To a 0.5 ml volume of sample, 5µl Triton X-114 (Sigma) was added, the mixture vortexed and placed in an ice bath for 5 min. The chilled sample was vortexed again then warmed to 37°C for 10 min, before being centrifuged at 10,000x g for 10 min. After centrifugation, the detergent phase was presented as an oily droplet at the bottom of the tube and the remaining supernatant was retained for endotoxin content analysis.

2.6.3 Sephacryl chromatography

Sephacryl® S-200 High Resolution gel filtration media (Pharmacia Biotech) was prepared by centrifugation at 6,000 x g and repeated re-suspension in 8M urea (Buffer B, Appendix C.2), until the volume of the packed slurry was approximately 20 ml. A volume of between 2 and 3 ml of urea buffer was added to the sephacryl slurry to enable packing of the column. The buffer was allowed to flow through the column and 1 ml of purified CyaA was applied followed by 20 ml of buffer. Twenty 1 ml aliquots were collected and protein content was estimated by measuring the OD_{280nm}. The samples with the highest protein content were analysed by SDS-PAGE

to determine the amount of CyaA and an aliquot of each column fraction was sent for endotoxin analysis.

2.6.4 END-X®

END-X® B15 (Associates of CapeCod Incorporated, USA) contains immobilised endotoxin neutralising protein from *Limulus polyphemus* as the affinity ligand which enables the removal of Gram-negative endotoxins from solutions. A volume of between 1 and 1.5 ml of purified CyaA was added directly to the tube containing the affinity resin and the mixture incubated at 4°C for 4 h with gentle agitation. After incubation, the sample was centrifuged at 12,000 x g for 2 min to pellet the resin. The supernate was transferred to an endotoxin-free receiver tube and sent for endotoxin analysis.

2.7 Protein quantification

The Coomassie® Plus-200 protein assay kit (Pierce, USA) provided a colourimetric method for total protein quantification, according to the microtiter plate protocol. When Coomassie® dye binds protein in an acidic medium, an immediate absorbance shift occurs from 465 nm to 595 nm with a simultaneous colour change of the reagent from green/brown to blue. Briefly, Bovine Serum Albumin (BSA) standards and urea-extracted test proteins were diluted in the range 100 to 1,500 µg/ml and 10 µl volumes added to wells of a microtiter plate (Nunc). To each well, 300 µl of Coomassie® Plus reagent was added and mixed on a plate shaker for 30 sec. The OD_{600nm} was measured with a plate reader and protein concentrations determined from a standard curve.

2.8 SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970) in a vertical gel electrophoresis tank (Hoefer Scientific Instruments, USA). Glass plates were assembled according to the manufacturer's instructions. Resolving gels, containing 7.5-10% acrylamide (Appendix D.1), were poured between the assembled plates until the gel was approximately 4cm below the top of the plates and 100% ethanol was used as an overlay. The gel was left undisturbed for approximately 1 hour at room temperature until set. A stacking gel containing 6% acrylamide (Appendix D.2) was poured onto the polymerised separating gel and a comb placed into the gel solution and allowed to set. The wells were filled with 1 X electrode buffer (Appendix D.3), protein samples loaded with sample buffer (Appendix D.4) and the gel assembled into the electrophoresis tank. The lower buffer reservoir containing 1 X electrode buffer was stirred with a magnetic stirrer during vertical gel electrophoresis. The gel was run at 20 mA until the tracker dye entered the separating gel at which point the current was increased to 50 mA. The gel was removed when the tracker dye reached the bottom of the separating gel and stained for protein visualisation or blotted as appropriate. For staining, 1 % (w/v) Coomasie blue stain solution (Appendix D.5) was used for a minimum of 2 h on a rotating platform before being replaced with de-stain solution (Appendix D.5) until the background was decolourised.

2.9 WESTERN BLOTTING

2.9.1 Western Blotting transfer

After SDS-PAGE, proteins were transferred to nitrocellulose membrane (Hybond-C, Amersham-Pharmacia Biotech) according to the method described by Towbin *et al.* (1979). Briefly, the stacking gel was removed and the separating gel soaked in electroblotting buffer (Appendix D.6) for 5 min. The blot was then assembled and placed in electroblotting apparatus (Trans-Blot cell, Bio-Rad, USA) containing electroblotting buffer and a voltage of 20 volts applied for several hours.

2.9.2 Development of blots

After transfer onto the membrane, proteins were visualised by staining in 0.5 % (w/v) Ponceau S (Sigma) solution in water for 5 min. Background colour was removed by de-staining in water for 2 min and the position of the molecular weight markers noted with indelible ink. The membrane was then de-stained in PBS (Appendix A.4) for 10 min and incubated in blocking buffer (Appendix D.7) for 1 h at room temperature with gentle agitation. Primary antiserum (kindly supplied by R. Parton), was obtained after innoculation i.m of a rabbit with purified CyaA followed by four booster injections at monthly intervals (Hormozi *et al.* 1999). Antiserum was diluted in blocking buffer and incubated with the membrane for 1 h at RT. The membrane was washed twice in fresh PBS for 15 min at RT. Horse-radish peroxidase-conjugated secondary antibody (Scottish Antibody Production Unit, UK) was then diluted 1 in 1000 in blocking buffer and incubated with the membrane for 1 hour at RT. The membrane was washed in PBS for 2 X 15 min at RT before being immersed in freshly prepared 0.05% (w/v) 3, 3'diaminobenzidine (DAB) (Sigma)

solution (Appendix D.8) for approximately 2 min. Washing the membrane in distilled water stopped the staining reaction.

2.10 CHARACTERISATION OF CyaA

2.10.1 Haemolysis assay

Turkey erythrocytes (Division of Virology, NIBSC) were washed in PBS and adjusted to give a final concentration of 0.7 % (v/v). CyaA samples were diluted in Hank's Balance Salt Solution (HBSS) (Gibco) containing 0.4M urea, starting at 1 /20 dilution of toxin. CyaA samples were added to 96-well u-bottomed plates (Nunc) and, to a final volume of 50 µl toxin sample, 50 µl turkey erythrocytes were added, the samples mixed and the plate incubated for 24 h at 37°C. A positive control of 1 % saponin was used to demonstrate 100 % haemolysis and a negative control containing only erythrocytes in HBSS plus 0.4M urea was used to demonstrate 0 % haemolysis. After incubation, samples were transferred to microfuge tubes and centrifuged at 600rpm for 5 min. The supernates were transferred to a fresh 96-well plate and the OD_{540nm} measured on an Anthos ELISA plate reader (Life Science Int, UK).

2.10.2 MTT dye reduction assay

Cytotoxic activity was determined by the MTT assay as described by Mosmann (1983) using the CellTitre 96™ assay (Promega) kit. This assay is based on the reduction of a yellow tetrazolium dye (3-(4,5-di-methylthiazol-2-3yl)-2,5-diphenol tetrazolium bromide, MTT) to insoluble purple formazan crystals by dehydrogenases in the mitochondria of living cells. The activity of toxin on cells inhibits this

reaction. Mouse macrophage-derived (J774.2) cells were prepared in phenol red-free Roswell Park Memorial Institute (RPMI) medium (Gibco, UK) plus additions (Appendix E.1) to a concentration of 5 X 10^5 cells/ml. Cell viability was checked by Trypan blue exclusion before and after incubation. For this, 50 µl of 0.2 % (w/v) Trypan Blue solution (Appendix E.2) was added to 50 µl cell suspension and incubated for 5 min at RT. Cells that remained clear and had not taken up the blue stain were counted using an improved neubauer haemocytometer (Webber, UK). Urea extracts were diluted in RPMI medium in serial 2-fold dilutions starting with 1:20 dilution. A volume of 50 µl of each dilution was transferred in duplicate to wells of 96-well flat bottom microtitre plates (Nunc), before the addition of 50µl/well of cell suspension. The plate was incubated at 37°C for 2 h in a humidified 5 % CO_2 atmosphere before the addition of 15 µl/well of MTT dye (Promega) and a further 4 h incubation. Following incubation, 100 µl/well of solubilization / stop solution (Promega) was added and incubation continued for a further hour. The contents of each well were mixed, avoiding bubble formation and the OD_{540nm} was measured using an Anthos ELISA reader (Life Science Int, UK). A negative control containing only the cell suspension in urea buffer was included. 100% cell viability was taken as the mean absorbance value for the control wells. This value was used to calculate the percentage cell viability of the different CyaA preparations.

2.10.3 AC enzymic assay

AC enzymic activity was measured according to the method of Salomon *et al.*, (1974). CyaA samples were diluted 1:1000 and 1:10,000 in Tris buffer, and 10 µl of

samples with 80 µl of reaction mix (Appendix F.1) were pre-incubated in a water bath at 30°C for 1 min. A control containing 10 µl of urea buffer without CyaA was also included. 10 µl of substrate mix (Appendix F.2) containing 20 mM ATP and approximately 3000Ci/mmol $^{32}\alpha$-P ATP and 50Ci/mmol ^{3}H-cyclicAMP (Amersham) was added and incubation continued for a further 5 min. The latter component was used as an internal control to calculate the recovery of cAMP after chromatography. In this first step ^{32}P-ATP was converted to ^{32}P cAMP. The reaction was stopped by the addition of 200 µl 0.5 M HCl before neutralising with 200 µl 1.5 M imidazole (Sigma). The neutralised solution (0.5 ml) was loaded onto a polypropylene disposable column (Bio-Rad) containing alumina powder (Merck), which acts to separate ^{32}P-cAMP from residual ^{32}P-ATP, the latter being retained on the column. ^{32}P-cAMP and ^{3}H-cyclicAMP were eluted from the column with 3 ml of 10 mM imidazole pH 7.6, and added to scintillation solvent. ^{32}P-cAMP and ^{3}H-cAMP radioactivity was counted in a liquid scintillation counter for all samples (Wallac). To calculate the moles of cAMP formed, a calulation must firstly be done to correct for variation in recovery between alumina columns. The value from this formula can be substituted into the equation below (Ladant *et al.*, 1988).

$$\text{Corrected value} = \frac{\text{Total input } ^{3}\text{H-cAMP}}{\text{Total recovered } ^{3}\text{H-cAMP}} \times \text{measured } ^{32}\text{P-ATP}$$

Example: over page

Sample	^{3}H cpm	^{32}P cpm
Reaction mix	10534	18
Substrate solution	7312	181028
CyaA dilution	8636	5780

$$\frac{10534}{8636} \times 5780 = 7050 \text{ cpm}$$

200 nmol ATP yields 181028 cpm, therefore 1000cpm corresponds to 1.1048 nmol
7050 cpm corresponds to 7.789 nmol cAMP formed during the incubation, therefore
7.789/5 = 1.558 nmol/min for 10µl of sample dilution,
which is equivalent to 155.8 nmol/min.ml of sample dilution.

2.10.4 Conductimetric assay

An alternative method for the measurement of adenylate cyclase activity employs a method where the conductance of a sample solution is measured. This assay was developed by Lawrence *et al* (1998). Briefly, the apparatus consists of 8 conductivity cells each containing platinum electrodes which have been fused into the walls of a glass tube (Figure 2.2). The cells are all linked to computer software that processes conductimetric readings and enables drawing of tangents to curves in order to determine slopes and reaction rates, and to measure total displacement between the initial and final baselines. A 2 ml volume of Bicine buffer (pH 8.0) (Appendix F.3), containing 0.5mM ATP, was added to each cell, followed by 0.5 µl pyrophosphatase (1 U/ µl, Sigma), 2 µl calmodulin (7.5mg/ml, kindly provided by Dr. A. Lawrence, Glasgow University) and the reaction was started by addition of 2 µl of CyaA

sample. 1 I.U inorganic pyrophosphatase hydrolyses the substrate at a rate of 1μmol/min. Hydrolysis of the substrate $(PPiMg)^{2-}$ generates four new mobile charged ions resulting in a conductance change (see below).

$(ATPiMg)^{2-} + Bicine^{-} \longrightarrow cAMP- + (BicineH) + (PPiMg)^{2-}$

$(PPiMg)^{2-} \longrightarrow 2\ Pi^{2-} + Mg^{2+}$

There is no change in conductance unless the $(PPiMg)^{2-}$ product is cleared to release the Mg^{2+} ion from chelation.

The total conductance changes and tangents to curves were measured by an on-screen line drawing. Thus for a substance concentration of 1mM giving a total change of x units and an initial slope of y units per minute, the initial rate is;

Initial rate = (y/x)μmol/ml/min

2.11 *In vivo* ASSAYS

2.11.1 Animals

Female NIH mice, age 3 to 4 weeks, were obtained from Harlan UK Ltd, Oxford, UK and maintained in pathogen-free conditions.

2.11.2 Vaccines

Whole cell pertussis vaccine (WCV) (NIBSC reagent, 88/522) and a commercially available three component acellular pertussis vaccine (ACV) containing 25μg of PT chemically detoxified with formaldehyde and glutaraldehyde, 25μg of filamentous haemagglutinin (FHA) and 8μg of pertactin (P.69) were used for immunisations at either 0.1 single human dose (SHD)/mouse or 0.025 SHD/mouse respectively.

Figure 2.2 Conductimetric apparatus used to measure AC activity

2.11.3 Immunisation

Mice were immunised intraperitoneally with 0.5 ml of CyaA, individual acellular pertussis components or whole cell or acellular pertussis vaccines. Control groups received 0.5 ml of PBS or PBS containing LPS where stated. For antibody assays, mice received 15 μg of CyaA per dose and either 10 μg/dose of each of detoxified PT (PTd), FHA and P.69 or native PT, FHA and P.69 at 120 ng/dose, 25 μg/dose and 10 μg/dose, respectively, singly or together. For protection assays, mice received 25 μg of CyaA per dose and in some cases, the other protective antigens as noted.

2.11.4 Preparation of *B. pertussis* suspension for aerosol challenge

B. pertussis 18.323 was grown on charcoal agar containing 10% (w/v) defibrinated horse blood for 18 h followed by 2 further sub-cultures at 37°C for 16 to 18 h. The bacterial cells were re-suspended in 1 % (w/v) casamino acid solution (CAS) (Appendix A.3) and adjusted to 0.2 OD_{625nm} using a spectrophotometer (MSE, Fisons) to give 4 X 10^8 cfu/ml. The suspension was kept on ice until used for aerosol challenge.

2.11.5 Aerosol infection of mice with *B. pertussis*

The mice were challenged at either 4 or 6 weeks after the initial vaccination using the aerosol apparatus described by Xing *et al.*, (1999) (Figure 2.3). Briefly, aerosol infection of mice with *B. pertussis* was performed as follows unless otherwise stated. Groups of 10 mice were exposed to aerosols produced from *B. pertussis* suspension by fitting the noses of mice into the 10 nose ports on the metal animal exposure section of the aerosol apparatus for a period of 5 min. Five mice from the PBS-

immunised group were sacrificed under anaesthesia on day 0 of challenge to determine the level of exposure. Mice from the remaining groups were sacrificed on day 7 post challenge. The lungs and trachea were removed together and homogenised in 1ml 1% (w/v) CAS with a mini-beadbeater (Biospec Products, PA, USA) with 2.5-3.5mm glass beads (BDH, Poole, UK) for 10 sec at 4600 rpm at RT. Viable counts were then performed on the homogenate by diluting in 1% (w/v) CAS across microtiter plates and transferring 4 x 25µl aliquots per dilution onto charcoal agar plates, which were then incubated for 5 days at 37°C. Agar plates showing clear individual colonies were used for viable count estimates.

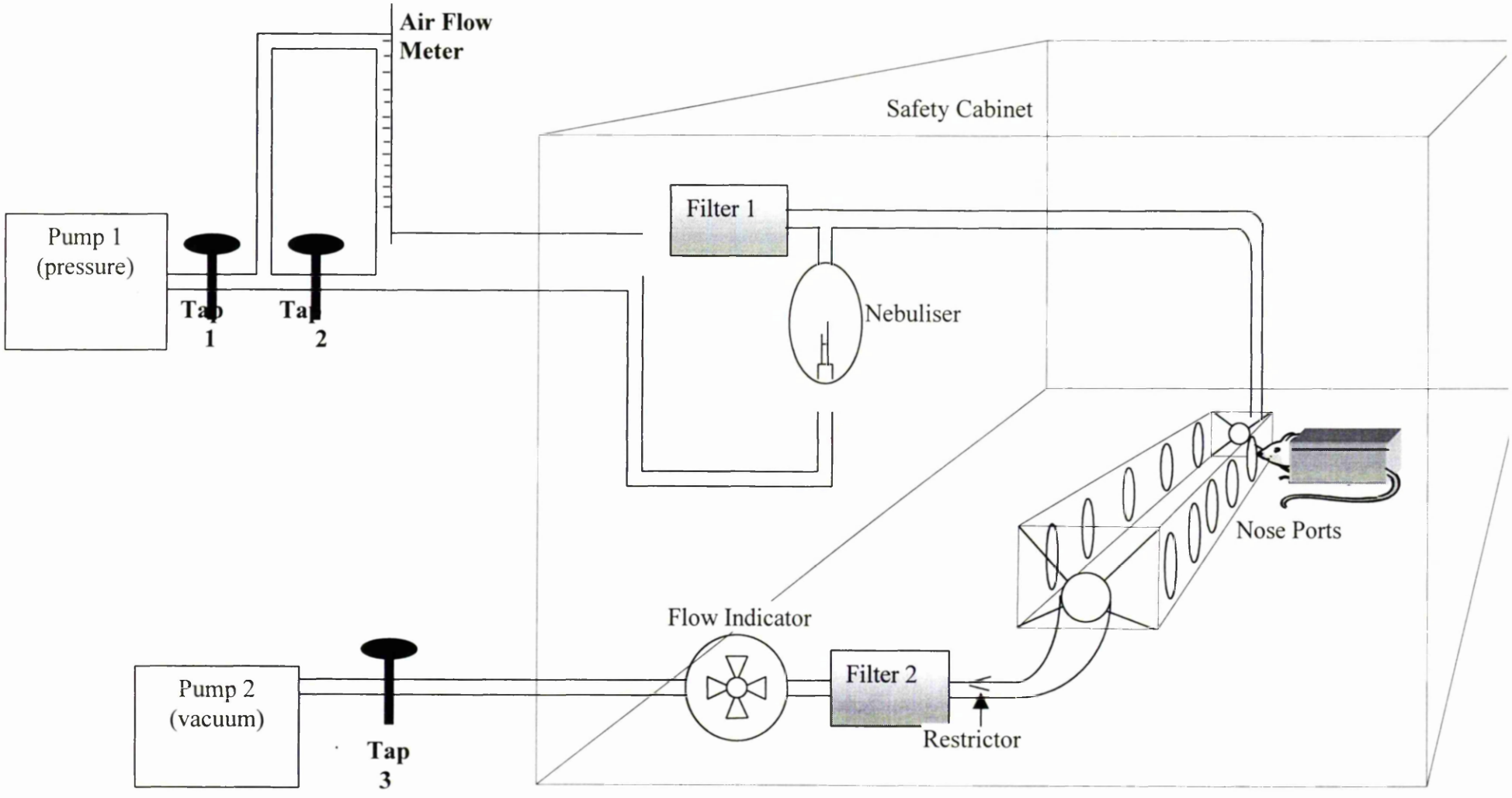
2.11.6 Preparation of *B. parapertussis* suspension for intranasal challenge

B. parapertussis CN5952 was prepared as for *B. pertussis*, except a final suspension of 1.5 X 10^9 cfu/ml was used.

2.11.7 Intranasal infection of mice

Mice were anaesthetised with a volume of 0.05ml/mouse Ketamine mixture (NIBSC) injected i.p, followed by an inoculation with a total volume of 10µl/nostril of *B. parapertussis* suspension containing 1.5 X 10^9 cfu/ml, using a 20µl pipette. Mice were sacrificed under anaesthesia on either day 0 post-challenge for the PBS-immunised control group, or day 5 post-challenge for the remaining groups. Viable counts were performed as described in section 2.11.5.

Figure 2.3 Aerosol Challenge Apparatus



2.11.8 Preparation of mammalian cells

Peritoneal lavage and spleen removal was performed 14 days post immunisation. Macrophage cultures were prepared according the method described by Beckerman *et al.* (1993). In brief, mice were terminally bled at different times. The peritoneal cavity was then lavaged with sterile PBS to recover macrophages. Cells were pooled from 5 mice and recovered by centrifugation at 800 x g for 10 min. The cell pellets were re-suspended in RPMI medium with L-glutamine (Gibco, UK) supplemented with 10 % (w/v) FCS, 1 % (w/v) penicillin and 1 % (w/v) streptomycin, placed in 24-well tissue culture plates at 2 X 10^6 cells per well, and incubated at 37°C under 5 % CO_2 in 90 % humidity for 2 h. The cells were washed 3 times with the RPMI medium to remove non-adherent cells.

Spleen cells were prepared from whole spleens by passing the tissue through a cell strainer (Falcon) with sterile PBS. The spleen cell suspension was centrifuged at 1000 x g for 20 min and pellets re-suspended in PBS and re-centrifuged as before. Cell pellets were re-suspended in RPMI medium and placed in 24-well tissue culture plates at 2 X 10^6 cells per well.

2.11.9 Culture and treatment of macrophages

Macrophages and spleen cells were cultured in a total volume of 1 ml per well with and without 2 X 10^6 heat killed *B. pertussis* cells/ml as described by Xing *et al.*, (1998) or CyaA antigens at 2 μg/ml in PBS. Cultures were incubated at 37°C under 5 % CO_2 in 90 % humidity for 24 h for macrophages and 48 h for spleen cells. Cell viability was checked by Trypan blue exclusion before and after incubation. For this, 50 μl of 0.2 % (w/v) Trypan Blue solution (Appendix E.2) was added to 50 μl cell suspension and incubated for 5 min at RT. Cells that remained clear and had not

taken up the blue stain were counted using an improved neubauer haemocytometer (Webber, UK).

2.11.10 Determination of nitrite production

Nitrite determinations were made on 50 µl volumes of either spleen or macrophage cell culture supernatants following treatment as described in section 2.11.9. A solution of $NaNO_2$ at 100µM was used to calculate the levels of NO generated. Doubling dilutions of the standard were prepared in sterile H_2O and 50µl volumes of each dilution were added to one column of a 96-well microtitre plate. The supernates and standard dilutions were mixed with 50 µl of Greiss reagent (Appendix E.3), and the OD_{540nm} measured after an incubation period of 5 min at room temperature using an Anthos ELISA reader (Life Science Int, UK). A standard curve was obtained which enabled the calculation of the nitrite production in the unknown samples.

2.12 RNA ANALYSIS

2.12.1 RNA extraction

The supernates from spleen cells taken from immunised mice were removed after the cells had been incubated with or without antigen as described in section 2.11.9. The cells were lysed with 500 µl Tri Reagent™ (Sigma) per 2 X 10^6 cells by repeated pipetting. After mixing, 200 µl chloroform per ml of Tri Reagent™ was added and the solution was then vortexed for 15 sec before being allowed to stand at room temperature for 15 min. The resulting mixture was centrifuged at 10,000 x g for 15

min at 4°C and the upper aqueous layer containing RNA was transferred into fresh tubes. The RNA was mixed with 200 µl isopropanol per ml of Tri Reagent™, allowed to stand at room temperature for 10 min, followed by centrifugation at 13,000 rpm for 10 min at 4°C. The supernate was removed and the RNA pellet washed with 1 ml of 75 % ethanol then centrifuged at 13,000 rpm for 5 min. The RNA pellet was dried by placing in a hot block at 60°C for approximately 10 min. Once dry, the pellet was re-suspended in 10 µl diethyl pyrocarbonate (DEPC)-treated water.

2.12.2 First-strand cDNA synthesis using Oligo (dT)

Following RNA extraction, 1 µl of Oligo (dT) (0.5 µg/ml) (Gibco, Life Technologies) and 1 mM dNTP solution (Gibco, Life Technologies) (containing dATP, dCTP, dGTP and dTTP at 1 mM concentration) was added to 10 µl of RNA solution and the mixture transferred to a 0.2 ml thin-walled PCR tube. The mixture was incubated at 65°C for 5 min and then placed in ice before 9 µl of reaction mix (see below) was added (all components Gibco, Life Technologies). Incubation was continued at 42°C for 2 min. To each tube, 1 µl of Superscript™ II Reverse Transcriptase (Gibco, Life Technologies) was added and then incubated at 42°C for 50 min. The reactions were terminated by heating to 70°C for 15 min and then chilled on ice. To each tube, 1 µl of RNase H (Gibco, Life Technologies) was added, followed by incubation at 37°C for 20 min, before proceeding to the amplification of target cDNA by PCR.

Reaction mix	**Volume per single reaction**
10 X Reverse Transcriptase (RT) buffer	2μl
25 mM $MgCl_2$	4μl
0.1 M DTT	2μl
RNase Out recombinant RNase inhibitor	1μl

2.13 Polymerase Chain Reaction (PCR)

PCR amplification of cDNA was performed on a Techne thermal cycler (Genius, Cambridge Ltd), using cytokine primer pairs (Applied Biosystems, UK) based on published sequence data (Appendix Table H). The following mastermix was prepared immediately before use (per reaction):

10 X PCR Buffer (minus $MgCl_2$)	5μl
50mM $MgCl_2$	1.5μl
10mM dNTP mix	1μl
5' primer (20μM)	1μl
3' primer (20μM)	1μl
Taq DNA polymerase 5U/μl (Gibco)	0.4μl
dH_2O	38.1μl
Total volume	48μl

2μl of each sample cDNA was added to a 48μl volume of mastermix.

The following thermocycling parameters were used on each 50μl sample:

Initial activation step	94°C	2 min
30 cycles of		
Denaturation	95°C	30 sec
Annealing	60°C	30 sec
Extension	72°C	45 sec
Followed by;		
Final extension step	72°C	5 min

2.14 IMMUNOLOGICAL METHODS

2.14.1 ELISA (IgG)

ELISA plates (Maxisorp, Nunc) were coated with 100µl per well of antigen (kindly supplied by GSK) diluted to 2 µg/ml in coating buffer (Appendix G.1) and incubated overnight at room temperature. The plates were then washed 3 times in washing buffer (Appendix G.2) containing 0.05% Tween in PBS then blocked with 100µl of diluent per well (Appendix G.3) for approximately 1 h. Plates were washed 3 times as above and dried thoroughly by repeated blotting on to paper towels. A volume of 100 µl of diluent per well was added into the plates from rows B to H and 148.5µl to row A. Serum samples in a 1.5 µl volume were added to the wells of row A, except for the first 2 columns, which were used for addition of reference serum containing known levels of antisera to PT, FHA and P.69 (97/642, NIBSC) (Gaines Das *et al.*, 2001) or diluent as a blank. Samples were mixed and 50 µl was transferred from row

to row making 3-fold dilutions down the plate (Rows B-H). The last 50 µl from row H was discarded and the plates incubated for 2 h at room temperature. Plates were washed 3 times as above and 100µl of Sheep anti-mouse IgG peroxidase conjugate (Sigma) at 1:1000 dilution was added per well. Plates were incubated for 2 h at room temperature before being washed and dried as above. Substrate solution (Appendix G.4.1) in 100 µl volumes was added to all wells and left to develop at room temperature for approximately 15 min. The reaction was stopped by the addition of 50µl of 1 M sulphuric acid per well and the OD_{450nm} was measured in an Anthos ELISA plate reader (Life Science Int, UK).

To calculate the titres, the OD_{450nm} reading for each dilution of mouse sera and reference was plotted against the $\log_3$ reciprocal dilution. This generated a straight-line graph to which a trend line, calculated by Microsoft Excel, could be applied. To obtain the $\log_3$ reciprocal dilution (x value) at an $OD_{450nm} = 0.5$ (cut-off level to standardise results), 0.5 was substituted for the y value in the trend line equation. An example of the calculation to obtain the $\log_3$ reciprocal dilution and hence the geometric mean is detailed below.

Example (Taken from Fig 3.8.3.1)

Trend lines obtained for 5 mice

$y = -1.0603x + 5.2295$	ie $0.5 = -1.0603x + 5.2295$ $\therefore x = 4.461$
$y = -0.9773x + 5.0399$	$x = 4.645$
$y = -0.9166x + 4.8123$	$x = 4.705$
$y = -1.0240x + 5.3238$	$x = 4.711$
$y = -0.9354x + 4.9504$	$x = 4.758$

Geometric mean (GM) = antilog (1/n X ∑log3 reciprical dilution)

GM = antilog (1/n X 23.28

= antilog (1/5 X 23.28)

= antilog 4.656

= 45289.76

When the reference serum containing known levels of antisera to PT, FHA and P.69 (97/642, NIBSC) was included, a further calculation was performed to obtain the ELISA units (EU)/ml.

The trend line for the reference serum was obtained in the same method as detailed previously and as before a value of 0.5 was substituted for the y value. The following calculation makes use of the same results in the previous example plus a further trend line for the reference.

Trend line for reference serum

$y = -1.071x + 3.9851 \quad \therefore x = 3.257$

GM = antilog 3.257

= 1807

GM for unknown samples (previous example) = 45289.76

Specific EU/ml for PT, FHA, P.69 = 17, 143, 30 respectively

$$\text{GM of antibody levels (EU/ml)} = \frac{\text{GM of unknown samples X specifc EU}}{\text{GM of refernce serum}}$$

$$\text{GM of anti-PT IgG} = \frac{45289.79 \text{ X } 17}{1807}$$

= 426.08 EU/ml

2.14.2 ELISA (IgG_1 and IgG_{2a})

The same procedure as section 2.14.1 for the measurement of IgG was performed except that sheep anti-mouse IgG subclass biotin conjugate (Pharmingen) in diluent was used in the primary reaction and streptavidin-horseradish peroxidase conjugate

(Pharmingen) at 1:1000 dilution in diluent was used as secondary antibody. Plates were washed 3 times as before followed by the addition of 100µl of substrate (Sigmafast, tablets of o-phenylenediamine di-hydrochloride (OPD) plus urea/hydrogen peroxide, dissolved in 20 ml of sterile distilled water, Sigma) to each well for an incubation period in darkness of 20 min. The reaction was stopped by the addition of 50µl of 3M HCl per well and the OD_{492nm} was measured in an Anthos ELISA plate reader (Life Science Int, UK). 97/642 has only been asigned unitage to total IgG and not the subclasses, therefore the specific EU/ml could not be calculated and instead only the geometric mean. As for IgG, the geometric mean was calculated as follows,

Geometric mean (GM) = antilog (1/n X ∑log3 recipricol dilution)

2.14.3 ELISA (IFNγ)

The same procedure as section 2.14.1 for the measurement of IgG was performed except that rat anti-mouse IFN-γ biotin conjugate (Pharmingen) in diluent was used in the primary reaction and streptavidin-horseradish peroxidase conjugate (Pharmingen) at 1:1000 dilution in diluent was used as secondary antibody. Plates were washed 3 times as before followed by the addition of 100µl of substrate (Sigmafast, tablets of o-phenylenediamine di-hydrochloride (OPD) plus urea/hydrogen peroxide, dissolved in 20 ml of sterile distilled water, Sigma) to each well for an incubation period in darkness of 20 min. The reaction was stopped by the addition of 50µl of 3M HCl per well and the OD_{492nm} was measured in an Anthos ELISA plate reader (Life Science Int, UK).

CHAPTER 3

RESULTS

3.1 PREPARATION OF CyaA FROM RECOMBINANT *E. coli* STRAINS

Recombinant CyaA toxin was produced using the *E. coli* BL21/DE3 strain (section 2.1). The CyaA pro-toxin and the CyaC protein were expressed in *E. coli* BL21/DE3 from two separate compatible plasmids, pGW44 and pGW54 (section 2.1). Co-expression of *cyaA* and *cyaC* in the presence of 1mM IPTG in *E. coli* BL21/DE3 harbouring both pGW44 and pGW54 resulted in the production of biologically-active CyaA. Expression of CyaA without CyaC from *E. coli* harbouring only pGW44, resulted in the production of biologically-inactive (non-acylated) CyaA. Active and inactive forms of CyaA, together with CyaC alone, were extracted as inclusion bodies that could be solubilised in 8M urea from IPTG-induced cultures of BL21/DE3 harbouring the relevant plasmids. The resulting urea extracts of strain BL21/DE3 (pGW44), BL21/DE3 (pGW54) and BL21/DE3 (pGW44,54) contained predominantly a 200 kDa protein, a 23 kDa protein and both a 200 and 23 kDa protein respectively, as shown in Figure 3.1.1. Lane 7 does not appear to show the presence of CyaC. This is due to the lower optical density of the culture at the point of IPTG-induction and therefore a lower level of expression of both CyaA and CyaC. A third compatible plasmid, pGW44/188, encoding the CyaA pro-toxin lacking AC activity due to a modification in the N-terminal end, was co-expressed with pGW54 to produce catalytically inactive CyaA (CyaA*). Urea extraction of BL21/DE3 (pGW44/188, pGW54) resulted in the production of 200 kDa and 23 kDa proteins as the major bands, as for BL21/DE3 (pGW44, pGW54) (Figure 3.1.2).

3.2 PURIFICATION OF CyaA FROM CRUDE UREA EXTRACTS

Crude extracts of CyaA from *E. coli* were purified initially by phenyl sepharose column chromatography alone (Section 2.5.2) (Figure 3.2.1). This method, however, was not effective in adequately removing other proteins or breakdown products of CyaA found in the crude extract. DEAE sepharose chromatography was more effective in removing lower molecular weight proteins (Figure 3.2.2). Ultimately a combination of DEAE sepharose column chromatography followed by phenyl sepharose column chromatography was found to be the best method (section 2.5.1, 2.5.2). Figure 3.2.3 shows the results of combined DEAE sepharose and phenyl sepharose column chromatography purification of CyaA from *E. coli* BL21/DE3 (pGW44, pGW54), indicating a predominant 200 kDa band along with only a limited amount of lower molecular weight proteins. An intermediate step, between DEAE sepharose and phenyl sepharose chromatography, was ultimately employed which made use of Centricon® centrifugal filter devices (Section 2.5.3). The filters used had a molecular weight cut-off of 100 kDa and functioned to concentrate proteins greater than this weight (Figure 3.2.4). SDS-PAGE analysis and Western blotting of the final purified CyaA with rabbit polyclonal antiserum raised against the 200 kDa protein is shown in Figures 3.2.5 and 3.2.6.

Figure 3.1.1 SDS-PAGE analysis of crude CyaA and CyaC preparations from *E. coli*.

Crude urea extracts, at approximately 2mg/ml in a 50µl volume, from two cultures of *E. coli* expressing CyaA pro-toxin, CyaC or acylated CyaA were subjected to electrophoresis on a 10 % polyacrylamide gel and stained with 1% (w/v) Coomassie blue solution (section 2.8). Arrows indicate the positions of the molecular weight standards.

Lane 1: 10kDa molecular weight marker

Lanes 2, 3: CyaA pro-toxin from *E. coli* BL21/DE3 (pGW44)

Lanes 4, 5: CyaC from *E. coli* BL21/DE3 (pGW54)

Lanes 6,7: CyaA and CyaC from *E. coli* BL21/DE3 (pGW44,54)

Lane 8: 10kDa molecular weight marker

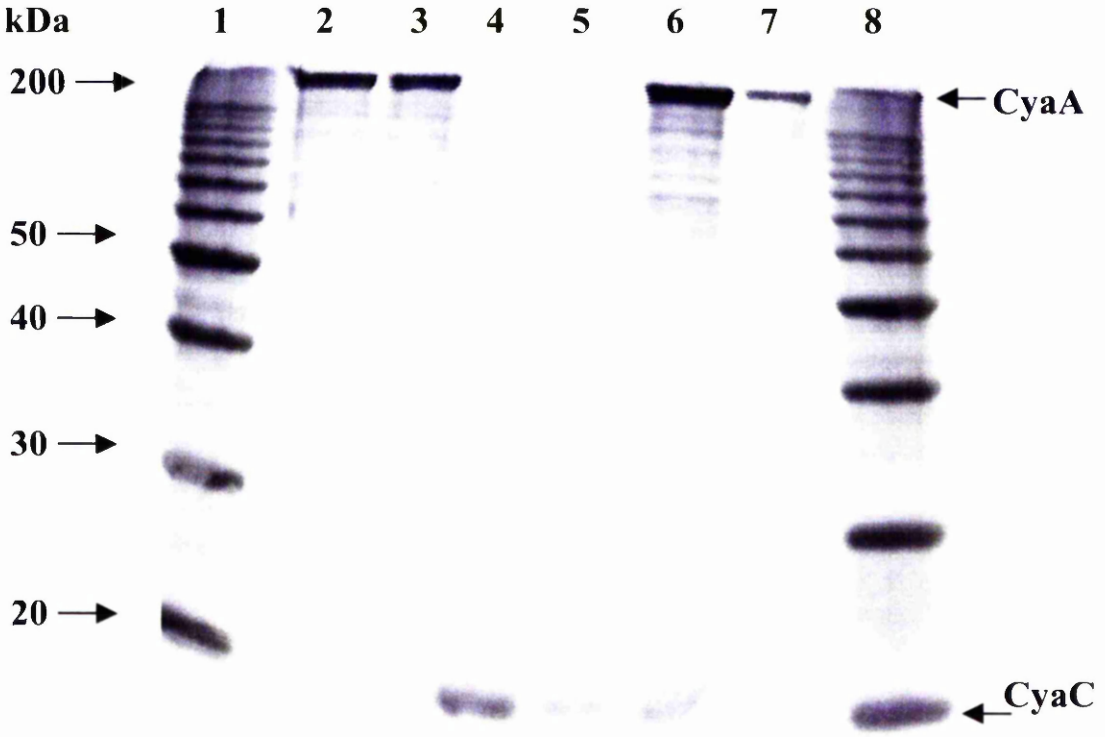

kDa
1
2
3
4
5
6
7
8
200
50
40
30
20
CyaA
CyaC

Figure 3.1.2 SDS-PAGE analysis of crude non-induced and induced CyaA preparations from *E. coli.*

Crude urea extracts of CyaA from *E. coli* BL21/DE3 (pGW44/188, pGW54) *and E. coli* BL21/DE3 (pGW44, pGW54) in a 50µl volume, were analysed by electrophoresis on a 10% polyacrylamide gel and stained with 1% (w/v) Coomassie blue. Aliquots for each preparation were removed from the flask prior to and after induction with IPTG for 3 hours (non-induced and induced) preparations respectively. Arrows indicate the positions of the molecular weight standards.

Lane 1: 10kDa molecular weight marker

Lane 2: Non-induced CyaA* from *E. coli* BL21/DE3 (pGW44/188,54)

Lane 3: Induced CyaA* from *E. coli* BL21/DE3 (pGW44/188,54)

Lane 4: Non-induced CyaA from *E. coli* BL21/DE3 (pGW44,54)

Lane 5: Induced CyaA from *E. coli* BL21/DE3 (pGW44,54)

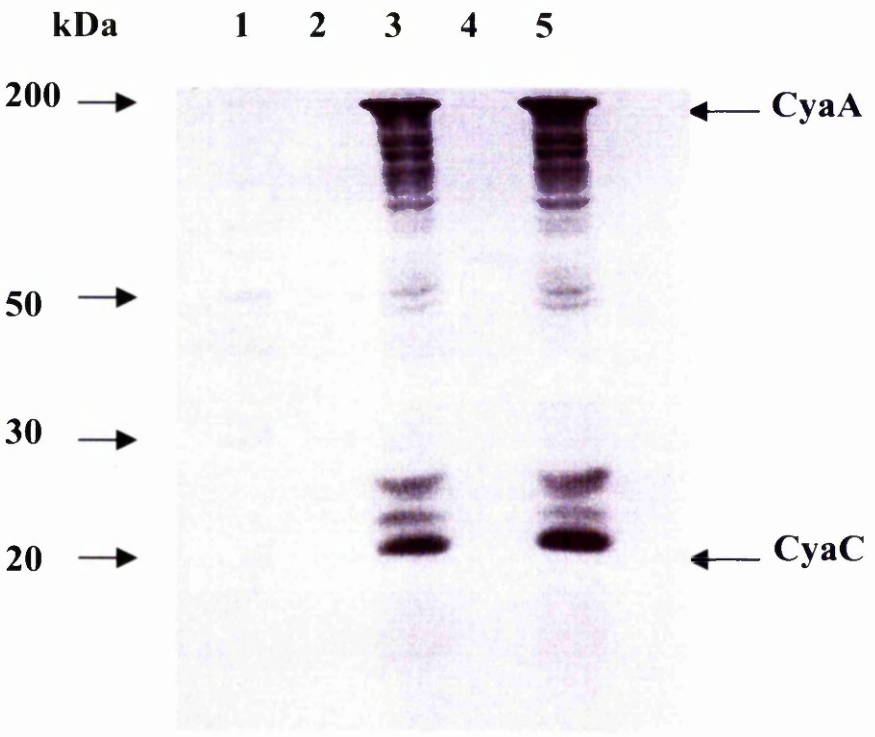

kDa
1
2
3
4
5
200
50
30
20
CyaA
CyaC

Figure 3.2.1 SDS-PAGE analysis of CyaA fractions prepared by phenyl sepharose column chromatography.

A crude urea extract of recombinant CyaA was purified using phenyl sepharose (section 2.5.2). The initial crude extract and aliquots from the column washes and elutions in a 50μl volume, were subjected to electrophoresis on a 10% polycacrylamide gel and stained with 1% (w/v) Coomassie blue. Arrows indicate the position of the molecular weight standards.

Lane 1:	Broad range molecular weight marker
Lane 2:	Crude CyaA from *E. coli* BL21/DE3 (pGW44,54)
Lane 3:	Flow-through from equilibrated column
Lane 4:	Flow-through from first column wash
Lane 5:	Flow-through from second column wash
Lanes 6, 7, 8:	First, second and third fractions eluted with 500mM NaCl (Solution B; Appendix C.4.3)
Lanes 9, 10, 11:	Fourth, fifth and sixth fractions eluted with 250mM NaCl
Lanes 12, 13:	Seventh and eighth fractions eluted with 8M urea without NaCl

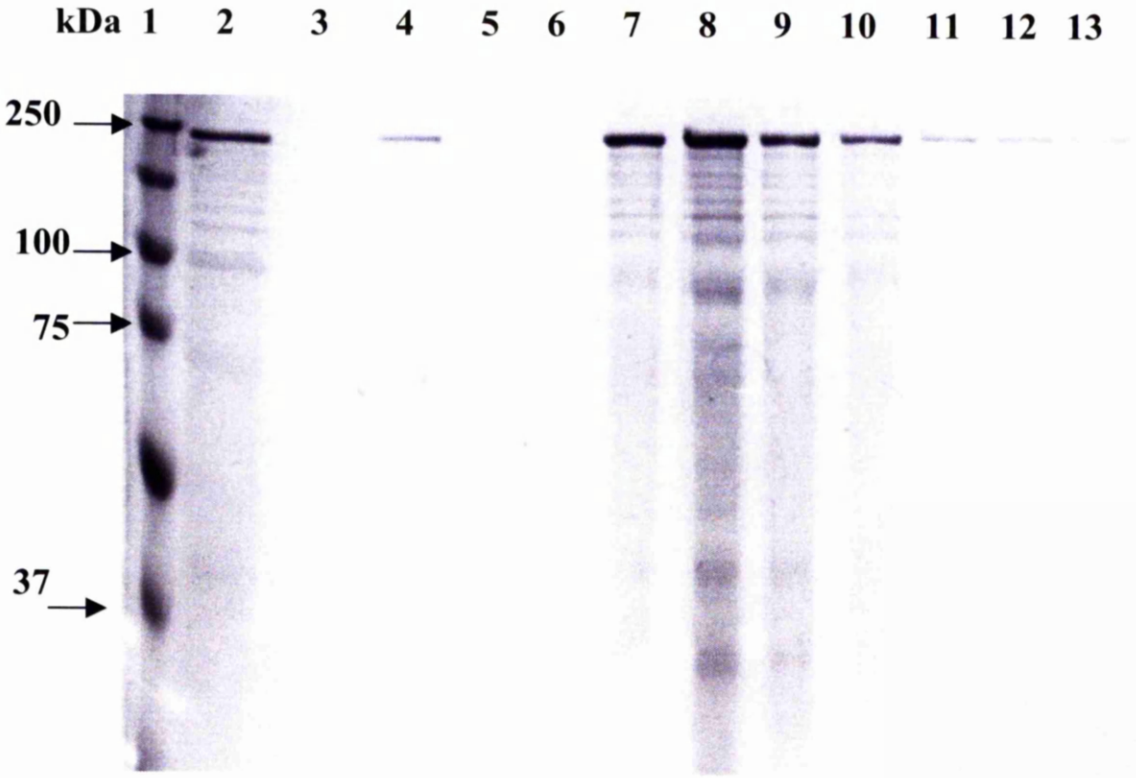

kDa 1 2 3 4 5 6 7 8 9 10 11 12 13
250
100
75
37

Figure 3.2.2 SDS-PAGE analysis of CyaA fractions prepared by DEAE sepharose column chromatography.

A crude urea extract of recombinant CyaA was purified using DEAE sepharose (section 2.5.1). The initial crude extract and aliquots from the column wash and different elutions in a 50µl volume, were subjected to electrophoresis on a 10% polycacrylamide gel and stained with 1% (w/v) Coomassie blue. Arrows indicate the position of the molecular weight standards.

Lane 1:	Broad range molecular weight marker
Lane 2:	Crude CyaA from *E. coli* BL21/DE3 (pGW44,54)
Lane 3:	Flow-through from column wash
Lanes 4, 5:	First and second fractions eluted with 250mM NaCl (Buffer 3; Appendix C.3.4)
Lanes 6, 7:	Third and fourth fractions eluted with 500mM NaCl
Lanes 8, 9:	Fifth and sixth fractions eluted with 1M NaCl
Lanes 10, 11:	Seventh and eighth fractions eluted with 1.5M NaCl
Lanes 12, 13:	Ninth and tenth elution eluted with 2M NaCl

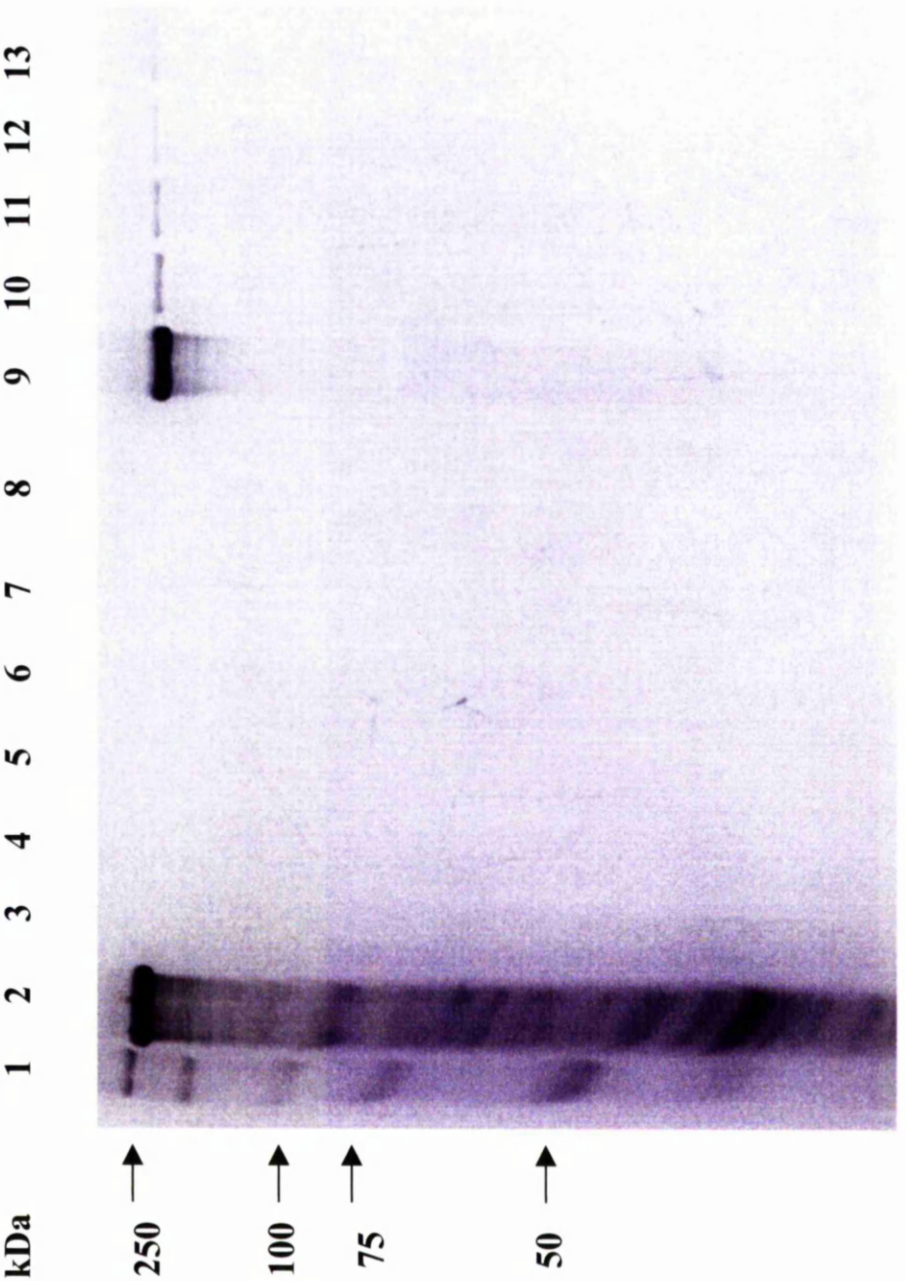

1 2 3 4 5 6 7 8 9 10 11 12 13
kDa
250
100
75
50

Figure 3.2.3 SDS-PAGE analysis of CyaA fractions prepared by phenyl sepharose column chromatography after purification by DEAE sepharose column chromatography.

The sixth and seventh elution fractions from the previous CyaA purification using DEAE sepharose (Figure 3.2.2) were pooled together and subjected to phenyl sepharose column chromatography. Aliquots in a 50µl volume from each elution fraction were subjected to electrophoresis on a 10% polyacrylamide gel and stained with 1% (w/v) Coomassie blue solution. Arrows indicate the positions of the molecular weight standards.

Lane 1:	Broad range molecular weight marker
Lanes 2, 3, 4, 5:	First to fourth fractions eluted with 500mM NaCl (Solution B; Appendix C.4.3)
Lanes 6, 7, 8, 9:	Fifth to eighth fractions eluted with 250mM NaCl
Lanes 10, 11, 12, 13, 14, 15:	Ninth to fourteenth fractions eluted without NaCl

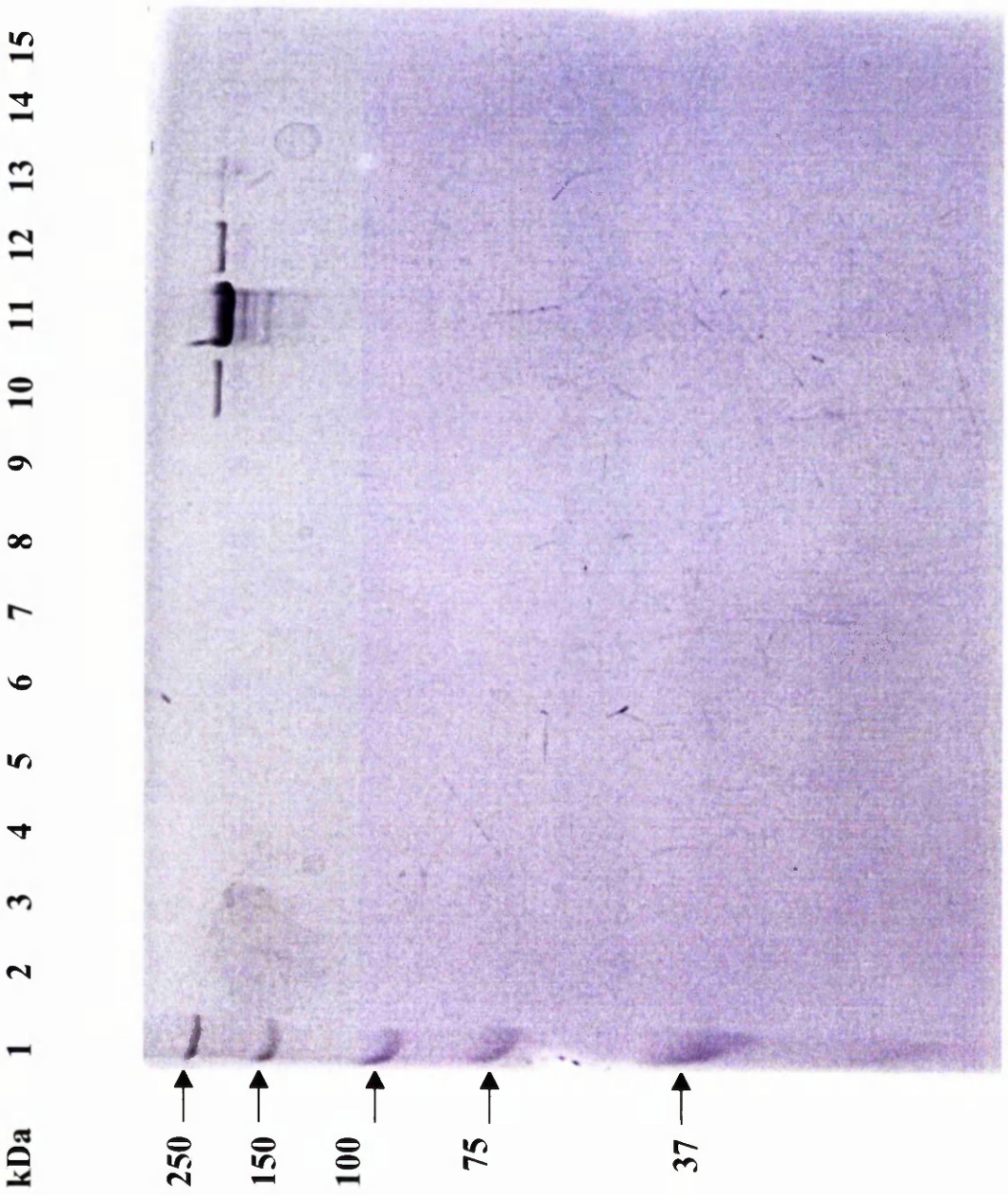

kDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
250
150
100
75
37

Figure 3.2.4 SDS-PAGE analysis of concentrated CyaA prepared by centrifugation with Centricon® centrifugal filter devices.

CyaA preparations purified by DEAE sepharose and phenyl sepharose chromatography were concentrated further using Centricon® centrifugal filter devices (section 2.5.3). Aliquots of 50µl volumes from the concentrated retentate and the residual filtrate were subjected to electrophoresis on a 10% polyacrylamide gel and stained with 1% (w/v) Coomassie blue solution. Arrows indicate the positions of the molecular weight standards.

Lane 1: Broad range molecular weight marker

Lane 2: Retentate of purified CyaA from *E. coli* BL21/DE3 (pGW44)

Lane 3: Filtrate of purified CyaA from *E. coli* BL21/DE3 (pGW44)

Lane 4: Retentate of purified CyaA from *E. coli* BL21/DE3 (pGW44/188,54)

Lane 5: Filtrate of purified CyaA from *E. coli* BL21/DE3 (pGW44/188,54)

Lane 6: Retentate of purified CyaA from *E. coli* BL21/DE3 (pGW44,54)

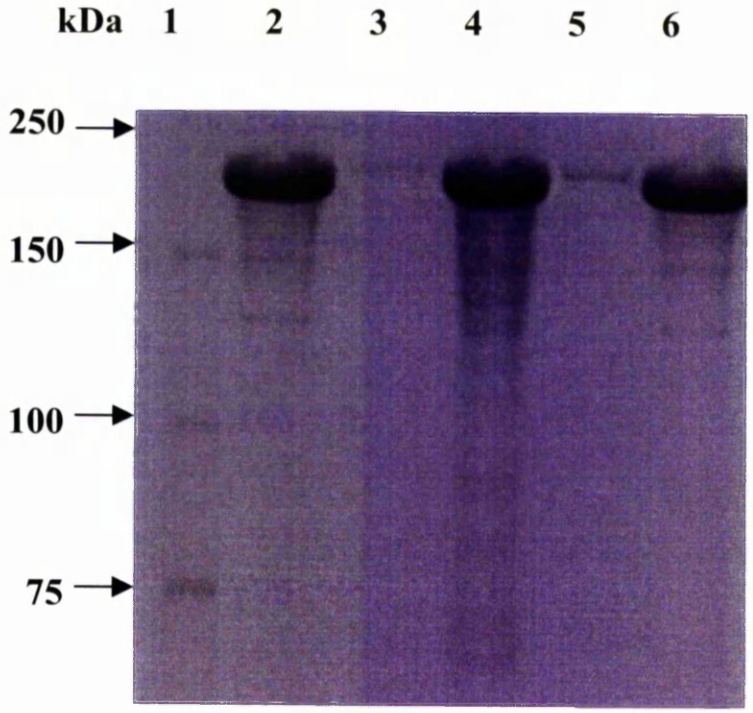

kDa
1
2
3
4
5
6
250
150
100
75

Figure 3.2.5 SDS-PAGE analysis of purified CyaA prepared by DEAE and phenyl sepharaose column chromatography, Centricon® column centrifugation and a further phenyl sepharose column chromatography stage.

CyaA preparations retained in the Centricon® columns after centrifugation were purified by a final phenyl sepharose column chromatography step. The eluted fractions in a 50µl volume were subjected to electrophoresis on a 10% polyacrylamide gel and stained with 1% (w/v) Coomassie blue solution. Arrows indicate the position of the molecular weight standards.

Lane 1: Broad range molecular weight marker

Lane 2: Purified CyaA from *E. coli* BL21/DE3 (pGW44)

Lane 3: Purified CyaA from *E. coli* BL21/DE3 (pGW44/188,54)

Lane 4: Purified CyaA from *E. coli* BL21/DE3 (pGW44,54)

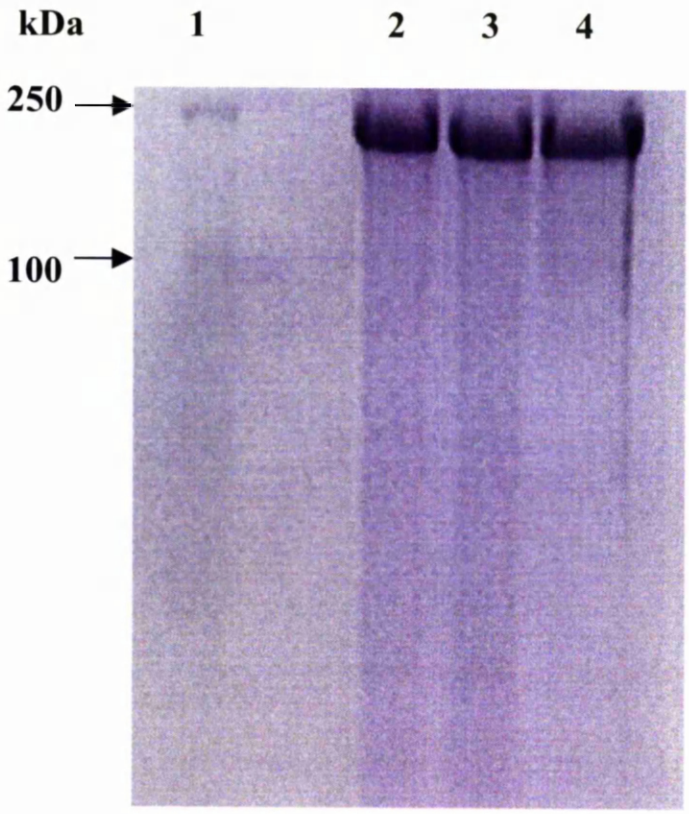

kDa
1
2
3
4
250
100

Figure 3.2.6 Western blot analysis of purified CyaA preparations using anti-CyaA polyclonal antiserum.

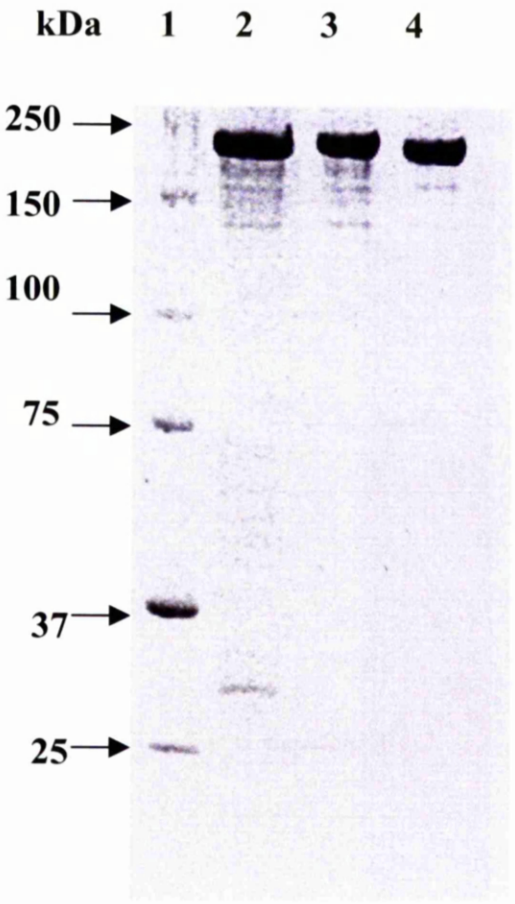
CyaA preparations purified by DEAE and phenyl sepharose column chromatography and Centricon® centrifugation methods as described in the legend to Figure 3.2.5 were subjected to electrophoresis on a 10% polyacrylamide gel and transferred to Hybond-C nitrocellulose membrane (section 2.9.1). The nitrocellulose blots were probed with rabbit anti-CyaA polyclonal antibody (section 2.9.2). Arrows indicate the position of the molecular weight standards.

Lane 1: Broad range molecular weight marker

Lane 2: Purified CyaA from *E. coli* BL21/DE3 (pGW44)

Lane 3: Purified CyaA from *E. coli* BL21/DE3 (pGW44/188,54)

Lane 4: Purified CyaA from *E. coli* BL21/DE3 (pGW44,54)


kDa
1
2
3
4
250
150
100
75
37
25

3.3 SUMMARY OF SDS-PAGE ANALYSIS

CyaC produced from plasmid pGW54 migrated on a 10% polyacrylamide gel at approximately 23kDa and CyaA proteins at approximately 200kDa (Figure 3.1.1). Crude urea extracts displayed additional bands that indicated the presence of contaminating proteins or CyaA degradation products. The majority of these were eliminated by further purification using DEAE and phenyl sepharose column chromatography followed by Centricon® concentration (Figure 3.2.5) and a further phenyl sepharose chromatography step. The three different preparations of CyaA (CyaA pro-toxin, CyaA* and CyaA) displayed very similar banding patterns on polyacrylamide gels although the extent of purification varied a little between preparations.

3.4 SUMMARY OF WESTERN BLOTTING

All purified CyaA proteins, when probed with anti-CyaA polyclonal antibody, displayed a major band at approximately 200kDa, although some minor bands were also detected at lower molecular weights (Figure 3.2.6). Given that the proteins had been through several purification procedures, the minor bands were likely to represent CyaA degradation products.

3.5 CHARACTERISATION OF CyaA TOXINS

3.5.1 Adenylate cyclase enzymic activity

Adenylate cyclase (AC) activity of purified urea extracts from *E. coli* BL21/DE3 (pGW44) (CyaA pro-toxin, non-acylated), *E. coli* BL21/DE3 (pGW44/188, pGW54) (CyaA*, acylated) and *E. coli* BL21/DE3 (pGW44, pGW54) (CyaA, acylated) were determined by two methods, the conventional $^{32}\alpha$P-ATP radioactive assay and a novel conductimetric assay (section 2.10.3, 2.10.4). The conductimetric assay can produce greater deviations between replicate assays, particularly when the enzymic activity is high. The results (Figure 3.5.11) show both CyaA pro-toxin and CyaA to be enzymically active as expected. The CyaA* toxin has a dipeptide amino acid insertion within the ATP binding site, and therefore should lack AC enzymic activity. This preparation did in fact demonstrate a very low, almost negligible value for cAMP production, presumably indicating a basal level of enzymic activity with some degree of experimental error in the measurement at such low levels.

3.5.2 Haemolytic activity of CyaA preparations on turkey erythrocytes

The haemolytic activities of purified CyaA pro-toxin, CyaA and CyaA* toxins were determined using turkey erythrocytes (section 2.10.1). Both forms of the acylated CyaA toxin showed a similar pattern of haemolysis (Figure 3.5.2) although the data in Figure 3.5.2 demonstrates the weak haemolytic nature of this toxin, as 50% haemolysis is only produced at protein concentrations of 30-40 μg/ml after incubation with erythrocytes for 24 hours (Figure 3.5.2; Table 3.5).

Figure 3.5.1

Comparison of CyaA adenylate cyclase enzymic activity from different toxin preparations

Enzymic activity in purified CyaA preparations was determined by two different methods, the conventional ^{32}αP-ATP radioactive assay and a novel conductimetric assay. Enzymic activity was measured in duplicate for each sample and the results represent the mean ± standard deviation for each method.

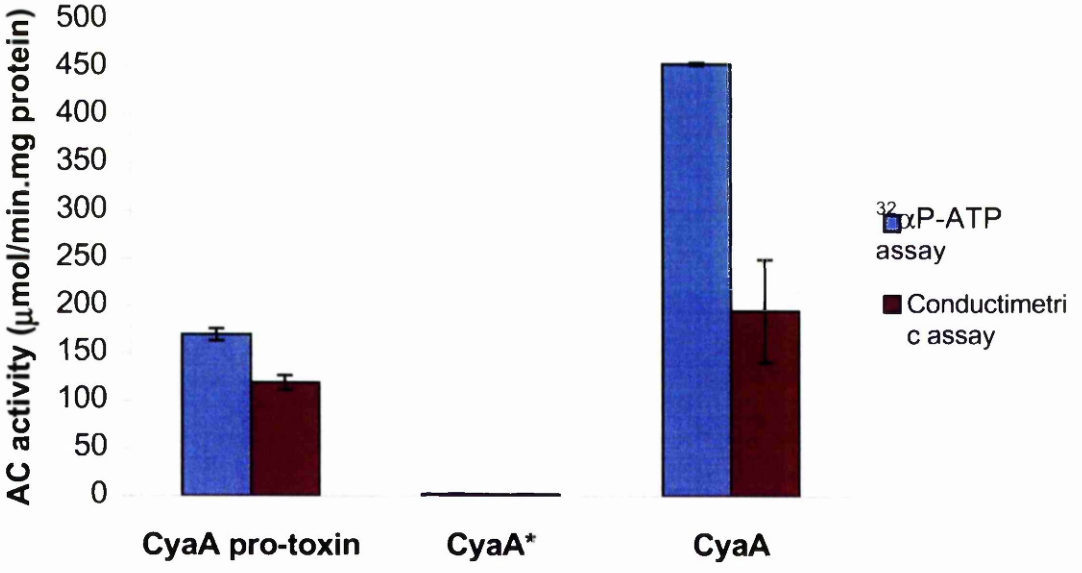
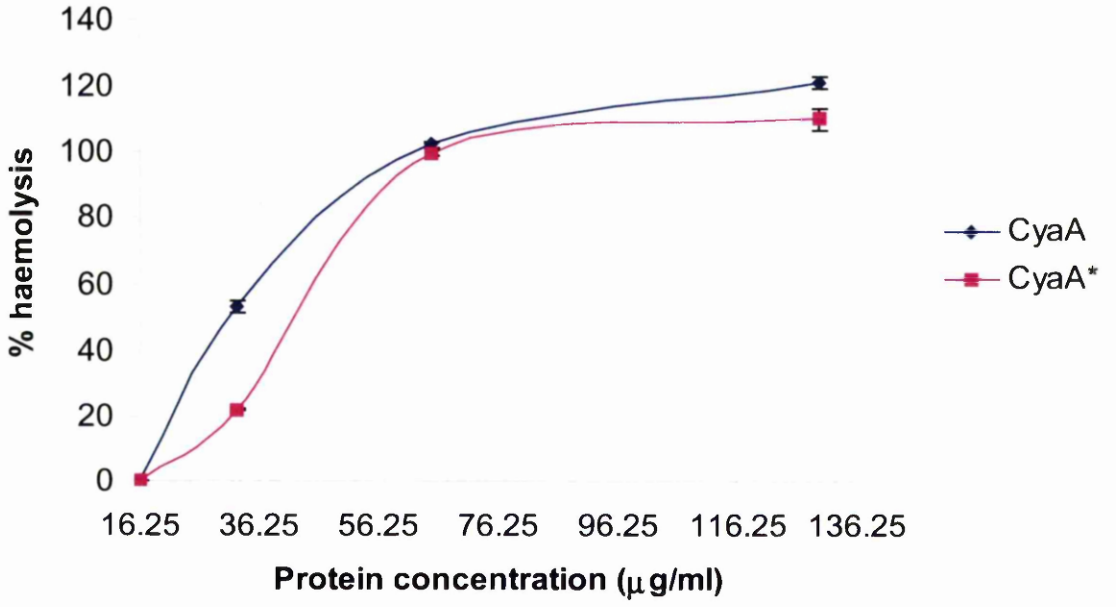


Figure 3.5.2

Percentage haemolysis of turkey erythrocytes by CyaA preparations

The haemolytic activities of the two purified CyaC-acylated proteins were determined in two fold serial dilutions using turkey erythrocytes. Percentage haemolysis was measured in triplicate for each sample and the results represent the mean ± SEM.



3.5.3 Cytotoxic activity of CyaA preparations on J774.2 macrophage cells

The effect of purified CyaA pro-toxin, CyaA* and CyaA preparations on cell killing of J774.2 mouse macrophage-derived cells was determined by the MTT dye reduction assay (section 2.10.2) (Table 3.5, Figures 3.5.3.1 and 3.5.3.2). CyaA pro-toxin is non-acylated and no cell killing was observed, as expected. CyaA demonstrated a high level of cytotoxic activity, which is in keeping with its enzymic, haemolytic and invasive activities. The N-terminally modified CyaA* toxin, with little AC enzymic activity, demonstrated some cytotoxicity at the highest protein concentrations tested. The possibility that this may have been due to an effect of urea in the preparation was excluded by the results obtained using CyaA pro-toxin. Both toxins were prepared in the same way and both contained the same concentration of urea, indicating that the cytotoxic effect of CyaA* on the cells was indeed a true reflection of the ability of this AC^- protein to kill J774.2 cells at high protein concentrations. The results of this assay would imply that cell killing by CyaA toxin is due mainly to the intrinsic AC activity of the protein. If this activity is removed, only residual toxicity is observed (100-fold less, Table 3.5), perhaps due to the very low, residual AC activity of the protein or, more likely, to its pore-forming activity as shown by haemolysis.

Figure 3.5.3.1 Cytotoxic activity of CyaA preparations on J774.2 mouse macrophage cells

The cytotoxic activity of the purified CyaA preparations was determined by the MTT dye reduction assay using mouse macrophage-derived J774.2 cells. The percentage cell viability at different CyaA protein concentrations was determined using duplicate samples (Section 2.11.9) and the results represent the mean absorbance at each dilution ± standard deviation.

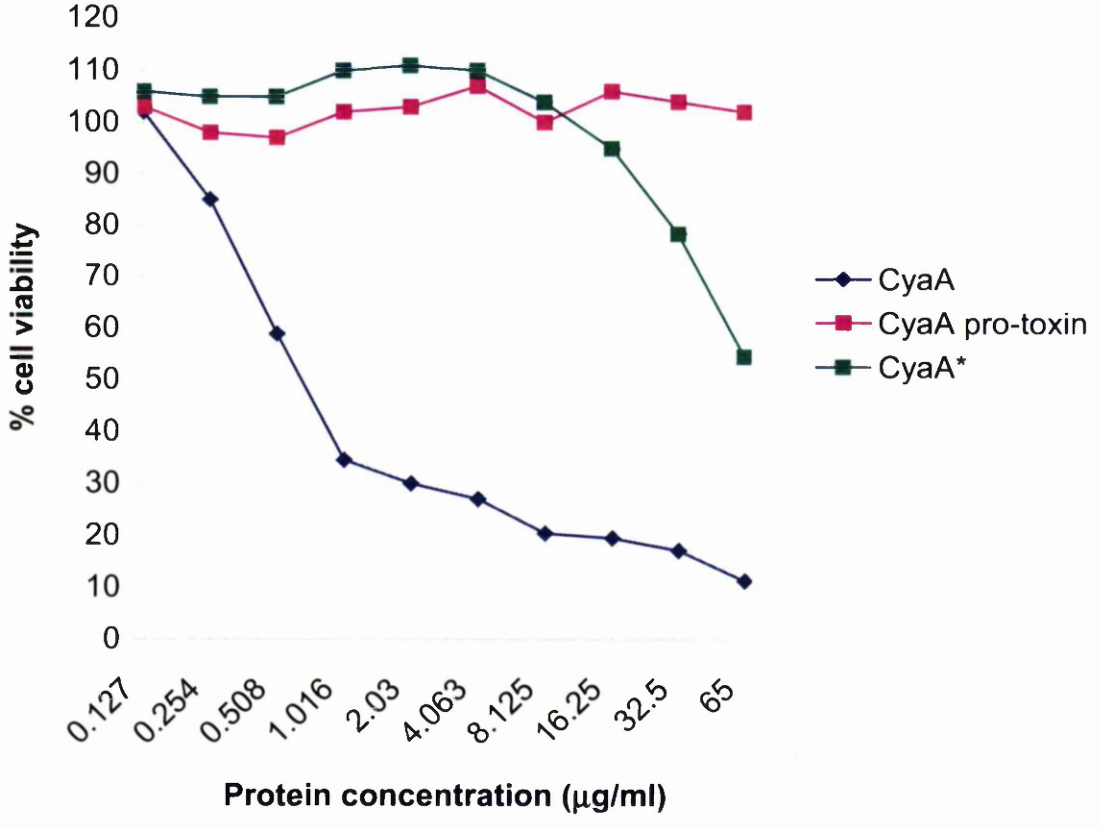
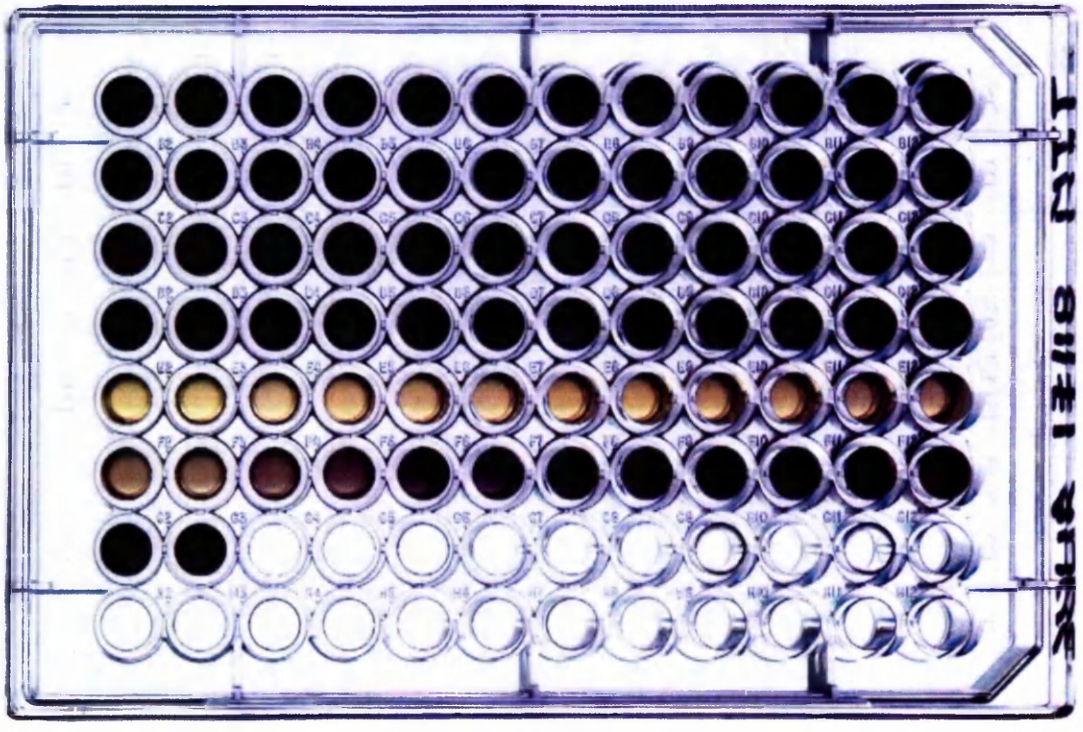


Figure 3.5.3.2 Demonstration of the MTT reaction

The MTT dye reduction assay was used to demonstrate the cytotoxic activity of CyaA on J774.2 mouse macrophage cells. Viable cells are indicated by the purple colour. Cells which have been incubated with the enzymic, invasive form of CyaA, are yellow, as the cells have been killed and were unable to convert the MTT to the purple formazan, following the reduction of the tetrazolium dye (Section 2.11.9).

Rows A+B: CyaA pro-toxin from *E. coli* BL21/DE3 (pGW44)
Rows C+D: CyaA from *E. coli* BL21/DE3 (pGW44/188,54)
Rows E+F: CyaA from *E. coli* BL21/DE3 (pGW44,54)
Row G (wells 1+2): RPMI medium only

All wells contain J774.2 mouse macrophage cells at 5 X 10^5 cells/ml and rows A-F contain doubling dilutions of the toxin starting at a protein concentration of 65µg/ml. Each dilution is done in duplicate.



3.5.4 Summary of characterisation of CyaA preparations

The different preparations were subjected to 4 different assays, to investigate adenylate cyclase enzymic activity, haemolytic and cytotoxic activities. Two different methods were investigated to assay enzymic activity, the conventional $^{32}\alpha$P-ATP radioactive assay and a novel conductimetric assay. Although the results for the 2 assays were not identical the overall pattern was similar. In preparations where greater amounts of cAMP were likely to be produced, measurement by the conventional method appeared to be more consistent as deviations between replicate samples were lower. However, the conductimetric assay is quicker and easier and is a good indication of whether AC activity is present or absent and is probably better suited for samples producing lower amounts of cAMP, as at these levels the deviations between replica samples were much smaller. The results in Table 3.5 show both CyaA pro-toxin and CyaA to be enzymically active and CyaA* to be only very slightly enzymically active.

The haemolytic activity was recorded as the amount of protein required to give 50% haemolysis of turkey red blood cells. CyaA pro-toxin was expected to be non-pore forming and, in fact, no haemolysis was detected. Both forms of CyaA gave similar levels of haemolysis, albeit very weak, indicated by the high protein concentrations required to obtain 100% haemolysis in 24 hours (Figure 3.5.2).

The cytotoxic nature of CyaA preparations was recorded as the amount of protein required for 40% killing of J774.2 macrophage cells *in vitro* in 4 hours. CyaA pro-toxin is non-acylated and therefore should be non-invasive, and, in fact, no cell killing was observed. CyaA was found to be highly cytotoxic, whereas CyaA* had only a degree of cytotoxicity at high protein concentrations.

Table 3.5: Properties of different CyaA preparations from *E. coli*

Preparation	AC activity (μmol cAMP.min/mg protein) $^{32}\alpha$P-ATP radioactive assay [a]	Enzymic activity (μmol cAMP.min/mg protein) Conductimetric assay [a]	Haemolytic activity (μg protein/ml required for 50% haemolysis of turkey red blood cells) [b]	Cytotoxic activity (μg protein/ml required for 40% killing of J774.2 macrophage cells) [a]
CyaA pro-toxin	169.53 ± 6.46	118.80 ± 7.87	not measured	non-cytotoxic
CyaA*	2.18 ± 0.60	1.84 ± 0.36	42.15 ± 0.42	58.50 ± 1.98
CyaA	453.49 ± 1.51	194.46 ± 54.60	30.83 ± 0.90	0.51 ± 0.06

[a] Results are the mean values ± standard deviation of 2 determinations

[b] Results are the mean values ± SEM of 3 determinations

3.6 ENDOTOXIN REMOVAL FROM CyaA PREPARATIONS

LPS is the term used to refer specifically to the endotoxin component of the outer membrane in Gram-negative bacteria. Outer membrane LPS is a complex molecule consisting of 3 covalently linked regions. Lipid A is a glycolipid that is largely responsible for the endotoxic activity of LPS and for the ability of LPS to act as a polyclonal activator. Core oligosaccharide is a complex oligosaccharide usually linked to lipid A via 2-keto-3-deoxyoctonate (KDO) and the O-specific chain is the immunodominant part of the LPS molecule (Section 1.4.8).

The *Limulus* amoebocyte lysate test (LAL test) is commonly used for the detection and quantification of LPS. The test depends on the ability of minute amounts of LPS to cause gelation of a lysate of the amoebocytes from the horse-shoe crab, *Limulus polyphemus*. In preliminary studies, all CyaA preparations were found to be highly contaminated with endotoxin as measured by the LAL test (Table 3.6), a fact that represented potentially serious problems for any immunological studies, due to the known immunostumulatory properties of LPS. Different approaches for endotoxin removal were considered.

3.6.1 Affi-Prep® polymyxin matrix as a method of endotoxin removal

The Affi-Prep polymyxin support (Bio-Rad) consists of polymeric macroporous beads with polymyxin B covalently attached. The main application of this support is the removal of endotoxin molecules from biological solutions by affinity chromatography. CyaA protein solutions in PBS were allowed to permeate through the affinity column (section 2.6.1) but, on final analysis by the LAL test, the samples

showed no significamt reduction in endotoxin levels, only a slight reduction in protein concentration.

3.6.2 Size exclusion chromatography as a method of endotoxin removal

LPS molecules consist of monomers with a molecular mass ranging between 10 and 20 kDa, but these normally form very stable aggregates, either in the form of micelles, with a molecular mass of between 300 and 1000 kDa, or vesicles which can have a mass greater than 1000kDa. Aggregation is mediated by non-polar interactions between the alkyl chains as well as by bridges among negatively-charged phosphate groups and bivalent cations. Size exclusion chromatography (section 2.6.3) was thought to be good candidate to remove the larger LPS aggregates, but no reduction in endotoxin content was obtained, indicating that perhaps the size of the aggregates was similar in size to CyaA or that the LPS was tightly bound to CyaA and could not be separated by size exclusion.

3.6.3 Addition of detergent as a method of endotoxin removal

Above the critical micelle concentration (CMC) of detergents, endotoxins can be accommodated in the micelle structure. In this way, detergents are able to aid in the disruption of larger LPS structures, vesicles and micelles, and also break protein-LPS links formed via calcium bridges or hydrophobic interactions. Triton X-114 was used to investigate this property (section 2.6.2). Triton X-114 is miscible with water and, at a temperature of 22°C, known as the cloud point, the detergent micelles aggregate. At a temperature of 4°C, the detergent is able to separate the protein and

LPS molecules and, at temperatures above the cloud point, the solution separates into aqueous and detergent-enriched phases (Aida and Pabst, 1990). CyaA was expected to be present in the aqueous phase but no phase separation was observed and as a result of this, further endotoxin analysis could not be completed, as CyaA was contaminated with Triton X-114, which in turn inhibited the LAL test.

3.6.3 END-X® B15 resin as a method of endotoxin removal

END-X® B15 is an immobilised affinity resin for the removal of Gram-negative bacterial endotoxins. The affinity ligand is the endotoxin-neutralising protein from *L. polyphemus* (one of the proteins present in the cascade reaction in the LAL test involved in gelation) and coated silica microspheres are the support. END-X® B15 resin was tested as a method of endotoxin removal (section 2.6.4) but was unable to reduce the endotoxin content of the CyaA preparations, presumably due to the high initial concentrations of endotoxin present in the samples, even after CyaA had been diluted, thereby leading to saturation of the resin. Unfortunatley the resin is supplied pre-packed in a micro-centrifuge tube and therefore no additional resin can be added to prevent saturation, and larger tubes with more resin were not available at the time of the investigation.

3.6.4 Summary of endotoxin removal methods

Purified CyaA pro-toxin, CyaA* and CyaA were all found to contain high levels of endotoxin which in turn would hamper any immunological studies performed in the future. Methods were performed to try and reduce the endotoxin levels in each preparation but these all proved unsuccessful, suggesting that the endotoxin was tightly bound to CyaA or was forming micelles of a similar size, thereby hindering any size exclusion chromatography. The endotoxin content of each sample preparation and the amount present in a 15μg immunisation dose, is given in Table 3.6. To overcome the problem presented by endotoxin contamination, an *E. coli* endotoxin standard (NIBSC, 94/580) was made available for immunological studies, along with a second control of boiled CyaA. Boiling the CyaA protein would denature the toxin but not inactivate the LPS, therefore acting as a suitable control. A value of 350 iu/ml of LPS was chosen for the control as this was an approximate average value between the endotoxin levels calculated for CyaA and CyaA* preparations and was used subsequently in vaccination experiments.

Table 3.6: Endotoxin content of different CyaA preparations

Preparation	**Endotoxin content per ml**	**Endotoxin content per 15μg protein**
CyaA pro-toxin	90,000 iu/ml	3857 iu
CyaA*	18,000 iu/ml	450 iu
CyaA	9,000 iu/ml	208 iu

3.7 IMMUNOLOGICAL PROPERTIES OF CyaA

The immunological properties of CyaA were investigated by firstly studying the effects that were generated when mice were vaccinated with different purified CyaA preparations. Specific IgG antibody levels at different time points following CyaA immunisation, protective abilities of the CyaA preparations in mice following aerosol challenge with *B. pertussis* and cytokine expression were measured.

3.7.1 Mouse IgG antibody responses to CyaA preparations

ELISAs were used to determine the antibody response from mice immunised with 15μg doses of protein. The mice were divided into two sets of four groups of five mice and each group received 0.5ml i.p of PBS, CyaA pro-toxin, CyaA* or CyaA preparations. Tail bleeds were taken at two, four and seven weeks post immunisation and a terminal bleed was done at 9 weeks from the first set of mice. The second set were boosted with an additional 15μg dose at seven weeks post immunisation and tail bleeds taken at 9 and 11 weeks before being terminally bled at 14 weeks post immunisation. Anti-CyaA IgG levels were determined by ELISA at each time point (section 2.14.1). The geometric mean titre of antibody to each antigen was calculated as described in Section 2.14.1

An example of the graph used to calculate the geometric mean titre is given in Figure 3.7.1.1 and the raw data in Appendix H.1. Substituting 0.5 for the y value in the trend lines shown in Figure 3.7.1.1 gives 3 values that can be used in the equation to obtain the geometric mean of the antibody titres. Figure 3.7.1.2 shows the geometric mean of the antibody titre at OD_{450nm} 0.5 and it can be seen that there is a difference in the pattern of results obtained with each CyaA preparation. CyaA pro-toxin

appears to generate an early anti-CyaA IgG response, apparent from the very first time point, whereas CyaA* and CyaA generate a more gradual increase in the antibody levels. There is no obvious reason as to why this should occur, as although the response being measured is to the active, acylated CyaA toxin and not specifically to the non-acylated pro-toxin cross-reaction should occur with the polyclonal anti-CyaA sera. One possibility could be related to the toxicity exhibited by the acylated forms of CyaA, which might, interfere with antigen presentation, by macrophages or dendritic cells. Alternatively, it could be that the non-acylated, non-active toxin is more immunogenic than the acylated form, although by 9 weeks the antibody levels were lower for the pro-toxin than acylated forms

The effect of boosting is shown in Figure 3.7.1.3. A significant increase in anti-CyaA IgG production was seen two weeks after a further dose of 15μg of each protein was given, indicating a good memory response. This increased up to 4 weeks post-boost with pro-CyaA and CyaA* but only up to 2 weeks post-boost with CyaA, before the levels started to decrease. The highest anti-CyaA IgG responses were generated by the 2-dose immunisations with CyaA*, followed by CyaA pro-toxin, and the poorest responses were generated with CyaA itself.

Figure 3.7.1.1

Example of calculation of anti-CyaA IgG antibody production in response to immunisation with CyaA preparations

Female NIH mice were vaccinated intraperitoneally with 15µg of pro-CyaA (prepared from pGW44) in PBS as described in Section 3.7.1. Anti-CyaA IgG levels were determined by ELISA (section 2.13.1) using CyaA (prepared from pGW44,54) as the coating antigen at a concentration of 2µg/ml. The absorbance at 450nm was recorded and the results for each dilution of the sera plotted using a log scale to enable the drawing of a trend line. The results of the ELISA for mice immunised with CyaA pro-toxin at a time point of 7 weeks is shown. The trend lines for three different mice from one group were used to interpolate the graph at a cut off point of OD_{450nm} 0.5 and obtain the geometric mean

$y = -0.852x + 3.6047$

$\log_3$ reciprocol dilution (x) = 3.644

Geometric mean (GM) = antilog (1/n X ∑log3 reciprical dilution)

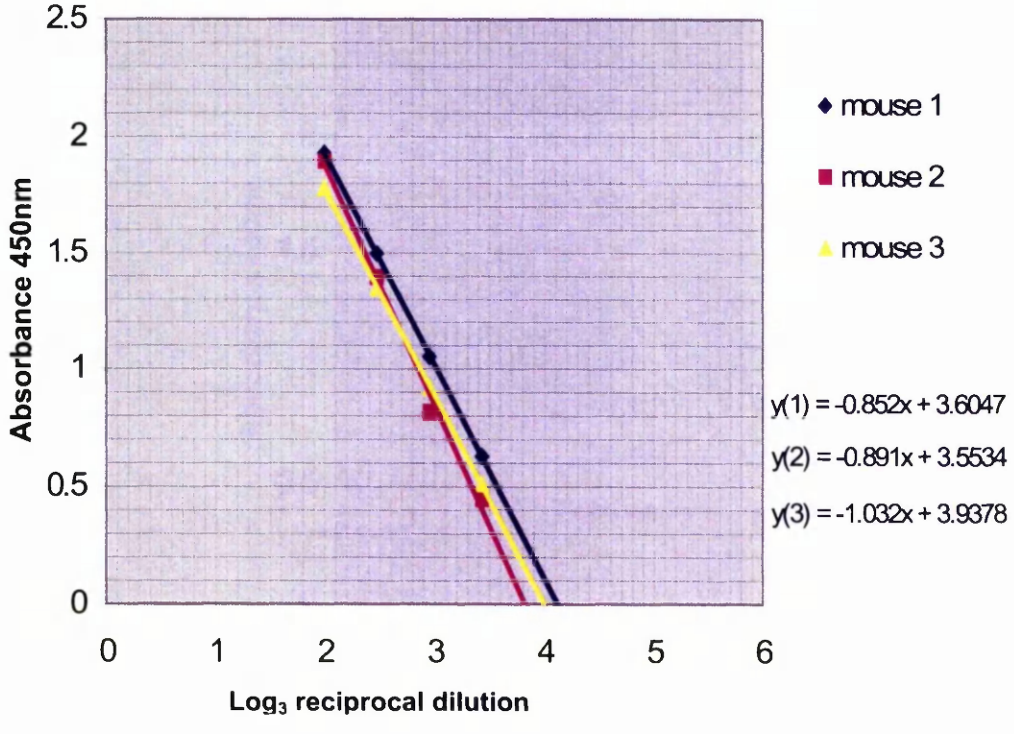


Figure 3.7.1.2

Time course showing the geometric mean of anti-CyaA IgG antibody titres

Anti-CyaA IgG levels for mice immunised with a single dose of 15µg of either CyaA pro-toxin, non-enzymic CyaA* and CyaA were determined at different time points over 9 weeks. Tail bleeds were taken at each time point to perform ELISAs using CyaA as a coating antigen (section 2.13.1). The geometric mean of the antibody titre for each CyaA preparation at different time points was calculated from the trend lines for each serum OD_{450nm} 0.5, as described in Figure 3.7.1.1.

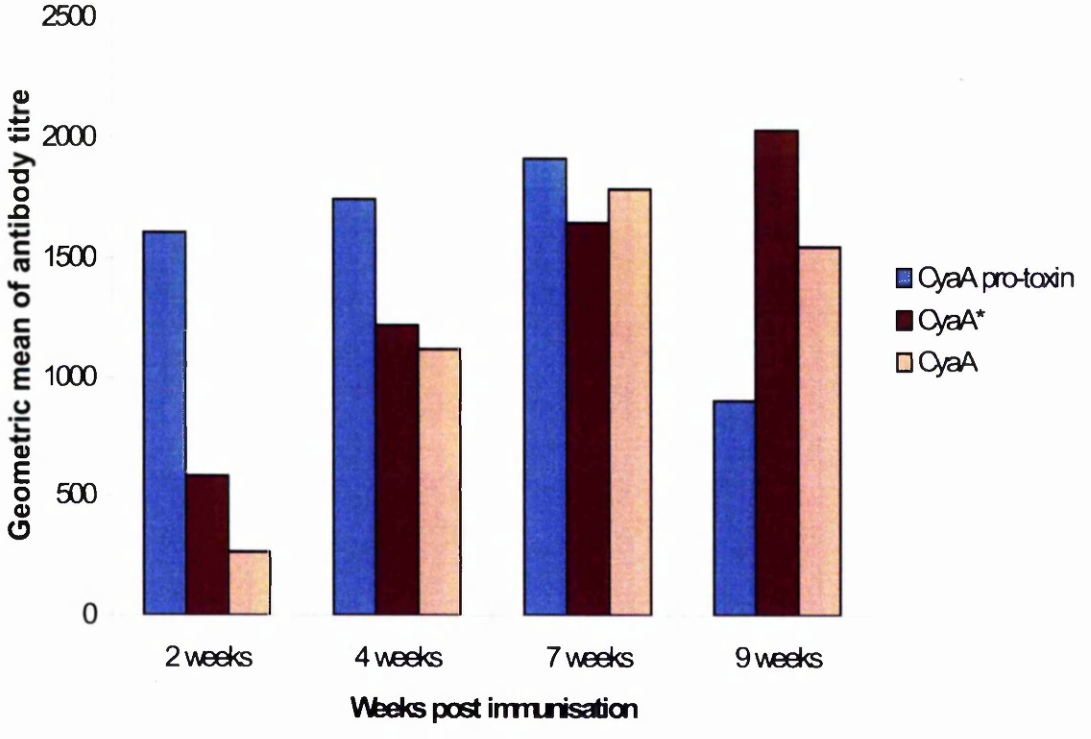
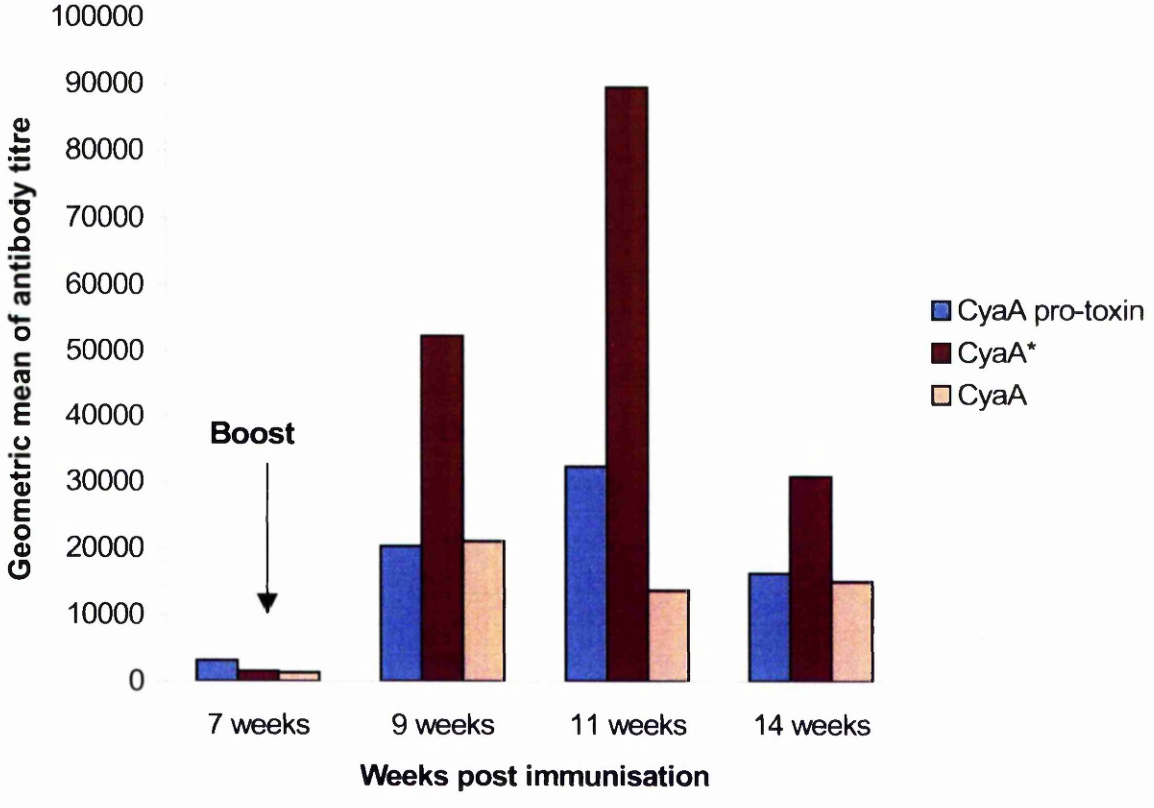


Figure 3.7.1.3

Effect of boosting on the anti-CyaA IgG antibody titres in response to immunisation with different CyaA preparations

The geometric mean titres were calculated as for Figure 3.7.1.1. Mice received a second immunisation of 15µg of protein at 7 weeks post immunisation and tail bleeds were taken at 9, 11 and 14 weeks.



3.7.1.1 Neutralisation effect of anti-CyaA sera on the cytotoxicity of CyaA

Sera obtained from tail bleeds at 7 weeks post immunisation from mice immunised with a single dose of 15μg of either CyaA* or CyaA preparations (section 3.7.1), were used to investigate any possible neutralisation of the cytotoxicity of CyaA on macrophage cells *in vitro*. The serum samples were pooled for each group of mice and then diluted in RPMI medium in doubling dilutions. A volume of 50μl of diluted sera, starting at 1:2 dilution, was added to a 50μl volume of CyaA toxin at 65μg/ml and incubated for 1 hour at 37°C prior to the addition to J774.2 macrophage cell suspension in 96-well plates. The cytotoxicity was evaluated by MTT assay (section 2.10.2). Figure 3.7.1.1.1 shows a photograph of the tissue culture plate at the end of the reaction and Appendix H.2 details the raw data. Rows A and B contain CyaA toxin alone, starting at an initial dilution of 1 in 20 in RPMI medium, equivalent to a concentration of 65μg/ml in the first well, and followed by a 2-fold dilution across rows A and B in duplicate for each dilution. The yellow colour across row A and at the start of row B is indicative of the cytotoxic nature of CyaA, which gradually decreases as the toxin becomes more dilute. Rows C and D contain the doubling dilutions of sera, taken at 7 weeks after a single dose, from PBS-immunised mice mixed with CyaA toxin at 65μg/ml in all wells. The continuous yellow colour throughout the wells confirmed that sera from PBS-immunised mice had no capacity to neutralise the cytotoxic effects of CyaA. Rows E and F contain the doubling dilutions of sera from CyaA*-immunised mice in duplicate with CyaA toxin at 65μg/ml in all wells. The first 4 wells of row E gave a purple colour, indicative of a non-cytotoxic response, the same as shown by the control wells that contained only J774.2 macrophages (H11 and H12). Row H, wells 9 and 10 were

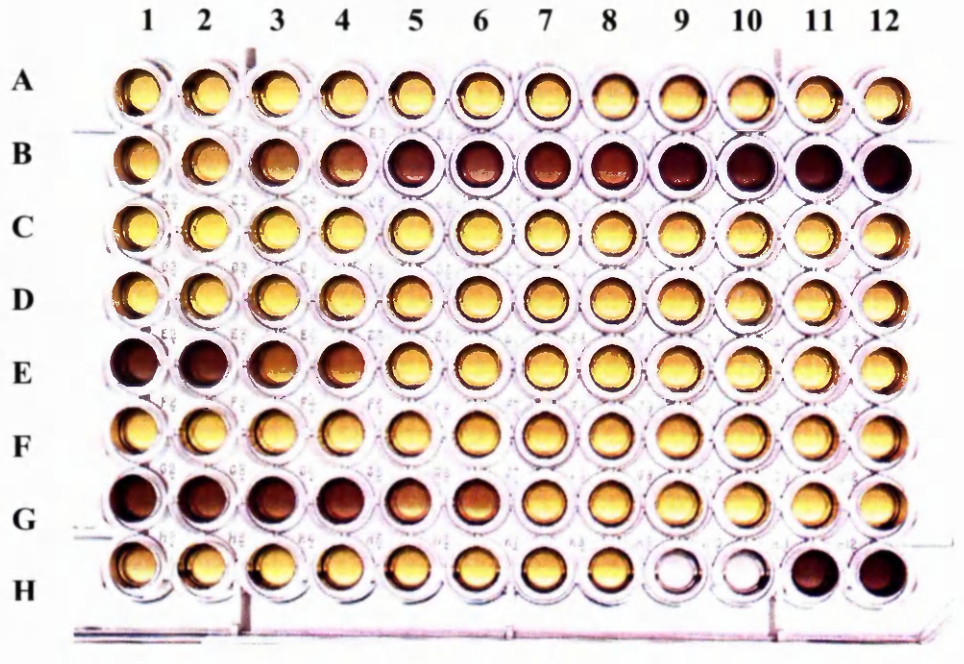
left blank to enable the control wells to be clearly identified. Rows G and H (except H9-12) contain the doubling dilutions of sera from CyaA-immunised mice in duplicate with CyaA toxin at 65µg/ml. The results are similar to those for CyaA* except that the 6 wells of Row G are purple, indicating that the sera from CyaA-vaccinated mice had a slightly stronger neutralising capacity. These results prove that antibodies generated from mice immunised with either CyaA or CyaA* were able to neutralise the cytotoxicity of CyaA on J774.2 macrophage cells *in vitro*. It was interesting to note that the antibodies generated to CyaA showed more neutralisation activity (down to a dilution factor of 1:8) than those generated to CyaA* (down to a dilution factor of 1:4), which is in agreement with the data in Figure 3.7.1.2 where at 7 weeks post-immunisation anti-CyaA IgG levels were highest in mice vaccinated with a single dose of CyaA compared to those given CyaA*.

Figure 3.7.1.4 **Visualisation of the neutralisation effect of anti-CyaA sera on the cytotoxicity of CyaA**

The MTT dye reduction assay was used to demonstrate the cytotoxic activity of CyaA on J774.2 mouse macrophage cells (Section 2.11.9). The neutralisation effect of anti-CyaA serum was demonstrated by the addition of immune sera to cells containing the cytotoxic CyaA.

Rows A + B: CyaA from *E. coli* BL21/DE3 (pGW44,54)
Rows C + D: CyaA plus serum from PBS-immunised mice
Rows E + F: CyaA plus serum from CyaA*-immunised mice
Rows G + H: CyaA plus serum from CyaA-immunised mice (except H9-12)
H9 + H10: CyaA only at 65µg/ml
H11 + H12: RPMI medium only

All wells contain J774.2 mouse macrophage cells at 5 X 10^5 cells/ml. Rows C-H contain CyaA at 65µg/ml plus the relevant serum starting at a neat concentration followed by doubling dilutions in duplicate.



3.7.2 Mouse protection with CyaA preparations

The different CyaA preparations were examined for their ability to protect mice against aerosol challenge with *B. pertussis* 18.323. Previous work carried out by Hormozi *et al.* (1999) showed that the active, acylated CyaA toxin was protective following intranasal challenge with *B. pertussis*, as indicated by the low lung weight and low cfu present in the lungs. The non-acylated CyaA protoxin was unable to protect and mice immunised with the preparation showed large, inflamed lungs and high bacterial counts after challenge. For this reason, only acylated CyaA toxins were examined in this study. The mice were immunised (ip) with two 25μg doses at a 4 week interval, with either CyaA* or CyaA, and subjected to a sublethal challenge from a suspension containing approximately 10^8 cfu/ml *B. pertussis* 18.323 two weeks after the second immunisation (section 2.14.4 and 2.14.5). Five mice from the PBS immunised groups were sacrificed on day 0 post-challenge and the remainder from all groups were sacrificed at day 7 post-challenge and the lungs and trachea removed together, to estimate *B. pertussis* colonisation (section 2.14.4). Figure 3.7.2.1 shows the results of the combined lung and tracheal counts. From this it can be shown that the mean counts on day 0 for the PBS-immunised group were 5.12×10^4, rising to 7.88×10^6 on day 7 post-challenge. The day 7 post-challenge counts for CyaA- and CyaA*-immunised mice were 1.05×10^5 and 3.15×10^4 respectively, indicating between a 1.5- and 2 log difference in colony numbers when compared with PBS-control mice. Although it appeared that CyaA* afforded a slightly better protection against colonisation than CyaA, there was no statistical significance at either the 5% or 10% student t-test level between CyaA- or CyaA*-immunised groups.

A further experiment using the same conditions was conducted to further confirm the protective effect of CyaA preparations by using a different *B. pertussis* challenge strain. *B. pertussis* W28 is a less virulent strain than *B. pertussis* 18.323 and this is reflected in the results shown in Figure 3.7.2.2. In this experiment only the enzymically inactive, acylated CyaA* preparation was used and again the same pattern of an approximate 1-2 log difference in cfu is observed after vaccination.

Figure 3.7.2.1

Mouse protection with CyaA preparations against aerosol challenge with *B. pertussis* 18.323

The protective activities of the two acylated forms of CyaA were examined in mice after an aerosol challenge with *B. pertussis* 18.323. Two groups of 5 mice received either 25µg (ip) of CyaA or CyaA* and 1 group of 10 mice received PBS as a control. At 4 weeks post immunisation, the groups all received a second injection and 2 weeks later were subjected to an aerosol challenge of *B. pertussis* 18.323 (section 2.11.5). The lungs and trachea were removed from 5 mice of the PBS group on the day of challenge and at day 7 post-challenge the remainder of the groups were sampled. Viable counts were performed on the combined lung and trachea homogenates and the mean log colony forming units (cfu) plotted at day 7 post-challenge for each group.

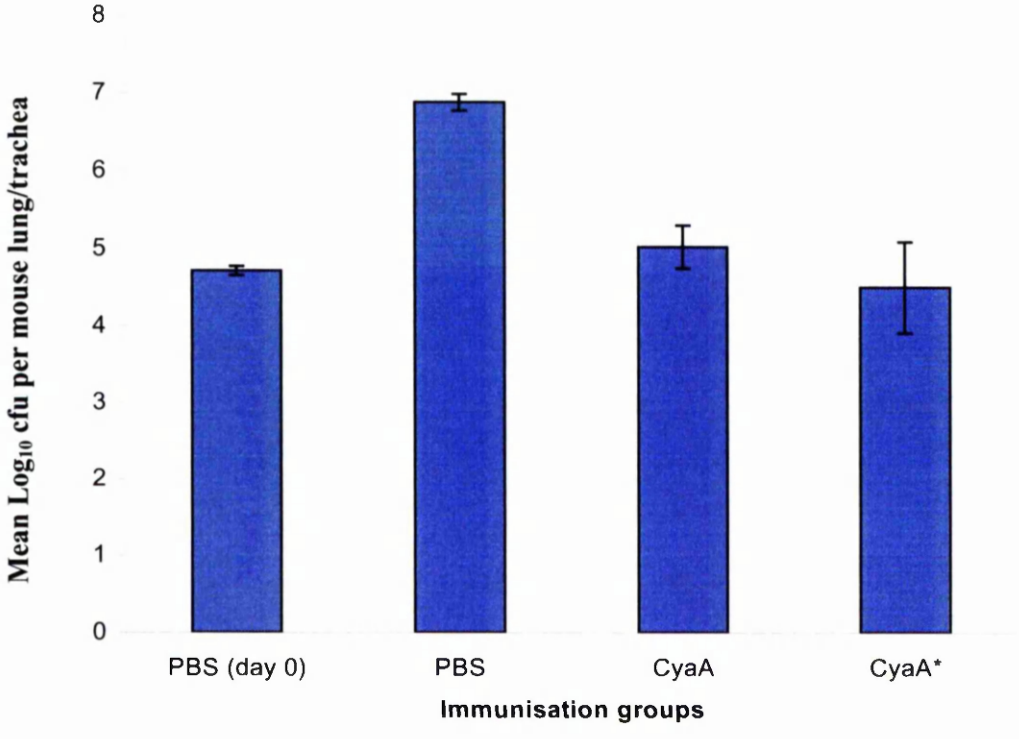
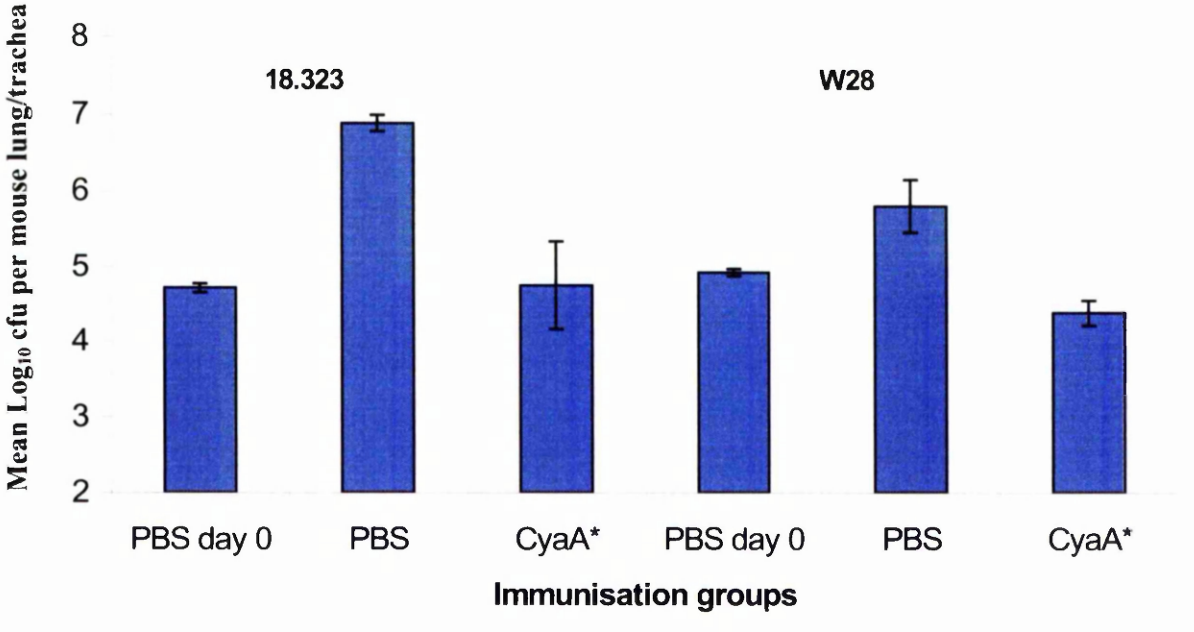


Figure 3.7.2.2

Mouse protection with CyaA preparations following aerosol challenge with *B. pertussis* 18.323 or *B. pertussis* W28

Two groups of 10 mice were immunised with either PBS or a single dose of 25µg CyaA* and then further divided to give 4 groups. At 4 weeks post-immunisation all groups received a second injection. Two weeks later one PBS-immunised group and one CyaA*-immunised group, were subjected to an aerosol challenge of *B. pertussis* 18.323. The other two groups were subjected to an aerosol challenge of *B. pertussis* W28. The mean lung and trachea log cfu was plotted at day 0 post-challenge for the PBS-immunised mice and at day 7 post-challenge for the remaining groups to investigate the difference in colonisation between the two *B. pertussis* strains.



3.7.3 Production of nitric oxide by macrophages from mice immunised with CyaA preparations

Nitric oxide (NO) is known to be an important regulator of the immune system and inflammatory cells including monocytes produce NO following incubation with bacteria and cytokines. Recently Xing *et al.* (1998) demonstrated that NO production served as a marker of macrophage activation in mice immunised with WCV. NO production from peritoneal macrophages obtained from groups of 5 mice immunised with a single 15μg dose of different CyaA preparations, was studied at day 14 post-immunisation (section 2.11.8, 2.11.9 and 2.11.10). In this experiment, control mice were immunised with either 15μg of boiled CyaA or 175 iu of purified LPS from *E. coli*. From the results shown in Figure 3.7.3 and Appendix H.3, it can be seen that when *B. pertussis* heat-killed cells were used as the *in vitro* re-stimulant the levels of NO production did increase even from control macrophages, although it is clear that immunisation with CyaA did not result in any significantly greater NO production when compared with the control groups of either PBS-or LPS-immunised mice.

3.7.4 Cytokine mRNA expression in mice immunised with CyaA preparations

Cytokines are important in modulating host responses to invasion by pathogens and therefore the detection and analysis of cytokine production can provide a greater understanding of the immune responses. RT-PCR technology is based upon the amplification of cDNA derived from mRNA extracted from cells of immunised mice. Cytokine-specific primers enable the amplification of cDNA samples to levels that can be visualised by agarose gel electrophoresis. Spleen cell cultures were

obtained from mice immunised with CyaA preparations and also control groups of mice immunised with boiled CyaA and *E. coli* LPS. They were re-stimulated *in vitro* with either *B. pertussis* heat-killed cells or CyaA* (section 2.11.9). Following RNA extraction and first strand cDNA synthesis (section 2.12), PCR amplification was performed using primers specific for IFNγ, IL-2 and IL-4 as well as β-actin. The intensities of the cytokine-specific amplicons were compared with the level of β-actin, a housekeeping gene that is known to have a constant level of expression (Walker, 1998), thus normalising for differences occurring between different samples and preparations.

Figure 3.7.4.1 shows an example of an agarose gel after PCR amplification of β-actin, IFNγ, IL-2 and IL-4 from different spleen cell preparations stimulated *in vitro* with *B. pertussis* heat-killed cells. It can be seen that the amplicon intensity for β-actin varied between 3 separate spleen cell mRNA preparations (Figure 3.7.4.1, lanes 2, 6 and 10). Digital analysis using Kodak 1D gel analysis software produces a numerical value of the PCR product intensity as a percent of the β-actin intensity (Appendix H.4).

Figure 3.7.4.2 displays a difference in cytokine response of spleen cells from mice immunised with CyaA* or CyaA. CyaA* leads to a mixed Th1/Th2 response, as indicated by the high levels of all 3 cytokines, whereas immunisation with CyaA shows a Th1 bias, as indicated by higher IFNγ and IL-2 levels and low IL-4 production. Surprisingly, stimulation of spleen cells with hkc from LPS-immunised mice produced very little cytokine mRNA response, although it should be noted that the LPS used in immunisations was from *E. coli* and the spleen cells were stimulated with *B. pertussis* heat killed cells. The concentrations of LPS used for immunisation, was similar to that present in CyaA preparations. The results could indicate that the

cytokine profile found in the CyaA and CyaA* groups were not caused by the LPS contamination of these preparations.

Figure 3.7.4.3 demonstrates how stimulation of spleen cells *in vitro* with CyaA* produced almost no increase in cytokine production from mice immunised with CyaA* but much higher levels of all 3 cytokines from mice immunised with CyaA. No firm conclusions can be drawn from these results as the experiment was only performed once using a limited selection of cytokines. However it does lead to the suggestion of possible differences between the immune response generated when using either the enzymically-inactive or enzymically-active forms of CyaA.

Figure 3.7.3

Production of nitric oxide by peritoneal macrophages from mice immunised with CyaA

Nitric oxide (NO) production was measured from macrophages obtained by peritoneal lavage from mice at 14 days post-immunisation with a single dose of 15µg protein of different preparations of CyaA. Cells were re-stimulated *in vitro* with either a suspension of *B. pertussis* heat-killed cells (hkc) at a concentration of 2 x 10^6 cells/ml, or the different CyaA preparations at 2µg/ml. After an incubation period of 24 hours, NO production for each immunisation group was determined (section 2.11.10). The results shown are the mean and SEM of three determinations.

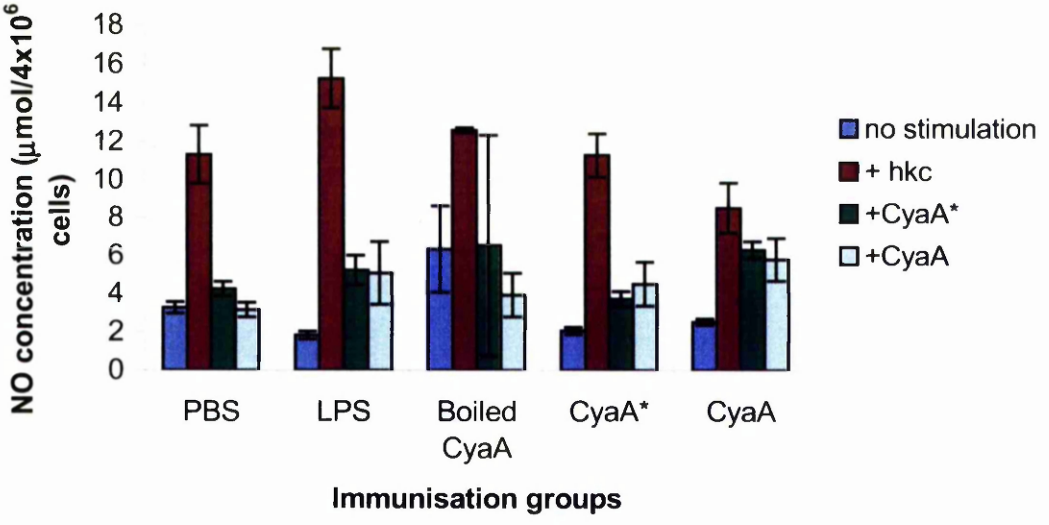


Figure 3.7.4.1

Example of agarose gel electrophoresis of PCR-amplified cytokine mRNA

Cytokine mRNA detection using RT-PCR was carried out on spleen cells taken at 2 weeks post-immunisation from immunised mice after *in vitro* stimulation with *B. pertussis* heat-killed cells. cDNA was amplified by PCR using cytokine specific primers which enabled visualisation of amplimers by agarose gel electrophoresis (section 2.12. 2.3).

Lanes 1 + 14	100 base pair ladder
Lanes 2 + 6 + 10	β-actin expression
Lanes 3 + 7 + 11	IFNγ expression
Lanes 4 + 8 + 12	IL-2 expression
Lanes 5 + 9 + 13	IL-4 expression
Lanes 2, 3, 4, 5	Spleen cells from PBS-immunised mice
Lanes 6, 7, 8, 9	Spleen cells from LPS-immunised mice
Lanes 10, 11, 12, 13	Spleen cells from CyaA*-immunised mice

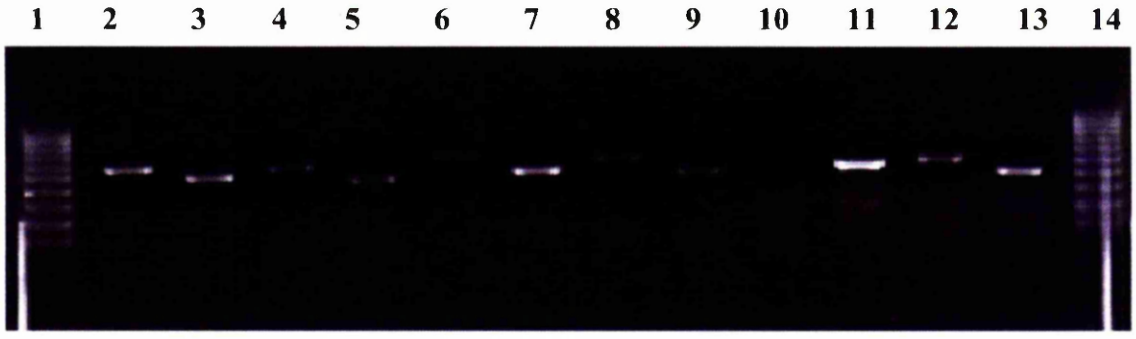


Figure 3.7.4.2

Cytokine expression in spleen cells derived from mice immunised with different CyaA preparations and stimulated *in vitro* with *B. pertussis* heat-killed cells.

Digital analysis of the RT-PCR products was carried out using Kodak 1D gel analysis software. The intensity of each band representing a different cytokine mRNA was calculated by the software package and given as a numerical value. The levels of cytokine mRNA expression from each immunised group, when spleen cells were stimulated *in vitro* with heat-killed cells, were expressed as a percentage of the intensity of β-actin band.

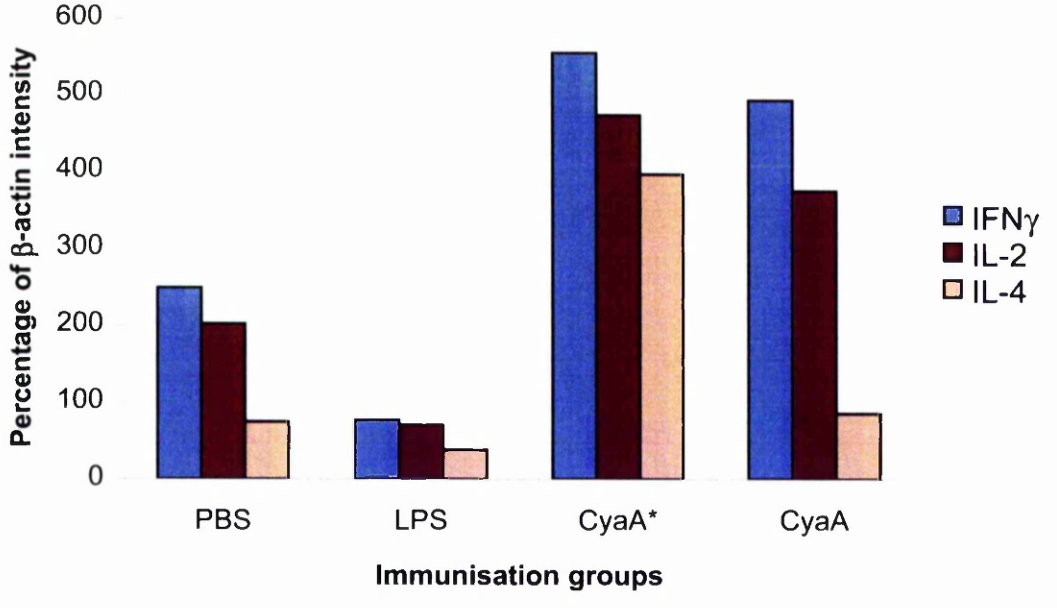
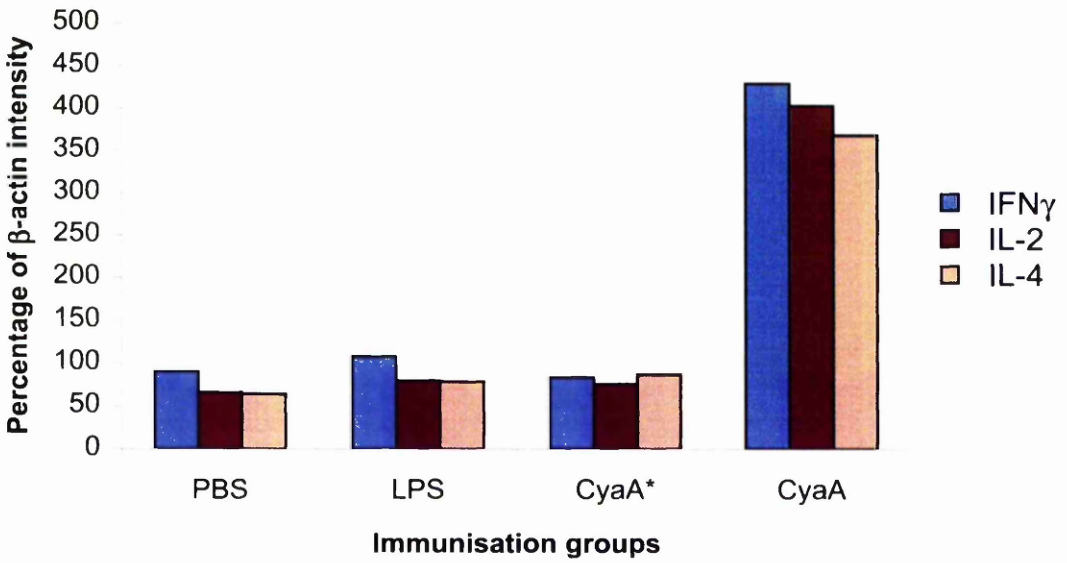


Figure 3.7.4.3

Cytokine expression in spleen cells derived from mice immunised with different CyaA preparations and stimulated *in vitro* with *CyaA*.*

The levels of cytokine mRNA expression from each immunised group when spleen cells were stimulated *in vitro* with CyaA* at a concentration of 2µg/ml were expressed as a percentage of the intensity of the β-actin band.



3.8 IMMUNOLGICAL PROPERTIES OF CyaA WHEN ADMINISTERED IN COMBINATION WITH PURIFIED PERTUSSIS ANTIGENS

Hormozi *et al.* (1999) had reported that CyaA acted as an adjuvant to boost the IgG response to ovalbumin (OA) when CyaA was co-administered with ova. In order to investigate whether CyaA could act as an adjuvant for protective antigens commonly included in ACV's, the different forms of CyaA were co-administered either with a combination of native PT, FHA and P.69 together or individually with detoxified PT, FHA and P.69. To assess any adjuvant effect, IgG antibody levels, protective capacities and cytokine expression were measured.

3.8.1 Mouse IgG antibody response to CyaA when co-administered with native PT, FHA and P.69 antigen mixture

Groups of 5 mice were immunised with a single 15μg ip dose of CyaA per mouse or 15μg of CyaA plus a mixture of native PT, FHA and P.69 at 120ng, 25μg or 10μg per dose respectively. Terminal bleeds were taken at 5 weeks post-immunisation and anti-CyaA IgG levels measured by ELISA (section 2.13.1). An example of the geometric mean calculation, by interpolating at OD_{450nm} 0.5, is shown in Figure 3.8.1.1. A difference in the anti-CyaA IgG levels was observed depending on whether the mice were immunised with CyaA* or the enzymically-active CyaA preparation (Figure 3.8.1.2). Mice receiving CyaA* plus the acellular components produced a lower level of anti-CyaA IgG antibody compared to mice immunised with CyaA* alone. In contrast, the opposite was found when CyaA was used, with an increase in IgG antibody levels with CyaA co-administered with native PT, FHA and P.69 compared to CyaA alone. It is difficult to draw conclusions from these

results as previous experiments with CyaA and CyaA* administered alone had also produced slight differences in anti-CyaA IgG levels at 4 weeks post-immunisation (Figure 3.7.1.2). In that case, however, immunisation with CyaA* alone produced only a slightly higher level of anti-CyaA IgG antibody compared to immunisation with CyaA alone.

3.8.2 Mouse IgG antibody response to native PT, FHA and P.69 when co-administered with CyaA

ELISAs were used to determine anti-PT, anti-FHA and anti-P.69 IgG levels in mice immunised with CyaA together with PT, FHA and P.69 (section 3.8.1). Again the geometric mean was calculated by interpolating at OD450nm 0.5. Figure 3.8.1.2 shows the differences in IgG antibody levels depending on the received immunisation. When CyaA* was co-administered with PT, FHA and P.69, higher titres of anti-PT, anti-FHA and anti-P.69 IgG were detected, compared to those obtained with PT, FHA and P.69 alone. However when CyaA was added to these components, only the anti-P.69 IgG titre showed a modest increase, whereas those for anti-PT and anti-FHA were lower.

Figure 3.8.1.1

Example of calculation of anti-CyaA IgG antibody titres after immunisation with CyaA

Anti-CyaA IgG levels were determined by ELISA (section 2.13.1) using CyaA as the coating antigen at a concentration of 2µg/ml. The absorbance at 450nm was recorded and the results for each dilution of each serum plotted on a log scale to obtain a trend line. The figure shows the results of the ELISA for sera taken from mice immunised with CyaA* alone at 4 weeks post immunisation. The trend line for each serum was used to interpolate the graph at a cut off point of OD_{450nm} 0.5.

$y = -1.0064x + 4.0036$

$\log_3$ reciprocol dilution (x) = 3.48

Geometric mean (GM) = antilog (1/n X ∑log3 recipricol dilution)

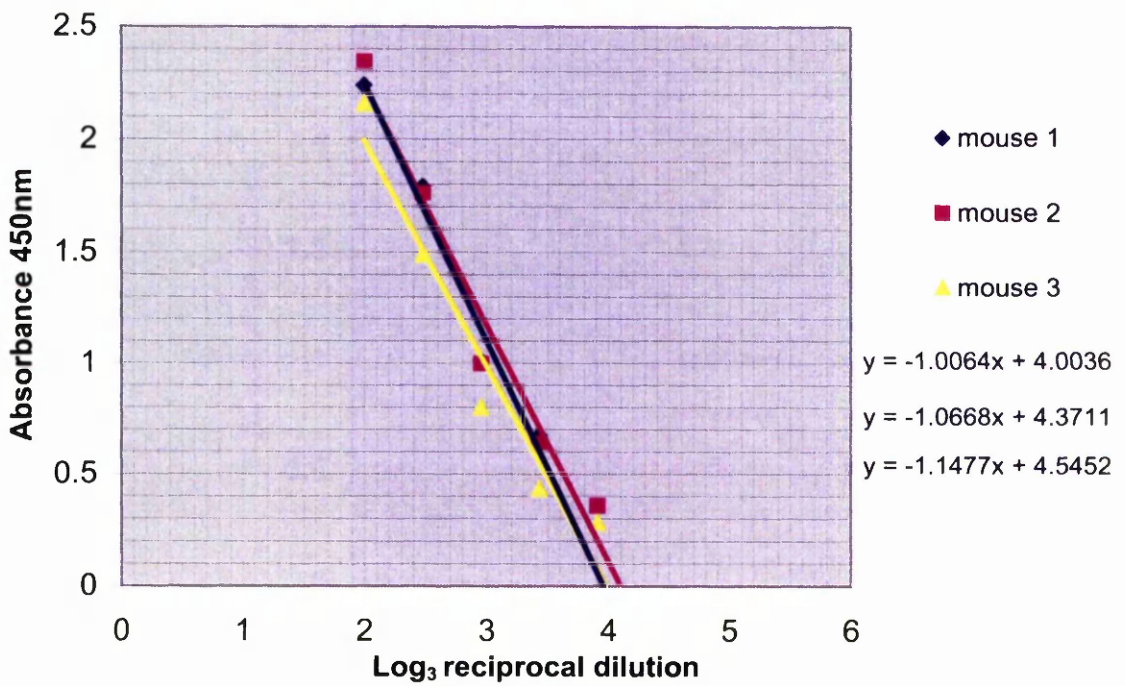


Figure 3.8.1.2

Anti-CyaA IgG antibody production in response to immunisation with CyaA in combination with native PT, FHA and P.69 antigens

Anti-CyaA IgG levels for groups of mice immunised with a single dose of 15µg of non-enzymic CyaA* or CyaA alone, or together with a combination of native PT, FHA and P.69 antigens at 120ng, 25 µg or 10µg/dose (PT, FHA and P.69 respectively) were determined. Terminal bleeds were taken at 4 weeks post-immunisation to perform ELISAs with CyaA as a coating antigen (section 2.13.1). The geometric mean of the antibody titre for each immunisation group was calculated from the trend lines for each serum dilution as described in Figure 3.8.1.1.

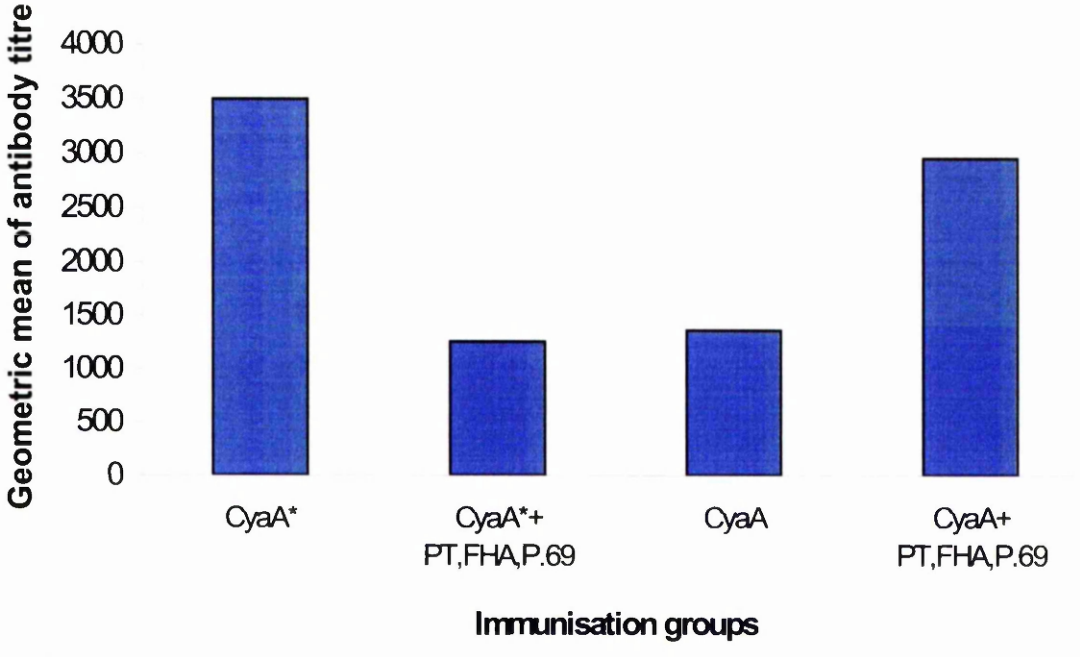
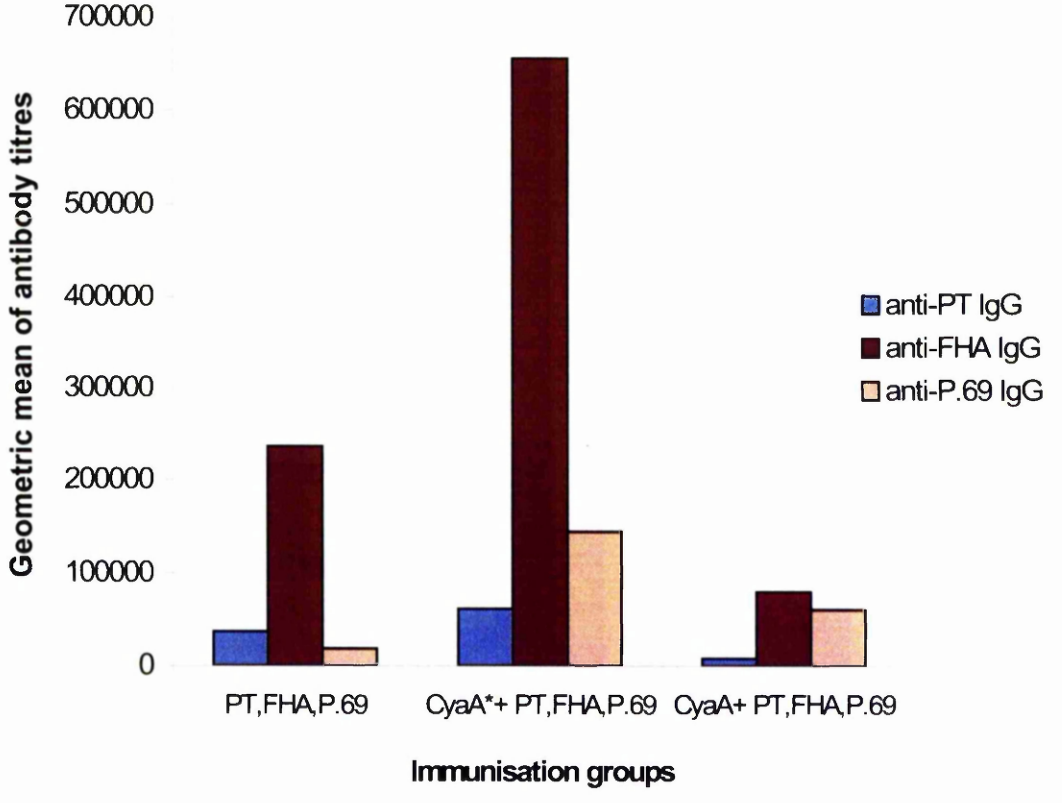


Figure 3.8.1.3

anti-PT, anti-FHA and anti-P.69 IgG antibody titres after immunisation with native PT, FHA and P.69 antigens with or without CyaA preparations.

Anti-PT, anti-FHA and anti-P.69 IgG levels were determined for the same groups of vaccinated mice used for the results of Figure 3.8.1.2. The relevant coating antigen of either PT, FHA or P.69 was used to coat ELISA plates at a concentration of 2 µg/ml (section 2.14.1). The geometric mean of the antibody titre for each immunisation group was calculated as described in Figure 3.8.1.1.



3.8.3 Mouse IgG antibody response to detoxified PT (PTd), FHA and P.69 antigens when co-administered with CyaA*

To investigate the effect CyaA* might have on the antibody response to each co-administered antigen, mice were immunised ip with detoxified preparations of PT (PTd), FHA and P.69 at 10μg per dose either on their own or in various combinations with each other, plus or without 15μg of CyaA* (section 2.14.3). Terminal bleeds were taken at 5 weeks post-immunisation and anti-PT, FHA and P.69 IgG and IgG_1 levels determined by ELISA. An internal reference standard (97/642, NIBSC) (Gaines Das *et al.*, 2001) containing known levels of anti-PT, FHA and P.69 was used on each ELISA plate to calculate the units of each antibody in the different samples. Again the absorbance at 450nm was plotted for each dilution of each mouse and reference serum and the geometric mean for each group was calculated by interpolating at OD_{450nm} 0.5. By substituting this value into the formula detailed on Figure 3.8.3.1, the IgG antibody levels for each antigen in the different samples was determined. As 97/642 has only been signed unitage to total IgG and not to antibody subclasses, only the geometric mean of the IgG_1 antibody titres could be measured, with no specific value, as described in the legend to Figure 3.7.1.1.

When CyaA* was co-administered with PTd or with FHA, the anti-PT IgG levels were lower than with PTd alone. However when CyaA* was co-administered with PTd in combination with FHA and P.69, the anti-PT IgG levels were found to be boosted from a value of 719.53 EU/ml with PTd alone to 857.53 and 903.75 EU/ml with CyaA* in combination with PTd and P.69, and CyaA* in combination with PTd, FHA and P.69 respectively. When boiled CyaA* was co-administered with PTd, FHA and P.69 the levels were lower than immunisation with CyaA* in

combination with PTd, FHA and P.69, which indicated that the enhanced anti-PTd response found in the latter response was due to the adjuvant effect of CyaA* rather than the LPS present in the preparation (Figure 3.8.3.2; Appendix H.6.1). As 97/642 had not been asigned unitage to IgG_1 titres only the geometric mean of antibody levels could be measured and therefore a direct comparison between IgG and IgG_1 levels could not be made, although, however, the general pattern of results could be interpreted. Anti-PT IgG_1 levels followed a slightly different pattern (Figure 3.8.3.2.1), in that immunisation with PTd alone produced the lowest level at 2513.7, and immunisation with PTd plus any other antigen increased the geometric mean levels. The greatest level was obtained when PTd was co-administered with P.69 and CyaA*.

Anti-FHA IgG levels showed a marked increase when CyaA* was included in the immunisation mixture; levels increased from 1.31EU/ml to 1237.47EU/ml when CyaA* was included. However, when a combination of antigens including PTd was given with CyaA* the IgG levels were not as elevated compared to the FHA plus CyaA* results. Immunisation with FHA in combination with PTd and CyaA resulted in a value of 1237.47EU/ml and immunisation with FHA in combination with PTd, P.69 and CyaA* gave an anti-FHA IgG value of 800.38EU/ml, although both greater than with immunisation with FHA alone (Figure 3.8.3.3; Appendix H.6.2). Anti-FHA IgG_1 levels paralleled the anti-FHA IgG results (Figure 3.8.3.3.1). Immunisation with FHA alone produced an anti-FHA IgG_1 geometric mean antibody level of 32.1. Again, when CyaA* was included in the immunisation mixture, the antibody levels reached their maximum of 5819.5, and gradually decreased to a value of 1709.8 when PTd and P.69 plus CyaA* were included in the mixture (Figure 3.8.3.3.1; Appendix H.7.2).

When CyaA* was co-administered with P.69 or FHA or PTd, the anti-P.69 IgG levels were greater than immunisation with P.69 alone which produced a value of 1.89EU/ml. When CyaA* was included in the immunisation mixture without PTd or FHA the greatest antibody level was obtained, a value of 1224EU/ml. The anti-P.69 IgG levels for immunisation with P.69 in combination with either PTd and CyaA* or PTd, FHA and CyaA* were 798 and 476EU/ml respectively, both greater than immunisation with P.69 alone but lower than the value of 870EU/ml which was obtained when mice were immunised with boiled CyaA* in combination with PTd, FHA and P.69 (Figure 3.8.3.4; Appendix H.6.3). Anti-P.69 IgG_1 levels produced a slightly different patterm of results. The greatest level was obtained when CyaA* and PTd were included in the immunisation mixture with P.69, a value of 997.5, although all were greater than immunisation with P.69 alone, which gave a geometric mean antibody level of 1.05. When P.69 was co-administered with CyaA*, the IgG_1 levels increased to 975.5 and then decreased to 178.8 and 1.14 when co-administered with PTd, FHA and boiled CyaA*, PTd and FHA respectively (Figure 3.8.3.4.1; Appendix H.7.3).

Figure 3.8.3.1

Example of calculation of IgG antibody titres after immunisation with CyaA plus PTd, FHA and P.69 antigens.

Anti-PT IgG levels were measured in each mouse serum after immunisation with either detoxified PT alone at 10µg/dose, in combination with other acellular pertussis vaccine components at 10µg/dose or with CyaA* at 15µg/dose. A reference standard (97/642 NIBSC) was included on the ELISA plate to give the ELISA units/ml (EU/ml) for each antigen. The absorbance at 450nm was recorded and the results for each dilution of the sample sera and reference sera were plotted using a log scale to enable the drawing of a trend line. Calculating the geometric mean of the samples using a cut off point of OD_{450nm} 0.5, dividing by the reference sera value, and multiplying by the ELISA units for the relevant antigen enabled the geometric mean antibody levels in EU/ml for each immunisation group to be plotted.

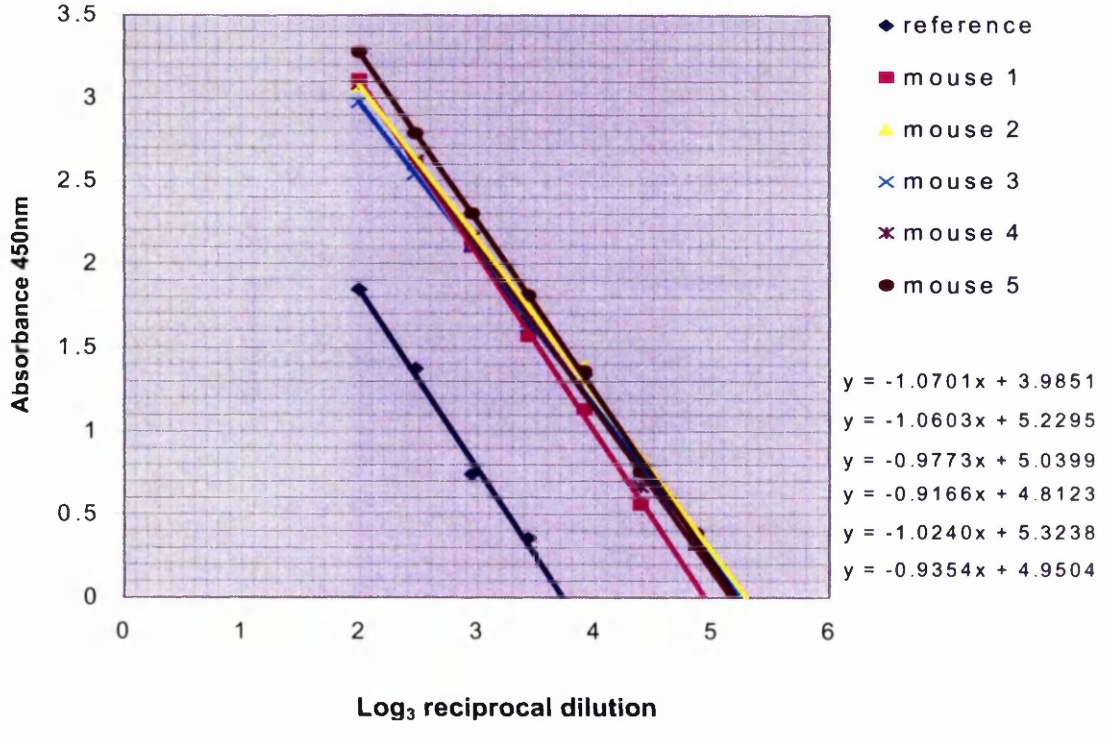


Figure 3.8.3.1 (continued)

Trend lines obtained for 5 mice

$y = -1.0603x + 5.2295$ ie $0.5 = -1.0603x + 5.2295$ $\therefore x = 4.461$

$y = -0.9773x + 5.0399$ $x = 4.645$

$y = -0.9166x + 4.8123$ $x = 4.705$

$y = -1.0240x + 5.3238$ $x = 4.711$

$y = -0.9354x + 4.9504$ $x = 4.758$

Geometric mean (GM) = antilog (1/n X ∑log3 recipricol dilution)

GM = antilog (1/n X 23.28

= antilog (1/5 X 23.28)

= antilog 4.656

= **45289.76**

Trend line for reference serum

$y = -1.071x + 3.9851$ $\therefore x = 3.257$

GM = antilog 3.257

= 1807

GM for unknown samples (previous example) = 45289.76

Specific EU/ml for PT, FHA, P.69 = 17, 143, 30 respectively

$$\text{GM of antibody levels (EU/ml)} = \frac{\text{GM of unknown samples X specifc EU}}{\text{GM of reference serum}}$$

$$\text{GM of anti-PT IgG} = \frac{45289.79 \text{ X } 17}{1807}$$

= **426.08 EU/ml**

Figure 3.8.3.2

Geometric mean of anti-PT IgG antibody responses after immunisation with detoxified PT and a combination of CyaA*, FHA and P.69 antigens

Geometric mean anti-PT IgG levels for the different immunisation groups were determined by ELISA as described in the legend to Figure 3.8.3.1.

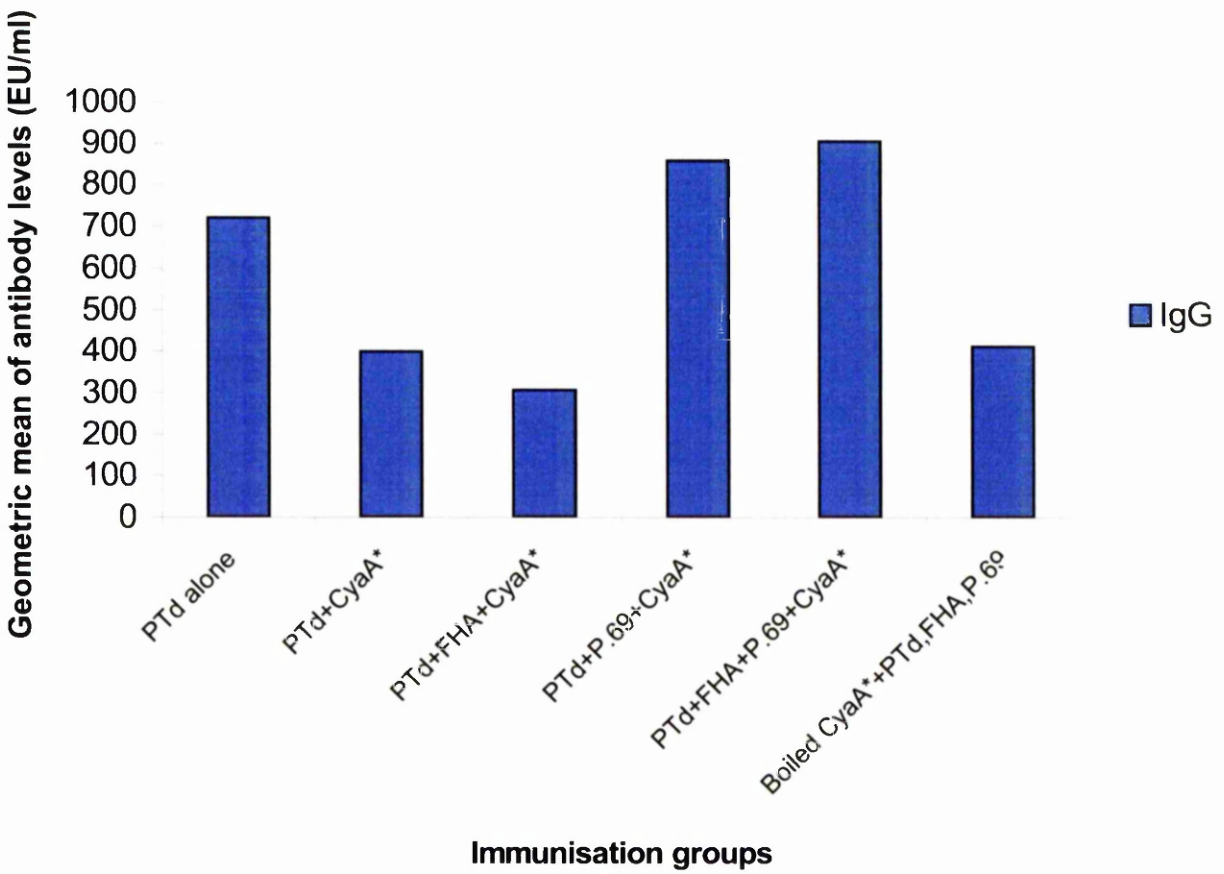


Figure 3.8.3.2.1

Geometric mean of anti-PT IgG_1 antibody titres after immunisation with detoxified PT and a combination of CyaA*, FHA and P.69 antigens

Geometric mean anti-PT IgG_1 levels for the different immunisation groups were determined by ELISA as described in the legend to Figure 3.7.1.1.

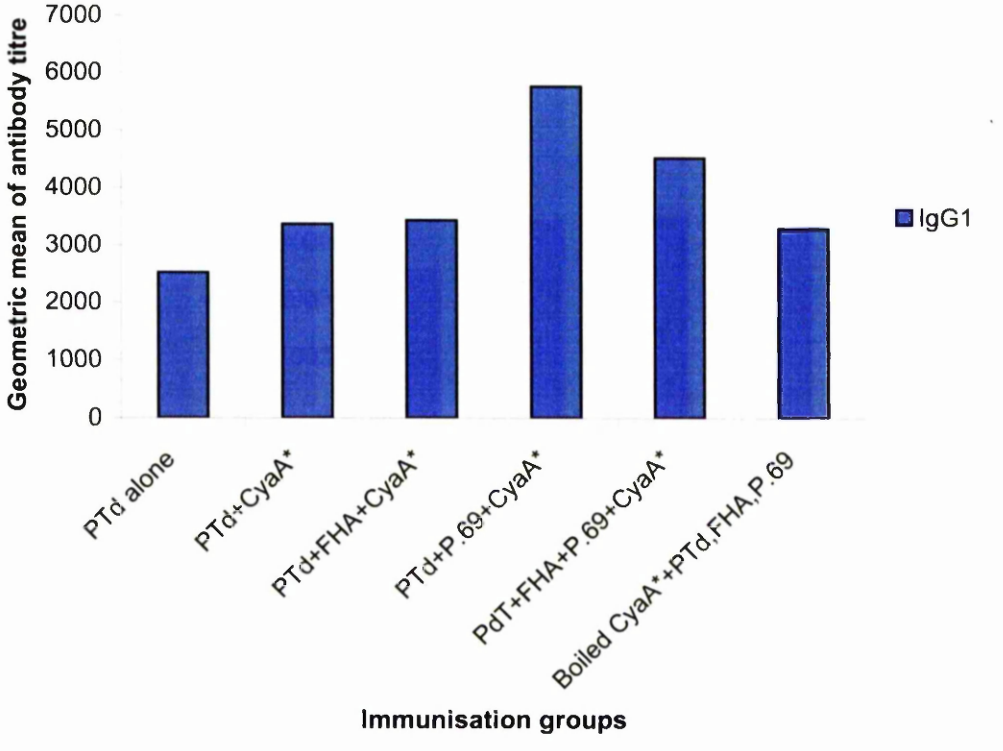


Figure 3.8.3.3

Geometric mean of anti-FHA IgG antibody titres after immunisation with FHA and a combination of CyaA*, detoxified PT and P.69 antigens

Geometric mean anti-FHA IgG levels for the different immunisation groups were determined by ELISA as described in the legend to Figure 3.8.3.1.

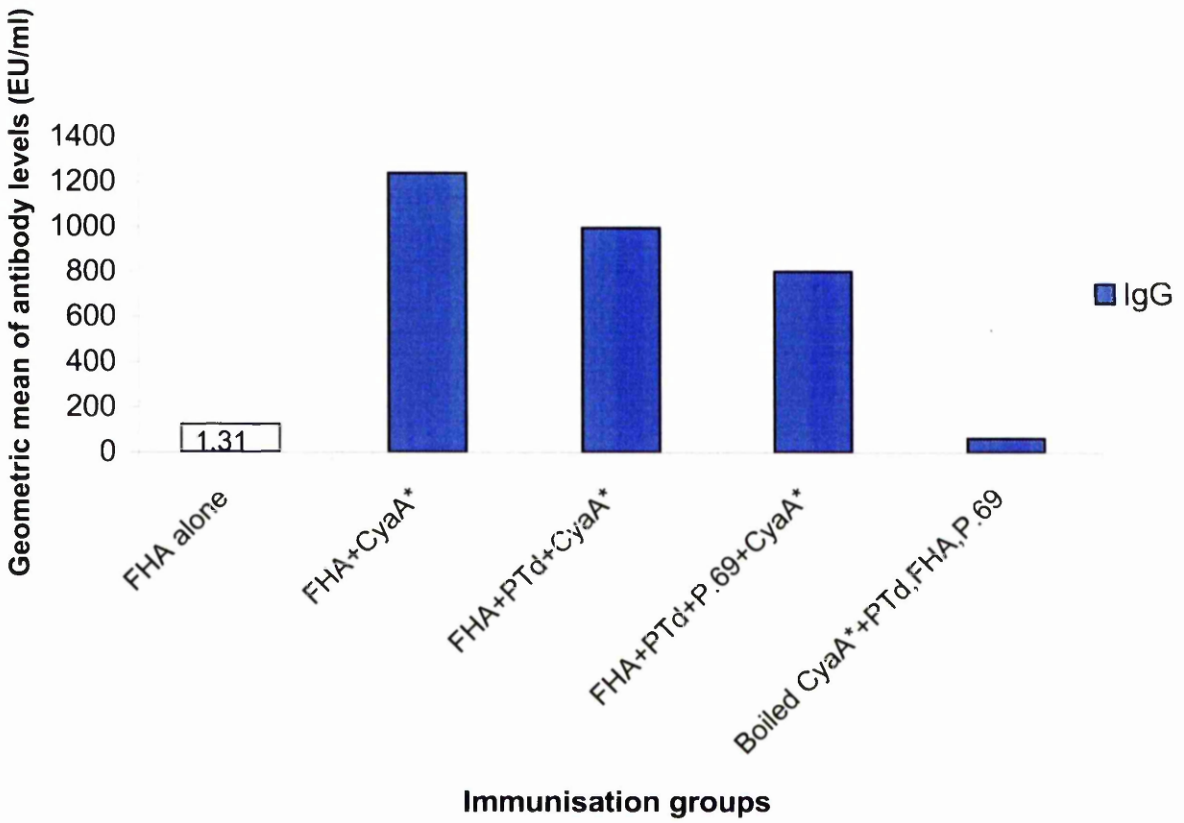


Figure 3.8.3.3.1

Geometric mean of anti-FHA IgG_1 antibody titres after immunisation with FHA and a combination of CyaA*, detoxified PT and P.69 antigens

Geometric mean anti-FHA IgG_1 levels for the different immunisation groups were determined by ELISA as described in the legend to Figure 3.7.1.1.

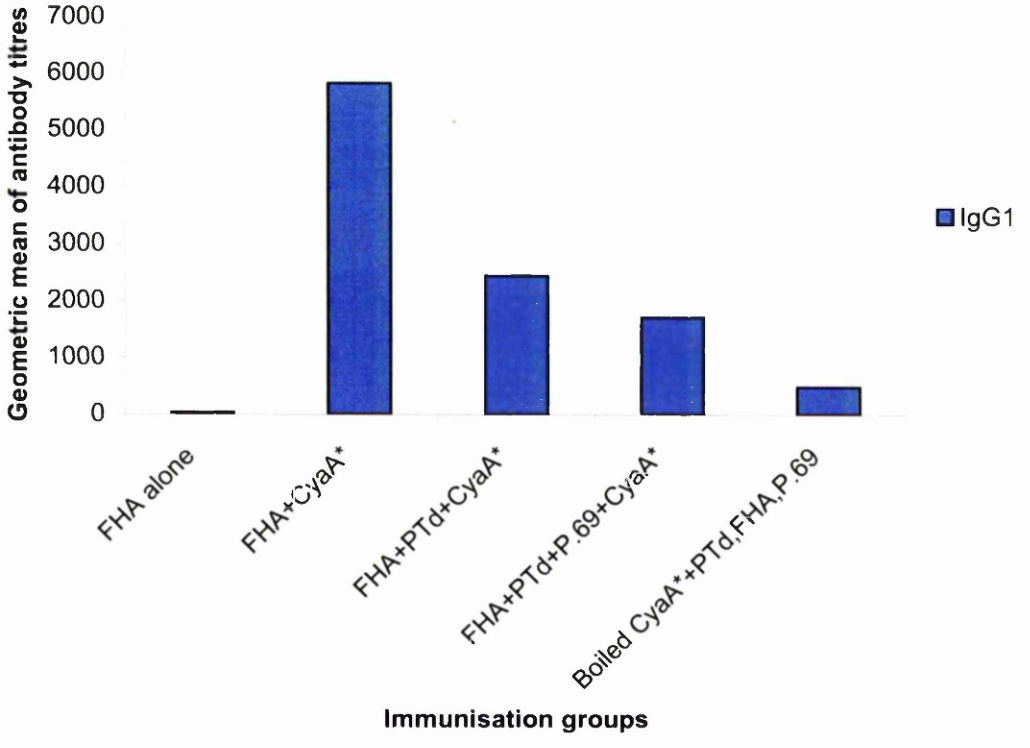


Figure 3.8.3.4

Geometric mean of anti-P.69 IgG antibody responses after immunisation with P.69 and a combination of CyaA*, detoxified PT and FHA antigens

Geometric mean anti-P.69 IgG levels for the different immunisation groups were determined by ELISA as described in the legend to Figure 3.8.3.1.

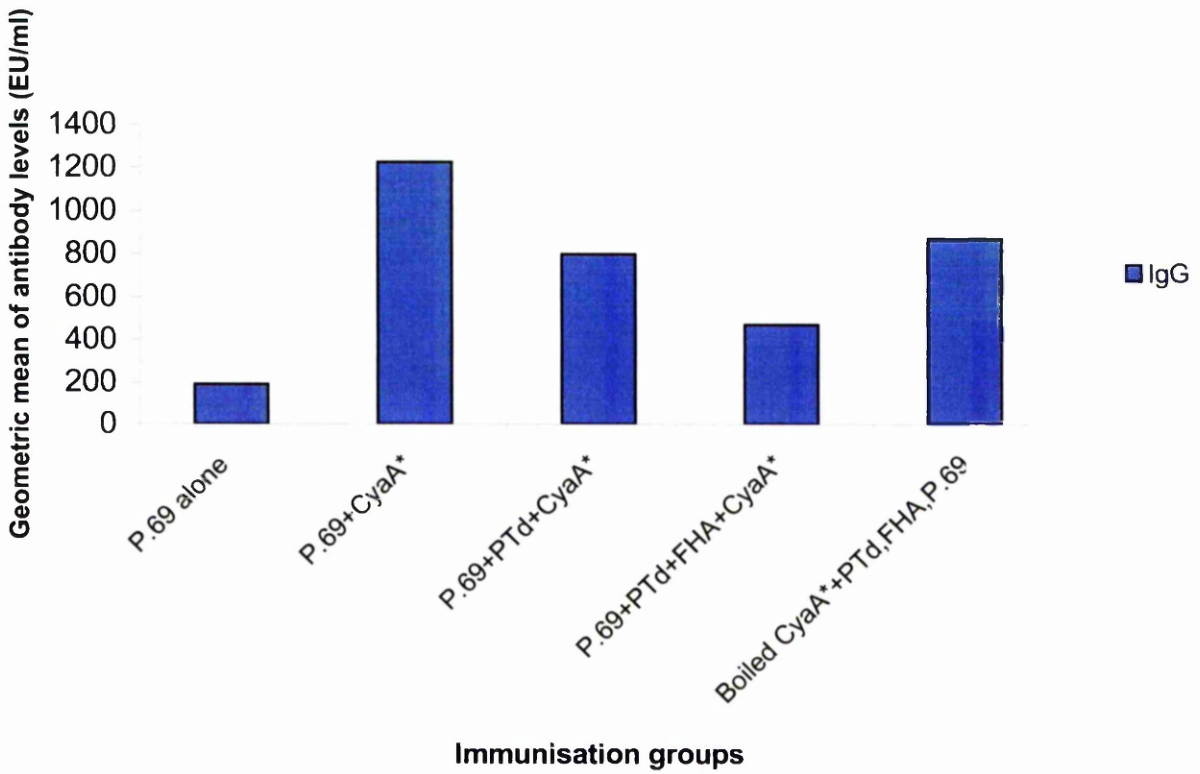
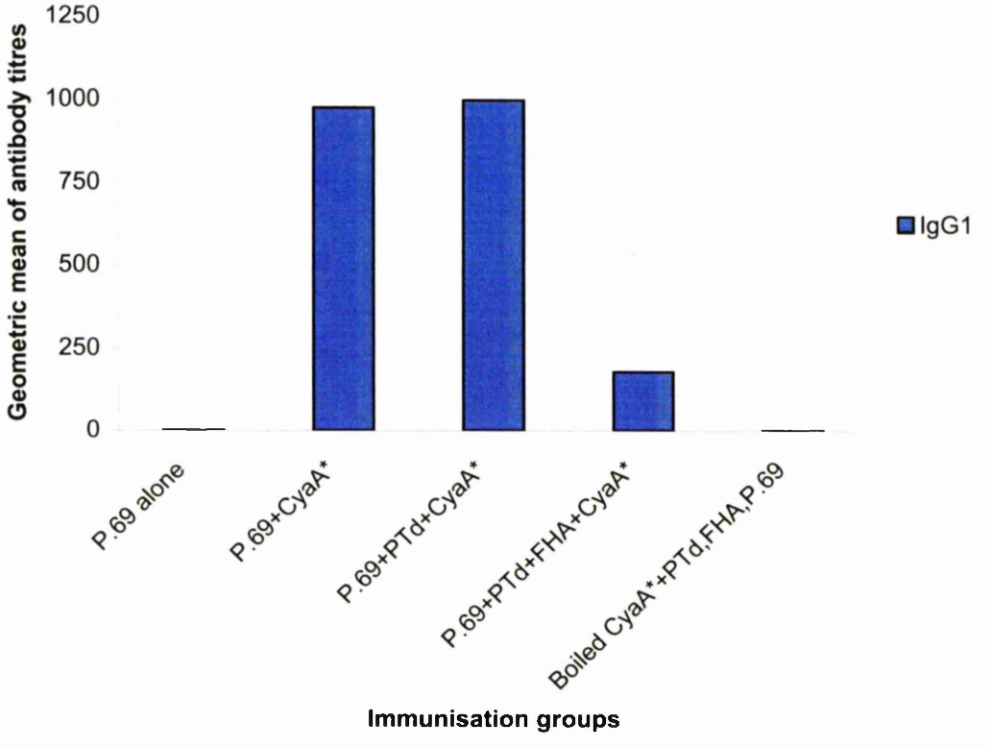


Figure 3.8.3.4.1

Geometric mean of anti-P.69 IgG_1 antibody titres after immunisation with FHA and a combination of CyaA*, detoxified PT and FHA antigens

Geometric mean anti-P.69 IgG_1 levels for the different immunisation groups were determined by ELISA as described in the legend to Figure 3.7.1.1.



3.8.4 Mouse protection with CyaA* in combination with native PT, FHA and P.69 antigen mixture against intranasal challenge with *B. parapertussis*

B. parapertussis causes a milder form of whooping cough in humans and, although not as severe as the infection caused by *B. pertussis*, it would be important when creating new acellular vaccines to ensure that any antigens known to be protective against infection from both species of *Bordetella* were included. To investigate the possibility of CyaA* protecting against *B. parapertussis,* three groups of 5 mice were immunised (i.p) with either 25μg of CyaA* or native PT, FHA, P.69 antigens at 120ng, 25μg and 10μg per dose respectively or a combination of CyaA* plus the antigen mixture. A fourth group of 10 mice received PBS by the ip route as a control. At 4 weeks post-immunisation, mice received an intranasal challenge of 10μl per nostril containing a *B. parapertusssis* suspension of $1.5x10^9$ cfu/ml. Five mice from the PBS immunised groups were sacrificed on day 0 of challenge and the remainder from all groups were sacrificed at day 5 post-challenge and the lungs and trachea removed together, to estimate *B. parapertussis* colonisation (section 2.11.6). Figure 3.8.4 shows the results of the combined lung and tracheal counts. CyaA* alone again offered some protection against challenge, in that the lung count was $1.97x10^7$ cfu/ml compared to that of $7.1x10^7$ cfu/ml in the control PBS-immunised mice but when included with the antigen mixture, the degree of protection against colonisation markedly increased. Mice receiving a mixture of native PT, FHA and P.69 had a mean lung count of $3.23x10^5$ cfu/ml and this was further reduced to $3.83x10^4$ cfu/ml when CyaA* was included in the immunisation mixture. The decrease in cfu when CyaA* was included with the ACV components, was tested using the Student's t-test at 5% level. The p value obtained was 0.003 which indicated that the decrease was significant, and highlighted the potential that CyaA*

may have to confer or enhance protection against both *B. pertussis* and *B. parapertussis* infections.

3.8.5 Effect of adding CyaA to a current commercially-available acellular vaccine on mouse protection following aerosol challenge

In order to test the possibility that CyaA* may enhance the protection afforded by current acellular pertussis vaccines (ACV), a further aerosol challenge experiment was conducted to investigate the effect of adding CyaA to a commercially-available 3-component ACV. Groups of 5 mice were immunised ip with a single dose of either, 0.125 IU/mouse (approximately equivalent to 0.03 SHD) of WCV, 0.25 SHD/mouse of a commercially available three-component acellular vaccine ACV (25μg PTd, 25μg FHA and 8μg P.69), 25μg of CyaA* or CyaA, 25μg of CyaA* or CyaA combined with ACV, and two control groups of either boiled CyaA or *E. coli* LPS at 175 IU/dose combined with ACV. A further group of 10 mice received 0.5ml ip PBS. At 4 weeks post-immuisation all groups were subjected to an aerosol challenge from a suspension containing approximately 10^8 cfu/ml *B. pertussis* 18.323. Five mice from the PBS immunised groups were sacrificed on day 0 of challenge and the remainder from all groups were sacrificed at day 7 post-challenge and the lungs and trachea removed together, to estimate *B. pertussis* colonisation. Figure 3.8.5 shows the results of the combined lung and tracheal counts. Mice receiving either CyaA* or CyaA immunisations gave mean lung counts of 7.78×10^6 cfu/ml and 5.54×10^6 cfu/ml respectively, slightly lower than the lung counts of 1.44×10^7 cfu/ml from PBS-immunised mice. WCV-immunised mice gave the greatest protection against colonisation, demonstrated by the low lung counts of

$3.83x10^3$ cfu/ml, and those receiving the ACV gave lung counts of $7.12x10^4$ cfu/ml. The lung counts showed a slight reduction when mice received either CyaA* or CyaA co-administered with ACV ($3.52x10^3$ and $2.49x10^4$ cfu/ml respectively), in comparison to those receiving ACV alone. However statistical analysis by the Student's t-test showed that the results between the ACV immunised group and the ACV co-administered with CyaA* or CyaA were not statistically significant at either the 5% or 10% level, indicating that addition of either CyaA* or CyaA to the ACV immunisation did not significantly enhance the protection afforded by the ACV alone under the experimental conditions used. Interestingly, both boiled CyaA* and LPS plus ACV gave quite high lung counts ($1.94x10^5$ and $1.54x10^5$ cfu/ml respectively), however when these were compared to CyaA* plus ACV the difference still was not significant at either the 5% or 10% level..

Figure 3.8.4

Mouse protection with CyaA* in combination with native PT, FHA and P.69 antigens against intranasal challenge with *B. parapertussis*

The protective activities of CyaA* in combination with native PT, FHA and P.69 antigens, were examined in mice after intranasal challenge with *B. parapertussis*. One group of 5 mice received a single dose of 25µg/dose of CyaA*, a second group of 5 mice received a combination of native PT, FHA and 69K antigens at 120ng, 25 µg and 10µg/dose respectively and a third group received a combination of CyaA* and antigen mixture. A fourth group of 10 mice received PBS as a control. At 4 weeks post immunisation the groups were subjected to an intranasal challenge of *B. parapertussis* (section 2.11.7). The lungs and trachea were removed from 5 mice of the PBS group on the day of challenge and at day 5 post-challenge the remainder of the groups were sampled. Viable counts were performed on the combined lung and trachea homogenate and the mean log colony forming units (cfu) and standard deviation plotted at day 5 post-challenge for each group.

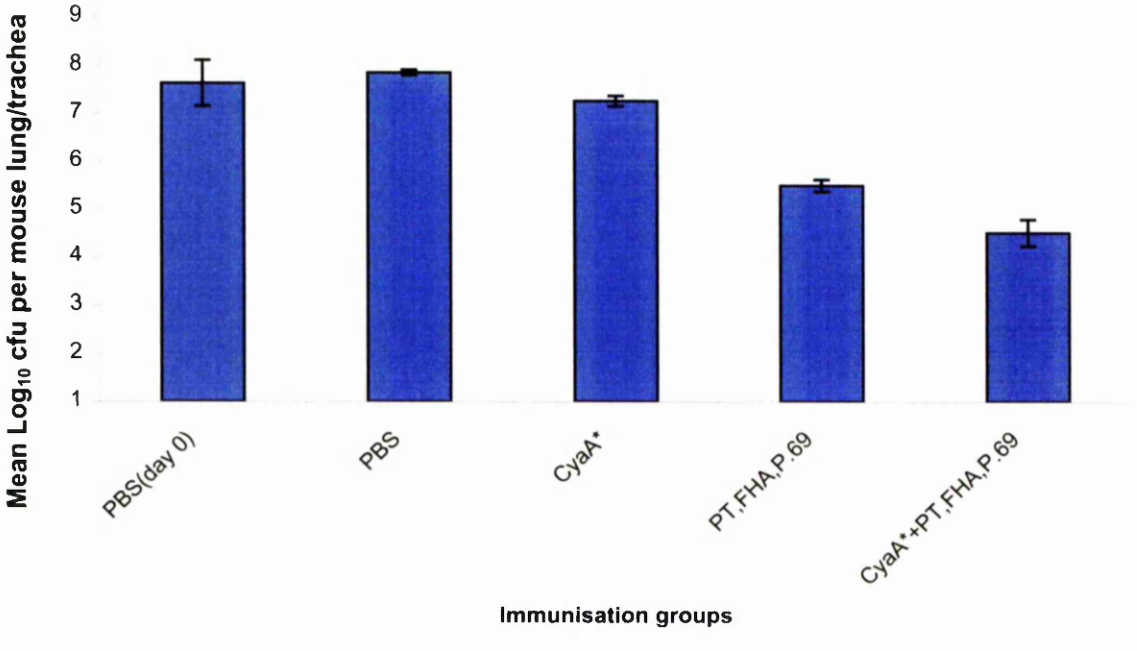
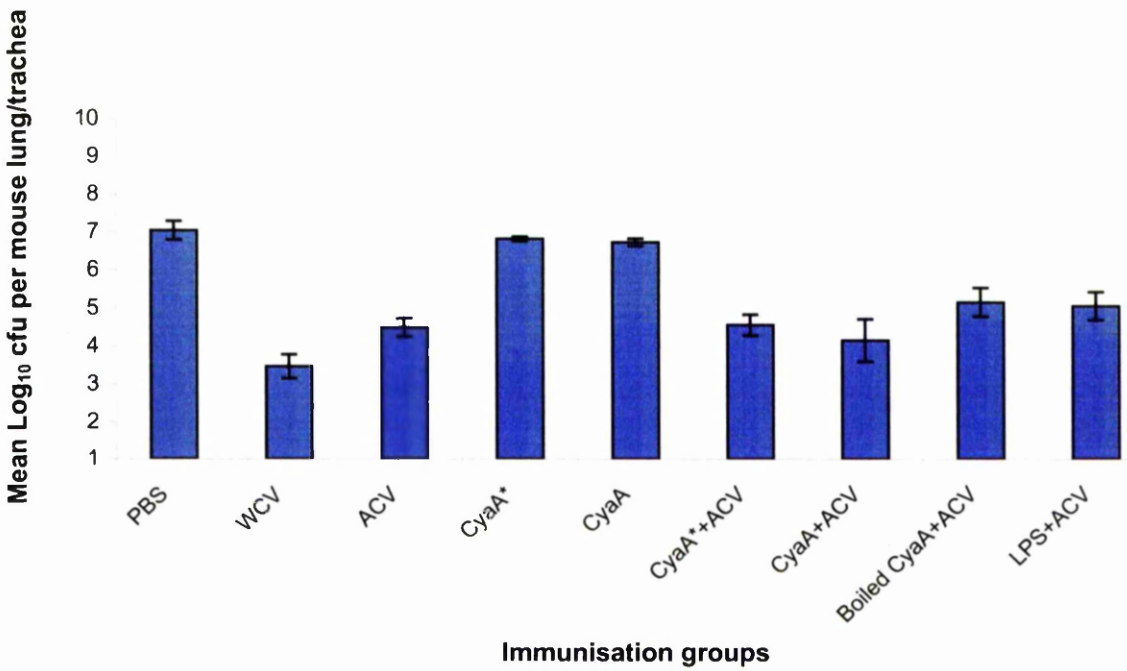


Figure 3.8.5

Comparison of the mouse protection with CyaA alone or in combination with a commercially available 3-component acellular vaccine against challenge with *B. pertussis* 18.323

Groups of mice were immunised with PBS as a control, pertussis WCV at 0.025 SHD/dose, ACV at 0.1 SHD/dose, CyaA* or CyaA alone at 25µg/dose or CyaA* and CyaA in combination with ACV. At 4 weeks post immunisation the groups were subjected to an aerosol challenge of *B. pertussis* 18.323 (section 2.11.5). The lungs and trachea were removed from 5 mice of the PBS group on the day of challenge and at day 7 post-challenge the remainder of the groups were sampled. Viable counts were performed on the combined lung and trachea homogenate and the mean log colony forming units (cfu) and standard deviation plotted at day 7 post-challenge for each group.



3.8.6 Production of nitric oxide by macrophages from mice immunised with CyaA in combination with native PT, FHA and P.69 antigen mixture

Nitric oxide (NO) levels measured from macrophages taken from mice that had been immunised with only CyaA preparations were found to be no greater than the control groups of PBS- or LPS-immunised mice (Figure 3.7.3), therefore the effect of adding CyaA to a combination of native PT, FHA and P.69 antigens was investigated. NO production was determined from peritoneal macrophages obtained from groups of 5 mice immunised (ip) with either, a single 15μg dose of different CyaA preparations, PT, FHA, P.69 at 120ng, 25μg and 10μg per dose respectively, 175 iu of purified LPS from *E. coli* plus the antigen mixture, or a combination of CyaA* or CyaA plus the antigen mixture. In this experiment, control mice were immunised with either PBS, 15μg of boiled CyaA or 175 iu of purified LPS from *E. coli*. Mice were terminally bled on day 14 post-immunisation and the peritoneal cavity was lavaged with sterile PBS to recover macrophages (Section 2.11.8). Nitrite determinations were made on supernates taken from the cultured macrophage samples after stimulation (Section 2.11.10). The effects of different stimuli *in vitro* on the macrophage cells from different immunisation groups, is displayed in Figure 3.8.6 and Appendix H.3. Stimulation with *B. pertussis* heat-killed cells, at a concentration of $2x10^6$ cells/ml, gave rise to an increase in NO production in all groups when compared with the levels from cells that did not receive any stimulation. Likewise, stimulation with CyaA* at a concentration of 2μg/ml also resulted in an increase in NO production, although not as marked as stimulation with *B. pertussis* hkc. In contrast, stimulation with CyaA demonstrated lower NO levels in all immunisation groups. This was probably due to the fact that, at 2μg/ml, the concentration of CyaA

is cytotoxic and may be killing the cells (Figure 3.5.3.1). As was demonstrated previously in Figure 3.7.3, immunisation with either CyaA* or CyaA did not result in an increase in NO production when compared to the PBS-, LPS- or boiled CyaA-immunised mice. However a marked increase in NO production was observed when macrophages from either CyaA* or CyaA in combination with PT, FHA, P.69 antigen mixture were tested, in comparison to macrophages from mice immunised with antigen mixture alone or in combination with LPS. The best results were obtained when CyaA* was used as the adjuvant. The overall increase in NO production would indicate that, although CyaA does not appear to stimulate macrophage activation when used on its own as a vaccine, when administered in conjunction with other acellular components, CyaA* in particular is able to enhance macrophage activation.

3.8.7 Cytokine mRNA expression in mice immunised with CyaA plus native PT, FHA and P.69 antigen mixture

The effect of adding CyaA to an immunisation mixture of native PT, FHA and P.69 antigens on cytokine expression in spleen cells was investigated as described for CyaA alone in section 3.7.4. The results shown in Figures 3.8.7.1 and 3.8.7.2 for mice vaccinated with CyaA or CyaA* alone support previous data (Figure 3.7.4.2 and 3.7.4.3). Spleen cells from mice immunised with CyaA* and stimulated with hkc, produced high levels of IFNγ, IL-2 and IL-4, whereas spleen cells from CyaA-immunised mice produced similar amounts of IFNγ and IL-2, but lower levels of IL-4 (Figures 3.7.4.2 and 3.8.7.1). Similarly, cells from CyaA*-immunised mice stimulated with CyaA* produced little of these cytokines, whereas cells from CyaA-immunised mice produced high levels of all these cytokines when stimulated with

CyaA* (Figures 3.7.4.3 and 3.8.7.2). Unexpectedly, the data show that addition of CyaA or CyaA* to PT, FHA, P.69 mixture, did not result in an enhancement in the levels of IFNγ, IL-2 or IL-4 cytokine expression from cells stimulated with hkc or CyaA* and, if anything, the cytokine response was repressed. This was an unexpected result as the addition of CyaA to native PT, FHA and P.69 antigen mixture in previous experiments had resulted in a clear adjuvant effect, by enhancing antibody production (Figures 3.8.2.1, 3.8.3.2, 3.8.3.3, 3.8.3.4), by enhancing protection against respiratory tract colonisation after *B. pertussis* or *B. parapertussis* challenges (Figures 3.8.4, 3.8.5) and by stimulating NO production from macrophage cells (Figure 3.8.6).

IFNγ production in spleen cells taken from mice immunised with CyaA preparations alone or in combination with pertussis antigens was also measured by ELISA (Figure 3.8.7.3). Unfortunately, due to time constraints, a direct comparison between all the groups and the *in vitro* stimuli could not be made, but, several points can be addressed. Firstly, a greater level of IFNγ was produced when mice had been vaccinated with the enzymically, active form of CyaA compared to CyaA* and the spleen cells were stimulated *in vitro* with CyaA*. This can be compared to Figure 3.8.7.2, in which a similar result was observed. However, when *B. pertussis* hkc were used as the stimulus the results differ from those obtained by RT-PCR. Mice vaccinated with both forms of CyaA gave quite high values for IFNγ production when stimulated with hkc, as was evident by RT-PCR (Figure 3.8.7.1). Interestingly the effect that the enzymic activity of CyaA had on IFNγ production was apparent when native PT was included in the immunisation protocol. When stimulated with hkc, spleen cells from mice given PT on its own gave IFNγ levels of 18368pg/ml, similar to the value for CyaA* vaccination but much lower than that of CyaA, which

were 13568 and 46336pg/ml respectively. However, when the different forms of CyaA were included in the immunisation with PT an interesting result was obtained. The non-enzymically, active form of CyaA* acted to raise IFNγ production to a value of 41344pg/ml, compared to PT alone at 18638pg/ml, but the enzymically, active form of CyaA apparently inhibited the production of IFNγ to a value of 224pg/ml when co-administered with PT. These results indicate a clear difference in the two forms of CyaA, most noticeably when native PT was co-administered with CyaA. This may be related to the fact that both toxins act to increase intracellular cAMP levels and may perhaps work in an antagonistic manner toward each other. As with the data obtained by RT-PCR, inclusion of CyaA* with the antigen mixture for vaccination produced spleen cells only marginally responsive to stimulation by CyaA*.

Figure 3.8.6

Production of nitric oxide by macrophages from mice immunised with CyaA in combination with native PT, FHA and P.69 antigen mixture

Nitric oxide (NO) production was measured from macrophages obtained from groups of 5 mice immunised ip with a single dose of 15µg CyaA or CyaA* alone, or in combination with a mixture of native PT, FHA and P.69 antigens at 120ng, 25 µg and 10µg/dose respectively. Cells were stimulated *in vitro* with either a suspension of *B. pertussis* heat-killed cells (hkc) at 2 x 10^6 cells/ml, or with the different CyaA preparations at 2µg/ml. After an incubation period of 24 hours, NO production for each immunisation group was determined (section 2.11.10).

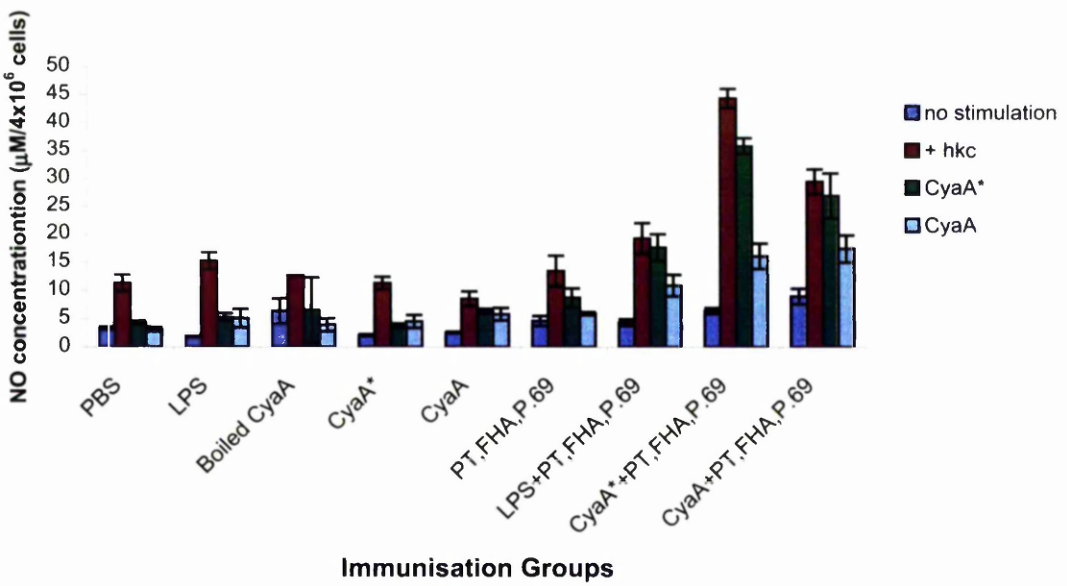


Figure 3.8.7.1

Cytokine expression from mice immunised with CyaA in combination with native PT, FHA and P.69 antigens, following stimulation *in vitro* with *B. pertussis* hkc

Cytokine mRNA expression using RT-PCR was carried out on spleen cells taken from mice immunised ip with a single dose of 15µg of different CyaA preparations alone or in combination with a mixture of native PT, FHA and P.69 antigens at 120ng, 25 µg and 10µg/dose respectively (section 2.11). cDNA from the different samples was amplified by PCR using cytokine specific primers which enabled visualisation by agarose gel electrophoresis (section 2.12. 2.3). The intensity of each band representing a different cytokine was calculated by the Kodak 1D gel analysis software package and given as a numerical value. The levels of cytokine mRNA expression from each immunised group, when spleen cells were stimulated *in vitro* with heat-killed cells, were expressed as a percentage of the intensity of β-actin band.

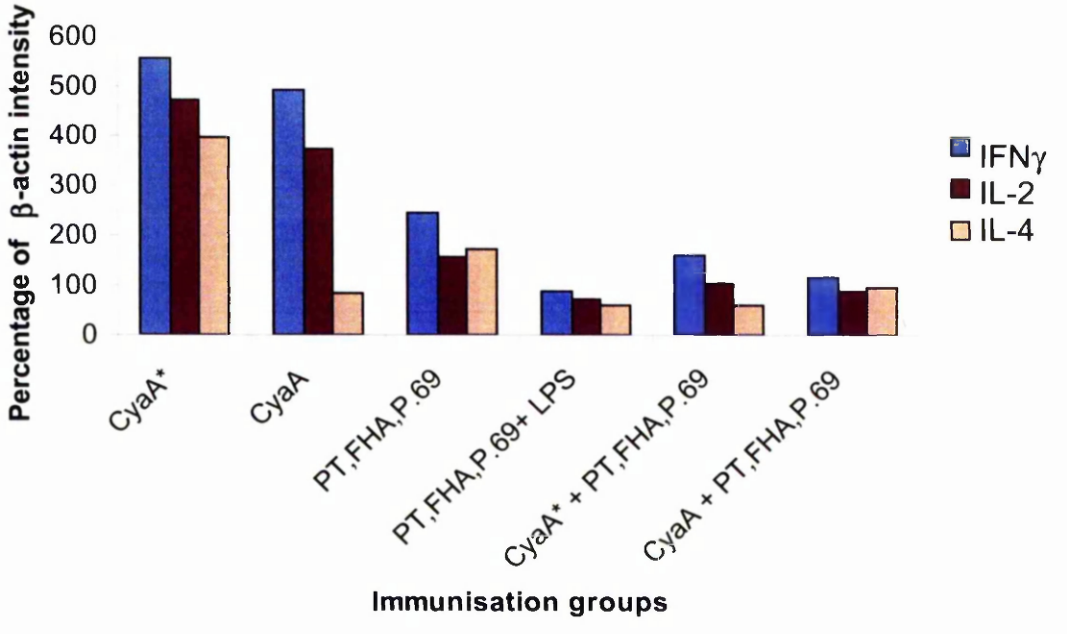


Figure 3.8.7.2

Cytokine expression from mice immunised with CyaA in combination with native PT, FHA and P.69 antigens, following stimulation *in vitro* with CyaA*

The levels of cytokine mRNA expression from each immunised group, when spleen cells were stimulated *in vitro* with CyaA* at 2µg/ml, were expressed as a percentage of the intensity of β-actin band.

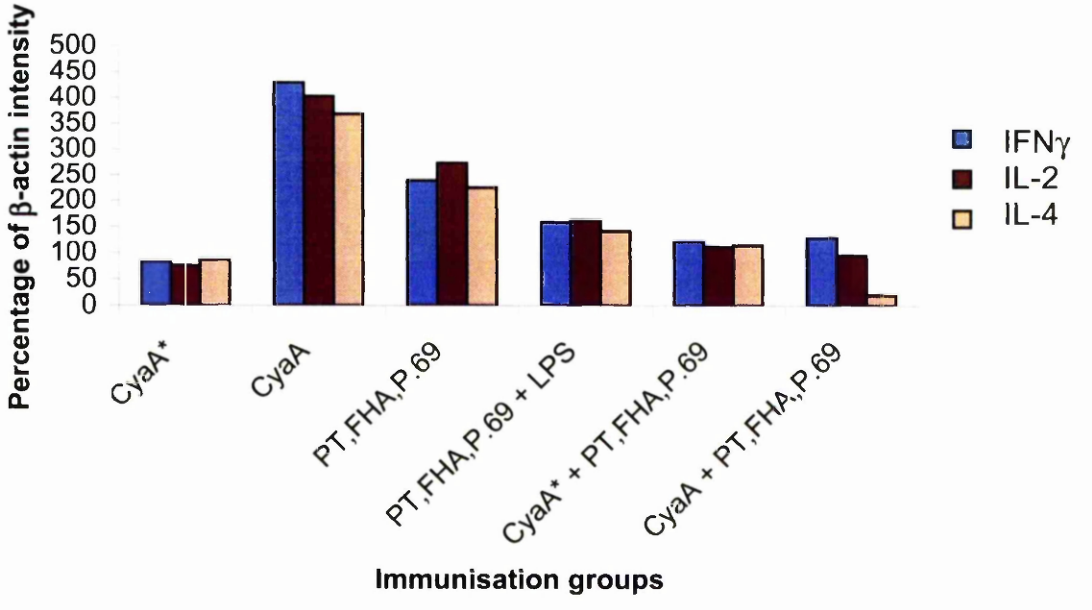
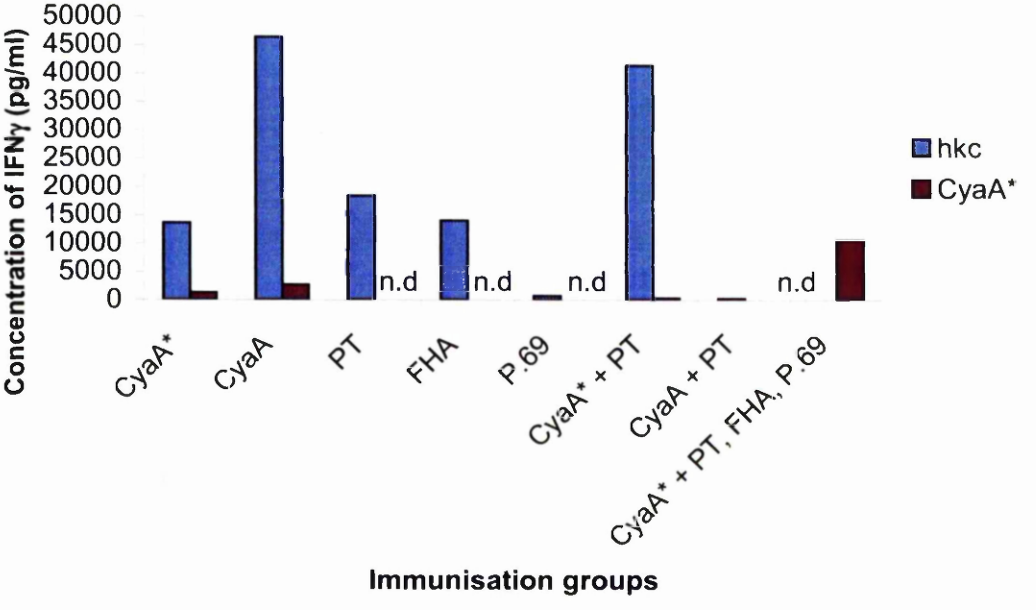


Figure 3.8.7.3

IFNγ expression from mice immunised with CyaA preparations in combination with PT, FHA and P.69 antigens, following stimulation *in vitro* with *B. pertussis* hkc or CyaA*

IFNγ expression was carried out on spleen cells taken from mice immunised ip with a single dose of 15µg of different CyaA preparations alone or in combination with native PT, FHA and P.69 antigens at 120ng, 25µg and 10µg/dose respectively (section 2.11). IFNγ production was measured by ELISA (section 2.14.3) and expressed as a concentration in pg/ml. (n.d = not determined).



CHAPTER 4

DISCUSSION

4.1 PRODUCTION OF CyaA FROM RECOMBINANT *E. coli* STRAINS

A high level production of active CyaA was demonstrated by Sebo *et al.* (1991) using a reconstructed *E. coli* system. Plasmid pACT7 was constructed to contain the *cyaA* gene expressed under the control of the transcription and translation signals of *lacZ*. The remaining four genes of the *cya* locus (*cyaB, cyaD, cyaE* and *cyaC*) were cloned into a low copy number vector that was compatible with pACT7 and included a strong inducible promoter and transcriptional terminators. The production of CyaA in *E. coli* from *cyaA* alone resulted in a non-invasive and non-haemolytic toxin. However, the presence of the CyaC gene product was found to be sufficient to render the CyaA pro-toxin invasive and haemolytic in the reconstructed system (Sebo *et al.*, 1991). In the study reported here, an efficient T7 RNA polymerase system, previously developed in this laboratory (Westrop *et al.*, 1996), was used for overexpression of *cyaA* and *cyaC* genes in a protease defective *E. coli* strain BL21/DE3, harbouring two separate compatible plasmids, pGW44 and pGW54. Westrop *et al.* (1996) demonstrated that strain BL21/DE3 (pGW44, pGW54) produced CyaA and CyaC in amounts that were similar to those obtained when the plasmids were carried separately, i.e. in strains BL21/DE3 (pGW44) and BL21/DE3 (pGW54), indicating that expression of one gene did not interfere with expression of the other gene. SDS-PAGE analysis of urea extracts produced in this study from strain BL21/DE3 (pGW44, pGW54) (Figure 3.1.1, lanes 6

and 7) suggested that the levels of CyaC were less when the two plasmids were co-expressed than when CyaC was expressed alone. This was contrary to the results of Westrop *et al.* (1996) but was likely to be an artifact of the gel, due to the difference in protein content of the urea extract loaded into these lanes. A third compatible plasmid, pGW44/188, was constructed from pACM188 (Ladant *et al.*, 1992) and encoded the CyaA pro-toxin that lacked enzymic activity due to a dipeptide amino acid insertion in the N-terminal end (Section 2.1). pGW44/188 was co-expressed with pGW54 to produce a full length invasive toxin with little enzymic activity (BL21/DE3 (pGW44/188,54). SDS-PAGE analysis demonstrated the same banding pattern for the enzymically-active form (CyaA) and the enzymically-inactive form (CyaA*) (Figure 3.1.2). These constructs enabled the production of the different forms of CyaA in *E. coli* without the inconvenience of growing *B. pertussis* and the knowledge that they were free from other *B. pertussis* components that could have displayed toxic or immunostimulatory properties.

4.2 PURIFICATION OF CyaA FROM RECOMBINANT *E. coli* STRAINS

To examine the immunogenic and immunostimulatory properties of CyaA highly purified preparations were desirable. The enzymically-active and inactive forms of CyaA were purified by several methods. The most effective method was a combination of DEAE sepharose column chromatography followed by phenyl sepharose column chromatography, as each method alone was not sufficient to remove contaminating

proteins. Large quantities of 200kDa CyaA proteins were obtained by these purification steps with only a few lower molecular weight proteins present. The majority of these were removed by including a further ultrafiltration and concentration step during the purification procedure (Figure 3.2.5). Any residual lower molecular weight proteins were assumed to be subsequent degradation products of CyaA as judged by immunoblotting with polyclonal antibody raised against 200 kDa CyaA in rabbits (Figure 3.2.6). The differences in molecular weight between the three forms of CyaA (CyaA pro-toxin, non-acylated; enzymically-active, acylated CyaA; and enzymically-inactive, acylated CyaA*) due to acylation or dipeptide amino acid insertion would not be expected to be detected by SDS-PAGE analysis and immunoblotting, and indeed it was not possible to differentiate the preparations by these methods (Figure 3.2.5 and 3.2.6).

4.3 ENDOTOXIN REMOVAL FROM CyaA PREPARATIONS

Recombinant proteins derived from *E. coli* are often contaminated with endotoxins due to the high quantities of LPS in the cell envelope of this Gram-negative bacterium. The levels of endotoxin in each of the three CyaA preparations were determined using the *L. amoebocyte* lysate (LAL) test (Table 3.5). In preliminary studies, all CyaA preparations were found to be contaminated with LPS, a fact that represented a problem for any immunological studies due to the highly immunogenic and immunostimulatory nature of LPS. Commonly used techniques for removing endotoxin contaminants are ultra-filtration and ion exchange chromatography although, in the case of large proteins,

ultrafiltration is not suitable and ion exchange methods are less specific. Other methods previously used include the use of polymyxin B and Triton X-114 phase separation (Morrison and Jacobs, 1976; Aida and Pabst, 1990). These methods were attempted to try to reduce the level of contaminating endotoxin but all proved unsuccessful (Section 3.6) which led to the conclusion that the endotoxin was probably tightly bound to the CyaA protein, as a reduction in endotoxin was accompanied by a reduction in CyaA content. A review by Petsch and Anspach (1999) found that chromatography with endotoxin adsorbers such as polymyxin B were ineffective in their ability to reduce endotoxins to an acceptable limit when large sample volumes were applied onto columns, and also that ultrafiltration with proteins greater in molecular weight than 150kDa was ineffective. Ultrafiltration will fail if interactions between endotoxin and proteins prevent endotoxin monomers from permeating through the membrane. RTX toxins tend to form aggregates in aqueous solutions through hydrophobic interactions, and since LPS forms similar aggregates it may form complex aggregates with CyaA. It may be that an RTX-LPS complex is actually the biologically active moiety, as suggested by Czuprynski and Welch (1995). Their data showed that *E. coli* HlyA activity and antigenicity were lost when HlyA preparations containing LPS were loaded onto a polymyxin B column. More recent data has shown LPS to complex with *P. haemolytica* leukotoxin, resulting in enhanced and stabilised leukolytic activity (Li and Clinkenbeard, 1999). In the present investigations, because complete removal of LPS from CyaA preparations was not possible, additional immunological effects due to LPS were taken into account by including *E. coli* LPS and heated CyaA controls in the immunological studies. Endotoxins are very stable molecules requiring temperatures of

between 180 and 250°C and alkali or acid of at least 0.1M to destroy them. Therefore, by heating the CyaA preparations to 100°C, it was assumed that only the CyaA toxin was destroyed and any effect due to endotoxin alone would be revealed. However, such experiments would not show whether the observed effects were due to an interaction between LPS and CyaA. This would only be possible by having pure preparations of CyaA to which different amounts of LPS could be added.

4.4 PROPERTIES OF DIFFERENT CyaA PREPARATIONS

4.4.1 Adenylate cyclase (AC) enzymic activity

Two methods were used to measure the specific enzymic activity of the purified CyaA preparations, the conventional $^{32}\alpha$P-ATP assay and a novel conductimetric assay (Figure 3.5.1). Although the conductimetric assay tended to produce greater deviations between replicate assays, particularly when the enzymic activity was high, it was nonetheless proven to be a much quicker and safer (non-radioactive) method for determining the levels of AC activity in different samples. Both CyaA pro-toxin and CyaA were enzymically active, although the pro-toxin displayed a lower level of activity than the acylated form in both methods (Table 3.5). This was not in keeping with the results reported by Westrop *et al.* (1996) and Hormozi *et al.* (1999), who, using the conventional $^{32}\alpha$P-ATP assay, found the pro-toxin to have a greater specific activity. The reasons for this discrepancy are not clear, but may reflect differences in the

accuracy of the measurement of protein concentrations in the purified samples in the presence of urea. The specific activity of CyaA from native *B. pertussis* was not tested in this study however, the enzymically-active, acylated CyaA had a specific activity when measured by the conventional $^{32}\alpha$P-ATP assay which was similar to earlier work by Hormozi *et al.* (1999); 433μMol cAMP formed per mg protein per minute in this study, compared with 626μMol cAMP formed per mg protein per minute (Hormozi *et al.*, 1999). The N-terminal modified CyaA* toxin demonstrated a very low, almost negligible value for cAMP production. Ladant *et al.* (1992) showed that modified CyaA proteins became highly susceptible to proteolysis in situ, except protein AC188LQ (from plasmid pACM188) from which pGW44/188 was derived. This was expressed at high levels and appeared to be as stable as the wild-type enzyme in terms of the lack of proteolysis, but was devoid of catalytic activity (less than or equal to 2.2μMol cAMP formed per mg protein per minute). The very low levels of enzymic activity obtained with CyaA* in the present work (less than 2.8μMol cAMP per mg protein per minute) presumably indicate some low residual activity and are in agreement with the data of Ladant *et al.* (1992).

4.4.2 Haemolytic activity of CyaA preparations

The homology of the genes for *B. pertussis* CyaA synthesis and secretion with those of *E. coli* alpha-haemolysin (HlyA), was extended by the finding of the *cyaC* gene (Barry *et al.*, 1991), a homologue of *E. coli hlyC* which is known to be required for the

production of a functional haemolysin molecule in *E. coli* (Wandersman and Delepelaire, 1990). The *cyaC* gene product was shown to be essential for invasive and haemolytic activities of CyaA (Barry *et al.*, 1991; Sebo *et al.*, 1991). This was supported by the present work where it was found that CyaA protoxin had no detectable haemolytic activity. Bellalou *et al.* (1990) characterised the molecular features of CyaA and demonstrated that the haemolytic activity of CyaA was calcium-dependent, like that of HlyA, but that its haemolytic activity was at least 100 times lower than that of *E. coli* HlyA. The weakly haemolytic nature of the CyaA preparations was demonstrated in Figure 3.5.2, where it can be seen that 50 % haemolysis was obtained only when CyaA at a protein concentration of between 30 and 40 μg/ml was incubated with turkey erythrocytes for a long incubation period of 18 hours. The lack of AC enzymic activity had little effect on the haemolytic properties of the toxin, as expected (Table 3.5).

4.4.3 Cytotoxic effect of CyaA preparations

The CyaA pro-toxin was shown to be non-cytotoxic against J774.2 mouse macrophage cells, which was in keeping with earlier findings that the toxin must be acylated by CyaC modification to bestow any toxicity (Rogel *et al.*, 1989). The acylated CyaA toxin demonstrated a high level of cytotoxic activity, which corresponded with the enzymic and haemolytic and activities (Table 3.5). Although CyaA is poor at forming pores and only weakly haemolytic, the primary biological function of CyaA is presumably to deliver its enzymic activity into cells, ultimately resulting in their intoxication by the

production of cAMP, rather than the formation of membrane channels. This may suggest why only single acylation at lysine 983 is sufficient for membrane insertion and cell-invasive activities of *B. pertussis* CyaA but with *E. coli* HlyA, acylation at two sites, lysine 564 and lysine 680, is optimal for the formation of membrane channels (Basar *et al.*, 2001). Stanley *et al.* (1994) demonstrated that both acyl modifications must be present for HlyA to form channels and that loss of either acyl groups results in the virtual abolition of cytolyitc ability.

Interestingly the N-terminally modified CyaA* toxin, which has little AC activity, also displayed a degree of cytotoxicity, as measured in the MTT assay, at higher protein concentrations, although to a much lesser extent compared to CyaA (Figure 3.5.3.1 and Table 3.5). This 100-fold difference between the cytotoxicity profiles of the two toxin preparations perhaps suggests that cell killing by CyaA toxin is due mainly to the intrinsic AC enzymic activity of the protein after it has entered the target cell and, if this activity is removed, only residual toxicity is observed. This effect of CyaA* could be due to either the very low residual level of adenylate cyclase activity of the protein or to its pore-forming haemolytic effect. Khelef *et al.* (1993) demonstrated that *B. pertussis* killed macrophages through apoptosis and that adenylate cyclase expression was essential for *B. pertussis* to promote cell death. It was suggested that apoptosis was induced by the increase in cAMP production, as this molecule has been shown to be responsible for changes in protein phosphorylation (Duprez *et al.*, 1993). The possibility that apoptosis may be induced by the pore-forming properties of CyaA was ruled out by Khelef and Guiso (1995). They determined the DNA fragmentation induced by different CyaA preparations in J774A.1 macrophages and were able to demonstrate that a mutated

CyaA lacking adenylate cyclase activity but retaining haemolysin activity, did not induce death of murine J774A.1 macrophages. The present study suggested that cell killing was due to intracellular AC activity provided by CyaA, but any future work should examine the mechanism of killing, whether by apoptosis or by cell lysis.

4.5 ANTIBODY RESPONSES TO CyaA

4.5.1 Anti-CyaA IgG response

Betsou *et al.* (1995) demonstrated that when anti-*B. pertussis* CyaA polyclonal antibody was used in Western blotting, the recombinant *E. coli* CyaA polypeptide of 200kDa was recognised. In this study, the mice developed specific anti-CyaA IgG antibody responses after vaccination with 15μg of the enzymically-active and inactive CyaA forms, the peak production occuring at 7 weeks post-immunisation for CyaA pro-toxin and enzymically-active, acylated CyaA. Interestingly, immunisation with the enzymically-inactive, acylated CyaA gave rise to the greatest specific IgG levels, with the peak production occuring at 9 weeks post-immunisation (Figure 3.7.1.2). There is no obvious reason as to why this should have occurred, as the IgG response was measured by ELISA, using the active, acylated form in all cases. One possibility could be the inherent toxicity of the acylated CyaA preparations which may impair the functioning of antigen presenting cells such as macrophages. This would affect antigen processing and presentation.

When a second immunisation dose was administered at 7 weeks post initial immunisation the effects of boosting were clearly observed 2 weeks later and indicated a significant rise in anti-CyaA IgG production, thereby demonstrating a good memory response (Figure 3.7.1.3).

Antibodies against both acylated forms of CyaA, whether enzymically active or inactive, were able to neutralise the cytotoxic effect of the recombinant active CyaA on J774.2 cells *in vitro*, following incubation of the toxins with the sera from mice immunised with either CyaA or CyaA* before addition to J774.2 cells (Figure 3.7.1.1.1). Rogel *et al.* (1988) reported that when purified CyaA was incubated with immune serum from guinea pigs, inhibition of CyaA activity was obtained, in comparison to incubation with non-immune sera, which had no effect on activity. Further work by Moullem *et al.* (1990) and Farfel *et al.* (1990) demonstrated that sera taken from pertussis patients was able to inhibit cAMP accumulation in comparison to control sera taken from a patient who did not contain any anti-PT or anti-CyaA antibodies. However, it was also shown that the enzymic properties of the toxin were not neutralised, when inhibition of enzymic activity and inhibition of penetration of the enzyme into target cells (Mouallem *et al.* 1990; Farfel *et al.* 1990) were compared. This disagrees with data presented by Betsou *et al.* (1995), who reported that antibodies against a truncated protein consisting of the N-terminal domain and the last 217 amino acids of CyaA were able to neutralise both the enzymic and cytotoxic activities of recombinant CyaA. However, a preliminary experiment carried out in this study suggested that the enzymic activity, as measured by the conductimetric assay, remained unaltered after incubation for 1 hour at 37°C with

mouse immune sera containing CyaA antibodies, although further investigation is required to confirm this.

4.6 ADJUVANT EFFECT OF CyaA ON OTHER PERTUSSIS ANTIGENS

Hormozi *et al.* (1999) demonstrated that co-administration of 15μg active recombinant CyaA with OA (subcutaneous route, (s.c) 2 doses at a two-week interval) enhanced the specific IgG response to ovalbumin compared to immunisation with ovalbumin alone, thereby indicating the potential adjuvant effect that CyaA may be able to exert. In a preliminary study, the levels of anti-CyaA IgG were monitored after a single vaccination (ip) with CyaA alone or in combination with native PT, FHA and P.69 (120ng, 25μg and 10μg respectively). Surprisingly anti-CyaA IgG was found to be lower when CyaA* was co-administered with the other antigens, but higher for CyaA (Figure 3.8.1.2). It is difficult to draw any direct conclusions from these results since they conflict with results of previous experiments when CyaA and CyaA* were administered alone under the same conditions and lower levels of antibody were produced against CyaA than against CyaA* (Figure 3.7.1.2.).

4.6.1 Anti-PT, anti-FHA and anti-P.69 IgG response

The effects that CyaA* and CyaA had when co-administered with each of the three ACV components was studied, firstly when administered in combination with native PT, FHA and P.69 as a mixture and secondly when co-administered with detoxified PT

(dPT), FHA and P.69 individually. When native PT was used in the immunisation mixture, mice received 120ng of PT per dose, plus 25μg of FHA and 10μg of P.69. When detoxified PT was used in the immunisation mixture, mice received 10μg of all three antigens, as there was no problem of toxicity with PT so the dose could be increased. In all cases a single dose of 15μg of CyaA was administered either on its own or in combination with the other antigens. When CyaA* was co-administered with the antigen mixture, anti-PT, anti-FHA and anti-P.69 IgG levels all increased in comparison to those obtained with the antigen mixture alone. However, when CyaA was co-administered with the mixture, only anti-P.69 IgG levels increased, while anti-PT and anti-FHA IgG levels decreased, compared to those for PT, FHA and P.69 alone (Figure 3.8.2.1). The difference between the two forms of CyaA is the lack of enzymic activity in CyaA*. It is possible that the difference in antibody production when co-administered with other purified pertussis antigens was due in part to the enzymic activity of CyaA. Alternatively, it may be related to mutually antagonistic interactions between active PT, a known adjuvant, and the potential adjuvant effects of CyaA as both these toxins act to raise intracellular cAMP levels. However, as the adjuvant effect of CyaA* seemed to be as good as, if not better than CyaA, only CyaA* was used in subsequent experiments to determine the adjuvant effect on responses to individual antigens (dPT, FHA and P.69) with PTd replacing active PT in these experiments. Anti-PT IgG levels decreased when either CyaA* or FHA was co-administered with PTd, but were boosted when P.69 was included in the immunisation, suggesting that P.69 is able to over shadow the effects of CyaA* and FHA and direct the antibody response back towards PT. CyaA* was shown to act as an adjuvant to PT but only when administered

in combination with P.69. This was demonstrated by the lower anti-PT IgG response generated to the control group which received boiled CyaA*, PTd, FHA and P.69 in comparison to a similar group that included CyaA* (Figure 3.8.3.2). Anti-PT IgG_1 levels increased with any combination of antigens compared to immunisation with PTd alone. The greatest effect was when P.69 and CyaA* were co-administered with PTd, again demonstrating the adjuvant effect that CyaA* exerts when in combination with P.69 (Figure 3.8.3.2.1). Unfortunately, no IgG_{2a} data was available so no IgG_1:IgG_{2a} ratio could be determined and compared between the groups. Both anti-FHA IgG and IgG_1 levels increased when CyaA* was included in the immunisation mixture, although an increase in the number of antigens in the mixture resulted in a reduction in the specific antibody response (Figures 3.8.3.3; 3.8.3.3.1). This may be related to antigen competition as for example, CD11b may act as a binding site for CyaA, FHA and P.69. Daum *et al.* (2001) compared the antibody response to *Haemophilus influenzae* type b capsular polysaccharide (PRP) after one, two or three doses of DTaP vaccine combined with a PRP-tetanus conjugate (PRP-T) vaccine and found that the anti-PRP and anti-tetanus toxoid antibody responses decreased with an increasing dose of DTaP/PRP-T. The mechanism by which the DtaP/PRP-T combination vaccine was able to depress the antibody responses is unclear, but may possibly reflect antigenic competition. Previously, Dagan *et al.* (1998) showed that recipients of a pneumococcal-tetanus toxoid conjugate vaccine had diminished PRP and anti-tetanus antibody responses compared to the pneumococcal-diphtheria toxoid conjugate vaccine recipients, and that both the anti-PRP and anti-tetanus toxoid antibody responses were inversely related to the total amounts of tetanus toxoid received in the combination vaccines. A similar pattern was

followed for anti-P.69 IgG and IgG_1, in that the greatest response was when CyaA* was co-administered with P.69 and as more antigens were included the antibody response decreased. Except, however when a mixture of boiled CyaA*, PTd, FHA and P.69 was given. In this instance the anti-P.69 IgG levels were greater in comparison to those that received FHA or PTd, but still not as great as the response to P.69 and CyaA* together (Figures 3.8.3.4; 3.8.3.4.12). The adjuvant effect cannot be explained by the presence of LPS in CyaA* preparations, as the antibody responses to these antigens in the boiled CyaA* group were poor but the possibility of an interaction between the effects due to LPS and CyaA* cannot be excluded. The overall conclusion was that inclusion of CyaA* in the immunisation mixture did seem to enhance the specific antibody responses to individual components, particularly when co-administered with P.69.

4.7 PROTECTIVE ACTIVITIES OF CyaA FOLLOWING IMMUNISATION WITH CyaA ALONE OR IN COMBINATION WITH OTHER PERTUSSIS ANTIGENS AGAINST *B. pertussis* INFECTION

Betsou *et al.* (1993) showed that CyaC-mediated modification of CyaA was essential for protective activity and Hormozi *et al.* (1999) also confirmed that only the acylated form of CyaA was able to afford any protection against an intranasal challenge of *B. pertussis* after receiving two 15μg subcutaneous doses of either native or recombinant CyaA. This has led to the suggestion that CyaA should be considered as a potential component of future ACV's. In addition it is known that *B. pertussis* mutants deficient in the

expression of CyaA are impaired in their ability to cause a lethal infection in mice (Weiss and Goodman, 1989; Guiso *et al.*, 1989) so neutralisation of CyaA toxin by antibodies present after vaccination should have a similar effect on *B. pertussis* virulence. This study therefore focused initially on the protection afforded to mice against *B. pertussis* infection when immunised with either the enzymically-active, acylated CyaA or the enzymically-inactive, acylated CyaA*. Mice received two 15μg doses of either CyaA or CyaA*, or PBS as a control, i.p. After aerosol challenge with *B. pertussis* 18.323, the number of bacteria in the lungs of mice immunised with CyaA showed an approximate 2-log reduction compared to the control group of mice given PBS alone. A further half-log reduction was obtained when mice were given CyaA* (Figure 3.7.2.1). These results are comparable to those of Betsou *et al.* (1993, 1995) who showed that mice immunised subcutaneously with two doses at a two-week interval of 15μg CyaA and infected intranasally at 2 weeks post-immunisation gave an approximate 1.5-log reduction in lung counts of *B. pertussis*. Similarly, Hormozi *et al.* (1999) demonstrated an approximate 2.5-log reduction when comparing the mean *B. pertussis* lung counts in mice given the active, recombinant CyaA in two doses.

Because the unmodified, toxic form of CyaA would be unlikely to be considered as an antigen for inclusion in an acellular vaccine preparation, subsequent work focused more on the capacity of the non-toxic CyaA* to enhance protection afforded by other known protective antigens of *B. pertussis,* particularly those included in the current ACV's, although CyaA was still included as a comparison. Mice received a single 25μg dose of either CyaA or CyaA* alone or in combination with 0.1 single human dose of a current commercially-available three-component ACV, or the acellular vaccine or WCV (0.025

single human dose) alone as a positive control (Figure 3.8.5). Mice that received PBS or *E. coli* LPS or boiled CyaA plus the ACV were included as control groups. As expected, WCV-immunised mice gave the greatest protection against aerosol challenge of *B. pertussis*, demonstrated by the low lung counts, while those receiving the ACV gave lung counts of approximately 1-log higher. The lung counts in mice given CyaA* or CyaA co-administered with ACV were slightly lower in comparison to those receiving ACV alone. However statistical analysis by the Student's t-test indicated that the results between the ACV-immunised group and the group where ACV was co-administered with CyaA* or CyaA were not statistically significant at either the 5% or 10% level. This showed that in this instance, the addition of either CyaA* or CyaA to the ACV immunisation did not significantly enhance the protection afforded by the ACV alone. However, this work should be repeated as the lung counts from mice immunised with ACV plus LPS or ACV plus boiled CyaA were noticeably higher than those from mice immunised with ACV alone, which would indicate an element of experemental variation that demands further analysis. Indeed, it should be noted that the level of protection afforded by CyaA or CyaA* alone was noticeably lower than that achieved in the previous experiment (Figure 3.7.2.1) with only an approximate half-log reduction in lung counts compared to the PBS-vaccinated control, although this can be explained by only a single vaccination of CyaA in the latter experiment. If, however, CyaA contributed an additive element toward protection against challenge, this would not have been so apparent in this experiment.

Xing *et al.* (2000) reported that results from an aerosol challenge demonstrated that with mice previously given a dose of 120ng of PT (ip), the lung counts were not much lower

when compared to the control group of mice given PBS. However, when PT was administered in combination with either FHA, P.69 or Fims (25μg, 10μg or 10μg respectively) the protection was greatly boosted, indicating that PT is expressing a synergistic effect on other *B. pertussis* antigens when given in small quantities. However, the PT included in the ACV formulations is in the detoxified form and the precise mechanism of interaction between dPT and other antigens still remains to be established.

4.8 PROTECTIVE ACTIVITIES OF CyaA FOLLOWING IMMUNISATION WITH CyaA ALONE OR IN COMBINATION WITH OTHER PERTUSSIS ANTIGENS AGAINST *B. parapertussis* INFECTION

Whooping cough can be caused by either *B. pertussis* or *B. parapertussis*. Ideally a pertussis vaccine should be efficacious against both species and this was tested during the study. Khelef *et al.* (1993) found that, despite a high degree of homology between *B. pertussis* and *B. parapertussis* species, no cross-protection was observed. They used a murine respiratory model to examine the efficacies of single doses of purified P.69, PTd, FHA and CyaA (8μg per dose of PTd, FHA or P.69, or 20μg CyaA) *B. pertussis* and *B. parapertussis* antigens against intranasal infection with *B. pertussis* or *B. parapertussis*. They found that *B. pertussis* antigens administered subcutaneously, protected against *B. pertussis* intranasal infection and similarly *B. parapertussis* antigens were able to protect against *B. parapertussis* infection. However, no cross-protection

was obtained. Willems *et al.* (1998) studied the efficacy of a WCV against *B. pertussis* and *B. parapertussis* infections in a mouse model and showed that vaccination with the Dutch WCV induced protection against a respiratory challenge with either *B. pertussis* or *B. parapertussis*, although the protection against *B. parapertussis* was less pronounced. More recently, Watanabe and Nagai (2001) demonstrated that mice immunised by aerosol infection with either *B. pertussis* or *B. parapertussis* and then allowed to recover for 6 weeks before each group was challenged with a second aerosol infection of either *B. pertussis* or *B. parapertussis*, showed a significant decrease in lung counts from both groups of infected mice two weeks later. This indicates that cross protection between the two species was apparent. Perhaps the reasons for the conflicting results were the different types of immunisation, i.e mice immunised with a bacterial suspension by the aerosol route compared with those immunised with a range of selected antigens administered subcutaneously.

In the present study, protection against an intranasal challenge with *B. parapertussis* was observed following an ip immunisation with a single dose of native PT, FHA and P.69 (120ng, 25μg and 10μg), and further enhanced when 25μg of CyaA* was included with the antigen mixture, as demonstrated by a further one-log reduction in lung counts (Figure 3.8.4). In this case CyaA* is having an additive effect and indicates the potential that CyaA* may have in affording increased protection against the two species. The results differ from those stated by Khelef *et al.* (1993) but there are two main differences in the experiment. Firstly, in this study, CyaA* was purified from the recombinant *E. coli* form and not from *B. pertussis*, although it is considered unlikely that this would account for the different results. Secondly, native PT at a low dose was

used in this immunisation compared to detoxified PT used by Khelef at a higher dose. As previously noted, Xing *et al.* (2000) had shown that native PT at small doses was able to have a synergistic effect on the protective properties of other pertussis antigens against aerosol infection, and this may be the case in this experiment with CyaA*. It would be interesting to repeat this experiment using a higher dose of dPT instead of the native PT or using a commercially available ACV.

4.9 EFFECTS OF CyaA ON NITRIC OXIDE PRODUCTION

NO and reactive nitrogen intermediates have been associated with the antimicrobial functions of macrophages (Ding *et al.*, 1988) and by using NO production as a marker of macrophage activation, Xing *et al.* (2000) have shown that it is possible to identify the relevant components of any vaccine that are responsible for activation of macrophages. Xing *et al.* (1997) demonstrated that macrophage activation might play an important role in the immunity produced by vaccination with whole cell pertussis vaccine. Macrophage and spleen cells taken from mice immunised with WCV were able to respond *in vitro* to selected antigens by synthesis of NO, a signal of macrophage activation. The relationship between NO production *in vitro* and the protection afforded *in vivo* against intra-cerebral challenge indicated that macrophage activation was probably involved in protective immunity and may play a role in controlling intracellular infection of mice caused by *B. pertussis*.

In this study, preliminary work used macrophages taken from mice immunised with the acylated forms of the toxin which were re-stimulated *in vitro* with CyaA or CyaA*. However, little additional increase in NO synthesis was observed in comparison with LPS- or boiled CyaA-immunised mice, control groups used to ensure any observed response was due to the effects of CyaA and not LPS (Figure 3.7.3). These results would imply that macrophage activation does not play a major role in the protective properties afforded by CyaA and suggests that perhaps the humoral side of the immune system is more important, as indicated by the high levels of anti-CyaA antibody. In addition, little NO production was seen by macrophages from mice vaccinated with a PT, FHA, P.69 antigen mixture when stimulated by hkc or CyaA preparations. This is in keeping with the reported lack of stimulation of the CMI response by ACV's. However, when mice received CyaA preparations in combination with native PT, FHA and P.69 antigen mixture, CyaA or CyaA* clearly acted as an adjuvant to enhance the production of NO by the cell-mediated arm of the immune response following *in vitro* stimulation with either *B. pertussis* hkc or CyaA* (Figure 3.8.7). Moreover, immunisation with CyaA* in combination with the PT, FHA, P.69 antigen mixture resulted in greater NO production from macrophages following stimulation with *B. pertussis* hkc or CyaA*, in comparison to immunisation with CyaA plus the antigen mixture. This may be related to the greater potential toxicity of CyaA for cells of the immune system. This can be seen when CyaA was used to stimulate macrophages at a concentration of 2μg/ml which was previously found to be cytotoxic to the cells (Figure 3.5.3.1; 3.5.3.2) and therefore no further enhancement of NO production was observed. These results therefore indicated that addition of CyaA* to the components of the ACV would not only enhance

its protective properties with regard to colonisation, but may also CMI responses in a similar manner to WCV. Canthaboo *et al.* (2001) suggested that CMI may have a pivotal role in activating macrophages to eliminate completely the bacteria that have escaped immune defences mediated by antibodies and this may explain why CyaA or CyaA* had an enhancing effect on protection afforded by dPT, FHA and P.69.

4.10 CYTOKINE mRNA EXPRESSION

Th1 cells are characterised by the secretion of IFNγ, IL-2 and TNFα and are adept at macrophage activation and stimulation of production of immunoglobulin isotypes that mediate antibody-dependent cellular cytotoxicity and complement activation. Such cells have been shown to activate appropriate defence mechanisms against intracellular pathogens. Th2 cells produce IL-4, IL-15 and IL-5 and are involved in the development of humoral immunity for protection against extracellular pathogens. He *et al.* (1998) examined cytokine mRNA expression, by reverse transcription PCR, for IFNγ, IL-2, IL-4 and IL-5 in the peripheral blood mononuclear cells (PBMCs) of schoolchildren and adults after natural infection or booster immunisation with diphtheria-tetanus-trivalent acellular pertussis vaccine (DTaP)(SKB, Belgium). The mRNA transcripts for both Th1 and Th2 cytokines were detected in the PBMC's of the vaccinated subjects and infected individuals after *in vitro* stimulation with *B. pertussis* antigens. These results are in agreement with earlier studies which found that induction of Th1 or Th2 cytokines is a vaccine- and antigen-dependent phenomenon related to the use of either whole cell or

acellular vaccines (Ausiello *et al.*, 1997). McGuirk and Mills (2000) used a murine respiratory-infection model to demonstrate that priming with a Th1 or Th2 vaccine can influence the local inflammatory response and immune effector cells in the lungs following aerosol challenge with *B. pertussis*. It is well known that *B. pertussis* can be taken up by macrophages and PMNs, therefore recruitment and activation of macrophages and PMNs may be a critical element of protective immunity to *B. pertusis* in the respiratory tract.

In this study, a difference was observed in the response of spleen cells after vaccination with the two forms of CyaA. Stimulation of spleen cells from CyaA* immunised mice with heat killed cells produced a cytokine mRNA response typical of a mixed Th1, Th2 profile, in that high levels of IFNγ, IL-2 and IL-4 were produced. On the other hand, CyaA-immunised mice produced a cytokine mRNA response biased towards a Th1 profile with high levels of IFNγ and IL-2 but very little IL-4 production (Figure 3.7.4.2, 3.7.4.3). In contrast, when spleen cells were taken from mice immunised with a combination of CyaA plus a mixture of native PT, FHA and P.69 antigens and stimulated *in vitro* with hkc or CyaA*, the total cytokine response was repressed. The levels of IFNγ, IL-2 and IL-4 all decreased in comparison with those of mice immunised with the antigen mixture alone (Figure 3.8.7.1, 3.8.7.2), which in turn were markedly less than those obtained when CyaA or CyaA* were used as vaccines. The reason for these unexpected results is not clear, as NO production and protection experiments displayed an adjuvant or additive effect respectively when CyaA was co-administered with the other purified *B. pertussis* antigens. The RT-PCR results for cytokine

expression do not represent conclusive data and need to be repeated and corroborated with cytokine determinations by ELISA.

The difference between the two forms of CyaA was also apparent in the levels of IFNγ produced as measured by ELISA. Spleen cells taken from mice immunised with either the enzymically, active or non-active form of CyaA, produced high levels of IFNγ when stimulated *in vitro* with hkc, 46336 and 13568pg/ml respectively, with the greatest level produced from cells taken from mice that had received the enzymically, active form of CyaA (Figure 3.87.3). However, when either form was co-administered with PT there was a clear difference in the levels of IFNγ produced. The non-enzymic form, CyaA*, acted to enhance the levels of IFNγ following co-administration with PT, with IFNγ levels rising from 18368pg/ml in spleen cells taken from mice immunised with PT alone, to 41344pg/ml in cells taken from mice immunised with CyaA* in combination with PT following stimulation *in vitro* with hkc. In contrast, when spleen cells were taken from mice immunised with CyaA plus PT and stimulated with hkc, the level of IFNγ produced decreased to 224pg/ml, a dramatic fall in the amount of IFNγ compared to the amounts when PT was given either alone or in combination with CyaA*. It is likely that both CyaA and PT, when administered together, were acting in an antagonistic manner, as both toxins have a similar mode of action related to cAMP production and therefore must compete with each other, as when either toxin was administered on its own, the levels of IFNγ produced were significantly higher compared to the value obtained when the two were co-administered.

4.11 CONCLUSIONS

The traditional vaccines against whooping cough, containing whole-killed *B. pertussis* cells, have been used successfully to control the disease in most developed countries. However, concerns over the reactogenicity of these vaccines and also the requirement of both humoral and cell-mediated responses, have motivated the development of newer, ACV's. The ACV's, however, are generally not as efficacious as most European whole-cell vaccines. The rationale for the present work was to investigate any benefits that CyaA might have when included in the current ACV's.

It was reported previously that only the enzymically-activate, acylated form of CyaA and not the enzymically-active, non-acylated pro-toxin form was protective when used to immunise mice against intranasal or aerosol challenge. The protective properties of CyaA were further investigated in the present work to show that the enzymic nature of CyaA was not important for protection as long as the toxin was acylated. This was demonstrated by the similar levels of protection afforded by the CyaA preparation regardless of the presence or absence of enzymic activity. Evidence for an adjuvant effect of CyaA on antibody response to OA had been reported but nothing had been described previously about the possible effects that CyaA might exert on immune responses to other *B. pertussis* antigens such as PT, FHA and P.69. This study aimed to investigate the immune responses generated upon immunisation with CyaA or CyaA* preparations, in combination with other *B. pertussis* antigens, and to determine a role, if any, for increased intracellular cAMP levels in target cells by noting any differences in responses when either CyaA or CyaA* was used. The activation of specific cytokines is important in the protection against infection and therefore the ability to bias the response

towards Th1 or Th2 cytokines is undoubtably a key factor in protection. Immunisation of mice with WCV has been demonstrated to selectively prime Th1 cells, whereas immunisation of mice with ACV generates T cells that secrete IL-4 an IL-5, plus low levels of IFNγ, features associated with a Th2 response.

The data presented here depicts a difference in the mRNA cytokine expression profile between immunisation with the two forms of CyaA. Immunisation with CyaA* followed by *in vitro* stimulation of spleen cells with *B. pertussis* heat-killed cells resulted in a mixed Th1/Th2 profile demonstrated by high levels of IFNγ, IL-2 and IL-4, However, immunisation with CyaA followed by the same treatment directed the cytokine response to Th1, indicated by high levels of IFNγ and IL-2, and low levels of IL-4 production. A similar difference between the two forms of CyaA was evident following IFNγ production as measured by ELISA. Immunisation with CyaA, CyaA* or native PT followed by in vitro stimulation with heat-killed cells resulted in high levels of IFNγ. However, when CyaA or CyaA* were co-administered with PT a difference related to the enzymic property of CyaA was apparent. CyaA* acted as an adjuvant towards PT to enhance the levels of IFNγ, whereas the enzymic form of CyaA served to dramatically reduce the amount of IFNγ produced. This difference is likely to be due to the action of the toxins working to increase cAMP levels and possibly having a mutually antagonistic effect upon each other. To further investigate cytokine production and understand the cellular response alternative methods of measurement could be employed. In addition to ELISA, enzyme-linked ELISPOT assay is available for the elucidation of Th1 and Th2 cytokine producing cells. The advantage over ELISA is that it is able to determine the frequency of cytokine producing cells in the single cell

population. Thus the frequency and number of Th1 and Th2 producing cells in different fractions ($CD4^+$ or $CD8^+$) from various tissues can be determined. The best results will undoubtedly come from performing at least two different assays, ELISA, ELISPOT or RT-PCR, thereby confirming the results describing the profile of Th1 and Th2 cytokine responses by immunisation. Alternative methods for measuring macrophage activation could be to calculate the levels of secreted products, such as superoxide or IL-12. In the same way that NO production was measured, superoxide levels could also be detected. A positive control would include macrophage cells that have been stimulated with phorbol-12-myristate 13-acetate (PMA). The release of superoxide from macrophages is tightly regulated, therefore freshly isolated cells produce negligible levels of superoxide in the absence of further stimulus. Bioassays are useful assays to measure bioactive TNFα, also an important regulator of macrophage activation.

Protection against both *B. pertussis* and *B. parapertussis* was enhanced when mice were immunised with CyaA or CyaA* co-administered with a purified pertussis antigen mixture compared to protection obtained with the antigen mixture alone. However the reduction in the number of bacteria in the lungs from mice immunised with CyaA in combination with a commercially-available three-component vaccine, was found not to be statistically significant when compared to the lung counts of those mice receiving the ACV alone. However taken in conjunction with the other experiments, CyaA does have an adjuvant effect on the antibody production to other protective antigens. It also has the effect of boosting NO production by macrophages when used in conjunction with other antigens for immunisation and this potential for boosting the cell-mediated arm of the immune system when used in conjunction with an ACV demands further work.

Moreover, AC enzymic activity does not appear to be essential for protective or adjuvant effects of the toxin, demonstrated by the results of immunisation with CyaA*.

Problems with endotoxin removal led to the necessity of including additional controls of *E. coli* LPS and boiled CyaA in immunological studies. This was not an ideal solution because any observed effects may have been caused by an interaction between LPS and CyaA, and ideally this would have been tested, by adding different amounts of LPS to a pure preparation of CyaA. It would have been preferable to reduce the amount of endotoxin in the crude lysate prior to further purification methods. Ultrafiltration is a a particularly effective method in eliminating large aggregates of up to one million daltons, however, monomers are also present and therefore this method can be ineffective. As endotoxin is negatively charged, the use of positively charged membranes has been employed for its removal. Such membranes include polyamides or bonded amines and in particular polymyxin B which has a very high binding affinity for the lipid A moiety of endotoxin. Unfortunately the levels of endotoxin could not be reduced sufficiently in this study, although additional work has since indicated that endotoxin levels could be further reduced. An alternative method could have been to investigate the potential of producing the recombinant protein strains in a Gram-positive bacterium or yeast, such as *Pichia pastoris*, thereby eliminating the problem of LPS contamination.

In summary, this study has demonstrated that CyaA, both in the enzymic and non-enzymic form, has an adjuvant effect on the antibody response to other protective pertussis antigens and has the potential to enhance protection via the cell-mediated arm of the immune response, an important response to ensure complete bacterial elimination,

in particular those bacteria that have avoided the immune response via a humoral mechanism. It will be of great importance to further investigate these adjuvant properties and take full advantage of the methods available to understand fully the nature of the immune response if CyaA is to be included in a vaccine formulation.

Appendix A: Media composition

A.1 Luria Bertani broth (LB)

Tryptone (BDH Laboratory supplies)	10 g
Yeast extract (BDH)	5 g
Sodium chloride	10 g

Add 1.2 % (w/v) agar for solid media

Made up to 1 litre in distilled water

A.2 SOC medium

Tryptone (BDH)	20 g
Yeast extract (BDH)	5 g
Salts I (250 mM KCL, 1 M NaCl)	10 ml

Made up to 1 litre in distilled water

After autoclaving, add 10ml of 2 M sterile glucose and 10 ml of Salts II (1 M $MgCl_2.6H_2O$, 1 M $MgSO_4.7H_20$)

A.3 Casamino acid solution

NaCl	6 g
Casamino acids (BDH)	10 g

Made up to 1 litre in distilled water, pH to 7.

A.4 Phosphate Buffered Saline (PBS)

NaCl	10.0 g
KCL	0.75 g
$Na_2HPO_4.12H_2O$	1.44 g
KH_2PO_4	0.125 g

Made up to 1 litre in distilled water

Appendix B: DNA Solutions

B.1 TAE Buffer (50X 1 L)

Tris	242 g
EDTA (0.5M, pH 8.0)	100 ml
Glacial acetic acid	57.1 ml

B.2 6 X DNA loading buffer

Tris	60 mM
EDTA	6 mM
Sucrose	40 % (w/v)
Bromophenol blue	0.25 % (w/v)

Appendic C: CyaA expression and purification buffers

C.1 Buffer A

Tris (1 M, pH 8.0)	5 ml
$CaCl_2$ (0.1 M)	0.2ml

Made up to 100 ml in distilled water

C.2 Buffer B

Add 9.6g Urea to 20 ml of buffer A to give an 8 M urea solution

C.3 DEAE Sepharose Chromatography

C.3.1 Buffer 1

Tris	12.12 g

Made up to 100ml, pH to 8.0

C.3.2 Buffer 2

Tris	0.606 g

Made up to 100ml, pH to 8.0

C.3.3 Buffer 3

Tris	0.606 g
NaCl	0.292 g
Urea	48 g

Made up to 100ml, pH to 8.0

C.3.4 Elution protocol

Buffer 3 with adjusted NaCl content,

		Elutant volume
NaCl	250 mM	2 X 1ml
NaCl	500 mM	2 X 1 ml
NaCl	1.0 M	1 X 2 ml
NaCl	1.5 M	1 X 4 ml
NaCl	2.0 M	4 X 4 ml

C.4 Phenyl Sepharose Chromatography

C.4.1 Solution A

Tris	0.303 g
NaCl	2.922 g

Made up to 50 ml, pH to 8.0

C.4.2 Wash Buffer

Tris	0.303 g
NaCl	2.922 g
Urea	6.0 g

Made up to 50 ml, pH to 8.0

C.4.3 Solution B

(1)	Tris	0.303 g
	NaCl	1.461 g
	EDTA	0.037 g
	Urea	6.0 g
(2)	Tris	0.303 g
	NaCl	0.705 g
	EDTA	0.037 g
	Urea	6.0 g
(3)	Tris	0.303 g
	EDTA	0.037 g
	Urea	24.024 g

All made up to 50 ml

Appendix D: Protein analysis solutions

D.1 Separating gel (10 %)

Acrylamide/Bis solution	10 ml
dH_2O	11.05 ml
Tris-HCl (1.5 M, pH 6.8)	7.5 ml
20 % (w/v) SDS	150 µl
TEMED	15 µl
10 % (w/v) Ammonium persulphate (APS)	300 µl

D.2 Stacking gel (6 %)

Acrylamide/Bis solution	4 ml
dH_2O	10.69 ml
Tris-HCl (0.5 M, pH 6.8)	5 ml
20 % (w/v) SDS	100 µl
TEMED	10 µl
10 % (w/v) Ammonium persulphate (APS)	200 µl

D.3 Electrode buffer (10 X)

Tris base	30 g
Glycine	144 g
SDS	10 g

Made up to 1 litre in distilled water, pH to 8.3

D.4 Protein sample buffer

Glycerol	5 ml
20 % (w/v) SDS	2.5 ml
2-mercaptoethanol	0.5 ml
Tris (0.5M pH 6.8)	2.5 ml
Bromophenol blue	0.25 % (w/v)

D.5 Coomassie gel stain

Coomassie blue	0.5 g
Methanol	500 ml
Acetic acid	100 ml
dH_2O	400 ml

For destain, omit the coomassie blue

D.6 Electroblotting buffer

Tris base	7.2 g
Glycine	33.4 g
dH_2O	2400 ml
Methanol	600 ml

pH to 8.0

D.7 Blocking buffer

Skimmed milk	6 g
dH_2O	200 ml
Tween 20	400 µl

D.8 DAB solution

3-3' Diaminobenzidine (DAB)	0.05 g
PBS	98 ml
$CoCl_2$	2 ml
H_2O_2	100 µl

Appendix E: Tissue culture

E.1 RPMI medium

Foetal Calf Serum (Gibco BRL)	50 ml
Glutamine	5 ml
Fungizone (Gibco BRL)	5 ml

Dissolve in 500ml RPMI-1640 medium

E.2 Trypan blue solution

Trypan blue	0.2 g
NaCl	4.25 g

Dissolve each component individually in 100 ml dH_2O, then mix 4 parts trypan blue with 1 part saline

E.3 Greiss reagent

Solution A

0.1 % (w/v) N-[1-naphthyl] ethylenadiamine dichloride in dH_2O

Solution B

1 % (w/v) Sulfanilamide in 5 % H_3PO_4

15 ml of solution A is mixed with 15 ml solution B and kept chilled until use.

Used within 12 h.

Appendix F: CyaA enzymic assays

F.1 Reaction Mixture

Tris-HCl pH 8.0	60 mM
$MgCl_2$	7 mM
$CaCl_2$	0.1 mM
BSA	1mg/ml
Calmodulin	100 nM
non-radioactive cAMP	0.1 mM
tritiated cAMP (Amersham Pharmacia)	0.1mM (50 Ci/nmol)

F.2 Substrate solution

non-radioactive ATP	20 mM
$^{32}\alpha$P-ATP (Amersham Pharmacia)	2mM (3000Ci/nmol)

F.3 Bicine buffer

Bicine	10mM
$Mg(C_2H_3O_3)_2$	1.5 mM
ATP	0.5 mM

pH to 8.0

Appendix G: Immunological assays

G.1 Carbonate Coating Buffer

Na_2CO_3 anhydrous	1.59 g
$NaHCO_3$	2.95 g
NaN_3	0.5 g

Made up to 1 litre in distilled water, pH to 9.5

G.2 Wash Buffer (IgG)

PBS

Tween 20	0.05 %

G.3 Diluent and Blocking buffer

PBS

Tween 20	0.05 %
Foetal Calf Serum	10 %

G.4 Acetate Buffer (X 10)

$NaCH_3COOH.3H_2O$	68.04 g
Glacial acetic acid	1632 µl

Made up to 1 litre in distilled water

G.4.1 Substrate solution

Acetate buffer (X 10)	25 ml
Distilled water	225 ml
3,3'- 5,5' Tetramethyl benzidine dihydrochloride (TMB) in Dimethylsulphoxide (DMSO)	2.5 ml
Hydrogen Peroxide	75 µl

G.5 Blocking buffer (IFNγ)

PBS	
BSA	1 % (w/v)

G.6 Diluent (IFNγ)

PBS	
Tween 20	0.05 % (v/v)
BSA	1 % (w/v)

Appendix H.1a **Anti-CyaA IgG levels**

	2 weeks post immunisation			4 weeks post immunisation		
Log_3 reciprocal dilution	pro-CyaA	CyaA*	CyaA	pro-CyaA	CyaA*	CyaA
2	1.534	0.878	0.821	1.784	1.541	1.634
	1.393	1.017	0.85	1.821	1.982	1.693
	1.450	1.734	1.076	1.242	1.428	1.878
2.477	1.085	0.479	0.269	1.346	0.915	0.973
	0.826	0.465	0.262	1.363	1.713	1.166
	0.900	1.213	0.409	0.696	0.719	1.290
2.954243	0.660	0.246	0.153	0.782	0.409	0.468
	0.438	0.247	0.162	0.78	1.060	0.573
	0.522	0.555	0.180	0.481	0.316	0.678
3.431364	0.400	0.175		0.458	0.152	0.211
	0.309	0.166		0.505	0.428	0.232
	0.325	0.234		0.399	0.176	0.258

	7 weeks post immunisation			9 weeks post immunisation		
Log_3 reciprocal dilution	pro-CyaA	CyaA*	CyaA	pro-CyaA	CyaA*	CyaA
2	1.682	1.685	1.702	1.754	1.566	1.632
	1.717	1.703	1.698	1.687	2.079	1.760
	1.624	2.266	1.869	1.678	2.004	1.893
2.477	1.086	1.240	1.202	1.136	0.915	0.906
	1.453	1.178	1.260	1.085	1.733	1.335
	1.162	1.818	1.569	1.047	1.642	1.580
2.954243	0.622	0.608	0.591	0.546	0.409	0.414
	0.921	0.585	0.678	0.479	1.128	0.710
	0.625	1.174	1.101	0.492	1.095	1.085
3.431364	0.321	0.218	0.168		0.243	0.185
	0.462	0.268	0.180		0.443	0.223
	0.369	0.520	0.429		0.466	0.414

Appendix H.1b **Anti-CyaA IgG levels at 2, 4, 7 and 9 weeks post immunisation**

	Log_3 reciprocol dilution at OD_{450nm} = 0.5		
Time post immunisation	**Pro-CyaA**	**CyaA***	**CyaA**
2 weeks	3.096	2.671	2.536
	3.032	3.117	2.373
	3.398	2.603	2.347
Mean ± s.d	**3.175 ± 0.160**	**2.797 ± 0.228**	**2.419 ± 0.084**
4 weeks	3.368	2.899	2.831
	3.338	3.430	3.174
	2.861	2.975	3.307
Mean ± s.d	**3.189 ± 0.232**	**3.101 ± 0.234**	**3.104 ± 0.201**
7 weeks	3.209	3.131	3.096
	3.427	3.482	3.455
	3.164	3.138	3.137
Mean ± s.d	**3.267 ± 0.115**	**3.250 ± 0.164**	**3.229 ± 0.160**
9 weeks	2.987	3.457	3.178
	2.937	3.449	3.434
	2.938	3.018	2.996
Mean ± s.d	**2.954 ± 0.023**	**3.308 ± 0.205**	**3.203 ± 0.180**

Appendix H.1c **Anti-CyaA IgG levels following boosting at 7 weeks post immunisation**

	7 weeks post immunisation			9 weeks post immunisation		
Log_3 reciprocal dilution	pro-CyaA	CyaA*	CyaA	pro-CyaA	CyaA*	CyaA
2	1.929	1.452	1.978	2.851	3.650	2.301
	1.895	1.347	1.653	2.793	3.136	2.718
	1.78	2.045		2.979	3.176	2.228
2.477	1.497	0.793	1.572	2.339	3.065	2.191
	1.392	0.602	1.03	2.320	2.662	2.353
	1.347	1.737		2.484	2.732	2.016
2.954243	1.053	0.352	0.594	1.925	2.479	1.886
	0.816	0.28	0.385	1.802	2.188	1.953
	0.921	1.185		1.989	2.288	1.682
3.431364	0.63	0.191	0.329	1.596	1.893	1.359
	0.447	0.161	0.164	1.457	1.714	1.458
	0.508	0.247		1.381	1.843	1.097

	11 weeks post immunisation			14 weeks post immunisation		
Log_3 reciprocal dilution	pro-CyaA	CyaA*	CyaA	pro-CyaA	CyaA*	CyaA
2	2.987	2.243	2.327	2.448	2.114	2.306
	2.586	2.214	2.252	2.225	2.266	2.002
	2.414	2.354	2.295	2.502	2.28	2.33
2.477	2.460	2.051	1.832	2.019	1.988	2.088
	2.161	2.049	1.839	1.941	2.164	1.624
	2.083	2.304	2.016	2.099	2.161	2.115
2.954243	1.932	1.927	1.980	1.795	1.863	1.894
	1.733	1.911	1.500	1.636	1.998	0.88
	1.752	2.126	1.785	1.838	1.978	1.954
3.431364	1.405	1.619	1.674	1.308	1.542	1.412
	1.307	1.631	0.791	1.07	1.655	0.351
	1.421	1.915	1.361	1.517	1.646	1.798

Appendix H.1d **Anti-CyaA IgG levels at 7, 9, 11 and 14 weeks post immunisation following boosting at 7 weeks**

	Log_3 reciprocol dilution at OD_{450nm} = 0.5		
Time post immunisation	**Pro-CyaA**	**CyaA***	**CyaA**
7 weeks	3.570	2.938	3.214
	3.334	3.541	3.003
	3.434	2.831	
Mean ± s.d	**3.446 ± 0.097**	**3.103 ± 0.313**	**3.109 ± 0.106**
9 weeks	3.800	4.654	4.901
	3.168	4.874	4.561
	4.082	4.566	4.324
Mean ± s.d	**3.683 ± 0.382**	**4.698 ± 0.130**	**4.595 ± 0.237**
11 weeks	4.250	6.206	6.545
	4.760	6.385	3.822
	4.334	8.062	4.753
Mean ± s.d	**4.448 ± 0.223**	**6.884 ± 0.836**	**5.040 ± 1.130**
14 weeks	4.257	6.285	5.080
	4.359	6.350	3.314
	4.259	6.182	6.249
Mean ± s.d	**4.292 ± 0.048**	**6.272 ± 0.069**	**4.881 ± 1.206**

Appendix H.2: MTT neutralisation data

	Absorbance (540nm)			
Protein concentraion (μg/ml)	**CyaA mean ± sd**	**CyaA + sera from PBS immunised mice mean ± sd**	**CyaA + sera from CyaA* immunised mice mean ± sd**	**CyaA + sera from CyaA immunised mice mean ± sd**
65.00	0.141 ± 0.0015	0.186 ± 0.0140	1.068 ± 0.0695	0.949 ± 0.0330
32.50	0.117 ± 0.0055	0.155 ± 0.0035	0.523 ± 0.038	0.756 ± 0.0570
16.25	0.122 ± 0.0050	0.140 ± 0.0028	0.190 ± 0.0015	0.450 ± 0.0225
8.13	0.159 ± 0.0300	0.147 ± 0.0070	0.169 ± 0.0206	0.166 ± 0.0035
4.06	0.168 ± 0.0130	0.172 ± 0.0012	0.153 ± 0.0115	0.126 ± 0.0025
2.03	0.226 ± 0.0120	0.136 ± 0.0030	0.140 ± 0.0030	0.119 ± 0.0000
1.02	0.672 ± 0.0480	0.139 ± 0.0051	0.134 ± 0.0005	0.170 ± 0.0085
0.51	1.034 ± 0.0410	0.146 ± 0.0042	0.132 ± 0.0007	0.114 ± 0.0035
0.25	1.147 ± 0.1240	0.143 ± 0.0085	0.121 ± 0.0010	0.117 ± 0.0000
0.13	1.277 ± 0.2100	0.147 ± 0.0068	0.135 ± 0.0035	0.061 ± 0.0005
J774.2 cells	1.243 ± 0.022			

Appendix H.3 Nitric oxide levels

Immunisation group	no stimulation mean ± sd	+ hkc mean ± sd	+ CyaA* mean ± sd	+ CyaA mean ± sd
PBS	3.260 ± 0.31	11.310 ± 1.52	4.240 ± 0.38	3.140 ± 0.38
LPS	1.830 ± 0.22	15.250 ± 1.53	5.230 ± 0.78	5.080 ± 1.65
Boiled CyaA	6.340 ± 2.28	12.590 ± 0.13	6.520 ± 5.81	3.931 ± 1.14
CyaA*	2.055 ± 0.19	11.281 ± 1.13	3.715 ± 0.41	4.505 ± 1.15
CyaA	2.525 ± 0.16	8.510 ± 1.32	6.290 ± 0.45	5.795 ± 1.12
PT,FHA,P.69	4.605 ± 0.90	13.445 ± 2.75	8.695 ± 1.68	5.905 ± 0.26
LPS+PT,FHA,P.69	4.295 ± 0.60	19.230 ± 2.77	17.575 ± 2.45	10.865 ± 1.91
CyaA*+PT,FHA,P.69	6.370 ± 0.52	44.362 ± 1.72	35.783 ± 1.49	16.077 ± 2.27
CyaA+PT,FHA,P.69	8.957 ± 1.38	29.420 ± 2.27	26.880 ± 4.08	17.400 ± 2.42

Appendix H.4 % β-Actin intensity

Immunisation group	+ hkc			+CyaA*		
	IL-2	IL-4	IFNγ	IL-2	IL-4	IFNγ
PBS	248.76	201.72	74.30	89.91	65.38	63.85
LPS	76.88	69.65	37.80	107.46	79.21	78.32
CyaA*	554.49	472.46	395.72	82.85	75.32	86.53
CyaA	491.99	373.85	85.44	428.85	402.46	367.94
PT, FHA, P.69	243.65	156.90	172.02	238.67	272.83	225.23
LPS+ PT, FHA, P.69	86.04	73.21	59.98	158.04	162.21	141.58
CyaA*+ PT, FHA, P.69	159.90	103.14	61.62	121.60	111.15	114.02
CyaA+ PT, FHA, P.69	114.22	88.63	97.26	128.78	94.47	20.32

Appendix H.5a **Anti-CyaA IgG results**

Log_3 reciprocal dilution	Immunisation groups			
	CyaA	**CyaA***	**CyaA+ PT, FHA, P.69**	**CyaA*+ PT, FHA, P.69**
2	2.239	1.978	2.003	1.954
	2.342	1.928	2.198	2.239
	2.155	2.039	1.634	1.919
2.477	1.790	1.291	1.378	1.300
	1.761	1.063	1.791	1.861
	1.487	1.239	0.874	1.453
2.954243	1.012	0.568	0.656	0.768
	0.997	0.491	0.768	1.236
	0.797	0.56	0.425	0.875
3.431364	0.673	0.210	0.211	0.356
	0.641	0.179	0.356	0.814
	0.431	0.230	0.089	0.571

Appendix H.5b Anti-CyaA IgG results

Immunisation Group	Log_3 reciprocol dilution at $OD_{450nm} = 0.5$	Mean ± s.d
CyaA	3.481	**3.545 ± 0.062**
	3.629	
	3.525	
CyaA*	3.004	**2.968 ± 0.038**
	2.916	
	2.984	
CyaA + PT,FHA,P.69	3.076	**3.071 ± 0.175**
	3.283	
	2.854	
CyaA* + PT,FHA,P69	3.248	**3.471 ± 0.194**
	3.721	
	3.443	

Appendix H.6.1: anti-PT IgG data

Immunisation groups	Log_3 reciprocal dilution	Reference	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
PT alone	**2**	1.079	2.286	2.209	2.323	1.873	2.465
	2.477	0.804	1.945	1.871	1.972	1.624	2.106
	2.954243	0.402	1.605	1.533	1.621	1.375	1.747
	3.431364	0.191	1.264	1.195	1.27	1.126	1.388
PT + CyaA*	**2**	1.079	2.081	1.957	1.874	1.995	2.03
	2.477	0.804	1.759	1.658	1.545	1.688	1.72
	2.954243	0.402	1.437	1.36	1.216	1.381	1.413
	3.431364	0.191	1.115	1.061	0.899	1.074	1.104
PT + FHA + CyaA*	**2**	1.34	2.231	2.198	2.708	2.208	1.989
	2.477	0.993	1.867	1.879	2.295	1.87	1.594
	2.954243	0.485	1.507	1.516	1.888	1.486	1.185
	3.431364	0.265	1.162	1.298	1.469	1.281	0.828
PT + P.69 + CyaA*	**2**	1.203	2.603	3.495	3.111	2.855	3.223
	2.477	0.899	2.189	2.979	2.653	2.419	2.758
	2.954243	0.403	1.773	2.463	2.194	1.983	2.292
	3.431364	0.19	1.358	1.947	1.736	1.547	1.826
PT + FHA + P.69 + CyaA*	**2**	1.203	3.178	2.836	3.277	2.603	2.565
	2.477	0.899	2.709	2.415	2.81	2.212	2.185
	2.954243	0.403	2.24	1.994	2.344	1.821	1.804
	3.431364	0.19	1.771	1.573	1.877	1.429	1.424
Boiled CyaA* + PT, FHA, P.69	**2**	1.845	3.11	3.071	2.979	3.085	3.276
	2.477	1.375	2.6	2.625	2.542	2.619	2.787
	2.954243	0.742	2.1	2.178	2.105	2.153	2.299
	3.431364	0.354	1.568	1.732	1.629	1.656	1.81

Appendix H.6.2 anti-FHA IgG data

Immunisation groups	Log_3 reciprocal dilution	Reference	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
FHA alone	2	1.1241	0.476	0.523	0.793	0.781	0.277
	2.477	0.97	0.283	0.341	0.491	0.496	0.179
	2.954243	0.836	0.182	0.24	0.288	0.264	0.129
FHA + CyaA*	2	1.439	1.898	2.105	2.235	2.722	1.787
	2.477	0.912	1.515	1.656	1.862	2.265	1.496
	2.954243	0.482	1.163	1.26	1.489	1.785	1.167
	3.431364	0.234	0.707	0.654	1.114	1.398	0.836
	3.908485		0.397	0.363	0.745	0.866	0.401
FHA + PT + CyaA*	2	1.445	1.995	1.884	1.861	2.016	1.956
	2.477	1.085	1.645	1.595	1.569	1.596	1.58
	2.954243	0.574	1.324	1.21	1.204	1.427	1.342
	3.431364	0.29	0.813	0.772	0.767	0.895	0.967
FHA + PT + P.69 + CyaA*	2	1.683	1.467	1.837	1.56	1.83	1.927
	2.477	1.085	1.214	1.45	1.293	1.511	1.594
	2.954243	0.574	0.96	1.096	1.025	1.192	1.261
	3.431364	0.29	0.726	0.613	0.779	0.895	0.967
Boiled CyaA* + PT,FHA,P.69	2	1.508	0.6	0.961	1.091	1.021	1.008
	2.477	1.094	0.404	0.645	0.771	0.609	0.574
	2.954243	0.647	0.289	0.455	0.582	0.404	0.403
	3.431364	0.329	0.192	0.297	0.339	0.243	0.243

Appendix H.6.3 anti-P.69 IgG data

Immunisation groups	Log_3 reciprocal dilution	Reference	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
P.69 alone	**2**	1.766	0.23	0.555	0.172	0.18	0.181
	2.477	1.537	0.185	0.413	0.148	0.11	0.154
	2.954243	1.044	0.14	0.271	0.124	0.1	0.127
	3.431364	0.529	0.095	0.129	0.1	0.091	0.1
P.69 + CyaA*	**2**	1.932	1.618	1.489	2.055	1.75	1.75
	2.477	1.537	0.886	0.801	1.612	1.291	1.196
	2.954243	1.044	0.448	0.391	1.287	0.785	0.659
	3.431364	0.529	0.231	0.212	0.746	0.383	0.331
P.69 + PT + CyaA*	**2**	2.271	1.239	1.541	1.936	1.623	1.784
	2.477	1.796	0.414	0.793	1.507	1.128	1.081
	2.954243	1.273	0.227	0.363	0.841	0.544	0.522
	3.431364	0.639			0.395	0.255	
P.69 + PT + FHA + CyaA*	**2**	2.271	1.64	0.997	1.738	0.857	0.835
	2.477	1.796	0.91	0.293	1.015	0.295	0.315
	2.954243	1.273	0.426	0.156	0.473	0.159	0.156
	3.431364	0.639	0.196		0.242		
Boiled CyaA* + PT,FHA,P.69	**2**	2.115	1.728	0.855	1.123	1.712	1.873
	2.477	1.475	0.962	0.309	0.379	0.783	1.262
	2.954243	0.763	0.513	0.172	0.21	0.367	0.681
	3.431364	0.366	0.26			0.21	0.325

Appendix H.6.1b Anti-PT IgG levels

	Log_3 reciprocol dilution at OD_{450nm} = 0.5					
Immunisation group	**Mouse 1**	**Mouse 2**	**Mouse 3**	**Mouse 4**	**Mouse 5**	**Mean ± sd**
PT alone	4.613	4.440	4.502	4.631	4.478	**4.535 ± 0.076**
PT + CyaA*	4.011	4.365	4.328	4.324	4.342	**4.274 ± 0.132**
PT + FHA+CyaA*	4.310	4.349	4.555	4.542	3.818	**4.315 ± 0.267**
PT +P.69+CyaA*	3.367	4.769	4.720	4.577	4.790	**4.445 ± 0.544**
PT + FHA+P.69+CyaA*	4.724	4.647	4.839	4.565	4.590	**4.673 ± 0.099**
Boiled CyaA* + PT,FHA,P.69	4.344	4.276	4.381	4.600	4.171	**4.354 ± 0.142**

Appendix H.6.2b Anti-FHA IgG levels

	Log_3 reciprocol dilution at OD_{450nm} = 0.5					
Immunisation group	**Mouse 1**	**Mouse 2**	**Mouse 3**	**Mouse 4**	**Mouse 5**	**Mean ± sd**
FHA alone	0.511	1.872	2.032	2.522	2.502	**1.888 ± 0.734**
FHA + CyaA*	3.751	3.707	4.220	4.316	3.840	**3.967 ± 0.251**
FHA + PT +CyaA*	3.881	3.767	3.828	4.045	4.417	**3.988 ± 0.234**
FHA +PT +P.69+CyaA*	3.856	3.603	3.930	4.045	4.108	**3.908 ± 0.176**
Boiled CyaA* + PT,FHA,P.69	2.256	2.909	2.983	2.846	2.826	**2.764 ± 0.260**

Appendix H.6.3b Anti-P.69 IgG levels

	Log_3 reciprocol dilution at OD_{450nm} = 0.5					
Immunisation group	**Mouse 1**	**Mouse 2**	**Mouse 3**	**Mouse 4**	**Mouse 5**	**Mean ± sd**
P.69 alone	0	0	0	0	0	**0**
P.69 + CyaA*	3.022	2.970	3.753	3.287	3.197	**3.246 ± 0.278**
P.69 + PT +CyaA*	2.596	2.800	3.320	3.110	2.953	**2.956 ± 0.249**
P.69 +PT +FHA+CyaA*	3.006	2.457	3.064	2.931	2.836	**2.859 ± 0.215**
Boiled CyaA* + PT,FHA,P.69	3.075	2.401	2.551	2.795	3.206	**2.906 ± 0.304**

Appendix H.7.1: anti-PT IgG1 data

Immunisation groups	Log_3 reciprocal dilution	Reference	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
PT alone	2	1.409	1.447	1.894	2.052	1.810	1.764
	2.477	0.657	1.1118	1.430	1.354	1.072	1.155
	2.954243	0.258	0.838	1.079	1.048	1.057	0.896
	3.431364	0.191	0.416	0.559	0.446	0.473	0.402
PT + CyaA*	2	1.409	1.172	2.490	1.938	1.274	2.233
	2.477	0.657	0.952	1.948	1.487	1.065	1.729
	2.954243	0.258	0.673	1.421	1.111	0.608	1.250
	3.431364	0.191	0.438	0.815	0.432	0.218	0.665
PT + FHA + CyaA*	2	1.113	2.325	2.859	2.325	2.052	1.698
	2.477	0.409	1.726	2.278	2.202	1.362	0.918
	2.954243	0.1886	1.063	1.479	1.458	0.542	0.425
	3.431364		0.649	0.855	0.777	0.242	0.127
PT + P.69 + CyaA*	2	1.218	2.342	2.121	1.453	3.990	2.060
	2.477	0.618	1.637	1.369	1.123	3.232	1.651
	2.954243	0.225	1.077	0.788	0.874	2.474	1.020
	3.431364		0.731	0.382	0.479	1.791	0.669
PT + FHA + P.69 + CyaA*	2	1.218	1.719	2.361	2.046	1.904	2.020
	2.477	0.618	0.937	1.863	1.534	1.440	1.422
	2.954243	0.225	0.785	1.459	1.086	0.982	1.109
	3.431364		0.326	1.033	0.383	0.502	0.368
Boiled CyaA* + PT, FHA, P.69	2	1.209	1.859	1.606	1.382	1.260	1.800
	2.477	0.651	1.503	1.307	1.146	1.026	1.460
	2.954243	0.274	1.267	0.836	0.675	0.636	0.887
	3.431364		0.651	0.413	0.365	0.307	0.424

Appendix H.7.2: anti-FHA IgG1 data

Immunisation groups	Log_3 reciprocal dilution	Reference	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
FHA alone	2	1.018	0.44	0.363	0.381	0.228	0.46
	2.477	0.693	0.365	0.309	0.261	0.206	0.299
	2.954243	0.545	0.29	0.255	0.141	0.184	0.138
FHA + CyaA*	2	1.018	0.981	1.532	1.303	1.151	0.809
	2.477	0.693	0.874	1.329	1.101	0.958	0.702
	2.954243	0.545	0.779	1.127	0.885	0.722	0.492
	3.431364		0.593	0.868	0.705	0.511	0.377
FHA + PT + CyaA*	2	1.052	0.798	1.032	1.18	1.111	0.948
	2.477	0.696	0.662	0.917	0.827	0.928	0.811
	2.954243	0.418	0.538	0.637	0.594	0.764	0.629
	3.431364	0.252	0.346	0.484	0.4	0.591	0.417
FHA + PT + P.69 + CyaA*	2	1.052	1.005	0.984	1.068	0.791	0.998
	2.477	0.696	0.777	0.74	0.83	0.676	0.813
	2.954243	0.418	0.617	0.631	0.588	0.533	0.631
	3.431364	0.252	0.356	0.376	0.365	0.351	0.456
Boiled CyaA* + PT,FHA,P.69	2	0.928	0.819	0.543	0.681	0.618	0.554
	2.477	0.779	0.706	0.477	0.575	0.479	0.47
	2.954243	0.62	0.496	0.352	0.467	0.342	0.319
	3.431364	0.45	0.379	0.281	0.314	0.285	0.268

Appendix H.7.3: anti-P.69 IgG1 data

Immunisation groups	Log_3 reciprocal dilution	Reference	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
P.69 alone	2	1.400	0.09	0.09	0.09	0.09	0.11
	2.477	0.681					
	2.954243	0.252					
P.69 + CyaA*	2	1.400	1.559	1.292	1.622	1.058	1.503
	2.477	0.681	0.894	0.617	1.205	0.406	0.856
	2.954243	0.252	0.463	0.354	0.696	0.213	0.460
	3.431364	0.283	0.213	0.359	0.120		
P.69 + PT + CyaA*	2	2.094	1.242	1.195	1.628	1.60	1.646
	2.477	1.231	0.591	0.731	0.957	0.962	1.025
	2.954243	0.723	0.231	0.452	0.521	0.468	0.578
	3.431364	0.18	0.098	0.399	0.347	0.128	
P.69+ PT + FHA + CyaA*	2	2.094	1.280	1.302	0.777	0.415	0.419
	2.477	1.231	0.596	0.636	0.428	0.338	0.329
	2.954243	0.723	0.407	0.383	0.3	0.283	
	3.431364						
Boiled CyaA* + PT,FHA,P.69	2	1.535	0.247	0.224	0.261	0.200	0.184
	2.477						
	2.954243						
	3.431364						

Appendix H.7.1b Anti-PT IgG_1 levels

	Log_3 reciprocol dilution at OD_{450nm} = 0.5					
Immunisation group	**Mouse 1**	**Mouse 2**	**Mouse 3**	**Mouse 4**	**Mouse 5**	**Mean ± sd**
PT alone	3.364	3.430	3.566	3.305	3.324	**3.398 ± 0.094**
PT + CyaA*	3.099	3.309	3.441	3.608	3.720	**3.435 ± 0.219**
PT + FHA+CyaA*	2.983	3.135	3.504	3.765	3.674	**3.412 ± 0.304**
PT +P.69+CyaA*	3.441	3.263	3.560	3.553	4.254	**3.614 ± 0.337**
PT + FHA+P.69+CyaA*	3.202	3.438	3.385	3.377	3.997	**3.480 ± 0.271**
Boiled CyaA* + PT,FHA,P.69	3.167	3.247	3.352	3.368	3.729	**3.373 ± 0.193**

Appendix H.7.2b Anti-FHA IgG_1 levels

	Log_3 reciprocol dilution at OD_{450nm} = 0.5					
Immunisation group	**Mouse 1**	**Mouse 2**	**Mouse 3**	**Mouse 4**	**Mouse 5**	**Mean ± sd**
FHA alone	1.527	0.790	1.618	1.823	0	**1.152 ± 0.673**
FHA + CyaA*	4.277	3.899	3.458	3.878	3.009	**3.704 ± 0.434**
FHA + PT +CyaA*	2.993	3.257	3.379	3.179	3.680	**3.298 ± 0.229**
FHA +PT +P.69+CyaA*	3.002	3.148	3.308	3.143	3.167	**3.154 ± 0.097**
Boiled CyaA* + PT,FHA,P.69	2.262	2.426	2.752	3.027	2.255	**2.544 ± 0.301**

Appendix H.7.3b Anti-P.69 IgG_1 levels

	Log_3 reciprocol dilution at OD_{450nm} = 0.5					
Immunisation group	**Mouse 1**	**Mouse 2**	**Mouse 3**	**Mouse 4**	**Mouse 5**	**Mean ± sd**
P.69 alone	0	0	0	0	0	**0**
P.69 + CyaA*	2.544	2.958	2.910	3.017	3.358	**2.957 ± 0.260**
P.69 + PT +CyaA*	2.767	3.063	3.121	2.997	2.798	**2.949 ± 0.142**
P.69 +PT +FHA+CyaA*	0.100	2.010	2.598	2.600	0	**1.827 ± 1.026**
Boiled CyaA* + PT,FHA,P.69	0	0	0	0	0	**0**

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