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Purification, formulation and characterisation of the adenylate cyclase toxin of *Bordetella pertussis*

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Presented for the degree of Doctor of Philosophy

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2006

Abstract

Adenylate cyclase toxin (CyaA) toxin is an important virulence factor of *Bordetella pertussis*, the causative agent of whooping cough, and a potential component of acellular pertussis vaccine. The work involved the production of three purified forms of CyaA with different enzymic and invasive properties. These were: the native enzymatically-active, invasive toxin (CyaA), an invasive derivative lacking AC enzymic activity (CyaA*) and a non-acylated, non-invasive form of CyaA (proCyaA). These were expressed in *E. coli* BL21/DE3 as recombinant proteins. After purification by a combination of chromatographic methods (Q-and Butyl-Sepharose) their properties were investigated by several assays. The LPS content of these preparations was very low. The AC enzymic activity was assayed by a conductimetric method. CyaA and pro-CyaA had a high level of enzymic activity but that of CyaA* was very low. The cytotoxic properties of CyaA* and pro-CyaA towards J774.2 cells were similar and were 100-fold less than that of CyaA, but these differences were shown to some extent to be Ca^{2+} -dependent for CyaA*. Caspase 3/7 activities were measured over a range of toxin concentrations. At these concentrations, neither urea buffer alone nor CyaA* induced any significant increase in caspase 3/7 from different mammalian cells. The greatest effect of CyaA was observed on J774.2 and RBL-2H3 cells where increasing concentration of toxin gave increasing activity. Different concentrations of CyaA and CyaA* were used to investigate dose-dependent effects of the toxins on phagocytosis and the oxidative burst in U937 human monoblastic cells, J774.2 mouse macrophage-like cells and fresh human granulocyte cells (whole blood used). Significant effects were seen with CyaA on both phagocytosis and oxidative burst, but CyaA* did not have a significant effect on either. The results of this first part of the study showed that both

enzymatic and invasive functions are required for the cytotoxic effects of adenylate cyclase toxin.

In the second part of the work, CyaA was formulated as protein-coated microcrystals (PCMCs) on the surface of microcrystals of DL-valine. The aims of this formulation were to remove the urea, normally used to stabilize the protein, and to determine the stability of the enzymic and cytotoxic activities of the protein in this form, as a dry powder and when the PCMCs were redissolved in aqueous solution. The CyaA in the PCMCs was shown not to be readily soluble in aqueous buffers, but could be resolubilised in urea buffer and retained high AC and cytotoxic activity. Many different types of PCMC formulation were prepared in attempts to increase solubility of PCMCs in aqueous solution. PCMCs were made with CyaA coprecipitated with different combination of CaM, BSA, $CaCl_2$ or ATP and crystals were dissolved in different buffers at various pHs. The most promising results were obtained with CyaA-CaM-BSA-PCMCs where the highest levels of both AC enzymic and cytotoxic activities were seen when the PCMCs were dissolved in 100mM Bicine (pH 8). CaM alone preserved only the AC enzymic activity of PCMCs when coprecipitated with CyaA and DL-valine. AC and cytotoxic activities of CyaA were stable in PCMCs for up to a week at 37°C.

CyaA-CaM-BSA-PCMCs induced a strong serum IgG response to CyaA and BSA when injected subcutaneously into mice. The results indicated that CyaA-CaM-BSA-PCMCs offer a promising way to preserve the activity and antigenicity of CyaA in a non-aqueous formulation. Neutralisation tests proved that the antibody generated from mice immunised with CyaA-BSA-CaM-PCMCs was able to neutralise the AC enzymic and cytotoxic activities. Such PCMCs could have application for presentation of protein antigens that normally require cold storage for stability.

Acknowledgments

This work would not have been possible without the financial support of The Ministry of Health and Education Sciences of Iran. I am especially indebted to Dr. John Coote and Dr. Roger Parton who worked actively to provide me with the protected academic time to pursue my highest academic degree. As my teachers and mentors, they have taught me more than I could ever give them credit for here. They have shown me, by their example, what a good scientist/ person should be.

I am grateful to Dr. Marie-Claire Parker with whom I have had the pleasure to work during my PhD.

I wish to thank Dr. Barbara Orr for her useful help during the first and the second year of my PhD.

I would especially like to thank Prof T.H. Birkbeck as an assessor.

I am also grateful to Dr A.R Rezaee, Susan Baillie and Denise Candlish for their cooperation during the course of my PhD. Other members of Infection and Immunity Division have also provided me with extensive personal and professional guidance and taught me a great deal about both scientific research and life in general.

Nobody has been more important to me in the pursuit of my PhD than the members of my family. I would like to thank my family, whose love and guidance are with me in whatever I pursue. They are the ultimate role models. Most importantly, I wish to thank my loving and supportive wife, Nasrin, and my three wonderful children, Shahin, Shohreh and Shahrokh, who provide unending inspiration.

Abbreviations

AC	Adenylate cyclase
ACV	Acellular vaccine
Amp	Ampicillin
ATP	Adenosine 5' –triphosphate
Bp	Base pair
BG	Bordet Gengou
BSA	Bovin serum albumin
Bvg	*Bordetella* virulence gene
CaM	Calmodulin
Chlor	Chloramphenicol
CMI	Cell-mediated immunity
CTL	Cytotoxic T lymphocyte
CyaA	Adenylate cyclase toxin
kDa	Kilo Daltons
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DNT	Dermonecrotic toxin
dPT	Detoxified pertussis toxin
DTPa	Diphtheria tetanus pertussis acellular vaccine
ELISA	Enzyme-linked immunoabsorbent assay
FCS	Foetal calf serum
FHA	Filamentous haemagglutinin
Fim	Fimbriae
mg/µg	Milligrams/ micrograms
h	Hours
Kb	Kilobase
KDO	2-keto-3-deoxyoctusonic acid
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IPTG	Isopropyl-1-thio-β-D-galactoside
ml/µl	Milliliters/microlitres
LAL	*Limulus amoebocyte* Lysate
LB	Luria-Bertani
LCMV	Lymphocytic choreomeningitis virus
LPS	Lipopolysaccharide
M/mM	Molar/millimolar
MHC	Major histocompatibility complex
min	Minutes
mRNA	Messenger RNA
MTT	3-(4,5-di-methylthiol-2-3yl)-2,5-diphenol tetrazolium bromide
MWT	Molecular weight
NO	Nitric oxide
OA	Ovalbumin
OD nm	Optical density at wavelength
OMP	Outer membrane protein
ON	Overnight
PRN	Pertactin

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMN	Polymorphonuclear leukocyte
P/S	Penicillin/streptomycin
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
SDS	Sodium dodecyl sulphate
TAE	Tris/ Acetic acid/EDTA buffer
TBE	Tris/Boric acid/ EDTA buffer
TCF	Tracheal colonisation factor
TCR	T cell receptor
v/v	Volume by volume
vag	Virulence activated gene
vrg	Virulence repressed gene
w/v	Weight by volume
WCV	Whole cell vaccine

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Chapter 1

Introduction

1-1 The genus *Bordetella*

There are nine named species of the genus *Bordetella, B. pertussis, B. parapertussis, B. bronchiseptica, B. avium, B. holmesii, B. trematum, B. hinzii, B. petrii* and *B. ansorpii.* They are mostly obligatory parasites and inhabit the surface of the respiratory tract of humans and warm-blooded animals, including birds (Roberts and Parton, 2001; Ko *et al.*, 2005). *B. pertussis* infects only humans and causes the acute respiratory disease known as whooping cough (Yeh *et al*, 2003). *B. parapertussis* strains can be divided into two genetically distinct types, those which infect humans causing a pertussis-like disease and those which cause respiratory infection in sheep (Cullinane *et al.*, 1987). *B. bronchiseptica* is a common respiratory tract coloniser in wild and domesticated mammals where it generally causes chronic and persistent respiratory disease and asymptomatic infection (Woolfrey and Moody, 1991). It has also been occasionally described as a cause of pneumonia and bacteraemia in humans (Reina, 1991; Dorittke *et al.*, 1995; Gueirard *et al.*, 1996). *B. avium* causes an upper respiratory tract infection called bordetellosis in turkey poults and other birds but it has rarely been reported to cause infection in humans (Vandamme *et al.*, 1995). *B. hinzii* was proposed as the species for some strains isolated from poultry with respiratory disease, but the aetiological role of the organism was unclear. Recently it was reported as being a causative agent of septicaemia (Vandamme *et al.*, 1995; Kattar *et al.*, 2000). *B. holmesii* has been isolated from human sputum and nasopharyngeal specimens from patients with symptoms similar to pertussis. Recently, *B. holmesii* was isolated from a patient with sickle cell anaemia (Tang *et al.*, 1998; Mazengia *et al.*, 2000; Njamkepo., *et al* 2000).

B. trematum contains isolates from human wound and ear infections but never from the respiratory tract and was described originally as atypical *Bordetella* or unidentified (Vandamme *et al.,* 1996). von Wintzingerode ***et al.*** (2001) described a novel *Bordetella* species which was isolated from an anaerobic, dechlorinating bioreactor culture enriched from river sediment. Comparative 16S rDNA sequence analysis and DNA-DNA hybridisation experiments demonstrated that *B. petrii* was the first member of the genus *Bordetella* to be isolated from the environment. It is capable of anaerobic growth and also has the ability to reduce selenate to selenium. Ko *et al.* (2005) have reported a Gram-negative bacillus (SMC-8986^T) which was isolated from an epidermal cyst. Genotypic and phenotypic characteristics of this bacterium with regard to 16S rRNA sequence, analyses of cellular fatty acid composition and two protein-coding genes (*ris*A and *omp*A) led to the proposal that SMC-8986^T should be recognised as a member of the *Bordetella* genus, *B. ansorpii*.

1-2 The species *Bordetella pertussis*

Before the introduction of the genus *Bordetella* by Moreno (1952), the first three recognised species were grouped in the genus *Haemophilus*. Later, after several changes of name, it was classified in its own genus, *Bordetella*, which was named in honour of Jules Bordet. In 1640, the French physician De Baillou noted an epidemic with the characteristic symptoms of whooping cough in Paris in 1587 and made the first clear description of the disease. Although the actiological agent had probably been observed independently by several physicians during the last decades of the 19th century, the modern history of the disease clearly started in 1904 with the cultivation of the organism by Bordet and Gengou (Bordet and Gengou, 1906). In 1931, before vaccination against whooping cough was introduced, a standard textbook of Bacteriology stated that the disease might be one of the major causes of death in

civilised countries. Now it remains a ubiquitous, serious infectious disease causing, world-wide, an estimated 40 million cases and 360,000 deaths each year, 90 percent of which occur in developing countries (Ivanoff and Robertson, 1997). *B. pertussis* is a small (0.2 to 0.5 × 1μm), non-motile Gram-negative coccobacillus. It is strictly aerobic with an optimal growth temperature of 35-37° C, having a respiratory metabolism (Porter *et al.*, 1994). Although *B. pertussis* is the most fastidious of the *Bordetella* genus, the organism can be cultured in a buffered simple salts medium containing several amino acids, an organic sulphur source, and some growth factors such as nicotinic acid (an essential vitamin for the micro-organism), L-glutamic acid and ascorbic acid (Rowatt, 1957; Stainer, 1988). Essential amino acids are L-glutamic acid and one of the sulphur containing amino acids such as L-cysteine. Supplementation of the medium with heptakis (2,6-O-dimethyl) β-cyclodextrin and casamino acids not only improves the growth but also greatly enhances the yield of surface antigens such as pertussis toxin (PT), fimbriae (FIM), filamentous haemagglutinin (FHA) and adenylate cyclase toxin (CyaA) in the culture supernatant (Hozbor *et al.*, 1994).

1-2-1 Clinical significance

B. pertussis is a strict human pathogen, and there is no evidence for the existence of an animal or environmental reservoir (Cotter and Miller, 2001; Cherry and Heininger, 2004). It colonises the human respiratory tract and is transmited through the aerosolised droplets produced by coughing. Whooping cough is acute, with severe coughing which can progress to vomiting, convulsions, coma and death (Mattoo and Cherry, 2005). After an incubation period of 7-14 days, it manifests itself initially in the new host as a cold or mild cough, with little fever (Hewlett, 1997). The clinical course of whooping cough can be divided into three stages, 1) catarrhal, 2) paroxysmal and 3) convalescent. The first stage is characterised by an irriating cough, slight fever and sneezing, lasting

for 1-2 weeks (Friedman, 1988). Coughing becomes more prominent in the paroxysmal stage with disjointed attacks, whooping and vomiting lasting 2 to 6 weeks (Cherry *et al.*, 1988). The convalescent stage is marked by decreased paroxysms, persistent cough and secondary bacterial infections. Severe consequences of pertussis infection include encephalopathy, irreversible brain damage, and pneumonia. Moreover, physical damage such as the rupture of the frenulum of tongue, rectal prolapse and inginal hernia can result from paroxysmal coughing (Hewlett, 1995; 1997). Pertussis may occur at any age, but most cases of serious disease and the majority of fatalities are observed in early infancy (Edwards *et al.*, 1999). In adults, the main manifestation is a persistent paroxysmal cough, which is largely unresponsive to treatment. Typical pertussis occurs mainly during primary infection in unimmunized children, but 25 to 30% of such children present with mild or atypical pertussis. They may not be suspected of suffering from pertussis but will spread the disease. Also, adults play an important role in transmission (Isacson and Trollfors, 1993). Secondary infections, such as pneumonia and otitis media, may accur during pertussis particularly in infants, resulting from impairment of clearance mechanisms by *B. pertussis* (Cherry, 1996; Hewlett, 2000).

1-2-2 Epidemiology of *B. pertussis*

It is believed that the transmission of *B. pertussis* occurs via acrosol droplets expelled by severe coughing that pass directly from the respiratory tract of infected individuals to those of susceptible hosts (Hewlett, 1997). Before the introduction of vaccines and in a population in which immunisation is not performed, pertussis is one of the ten most common causes of death and is an epidemic disease with cycles every 2-5 years. Although the incidence has decreased significantly since the widespread use of vaccination, outbreaks of pertussis occur periodically (Cattaneo *et al.*, 1996). It appears that *B. pertussis* infections are endemic in adults and adolescents even when they have

mild or unrecognised disease, therefore these infections might serve as the source of the epidemic cycles involving unvaccinated children (Rappuoli, 1994; Black, 1997). Now that acellular vaccines are available, it may be possible to give booster immunization to adults to attempt to eliminate the reservoir of infection (Cherry, 1997).

1-2-3 Diagnosis

Isolation of *B. pertussis* from clinical specimens is the gold standard for the diagnosis of pertussis (Muller *et al.*, 1997). The standard laboratory method for the diagnosis of *B. pertussis* infection is culture of the organism from a nasopharyngeal swab. Clinical diagnosis of *B. pertussis* is often possible due to the characteristic and prolonged paroxysmal coughing in the typical disease. However, clinical features tend to vary with age and immune status, in adolescents and adults (Hallander *et al.*, 1999). Unfortunately, the catarrhal stage of the pertussis resembles a common cold, meaning that diagnosis may not be possible until the second stage (paroxysmal stage). Laboratory methods include direct detection of *B. pertussis* from nasopharyngeal aspirates and then by culture (Muller *et al.*, 1997). PCR assay has been used as an additional method for detecting and differentiation of *B. pertussis* and *B. parapertussis*. This method is highly sensitive and more rapid than culture to detect *B. pertussis* in specimens containing only a few cells (Aoyama *et al.*, 1997; Sloan *et al.*, 2002). PCR is particularly useful for pertussis diagnosis in pre-vaccinated infants. A number of serological tests, such as agglutination, complement fixation, fluorescent antibody test and the ELISA are available for diagnosis of *B. pertussis* (Hallander, 1999). The success of culture and serology is limited, especially among pre-vaccinated and recently-vaccinated children. In pre-vaccinated infants with whoop and less than 2 weeks of cough, PCR testing should be implemented promptly (Bamberger *et al*, 2005).

1-2-4 Treatment

Despite a dramatic reduction in the incidence of pertussis caused by universal immunization, outbreaks still continue to occur in countries with a high level of vaccine coverage. Treatment of infection with antibiotics may improve symptom severity during the catarrhal phase of pertussis but has no effect on established paroxysms, vomiting, or apnea if given during the paroxysmal or convalescent phases (Langley *et al*, 2004). The treatment for pertussis is primarily supportive, with nursing supervision during the paroxysmal and convalescent stages of illness. Antimicrobial therapy of *B. pertussis* established that several antibiotics are effective. Erythromycin is recommended for treatment of pertussis to prevent transmission of infection, but it is poorly tolerated because of gastrointestinal side effects. In addition, chloramphenicol, trimethoprim-sulphamethoxazole and tetracycline are effective. However, antibiotic treatment has limited value because the illness is usually unrecognised during the peak of contagiousness (Hoppe, 1996). It has been found recently that azithromycin is as effective as erythromycin estolate for the treatment of pertussis in children. Gastrointestinal adverse events were much more common with erythromycin treatment than azithromycin (Langley *et al.*, 2004).

1-3 Regulation of virulence

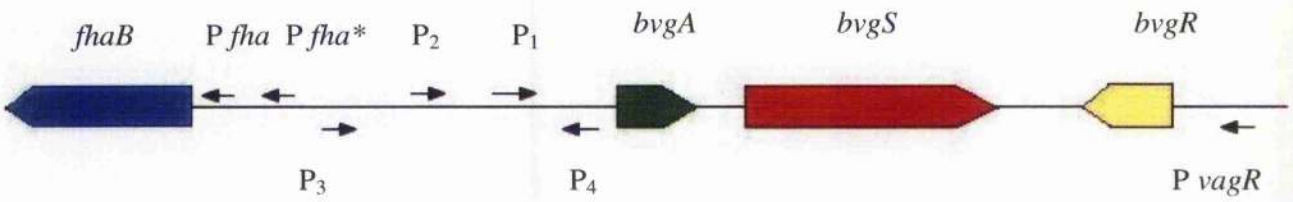
It was recognised that certain members of the *Bordetella* genus share a genetic locus *Bvg*AS that encodes a biological switch enabling them to mediate transition between different phases (Bvg^+, Bvg^i and Bvg^-) that are characterised by distinct patterns of gene expression. Although in the laboratory BvgAS activity can be inhibited by low temperature (<25°C), nicotinic acid and sulphate ions, the true signals sensed in nature or in the mammalian hosts are unknown (Deora *et al*, 2001; Mattoo *et al*, 2001). The BvgAS signal transduction system controls a highly-regulated programme of gene

expression in response to environmental stimuli. This regulatory cascade mediates the co-ordinated expression of almost all of the known or suspected colonisation and virulence factors currently associated with the infectious cycle of *Bordetella*. Studies on the *bvg*AS locus have shown that its transcript is controlled by a 350 bp DNA sequence having four promoters (Figure 1.1) promoters P_1, P_2 and P_3 are responsible for the transcription of the *bvg* locus, whereas the fourth, P_4 directs the synthesis of an antisense RNA of unknown function (Scarlato *et al*, 1990). P_{FHA} promoter, located near the *bvg* locus is responsible for the transcription of the adhesion factor FHA of *B. pertussis*. P *fha** has also been shown to express low levels of the FHA precursor protein, FhaB, *in vitro* (Boucher *et al.*, 1997).

BvgA and BvgS are members of a group of two-component regulatory signal-transducing proteins that communicate by a four-step His-Asp-His-Asp phosphorelay. According to the studies of Cotter and Jones (2003) and Deora (2004), phosphorylated BvgS can phosphorylate BvgA, which is then able to activate or repress transcription of the cognate genes. Akerley and Miller (1993) demonstrated that whilst BvgAS is activated, *Bordetella* are in the Bvg^+ phase, characterised by the expression of a number of Bvg-activated protein factors, including the adhesins and toxins encoded by the *vag* genes (Bvg-activated genes). They also reported that inactivation of BvgAS by modulating signals or by mutation results in the transition to the Bvg^- phase. In this phase, the *vag* genes are repressed and *vrg* genes (Bvg-repressed genes) are expressed. The role of the Bvg^i phase in *Bordetella* infection is unclear but it has been suggested that this intermediate phase might be involved in aerosol transmission (Cotter and Miller, 1997).

Figure 1.1 Schematic representation of *BvgAS* of *B. pertussis*

Schematic representation of the genetic region comprising the *bvg* operon and the *fha*B gene. The genes encoding BvgA, BvgS, BvgR and FhaB are shown (coloured boxes) and the direction of transcription from each promoter is shown by arrows (Adapted from Cotter and Allison 2003)


fhaB
P fha
P fha*
P2
P1
bvgA
bvgS
bvgR
P3
P4
P vagR

BvgS is a 135 kDa periplasmic sensor protein which resides in the cytoplasmic membrane and autophosphorylates using the γ-phosphoryl group of ATP (Boucher *et al*, 1994). It is often termed an unorthodox sensor protein because it contains a number of extra functional domains. BvgS consists of a periplasmic input domain and several cytoplasmic domains, the linker, transmitter, receiver and the C-terminal domain (Roy *et al.*, 1990; Uj and Miller, 1995). It was shown that deletion of these extra domains made BvgS non-functional. BvgA, a response regulator, is a 23-kDa cytoplasmic protein comprising a N-terminal receiver and a C-terminal output domain, which contains a helix-turn-helix (HTH) DNA binding motif. The transfer of a phosphate group from the C-terminal output domain of BvgS activates BvgA. A heptameric sequence-specific BvgA binding site, TTTCCTA, first proposed by Roy and Falkow (1990), is present as either direct or inverted repeats upstream of various *bvg*-regulated genes. These heptameric repeats are involved in the binding of BvgA to virulence-activated promoters, as shown by DNase I protection studies (Boucher and Stibitz, 1995; Marques and Carbonetti., 1997). Merkel and Stibitz (1998) recognised that the *bvgA* gene product is responsible for repression of the bvg-repressed genes. A locus required for expression of repressor activity was identified, designated as *bvgR,* that is located immediately downstream of the *bvgS* gene (Merkel *et al.*, 1995). The BvgR protein was shown to be responsible for the regulation of the *vrg* genes whose functions are unknown. It was speculated that the *bvg*R locus might be involved in the establishment or persistence of *B. pertussis* in the host (Merkel *et al.*, 1998). The results from studies with a *bvg*R-defective mutant strain indicated that *bvg*R-mediated regulation of gene expression contributed to respiratory infection of mice. A recent study demonstrated that expression of BvgR is activated by binding of phosphorylated BvgA to the *bvg*R promoter (Merkel *et al.*, 2003).

A second two-component regulatory system has been identified in *B. bronchiseptica*, which is required for the expression of certain *bvg*-repressed genes. This locus was designated *risAS* due to its association with reduced intracellular survival of *B bronchiseptica* within eukaryotic cells (Jungnitz *et al.*, 1998). Recently, it was found that RisA was essential for expression of *vrg*-repressed genes and that the RisA protein binds to the promoter region of both the *vrg6* and *vrg18* genes (Croinin *et al.*, 2005), but that, the *risS* gene does not encode a functional RisS protein (Stenson *et al.*, 2005).

1-4 Virulence factors of *Bordetella pertussis*

B. pertussis produces several potential virulence factors which play an important role in pathogenesis. These factors can be grouped into two major categories: adhesins, such as filamentous haemagglutinin, pertactin and fimbriae, and toxins, such as pertussis toxin, adenylate cyclase toxin, dermonecrotic toxin and tracheal cytotoxin (Figure 1.2). The adhesins and toxins act in concert to establish infection. The bacteria bind to ciliated epithelial cells in the upper respiratory tract. Once attachment is initiated, toxins produced by the bacterium enable colonisation to proceed by interfering with host clearance mechanisms (Goodwin and Weiss, 1990; Smith *et al.*, 2001). Other virulence factors of *B. pertussis*, which include LPS and the products of serum resistance loci, also need to be considered. As Kerr and Matthews (2000) observed, the pathophysiological sequence consists of attachment, evasion of host defences, local effects and systemic effects. Our understanding of *Bordetella* virulence at the molecular level has led to the development of new acellular vaccines against whooping cough. In the following pages, the properties of *B. pertussis* virulence factors will be explained.

Figure 1.2 Virulence factors of *B. pertussis*

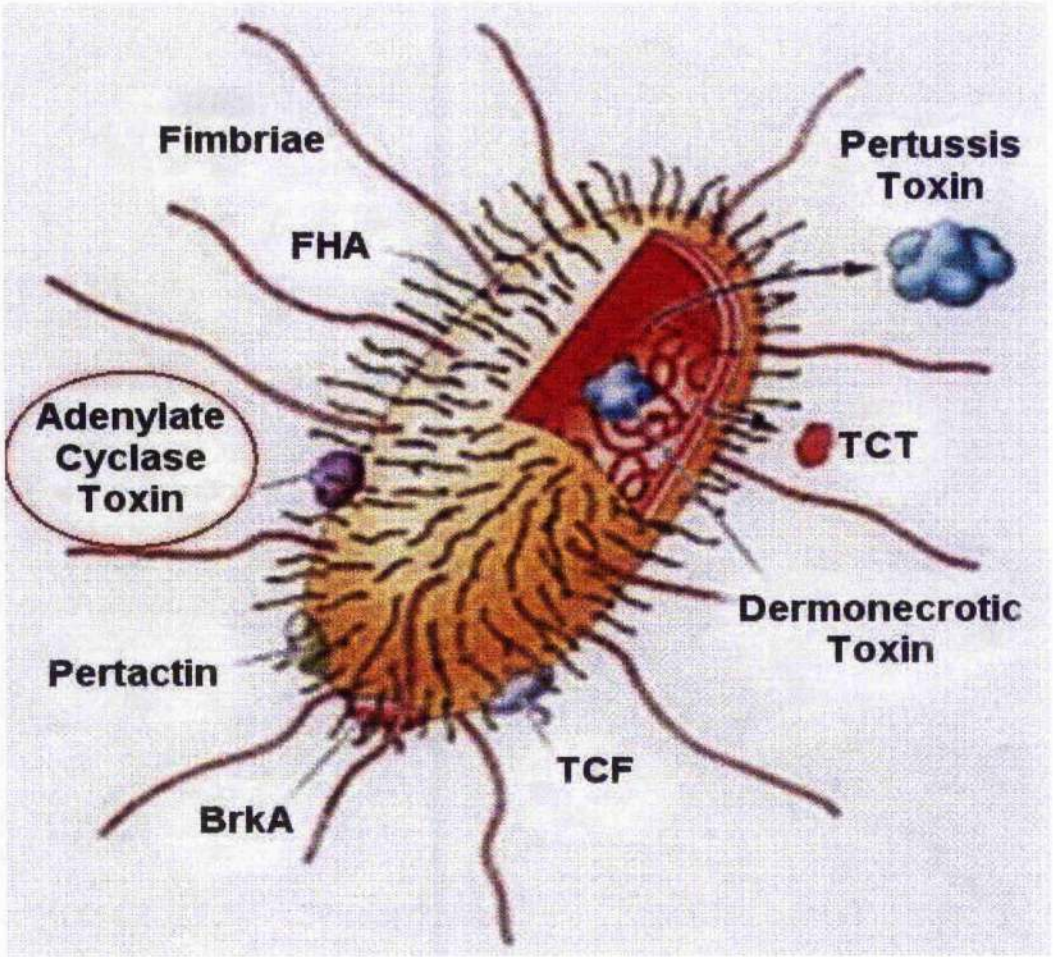
Some important virulence factors of *B.pertussis* are shown in the Figure and their properties are described in the text pages (Adapted from Weiss, 1997).

FHA: filamentous haemagglutinin

TCF: tracheal colonisation factor

BrkA: Bordetella resistance to killing protein

TCT: tracheal cytotoxin


Fimbriae
FHA
Pertussis Toxin
Adenylate Cyclase Toxin
TCT
Dermonecrotic Toxin
Pertactin
TCF
BrkA

1-4-1 Filamentous haemagglutinin

The major adhesion protein is FHA, a 220kDa protein expressing at least three different binding activities 1) carbohydrate binding, 2) heparin sulphate binding and 3) integrin binding via an RGD site which comprises the amino acid sequence Arg-Gly-Asp (Locht *et al.,* 1993). Carbohydrate binding mediates attachment to ciliated cells present in the respiratory epithelium that is the main binding site for *B. pertussis* in the respiratory tract (Tuomanen and Weiss, 1985). FHA also binds to sulphated carbohydrates of sulphatides and peptidoglycans on the surface of epithelial cells or in the extracellular matrix (Brennan *et al.,* 1991; Menozzie *et al.*, 1994). The heparin binding activity of FHA may allow the bacterium to adhere to non-ciliated cells as demonstrated using WiDr (human colon carcinoma cell), Hela and Vero cells (Hazenbos, *et al*, 1993; 1995). The FHA tail region contains an RGD sequence, which is present in a number of other bacterial adhesins and is a region of attachment of many eukaryotic integrin-binding proteins. It has been realised that it is this RGD tripeptide which facilitates binding to the integrin receptor CR3 which might allow entry of *B. pertussis* into phagocytes without an oxidative burst (Relman *et al;* 1990).

FHA is initially produced as a large 367 kDa precursor FhaB, which undergoes both amino-terminal (Lambert-Buisine *et al.,* 1998) and carboxy-terminal maturation (Renauld-Mongenie *et al.,* 1996). FHA is present on the surface of *B. pertussis* and is also secreted into the medium in liquid culture (Roberts and Parton, 2001). FHA is also associated with the outer membrane of the bacterial surface where it is thought to be responsible for autoagglutination of the bacteria by FHA-FHA homotypic interaction (Menozzi *et al.,* 1994). The synthesis of FHA depends on an outer-membrane-associated accessory protein named FhaC (Jacob- Dubuission and Hamel, 1999). FhaC is a pore-forming outer-membrane protein that interacts with FhaB, allowing for

secretion of the FhaB. An FhaC mutant exhibited a slight, transient defect in colonisation of the nasal cavity and trachea of mice (Geuijen *et al.*, 1997).

FHA is also highly immunogenic in humans and is a protective antigen in animal models (Shahin *et al.*, 1992; Amsbaugh *et al.*, 1993; Cahill *et al.*, 1993). McGuirk and Mills (2000) demonstrated that interaction of FHA with receptors on macrophage results in suppression of synthesis of the pro-inflammatory cytokine, interkeukin-12 (IL-12), via an IL-10 dependent mechanism. These data reveal a role for FHA in facilitating persistence by curbing protective Th1 immune responses (Zaretzky *et al.*, 2002). FHA along with CyaA was recently shown to be necessary to inhibit monocyte-dependent T-cell proliferation in vitro (Boschwitz *et al.*, 1997). A recent study showed that a direct physical association can occur between CyaA toxin and FHA. It was suggested that FHA might play a role in CyaA toxin retention on the surface of *B. pertussis* and raises the possibility of an involvement of adherence mediated by FHA in delivery of AC toxin from the bacteria to the target cells (Zaretzky *et al.*, 2002). Furthermore, as a dominant adherence factor it may play a role in human host species tropism and receptor recognition.

1-4-2 Fimbriae (FIM)

B. pertussis has two antigenetically distinct types of fimbriae, features of serotype 2 and serotype 3 strains, each of which are made up of major and minor subunits and are believed to cause persistency of infection (Locht *et al.*, 1992; Willems *et al.*, 1992). The main body of each fimbria is composed of a major subunit protein, Fim2 or Fim3 also known as agglutinogens (AGG2 and AGG3), of 22.5 kDa or 22 kDa encoded by unlinked chromosomal loci *fim2* and *fim3*, respectively (Livey *et al.*, 1987). The same 40 kDa minor fimbrial protein, FimD, is present at the tip of both types of fimbriae

(Geuijen *et al.*, 1997). Different strains of *B. pertussis* express fim2 and fim3 singly or together or not at all. A third unlinked locus, *fim*X, is expressed only at very low levels in *B. pertussis* and recently a fourth fimbrial locus, *fim*N was identified *in B. bronchiseptica* (Kania *et al,* 2000). It was reported that a fifth gene, *fim*A, was identified *in B. parapertussis* and *B. bronchiseptica,* located immediately upstream of the fimbrial biogenesis operon *fimBCD* and 3' of *fha*B, that is expressed and capable of encoding a fimbrial subunit type, FimA (Boschwitz *et al.*, 1997).

The major fimbrial subunits Fim2 or Fim3 bind to sulphated sugars which are ubiquitous in the respiratory tract, whereas the minor subunit FimD shows affinity for VLA-5, an integrin that is found on macrophages (Geuijen *et al.,* 1996). Fimbriae stimulate agglutinating antibodies and there is evidence that agglutination elicited by whole-cell vaccines is correlated with immunity against pertussis in humans (Medical Research Council 1954). Binding of fimbriae to monocytes results in activation of CR3 (complement receptor type3), the monocyte receptor of FHA, reflecting their synergistic action during infection (Menozzi et al., 1991). *In vivo* studies have shown that Fim$^-$ *B. pertussis* strains are unable to multiply in the nasopharyx and trachea of mice (Mooi *et al.,* 1992; Geuijen *et al.*, 1997). It has been shown that fimbriae are protective immunogens in mice and that a five-component acellular *B. pertussis* vaccine containing Fim2 and Fim3 is very efficacious in the human infant (Jones *et al.,* 1995; Steffen *et al.,* 1996; Willems *et al.,* 1998). Another study showed that when vaccine containing Fim2, Fim3, PT, FHA and PRN was compared with vaccine containing PT, FHA and PRN the first vaccine displayed significantly greater efficacy (Olin *et al,* 1997). It has been shown by Mattoo *et al.* (2000) that fimbriae are essential components for persistence of *B. bronchiseptica* in the trachea in mouse and rat models.

1-4-3 Pertactin (PRN)

Autotransporters are outer-membrane proteins which are secreted by gram-negative bacteria. They consist of an N-terminal signal sequence, to direct translocation across the inner membrane, a passenger domain to be delivered to the cell surface and a C-terminal transporter domain that is proposed to mediate secretion across the outer membrane. A number of related surface-associated proteins belong to the autotransporter secretion family (Henderson and Nataro, 2001). PRN was the first member of this family identified and characterised in *Bordetella* and its expression is under the control of the *bvg* locus. It is partly responsible for the adhesion of the bacteria to host cells (Montaraz, *et al*, 1985; Gotto *et al.*, 1993).

The PRN gene (*prn*) of *B. pertussis* encodes a large precursor of 93.5 kDa which is processed at both the N-and C-terminal ends to generate mature PRN. PRN was originally referred to as P69 or 69kDa protein, a size determined by SDS-PAGE analysis. Similar molecules are produced as proteins of 68 and 70-kDa by *B. bronchiseptica* and *B. parapertussis* respectively (Kobisch and Novotny, 1990). PRN is an outer membrane protein and one of the agglutinins of *B. pertussis* which has also been observed to contribute to the binding to target cells, at least in part by its RGD sequence (Leininger *et al.,* 1991). Studies on Chinese hamster ovary (CHO) cells using purified PRN in the presence of synthetic peptides containing RGD sequences showed that these three residues were critical for cell adhesion as the synthetic peptides decreased the ability of PRN to adhere to the cell surface. A mutant deficient in PRN was 30-40% less adhesive than wild type *B. pertussis* to HeLa and CHO cells (Leininger *et al.*, 1991).

PRN is one of the few *B. pertussis* proteins that exhibit antigenic variation, and antigenic divergence between vaccine strains and clinical isolaties has been observed in

a number of vaccinated populations (Mooi *et al.*, 1998; Mastrantonio *et al.*, 1999). According to Hewlett (1997), 3-component acellular vaccines containing FHA, PT, and PRN have been shown to be significanttly more effective than 2-component vaccines with FHA and PT. Serological analysis has shown that pertactin is highly immunogenic in human infants and also may be the most important component when used in acellular pertussis vaccines (Cherry *et al.*, 1998; Hewlett, 1998; Storsaeter *et al.*, 1998). It has been reported to protect mice against aerosol challenge with *B. pertussis* when adminstered parenterally or intranasally (Roberts *et al* 1992; 1993).

1-4-4 Bordetella resistance to serum killing protein (BrkA)

The autotransporter protein BrkA is expressed as a 103kDa precursor that is processed during secretion to yield a 73kDa N-terminal α-domain and a 30kDa C-terminal β-domain. It shows some similarities to PRN, possessing two RGD motifs, an outer membrane localisation signal, a proteolytic cleavage site. The RGD sequences, as with PRN, have a role in adhesion to epithelial cells and invasion of eukaryotic cells (Fernandez and Weiss, 1994). After translocation, the α-domain is tightly associated with the bacterial surface and it is not detected in *B. pertussis* culture supernatants whereas the β-domain is isolated from *B. pertussis* outer membrane fractions (Passerini *at al.*, 1999; Oliver and Fernandez, 2001). BrkA has been proposed to play a role in serum resistance and contribute to the adherence of *B. pertussis* to target cells *in vitro* and *in vivo*. The protein also protects against lysis by certain classes of antimicrobial peptides. However, it is not required for serum resistance of *B. bronchiseptica* (Rambow *et al.*, 1998). There is no information on the ability of BrkA to function as a protective immunogen and it is not included in any current acellular vaccines. It has been shown that a recombinant form of the β-domain of BrkA is able to create channels in artificial membranes (Oliver *et al.*, 2003).

1-4-4-1 Other autotransporters

On the basis of amino acid similarity, two proteins, Tcf and Vag8, have been classified as autotransporter proteins. Tracheal colonisation factor (Tcf) is a virulence-associated factor expressed only in *B. pertussis*. The protein is encoded by the *tcf* gene and the derived amino acid sequence produces a 68kDa protein which contains a RGD sequence and which, after cleavage of a prokaryotic signal sequence, would give a 64kDa protein. The C-terminal (30kDa) domain of the protein has 50% identity to the C-terminal end of pertactin (Finn and Stevens, 1995). The exact role of Tcf is not clear but a mutant lacking Tcf had reduced ability to colonise the mouse trachea compared with the parent strain. Hence, Tcf appears to be a unique virulence factor of *B. pertussis*. Finn and Stevens (1995) first described the Vag8 protein. The 95kDa autotransporter shows homology with the C-terminal ends of PRN, BrkA and Tcf. This protein appears to be involved in type III secretion in *B. bronchiseptica* (Finn and Amsbaugh, 1998).

In addition to Tcf and Vag8 proteins, *Bordetella* autotransporter C (BapC) is a newly identified member of the autotransporter family. The *B. pertussis bapC* gene sequence was originally submitted to GenBank as a putative autotransporter protein gene (accession number AF081494) named *bap5* (Blackburn, 2000) and later submitted independently as *bapC* (AJ277634). BapC is predicted to be 79.5kDa. No N-terminal signal sequence was detected but the C-terminal domain is characteristic of the autotransporter protein family (Blackburn, 2000). Removal of this domain would yield a mature passenger protein of 49 kDa and the predicted protein sequence also has a RGD motif. It was found to be regulated by the *bvg* locus (Bokhari, 2002) and is predicted to be also encoded by *B. parapertussis* and *B. bronchiseptica* (Parkhill *et al.*, 2003). This protein has properties in common with the other members of the

autotransporter family, in particular with BrkA (Bokhari, 2002). The potential role of BapC was identified using a *B. pertussis* BapC mutant and, like BrkA, it also appears to confer serum resistance to *B. pertussis* and act as an adhesin to host cells. However, to date, no information on the protective abilities of BapC have been reported.

1-4-5 Tracheal cytotoxin

Gram-negative bacteria produce a disaccharide tetrapeptide monomer of peptidoglycan. However, only *B. pertussis* and *Neisseria gonorrhoeae* have been reported to release this fragment extracellularly, in the form of muramyl peptides (Cookson *et al*, 1989). Tracheal cytotoxin (TCT) is a muramyl peptide and is the most recently discovered of the *Bordetella* toxins. It is produced by at least 4 species *(B. pertussis, B. parapertussis, B. bronchesiptica* and *B. avium*) (Goldman *et al.*, 1988). It is therefore an unusual, low molecular weight toxin (Cookson *et al.*, 1989). The toxin targets the ciliated epithelial cells in the respiratory tract, probably by inhibiting DNA synthesis and inducing the production of interleukin-1 (IL-1α), which causes an increase in body temperature and nitric oxide production in hamster tracheal epithelial (HTE) cells (Heiss *et al.*, 1993; Masure *et al.*, 1992). It is proposed that, *in vivo,* the toxin activates host cell nitric oxide synthesis and the resulting high levels of nitric oxide diffuse to epithelial ciliated cells which are much more susceptible to its damaging effects (Heiss *et al.*, 1994; Flak and Goldman, 1996;). It is involved in initiation of disease and has the ability to cause ciliostasis (restriction of the free movement of cilia) and the killing and extrusion of the ciliated epithelial cells from the mucosa (Masure, 1992). Such effects could well explain some of the pathological events in pertussis infection such as stimulation of excessive mucus production in the airways, coughing, the persistence of respiratory tract abnormality in pertussis long after elimination of bacteria and tendency to secondary bacterial infections (Wardlaw and Parton, 1988). It also has a toxic effect on

other cells, impairing neutrophil function at low concentrations and causes cytotoxicity when present in large quantities (Cundell *et al.*, 1994). Other studies indicated that endotoxin of *B. pertussis* plays an important role, in association with TCT, to damage the airway epithelium. Endotoxin and TCT were synergistic in induction of IL-1α and production of nitric oxide (Flak and Goldman, 1999; Flak *et al.*, 2000). Moreover, although TCT could just be termed a breakdown product of peptidoglycan manufacture, it is neverthless considered an integral component in the pathogenesis of pertussis infection (Smith *et al.*, 2001).

1-4-6 Lipopolysaccharide (LPS) or endotoxin

Different LPS molecules are expressed by different *Bordetella* spp. which might be a factor in the high level of species specificity within this genus (van den Akker, 1998). The endotoxin of *B. pertussis*, like that of some other bacterial pathogens, is generally referred to as a lipooligosaccharide (LOS) (Brodeur *et al.*, 1993). *B. pertussis* LOS lacks O-antigen side-chains, and is therefore more similar to rough LPS type, whereas the LPS produced by *B. bronchiseptica* is a smooth type and is similar to that of *B. parapertussis*, as both express temperature-dependent O antigen. They show chemically and immunologically identical O-polysaccharide structures, but their core oligosaccharide components differed. The core oligosaccharide of *B. parapertussis* was chemically distinct from the core of *B. bronchiseptica* that appeared to be structurally and immunologically similar to a core oligosaccharide of *B. pertussis* (Di Fabio *et al.*, 1992).

B.pertussis actually produces two distinct LPS molecules which resolve as two bands (A&B) on silver-stained SDS-PAGE (Peppler at al, 1984; Lasfargues *et al.*, 1993). The B band consists of a lipid A molecule linked via a single keto-deoxy-octulosonic acid (KDO) residue to a branched oligosaccharide core sructure containing glucose, heptose,

glutamic acid, glucosamine and galactosaminuronic acid (Caroff *et al.*, 1988; 1990). Band A consists of band B plus a trisaccharide consisting of *N*- acetyl-N-methylfucose, 2,3 di-*N*-acetyl-mannosaminuronic acid and *N*-acetyl glucosamine. Several studies have reported that the *wlb* locus, composed of 12 genes (*wlbA-wlbL*), is required for the biosynthesis and assembly of the band A trisaccharide and, from mutational analyses, certain functions have been assigned to these genes (Preston *et al.*, 2002). In *B. pertussis*, deletion of *wlb* locus leads to the expression only of the B band, indicating that these genes are necessary for the addition of the trisaccharide. In *B. parapertussis* and *B. bronchiseptica*, these mutations result in the loss of both the band A and the O antigen (probably due to loss of the core acceptor site), leaving a lipid A-inner core molecule (Allen *et al.*, 1998; Peterson and Maskell., 2001).

Both LPS molecules of *B. pertussis* can activate the alternative complement pathway and stimulate cytokine release. However, the role which LPS plays in the pathogenesis of *Bordetella* infection is unclear (Amano *et al.*, 1990; Watanabe *et al.*, 1990). It has been demonstrated that *B. pertussis* endotoxin can act synergistically with tracheal cytotoxin in the whooping cough syndrome to induce inflammation in the respiratory mucosa and damage to ciliated epithelial cells (Flak and Goldman, 1999; Flak *et al.*, 2000). Also LPS has a role in inducing immunity to pertussis infection. Nevertheless, it is probably the main cause of the reactogenicity of the whole-cell pertussis vaccine and one of the aims of developing an acellular vaccine was to eliminate this toxin and reduce significantly the reactogenic side effects of the vaccine (Cherry *et al.*, 1988).

1-4-7 Dermonecrotic toxin (DNT)

DNT is a cytoplasmic component and it was one of the first toxins described in *B. pertussis*. DNT toxin is completely inactivated by heating at 56°c for 10 min and for

this reason it was originally termed heat-labile toxin (HLT) (Livey and Wardlaw, 1984). DNT causes necrotic skin lesions, reduced weight gain and spleen atrophy in mice. DNT is responsible for mouse lethality when injected intravenously at high doses (Endoh *et al.,* 1986; 1990). The DNTs of three subspecies of *Bordetella* (*B. pertussis*, *B. parapertussis* and *B. bronchiceptica*) are much more highly related genetically and biologically than the *B. avium* DNT (Walker and Weiss, 1994). It has been reported that DNT inhibits the elevation of alkaline phosphatase activity and reduces the expression of type-I collagen in an osteoblast-like cell line and was also found to stimulate DNA and protein synthesis in these cells without cell division, leading to polynucleation (Horiguchi *et al.*, 1989). The genes for DNT of *B. pertussis* and *B. bronchiseptica* have been cloned and sequenced and the ORF of DNT shown to consist of 4395 bp coding for 1464 amino acids (aa) with isoelectric point of 6.63 (Pullinger *et al.*, 1996). Kashimoto *et al* (1999) demonstrated that cysteine in the catalytic domain at position 1305 is an essential amino acid for the enzymatic activity of dermonecrotic toxin. Horiguchi *et al.* (1997) showed that DNT catalyzes the deamidation of Gln-63 of RhoA and converts it into Glu. They concluded that the deamidated RhoA bearing Glu-63 becomes constitutively active and stimulates the formation of actin stress fibres in DNT-treated cells.

The exact role of DNT in whooping cough is still unclear. It seems that the DNT is not required for lethal infection in the infant mouse model, also suggesting that DNT does not play an important role in virulence for humans (Gueirard and Guiso, 1993). However, the production of DNT by *B. bronchiseptica* strains may play an important role in their ability to produce turbinate lesions and pneumonia in neonatal piglets (Roop *et al.*, 1987).

1-4-8 Pertussis toxin (PT)

PT is a major colonising factor and a protein exotoxin produced uniquely by *B. pertussis*. It remains cell-bound as well as being released into the extracellular environment. It is an AB_5 toxin of 106-kDa consisting of an A subunit (S1) and a complex B pentamer comprising S2-S5 in a ratio of 1:1:2:1 (Saukkonen *et al*, 1992). Each subunit is synthesised with an N-terminal signal sequence that allows transport of the PT subunits into the periplasmic domain via the general export pathway. The five structural genes for S1-S5 are followed by nine *Ptl* (pertussis toxin liberation) genes (*ptlA-ptlI*) which encode the secretion complex for export from the cell and are co-transcribed from a single operon, regulated by the Bvg regulatory system (Gross and Rappuoli, 1988, Kotob *et al.*, 1995). It has been demonstrated that the A subunit and B oligomer have different biological activities *in vivo*. The enzymatically-active A subunit (S1) ADP-ribosylates the alpha subunit of several heterotrimeric G proteins in mammalian cells and the B oligomer binds to unidentified glycoconjugate receptors on cells (Katada *et al*, 1983; Alonso *et al*, 2001). *In vitro*, the combination of the two subunits could induce the toxin activities exhibited by native PT which shows toxicity in a CHO cell clustering test and haemagglutination assay (Sheu *et al.*, 1997). PT is involved in a number of activities such as histamine sensitisation, lymphocytosis promotion, and insulin secretion *in vivo* (Weiss *et a.l*, 1984). Recent research by Carbonetti *et al.* (2003) shows that the PT acts as colonisation factor and it was found that its enzymic activity rather than its cell binding activity was necessary for mouse model colonisation. When the S1 subunit ADP-ribosylates the G proteins, it prevents inhibitory signals to the adenylate cyclase complex, and thus induces intracellular cAMP accumulation (Burnette, 1992). PT, like cholera toxin and *Escherichia coli* heat-labile enterotoxin, enters target cells via endocytosis and passes through the Golgi into the endoplasmic reticulum (Hazes and Read, 1997). Recent studies have demonstrated

that the leukocytosis induced by PT is G protein dependent and its effects inhibit the increase in intracellular Ca^{2+} levels in $CD4^+$ and $CD8^+$ cells. It also causes enhancement of both Th1 and Th2 cytokine production (Loetscher *et al.*, 1994; Ryan *et al*, 1998). Many studies have shown that PT and FHA are necessary for colonisation of the upper respiratory tract by *B. pertussis* (Tuomanen and Weiss, 1985; Alonso, 2000). PT acts as a major virulence factor as it enters the bloodstream, then binds to specific receptors on host cells after which it disrupts several functions of cells (Weiss, 1997).

PT has an important contribution to the disease process of *B. pertussis* and, in recognition of that fact, is a component in toxoided form in all acellular pertussis vaccines developed and licensed thus far (Hewlett and Cherry, 1997; Castro *et al.*, 2001). However, the exact role of pertussis toxin in the pathogenesis of whooping cough and its main site of action in the host, whether locally in the respiratory tract or systemically, remains unclear. The fact that *B. parapertussis* can also cause whooping cough but does not produce PT implies that it may not have a major role in causing the typical whooping cough symptoms (Hewlett, 1997; Hoppe, 2000).

1-4-9 Adenylate cyclase toxin (CyaA)

CyaA is one of the major virulence factors of *B. pertussis* and will be disscused in detail in section 1-6.

1-5 Repeat in toxin (RTX) family

RTX (repeat in toxin) toxins, so named because of their amino acid repeat motifs in the structural protein, are a family of pore-forming proteins which are produced by a broad range of Gram-negative bacteria (Frey and Kuhnert, 2002). They are proteins with molecular weights of 102 –177 kDa and have a series of glycine and aspartate-rich nonomeric repeats near the C-terminal end of the proteins. The number of repeats varies

between 9-40 in tandem arrays with the sequence UXGGXG (N/D) DX, where U is a large hydrophobic amino acid and X is an arbitrary amino acid (Welch, *et al*, 1992, Coote, 1992). RTX toxins lack a cleavable N-terminal signal peptide and their secretion is not *sec* dependent and they are not exported via the general secretory pathway. The extracellular secretion of RTX toxins rather proceeds via the type-1 secretion pathway, which allows direct translocation of the toxins across both the inner and outer membranes in one step, without any detectable periplasmic intermediate. Inner membrane ATPase is one of the three envelope proteins which affect the secretion of RTX toxins. The ATP-binding cassette (ABC) protein provides energy for the secretory process through hydrolysis of ATP. A membrane fusion protein (MFP), one of the transport accessory proteins found mostly in Gram-negative bacteria where they function in conjunction with the inner membrane transporters such as ABC proteins (Dinh *et al.*, 1994), is also required. The third protein 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.

All RTX toxins create pores in the cell membrane of target cells leading to eventual cell lysis and they appear to require Ca^{2+} for cytotoxic activity (Coote, 1992). Based on differences in target cell specificity, RTX toxins can be divided into two major groups. The first group, comprised of the haemolysin of *Escherichia coli* (HlyA) and the adenylate cyclase toxin of *B. pertussis* (CyaA), have low specificity and are active on many mammalian cell types. The second group includes leukotoxins of *Mannheimia* (*Pasteurella*) *haemolytica* (LktA), *Actinobacillus actinomycetemcomitans* (LtxA) and *A. pleuropneumoniae* which have high specificity and are able to lyse only leukocytes of ruminant, primate or porcine origin, respectively (Coote, 1992). It has been shown that two RTX toxins, *Escherichia coli* α-haemolysin and CyaA toxin from *B. pertussis*, are

activated by covalent acylation of specific internal lysine residues by a separate gene product. Four proteins are actually required for the synthesis, activation and secretion of RTX toxins (Coote, 1992) and these are normally encoded by an operon consisting of four genes, *CABD*.

1-5-1 Haemolysin of *E. coli*

Escherichia coli haemolysin (HlyA) is one of the best-characterised members of the RTX toxin family and lyses a wide variety of target cells from many animal species (Welch *et al.,* 1995). HlyA is secreted from *E. coli* strains causing urinary tract infection in humans and animals and these strains are able to produce characteristic zones of haemolysis around colonies on blood agar (Cavalieri *et al.*, 1984). *E. coli* haemolysin (HlyA) is expressed from the *hlyCABD* operon, which encodes proteins of 20kDa, 110kDa, 80kDa and 55kDa, respectively. HlyA is produced as an inactive protoxin (pro-HlyA) which is activated by acylation by HlyC and secreted through a sec-independent pathway involving HlyB and HlyD (an inner membrane traffic ATPase and inner membrane protein) as well as the genetically-unlinked TolC (Welch *et al.,* 1995). Pro-HlyA is activated intracellularly by acylation of two internal lysine residues, Lys 564 and Lys 590. Activation is mediated by the co-synthesized cytoplasmic protein HlyC, which acts as an acyltransferase using acylated acyl carrier protein (ACP) as the fatty acid donor (Issartel *et al.* , 1991; Trent *et al.*, 1998). It has been demonstrated that the α-haemolysin lyses erythrocytes from many species and also shows strong cytotoxic and cytolytic activity against a variety of nucleated cells. In addition, HlyA kills immune cells involved in first line defence mechanisms, including polymorphonuclear (PMN) leukocytes and monocytes (Calvalieri and Snyder, 1982; Bhakdi *et al.*, 1989; 1990). Bauer and Welch (1996) have reported that HlyA impairs the ability of macrophages to process antigens.

1-5-2 Leukotoxins of *Mannheimia haemolytica* and *Actinobacillus actinomycetemcomitans*

M. haemolytica, the causative agent of bovine pneumonic pasteurellosis, produces a 104kDa leukotoxin (LktA), a member of the RTX toxin family, that kills only ruminant leucocytes (Shewen and Wilkie 1982; Brown *et al.*, 1997). The genetic organisation of the *lktA* operon (*lktCABD*) is similar to the *E.coli haemolysin (hlyCABD)* operon. *lktC* encodes a protein (LktC) that is responsible for activation of leukotoxin (pro-LktA) by acylation (Forestier and Welch, 1990). At low concentration, LktA stimulates bovine neutrophils to produce reactive oxygen intermediates, degranulate and release leukotriene B4 (LTB_4). As the LktA concentration is increased, target cells are stimulated to undergo apoptosis (Stevens and Czuprynski, 1996). At high concentrations of LktA, necrosis occurs, consequent to the membrane damage resulting from pore formation, suggesting that the LktA helps the bacteria to evade phagocytes and promote bacterial proliferation and survival at the site of infection (Czuprynski *et al.*, 1991; Henricks *et al.*, 1992; Wang *et al.,* 1998).

The 116-kDa-protein leukotoxin (LtxA) synthesised by *A. actinomycetemcomitans,* one of the actiological agents of periodontosis in humans, belongs to the RTX family. Structural, genetic organisation and functional domains of LtxA are close to the LktA of *P. haemolytica.* Both LktA and LtxA share a 40-50% amino acid homology with *E.coli* HlyA (Lally *et al* 1991). LtxA differs from other RTX toxins as it disrupts only human and some non-human primate PMNs (Mangan *et al.*, 1991; Lally *et al.*, 1999). Again, it is presumed that LtxA helps bacteria to evade phagocytes at the site of infection (Lear *et al.,* 1995; Karakelian, 1998). A recent study showed that *A. actinomycetemcomitans* has the potential to secrete abundant leukotoxin, and it is therefore appropriate to consider a possible role for leukotoxin secretion in the pathogenesis of the bacterium (Kachlany *et al.*, 2000).

1-6 Adenylate cyclase toxin (CyaA)

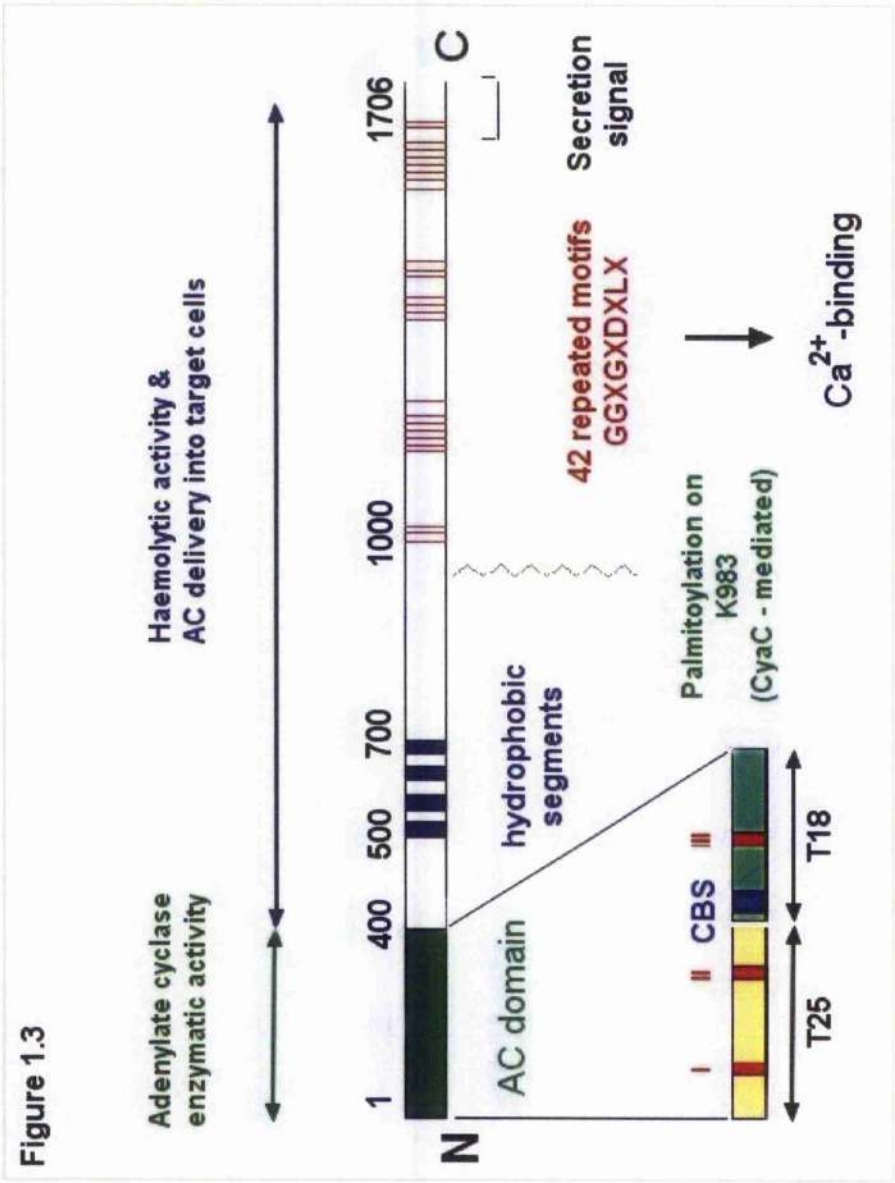
Adenylate cyclase toxin (*CyaA*) of *Bordetella* species is also a member of the RTX toxin family (Welch, 1991; Coote, 1992). It is a single protein with a molecular mass of 177 kDa as calculated from the amino acid sequence, but has an apparent molecular weight of 200-216 kDa displayed by SDS-PAGE (Rogel *et al.*, 1989). It is able to release a catalytically-active adenylate cyclase (AC) 45 kDa fragment by proteolysis (Bellalou *et al.*, 1990). The gene for CyaA toxin was cloned and sequenced by Glaser *et al.*, (1988). The 400-residue amino acid N-terminal domain (Figure 1.3) corresponds to the calmodulin-activated catalytic moiety (Glaser *et al.*, 1988). Calmodulin binds with high affinity to the enzyme and stimulates its activity up to 1000-fold. The catalytically-active region of CyaA is organised into two domains, an N-terminal domain of 25 kDa (T25) which carries the catalytic site and an 18kDa C-terminal domain (T18) that carries the calmodulin binding site. T25 is located between amino acid residues 1-224 and T18 is located between amino acid residues 225-399 (Mock and Ullmann, 1993). The haemolysin domain between amino acid residues 400-1706 displays structural characteristics that link CyaA to the RTX family (Coote, 1996). It shows 25% similarity with *E. coli* HlyA and 22% similarity with *M. haemolytica* LktA and is responsible for the haemolytic phenotype of *B. pertussis* (Glaser, 1988). The haemolysin domain mediates the binding and internalisation of the toxin into eukaryotic cells (Ladant and Ullman, 1999).

CyaA toxin is initially synthesised as an inactive precursor and activated by CyaC-mediated palmitoylation of lysine 983. Acylation facilitates binding to target cells and pore formation (Betsou *et al.*, 1993). The C-terminal haemolytic domain (residues 400-

Figure 1.3 Structural organization of *B. pertussis* adenylate cyclase (CyaA) toxin

Numbers represent amino acid residues. The catalytic domain (AC) is enlarged to show the T 25 and T 18 subdomains. CBS corresponds to the main calmodulin-binding site, and boxes I, II and III represent regions involved in catalysis. A pore forming region with four hydrophobic segments, a region of CyaA-dependent palmitoylation, 42 copies of Ca^{2+} binding site and a non-processed carboxy terminal secretion signal is termed the haemolytic domain. (Adapted from Ladant and Ullmann 1999).

Figure 1.3


Adenylate cyclase
enzymatic activity
Haemolytic activity &
AC delivery into target cells
1
400
500
700
1000
1706
N
C
AC domain
hydrophobic
segments
42 repeated motifs
GGXGXDXLX
Secretion
signal
I
II
CBS
III
Palmitoylation on
K983
(CyaC - mediated)
Ca2+-binding
T25
T18

1706) is composed of four hydrophobic segments (residues 500-700), the palmitoylation region at lysine 983, and 46 characteristic glycine and aspartate rich nona-peptide GGXGXDXLX repeats which are located between amino acid residues 1006-1638 that represent Ca^{2+} binding sites. The final C-terminal 106 amino acids residues of the toxin contain a signal which is required for secretion by the CyaB, D and E apparatus. As described earlier this signal motif is common to the other RTX toxins (Sebo and Ladant, 1993).

A number of *Bordetella* species such as *B. parapertussis* and *B. bronchiseptica* produce adenylate cyclase toxin (Preston *et al.*, 2002). Donato *et al.* (2005) have reported that *B. hinzii* produces a full-length CyaA protein in quantities that are comparable to those made by *B. pertussis*. In contrast to *B. pertussis* CyaA, however, CyaA from *B. hinzii* is less extractable from whole bacteria, non-haemolytic, has a 50-fold reduction in adenylate cyclase activity, and is unable to elevate cyclic AMP levels in host macrophages (non-toxic). The decrease in enzymatic activity is attributable, at least in part, to a decreased binding affinity of *B. hinzii* CyaA for calmodulin, the eukaryotic activator of *B. pertussis* CyaA. In addition, the authors demonstrated that the lack of intoxication of target cells by *B. hinzii* CyaA may be due to the absence of expression of *cyaC*, the gene encoding the accessory protein required for the acylation of CyaA from *B. pertussis*. The inability of purified *B. hinzii* CyaA to penetrate target cells could explain the lack of toxin activity. CyaA is unique among RTX toxins as it has both AC and haemolytic activity. The protein has the ability to enter into eukaryotic cells and deliver a part of its catalytic domain into the cell (Ladant and Ullman, 1999) where, upon activation by endogenous calmodulin, it catalyses formation and production of unregulated cAMP levels. CyaA intoxication leads to evident toxic effects on such cells as neutrophils and macrophages and prevents phagocytosis and chemotaxis.

CyaA of *B. pertussis* penetrates target cells by binding to a specific receptor (CD11b/CD18). CyaA displays a selective cytotoxicity towards cells that possess the integrin receptor CD11b/CD18, indicating that its interaction with CD11b favours translocation of the catalytic domain and the subsequent increase of intracellular cAMP concentration and cell death (Guermonprez *et al.*, 2001). These authors also noted that Ca^{2+} ions were required for translocation of the catalytic domain into cells suggesting that Ca^{2+} binding to CyaA is necessary for interaction of CyaA with CD11b. A recent study demonstrated that the acylation of CyaA is required for interaction of the toxin with cells expressing CD11b. It was shown that pro-CyaA is still able to bind the CD11b receptor, but this interaction is weak and reduces markedly membrane penetration of the pro-toxin. Hence, toxin acylation may be needed to confer a conformation on CyaA that is required for cell binding and the transmembrane delivery of the catalytic domain to the cell cytosol where it can catalyze the conversion of ATP to cAMP (El-Azami-El-Idrissi *et al*, 2003). However, the catalytic domain is not required for binding of toxin to CD11b because the interaction of toxin with CD11b/CD18 and with eukaryotic membranes is mediated through the C-terminal domain of the protein. These studies open new prospects for understanding the interaction of *B. pertussis* with innate and adaptive immune systems (El-azami-El-Idrissi *et al.*, 2003). The reason for this is that immunization of mice with purified CyaA or anti-CyaA specific antibodies reduced the time course of respiratory tract colonisation by *B. pertussis* and protected the mice against a lethal intranasal infection (Guiso *et al.*, 1991; Hormozi *et al.*, 1999). Other studies have indicated that specific antibodies to CyaA were detected in the sera of human infants infected with the bacteria (Guiso *et al.*, 1993).

In addition to CyaA of *B. pertussis,* a number of pathogenic bacteria secrete toxins that alter the intracellular concentration of cAMP (Ide, 1971; Aseeve, 1987). These toxins either disrupt the normal regulation of the host cell's adenylate cyclases/phosphodiesterases or they themselves catalyze the synthesis of cAMP in the host cell. With regard to the latter activity, three such toxins have been identified: the edema factor of *Bacillus anthracis*, ExoY of *Pseudomonas aeruginosa*, and the adenylate cyclase of *Yersinia pestis*. These adenylate cyclase toxins enter the eukaryotic host cells and is activated by eukaryotic cofactors, like calmodulin, to trigger the synthesis of cAMP in these cells and the immune effector cells appear to be the primary target of these adenylate cyclase toxins (Ahuja *et al.*, 2004). Both CyaA and Edema factor (EF) are activated in host cells by calmodulin (CaM). CyaA has 100-fold higher affinity for CaM than EF. CyaA and Edema factor have about three orders of magnitude higher AC activity than the AC activity of host cells (Drum *et al.*, 2002), and thus the entry of EF and CyaA would be expected to raise the cAMP concentration of host cells to the supra-physiological. The optimal activation of both toxins by CaM occurs at the resting cell intracellular calcium concentration when CaM is not limited (e.g 10 μg/ml protein). However, there is a major difference between these two toxins at relatively low free CaM concentrations (i.e. 0.1 μM). Shen *et al.* (2002) showed that the apparent affinity of EF for CaM is low (> 10 μM) at the resting calcium concentration (20-50 nM) so that EF may be minimally activated. In contrast, CyaA is still optimally activated at the resting calcium concentration.

1-6-1 Interaction of CyaA with target cells

Among bacterial toxins, *B. pertussis* CyaA has a unique mechanism of entry that consists in the direct translocation of its catalytic domain across the plasma membrane of target cells. After binding of CyaA to the eukaryotic cell receptor, mediated by the C-

terminal domain (residues 400-1706), the N-terminal catalytic domain (residues 1-399) is directly translocated through the plasma membrane into the cell. The exact mechanism of how *B. pertussis* AC enters targets cells has not been completely determined. However, the penetration through the cell membrane depends on a conformational change of CyaA, which is induced by Ca^{2+} concentrations in the millimolar range (Hanski and Coote, 1991).

CyaA has a large number of low affinity (K_D = 0.5-0.8 mM) Ca^{2+} binding sites located in the Asp-Gly rich repeat region of the toxin. Binding of the toxin to low affinity sites induces major structural rearrangments of CyaA that may be involved in delivery of AC domain into target cells (Rose *et al.*, 1995). On the other hand, binding of calcium to a small number of high affinity binding sites might be necessary for haemolytic activity of CyaA. The minimal stimulatory concentration of calcium ions ranged from 0.6 to 0.8 mM, depending on the ionic strength of the aqueous phase. Half-maximal channel activity of CyaA was observed at 2-4 mM, and saturation was reached at 10 mM calcium concentration, respectively (Knapp *et al.*, 2003). It has been shown that calcium binding to the low affinity sites induces a large conformational change of CyaA, as revealed by an important increase in the α-helical structures (Rose *et al*, 1995). This conformation might be directly involved in the Ca^{2+}-dependent translocation of the catalytic domain of CyaA through the plasma membrane of target cells. On the other hand, addition of Ca^{2+} increases the β-sheet content of the repeat domain (RD) of CyaA indicating that binding of calcium to the repeat motifs stabilizes the secondary structure of the protein (Rose *et al.*, 1995).

It has been noted by Locht and Antonine (1999) that at least three consecutive steps can be distinguished in the entry process of CyaA, 1) membrane insertion, 2) translocation, and 3) intracellular cleavage. Membrane insertion occurs even at low temperatures,

whereas translocation only occurs at a temperature above 20°C. In addition, translocation requires higher calcium concentrations than membrane insertion. The N-terminal fragment of toxin is cleaved in the cell, releasing the enzymatically-active 45kDa protein 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 CyaA toxin. This supports the theory of direct translocation, as entry of toxin through a trafficking mechanism or endocytosis would required a much longer exposure time (Rogel and Hanski, 1992). Intoxication of target cells by *Bordetella pertussis* can be blocked by monoclonal antibodies against CyaA or CD11b, the receptor for the toxin. It was also shown that the increase in cAMP in target cells was not correlated with the total number of bacteria present in the medium, but depended on the number of bacteria adherent to the target cells. Thus attachment of the bacteria was very important for efficient delivery of CyaA toxin (Gray *et al.*, 2004).

1-6-2 Haemolytic activity of CyaA

CyaA possesses haemolytic activity which is closely associated with the capacity of the protein to penetrate target cells (Bellalou *et al.,* 1990, Rogel *et al.,* 1991). The haemolytic domain of CyaA plays a critical role in the cell binding and entry of CyaA. Furthermore, recombinant AC molecules that lack the catalytic domain still exhibit full haemolytic activity (Sakamoto *et al.,* 1992). It is suggested that the main role of the haemolytic domain is not to lyse cells but to form channels for translocation of the catalytic domain into target cells such as macrophages (Bellalou *et al.,* 1990). A complete C-terminal domain is required for the tight association of AC with membranes as well as for its toxic and haemolytic activities (Iwaki *et al.,* 1995). It was shown that the last 217 residues of the C-terminal domain are required for toxicity and the domain between residues 624 and 780 is necessary for transfer of the catalytic domain through

the target cell membrane. The haemolytic functions of CyaA are separable from the above function and probably mediated by different domains on the CyaA toxin polypeptide (Sebo *et al.*, 1991; Rogel *et al.*, 1991). The haemolytic activity of CyaA required significantly lower Ca^{2+} concentrations than for membrane binding (Hewlett *et al.*, 1991; Rogel *et al.*, 1991). It is suggested that the high affinity binding sites of Ca^{2+} present in CyaA might be required for the binding of the toxin to the membrane and for the lysis of red blood cells whereas Ca^{2+} binding to low affinity sites is involved in the translocation of the toxin. Translocation of the CyaA domain into the cell probably does not proceed through the small transmembrane pore that is formed by the haemolysin moiety of CyaA (Rogel and Hanski, 1992; Rose *et al.*, 1995).

1-6-3 CyaA as a virulence factor

Among the virulence factors produced and secreted by *B. pertussis*, CyaA toxin and PT are able to invade mammalian cells to impair intracellular functions. In addition, mutants deficient in both PT or CyaA or both toxins, had a reduced ability to cause lethal in infant mice (Weiss *et al.*, 1984). It has also been shown that CyaA is an essential factor for the initiation of *B. pertussis* infection (Khelef *et al.*, 1992). Both PT and CyaA may act directly to alter immune cell functions and promote the ability of *B. pertussis* to cause a lethal infection in infant mice (Weiss and Goodwin, 1989; Khelef *et al.*, 1994). It was noted that a CyaA mutant was rapidly cleared from the lungs with no viable bacteria remaining at 10 days post-challenge, suggesting that CyaA is an important colonization factor required for the bacteria to initiate infection (Goodwin and Weiss, 1990). A number of studies have confirmed that phagocytic cells are a primary target of CyaA. The toxin is able to induce apoptosis in mouse alveolar macrophages *in vitro* and *in vivo* (Khelef *et al.*, 1993; Gueirard *et al.*, 1998).

Recent studies have suggested that expression of CyaA inhibits uptake and possible destruction of *B. pertussis* by human respiratory epithelial cells and human neutrophils (Bassinet *et al.*, 2000; Weingart and Weiss, 2000). Interaction of *Bordetella pertussis* with tracheal epithelial cells results in the secretion of high levels of interleukin-6 (IL-6) and very low levels of IL-8, which is dependent on the expression of CyaA by the bacterium but not dependent on other toxins or adhesins. This study showed the important role of the toxin in the pathogenicity of the bacterium (Bassinet *et al.*, 2004).

1-7 Immune response to infection

The physiological function of the immune system is defence against microbes, mediated by the early reactions of innate immunity and the later responses of adaptive immunity. Innate or natural immunity consists of a mechanism that exists before infection, which is capable of rapid response to microbes and will react in essentially the same way to repeated infections. The components of natural immunity include physical and chemical barriers such as the epithelial cell barrier, phagocytic cells (macrophages, neutrophils and NK cells) that can bind to microbes, internalise them and then kill them, blood proteins such as the complement system and cytokines that regulate and co-ordinate many of activities of the cells of innate immunity. The mechanism of innate immunity provides the initial defence against infections. However, many microbes have evolved numerous mechanism for evading these reactions (Workman, 2003).

Adaptive immunity develops as a specific response to infection and the defining characteristics of this immunity are specificity for distinct macromolecules and an ability to remember and respond more vigorously to repeated exposure to the same microbe. The mechanisms of adaptive immunity develop later in infection and consist of activation of lymphocytes and their products (Paul and Seder, 1994). An important

link between innate and adaptive immunity is that the innate responses participate in initiation of the adaptive responses and influence the nature of the responses and, secondly, adaptive immune responses use many of the mechanisms of innate immunity to eliminate microbes. There are two types of adaptive immune responses, humoral and cell-mediated immunity (CMI), which are mediated by different components of the immune system and function to eliminate different types of microbes (Paul and Seder, 1994). T lymphocyte and their products, which are important for defence against intracellular bacteria mediate CMI, whereas humoral immunity is mediated by B-lymphocytes and their products such as antibodies which defend against extracellular microrganisms. Both B and T lymhocytes are derived from the bone marrow but T cell development proceeds in the thymus. After they leave the bone marrow and thymus, they enter the circulation and populate the peripheral lymphoid organs (Abbas et al., 2000).

T cells can also be distinguished by their different antigen receptors. T cell receptors (TCRs) divide into two defined types: one type is a heterodimer of two disulphide-linked polypeptides, TCR αβ, and the other consists of γ and δ polypeptides. These two types of TCRs receptors (αβ and γδ) show quite distinct anatomical locations. 95% of αβTCRs exist in peripheral T cells, although there is a small proportion of γδ T cells in the thymus and lymphoid organs. The αβ T cells are subdivided into two different forms; one is $CD4^+$ marked and mainly acts as T helper cells which help induce immune responses after interaction with other immune cells and recognition of their specific antigens related to the MHC class II. The second is $CD8^+$ T cells, which recognize antigens in association with MHC class I molecules. In addition, two groups of T helper cells (Th1&Th2) develop from the $CD4^+$ lymphocytes, which have different cytokine profile production. Th1 cells mainly produce IFN-γ and IL-2 and are important for development of CMI and eliminating intracellular microbes (Murphy *et al*, 2000), while

Th2 cells produce IL-4 and IL-10 and are important for developing humoral immune responses.

The Th1 differentiation pathway is a response to micro-organisms that infect or activate macrophages and to those that activate NK (natural killer) cells. Th2 differentiation occurs in response to foreign antigen and allergens, which causes chronic T cell stimulation, often with little macrophage activation. IFN-γ and IL-2 produced by Th1 cells stimulate phagocytosis, opsonization, and IgG2a production. On the other hand, Th2 cells produce IL-4 and IL-10 that stimulates IgG1 production and can result in IgG1–mediated allergic reactions in mice. In the majority of cases, an immune response elicits both Th1 and Th2 activities. Moreover, Th1 and Th2 are both able to induce B cells to produce immunoglobulin (Ig) (Murphy *et al*, 2000).

$CD8^+$ T cells can be divided into two subsets, one of which express CD28 molecules and produces IL-12 in response to activation signals and the other subset which expresses the CD11b/CD18 cell receptor molecules. CD11b/CD18 or CR3 belongs to the α-intergrin family and is expressed on monocytes, PMNs, B and T cells.

B-lymphocytes produce immunoglobulins that are present in biological fluids and are also found on the surface of a limited number of cells types of all mammalian cells. One of the most important functions of immunoglobulin is to bind antigen, which can result in antigen neutralisation.

NK cells are a third population of lymphocytes where receptors are different from those of B and T cells. They play an important role in the recognition and killing of certain tumour cells. NK cells are also able to kill targets cells coated with IgG antibodies (opsonised). Through their receptor for IgG, NK cells can release interferon-α, IL-2 and

other cytokines when activated, which may be important in the regulation of immune responses (Biron *et al*, 1999).

1-8 Pertussis vaccination

Pertussis (whooping cough) is a ubiquitous, serious infectious disease with 40 million cases and nearly 300,000 deaths in children worldwide in 2000. Most cases and deaths occur in developing countries (Ivanoff and Robertson, 1997; WHO, 2001). One way to reduce these numbers or even to eradicate pertussis is to find more effective vaccines. Both curently available WCVs and ACVs only protect infants from severe pertussis, their efficacy is somewhat variable, and they fail to protect the adult population against contagious pertussis. It is now thought that the primary reason for the lack of efficacy of pertussis vaccines is that they give protection for only a limited period of time (Nennig *et al.*, 1996).

Immunisation against whooping cough was introduced in the UK in 1950s. The incidence of whooping cough in England and Wales dropped steadily as the vaccination rate rose to around 80% in the 1970s (Pollard, 1983). Unfortunately, after public concerns over the safety of the vaccine, the incidence rose again as the vaccination rate dropped between 1975 and 1988. This resulted in the 1977-1979 epidemics, when 102,000 children were seriously ill and 32 died. In contrast, in countries such as Fiji, where the vaccination programme was not interrupted, the disease was practically eliminated (Pollard, 1983). In the UK, when vaccination coverage was restored the disease incidence declined. Nonetheless, despite the high vaccine coverage levels, the disease is still an important cause of morbidity and mortality in England and Wales, especially among infants (Crowcroft *et al.*, 2002). In recent years, several countries have reported a resurgence of pertussis, despite maintaining high vaccination coverage. According to a survey by the US Health Department, a total of 35,508 cases of pertussis

were reported to the Center for Disease Control (CDC) through the National Notifiable Disease Surveillance System (NNDSS) during 1990-1996. The incidence generally decreased over time from 108 cases per 100,000 population in 1990 to 2.9 cases per 100,000 population in 1996, although large outbreaks were reported in Cincinnati and Chicago and in Idaho, Massachusetts and Vermont. In 1996, 7796 cases of pertussis were reported, the highest number of disease cases reported since 1967 (Guris *et al.*, 1999).

Although pertussis vaccination coverage is very high in France, the organism is still circulating, affecting the pediatric population, mostly non- or incompletely vaccinated infants. The results of Grimprel *et al.* (1998) study strongly support the importance of adhering to the immunization schedule and suggest introducing booster dose(s) to prolong vaccine immunity and reduce the exposure to *Bordetella pertussis* of infants too young to be immunized. In Australia the incidence of disease dramatically increased from 2.5 per 100,000 in 1991 to a peak of 30.5 per 100,000 in 1994 (Andrews *et al.*, 1997). In the Netherlands, 2771 cases of pertussis were reported to the Inspectorate of Health from a population 15 million, compared with 319 cases in 1995. In recent years, there has been a dramatic increase in the disease in older children and adults. This is attributed to waning immunity over time in vaccinated individuals (Melker *et al.*, 1995).

1-8-1 Whole cell vaccines (WCV)

Effective killed whole-cell vaccines (WCV) were introduced in the 1950s. They played an important role in prevention of pertussis in many countries (Pittman, 1991). WCVs against pertussis have now been used in developed countries for approximately 50 years and have considerably reduced mortality and morbidity. Vaccines produced by different countries differ in potency, immune response and reactogenicity (Edwards *et al.*, 1999).

WCV is usually a combination of diphtheria and tetanus toxoids and a suspension of killed *B. pertussis* cells (Department of Health., 1990). In countries with good vaccination programmes, DTP is routinely started at two months of age and given as 3 equal doses at 4-8 week intervals followed by a booster vaccination at 12 to 18 months after the first vaccination or before 6 years of age. This schedule should give more than 90% protection (Preston, 1987). However, the immunisation schedules vary from country to country. The efficacy of pertussis WCV has gradually reduced the morbidity and mortality of pertussis but, in recent years an increase in incidence of pertussis has been reported in USA, Canada, Australia and many of the countries in Europe (see section 1-8).

In spite of the efficacy of WCV it may cause local and systemic reactions in children such as pain, redness or swelling, mild fever, drowsiness, anorexia, prolonged crying and convulsions. More severe reaction such as high fever, permanent brain damage and death may occur at an estimated rate of 1 in 100,000 vaccinations (Marcuse and Wentz, 1990; Gale *et al,* 1994; Cherry, 1996). Recent research by Ennis *et al.* (2005) shows no evidence that WCV contributes to allergic asthma, but provides evidence of a mechanism whereby WCV vaccination has a protective role against this disease. Because of the efficacy and low cost of WCV it will probably be widely used for some time to come, particularly in developing countries (Decker and Edwards, 2000). Despite this efficacy some countries with high coverage of vaccination have experienced increases in pertussis, which may be the result of antigenic differences between the WCV strain and circulating strains (Willems and Mooi, 1996). A study in Netherlands (1996), showed polymorphisms in the genes coding for *B. pertussis* virulence factors PRN and PT and this was cited as evidence for a vaccine-driven evolution in circulating strains that has led to a fall in vaccine efficacy (Mooi *et al.*, 1998).

1-8-2 Acellular vaccines (ACV)

Acellular vaccines (ACV) are composed of purified components of *B. pertussis* (Mahon *et al.*, 1997, 2000; Murphy *et al.*, 1997). ACVs against *B. pertussis* have been used in Japan since 1981 (Sato *et al.*, 1984). Two types of acellular vaccines B (Biken) and T (Takeda) were produced in Japan. The B-type contains an equal amount of toxoided PT and FHA, whereas the T (Takeda) type vaccines is a co-purified suspension of PTd, and FHA, and also contained pertactin and Fim. It has been reported that both ACVs were able to protect children with similar efficacy and with a low incidence of reaction (Sato, 1988; Kimura *et al.*, 1988; Edwards *et al.*, 1999).

In 1986, ACVs which contained either PT toxoid alone or FHA and toxoided PT were trialled in Sweden. They showed lower efficacy and lower reactogenicity than the WCV although they still had some side effects (AD HOC group, 1988; Tuomanen *et al.*, 1993). A number of other ACVs were selected for subsequent trials in USA and Europe. All included toxoided PT, either alone or with up to 5 components including FHA, fimbrial proteins 1 and 2 and pertactin (Storsaeter *et al.*, 1990). In November 2001 an ACV in combination with diphtheria and tetanus vaccines, was introduced in to the UK to provide long lasting pertussis immunity into adulthood (Pigott *et al.*, 2002). According to the study of Gustafsson *et al.* (1996) the five component ACV (PTd, FHA, PRN, Fim2 and3) that they assessed could be recommended for general use, since it had a favorable safety profile and conferred sustained protection against pertussis whereas a two-component ACV and the North American whole-cell vaccine were less efficacious. Le *et al.* (2004) showed that immunisation of adolescents and adults with an ACV induced strong IgG and IgA antibody response against PT, FHA and pertactin, suggesting that booster vaccination with ACVs could help to reduce the incidence of the disease in adults. Rappuoli *et al.* (1991) produced a third generation of ACVs with high

efficacy that contained genetically-toxoided PT, alone or with FHA and pertactin. These were clinically tested in 1000 infants that received three doses of the vaccines and it was shown that both vaccines were safe and highly immunogenic.

In general, results from various human trials in different countries have shown that all of the ACV preparations were effective, with estimates of efficacy against severe pertussis ranging from 59-93%. Moreover, the multi-component vaccines (containing 3-5 antigens) were better than one and two components (Olin *et al.*, 1997). Thus, the best ACVs have the potential to control the spread of disease. Decker and Edwards (2000) noted that the number of components, the quantity of each and the method of production, particularly toxoided PT, seems to influence the efficacy of the ACV vaccines. Morever, results from animal model studies have been beneficial. A study in mice indicated that PT was necessary as a vaccine component for protection against an intracerebral challenge with *B. pertussis* and it appeared that FHA could also play an important role, as a vaccine containing a small amount of FHA with a large amount of PT (1: 11) was strongly protective in both the aerosol challenge and the intracerebral model (Watanabe *et al.*, 2002). Denoel *et al.* (2005), in a recent study of immunity in the mouse model, compared immune responses induced by three and five component vaccines and their ability to induce clearance of *B. pertussis* from the lungs of infected mice. Their findings also showed that the effect of a bicomponent DTPa vaccine on bacterial clearance was lower when compared with tri- or pentavalent DTPa vaccines.

1-9 Immunity to pertussis infection

Acquired immunity against *B. pertussis* develops after natural infection or immunisation with vaccines. Antibodies (Abs) may function either by neutralising bacterial toxins, inhibiting extracellular bacteria from binding to cells in mucosal tracts or by enabling bacterial uptake and destruction by macrophages and neutrophils (Storsaeter, 1998;

Cherry, 1998; Taranger, 2000). There is evidence based on passive immunisation, which shows that antibodies (Abs) play an important role in protection against pertussis disease. Passive immunisation with mAbs specific for PT, pertactin or LPS were shown to protect infant mice from lethal *B. pertussis* infection (Sato, 1990; Shahin *et al.*, 1990; 1994). IgG and IgA isotypes are involved in host defence against bacterial infection. Infection of mice or humans with *B. pertussis* induced secretory IgA in the respiratory tract or nasopharyngeal secretions and also demonstrated IgA in convalescent serum which was able to inhibit adherence of *B. pertussis* to human ciliated epithelial cells (Tuomanen *et al.*, 1984; Mills *et al.*, 1993). The importance of Abs in immunity to *B. pertussis* was demonstrated using naïve Ig $^{-/-}$ mice where infection by respiratory challenge with *B. pertussis* caused a chronic infection. B cell-deficient mice were unable to clear the bacteria from their lungs even after 6 months, whereas the wild-type mice could clear infection within 8-10 weeks (Mahon *et al*, 1997). According to Leef *et al.* (2000), intranasal immunisation of Ig $^{-/-}$ mice with inactivated *B. pertussis* resulted in partial protection which was restored to full protection after transfer of *B. pertussis* immune B cells. Furthermore, B cells may function in protection against *B. pertussis* independently of antibody production, probably via a role in mucosal immunity or in antigen presentation to T cells (Golvokina *et al.*, 1999). Moreover, opsonization of *B. pertussis* by antibodies is an important mechanism of host defence required for optimal phagocytosis, and phagocytic cells are thought to play an important role in clearance of the organism.

Other studies have focused on the induction of $CD4^+$ T cells in immunised or infected mice. These findings have reported that respiratory infection or immunisation of mice with WCV selectively primes Th1 cells. However, ACV immunisation of mice generated T cells that produced IL-4, IL-5 and low levels of IFN-γ. These results

generated using spleen or lymph node T cells stimulated *in vitro* with *B. pertussis* antigens were representative of a more Th2-orientated response (Mills *et al.*, 1993; 1992; Redhead *et al.*, 1993; Barnard *et al.*, 1996). In one report, immunization with FHA was found to be the most effective and resulted in complete clearance of the bacterial infection from the lungs within 14 days (Cahill et al., 1993). In a recent study, FHA induced secretion of IL-10 and IL-6 by macrophages, which might explain the suppression of T cell responses in the lungs of mice during infection. IL-10 is a potent suppresser of CMI, and reduces Th1 immune responses (McGuirk *et al*, 2000).

It has been demonstrated by Mills *et al.* (1998) that the mechanism of immunity against *B. pertussis* might involve both humoral and cellular immune responses. Several studies have shown that *B. pertussis* can persist intracellulary within murine alveolar and human macrophages. Internalisation of the bacteria is immunoglobulin G (IgG) dependent, and evidence suggests that survival depends, at least in part, on inhibition of phagosome-lysosome fusion (Cheers and Gray, 1996 and Steed *et al.*, 1991).

Bromberg *et al.* (1991) indicated that cell-mediated immunity might be a crucial component of long term immunity against *pertussis* as it has been reported that *B. pertussis* can invade and survive within eukaryotic cells including macrophages and neutrophils. The extra and intracellular location of *B. pertussis* is consistent with a role for cellular as well as humoral immune mechanisms for bacterial elimination from respiratory tract infection (Mills, 2001).

1-9-1 Protection by CyaA

Brezin *et al.* (1987) showed that antibodies to CyaA prevented the effects of haemorrhagic alveolitis in mice infected by *B. pertussis*. Protection against pertussis infection of mice by CyaA was shown to depend on the post-translational modification mediated by the *cyaC* gene product (Betsou *et al.*, 1993; 1995; Hormozi *et al.*, 1999).

CyaA has been shown to act as a protective Ag against bacterial colonisation in a murine respiratory model where two immunisations with CyaA at a two-week interval induced protection against colonisation of *B. pertussis* (Guiso *et al.*, 1991; Hormozi *et al.*, 1999). The efficacy of protection by CyaA was reported to be less than the efficacy of the WCV. However, it seems that CyaA could be a valuable component of an acellular vaccine. The period of bacterial colonisation of the mouse respiratory tract could be shortened by both active and passive immunisation with anti-CyaA antibodies (Guiso *et al.*, 1991). Hormozi *et al.*, (1999) showed that purified native CyaA or recombinant CyaA produced in *E. coli* exhibited protective activity in mice against intranasal challenge with *B. pertussis* 18323. It has also been demonstrated that anti-CyaA antibodies can be detected in humans after infection with *B. pertussis* (Arciniega *et al.*, 1991) and such antibodies are also produced after vaccination with WCV. Betsou *et al.* (1995) located the protective epitopes of CyaA to the 800-residue C-terminal repeat portion of CyaA. Weingart and Weiss (2000) demonstrated that CyaA blocks phagocytosis by human neutrophils and Weingart *et al.* (2000) showed that neutralising antibodies to CyaA promoted phagocytosis of *B. pertussis* through inhibition of the activity of CyaA.

In short, the results of these studies demonstrated the potential for CyaA as a protective immunogen and that neutralising Ab to CyaA could be helpful in preventing infection by *B. pertussis,* by preventing the action of CyaA on phagocytes. In spite of such properties, none of the present acellular pertussis vaccines contains CyaA. As recombinant CyaA, expressed in *Escherichia coli,* is capable of inducing protective immune responses in mice, against subsequent challenge with *B. pertussis*, it is therefore a potential candidate for inclusion in ACVs.

1-9-2 CyaA as an adjuvant

Immune responses against antigens can be potentiated by co-adminstration of adjuvant, such as alum or Freund's complete adjuvant (FCA). CyaA has been shown to enhance antibody levels to co-administered ovalbumin (Hormozi *et al.*, 1999). In a recent investigation by MacDonald-Fyall *et al.* (2004), it was shown that CyaA could raise the specific IgG responses to PT, FHA and PRN. In addition, it was noted that CyaA*, lacking AC enzymic activity, acted as a better adjuvant than CyaA. The adjuvant activity of CyaA may reside in its ability to activate cells of the innate immune system through increased levels of cAMP or, in the case of CyaA*, it may be more related to binding to the receptor CD11/CD18. It has been reported that CyaA can promote maturation of monocyte-derived dendritic cell and suppress inflammatory cytokine production by human MDDC (Gentile *et al.*, 1990; Guermonprez *et al.*, 2001; Bagley *et al.*, 2002). Recent research by Ross *et al.* (2004) showed that CyaA may act as an adjuvant to enhance specific subsets of $CD4^+$ T cells by promoting activation of DC, promoting Th2 and Tr1 responses, with significant enhancement of antigen-specific IL-10-producing T cells. This effect appears to be mediated in part by its ability to activate cells of the innate immune system, including DC. However, their data also reveal that CyaA can synergize with and modulate TLR4-mediated responses of DC to LPS. The adjuvant properties of CyaA* may be more closely linked to the binding of the CD11b/CD18 receptor and subsequent unknown signalling events. A CyaA* derivative was shown to have the capacity to deliver its catalytic domain into the cytosol of target cells, and to deliver $CD8^+$ T-cell epitopes, resulting in intracellular processing and presentation of the epitope by MHC class I molecules at the surface of antigen-presenting cells (APC) (see below section 1.9.3).

1-9-3 CyaA as a vaccine delivery vehicle

The main mechanism for elimination of viruses, parasites, and intracellular bacteria that reside within cells is by cytolytic T lymphocytes (CTLs), which kill the infected cells. Most Ag-specific CTLs are $CD8^+$ T cells that recognise cytosolic, usually endogenously-processed, foreign antigens in association with class I major histocompatibility complex (MHC) molecules on any nucleated cell. Full differentiation of CTLs like other lymphocytes requires at least two signals. The first is the presence of the antigenic peptide-MHC complex and the second being either cytokines produced by $CD4^+$ T cells or costimulators expressed by infected cells. The antimicrobial effects of CTLs are mainly concerned with destroying the infected cells before the pathogens are reproduced. The mechanisms include activation of nucleases within infected cells that degrade microbial DNA and secretion of cytokines with IFNγ activity (Abbas *et al.*, 2000; Paul, 1999). Exogenous antigens enter the endosomal pathway and are presented to $CD4^+$ T cells in association with Class II MHC molecules whereas endogenously synthesised Ags, such as viral proteins, are presented to $CD8^+$ T cells in association with MHC Class I molecules.

Vaccination strategies aimed at generating CTL responses *in vivo* have been actively sought. To date most CTL activation strategies are based on the use of live vectors (Fayolle *et al* 1996). An alternative possibility would be to introduce an antigen into the cell cytosol by targeting it via an invasive non-replicative vector. The invasive CyaA of *B. pertussis* is suited for such a purpose as it can deliver its catalytic domain into the cytosol of a large number of eukaryotic cells and particularly into antigen-presenting cells. It has therefore been used as an appropriate vector to carry foreign peptides into the cytosolic pathway (Ladant *et al.*, 1992). In recent studies, researchers analysed the possibility that CTL epitopes delivered to cells by recombinant CyaA toxins could

associate with MHC class I molecules and induce cytolytic T cell responses *in vivo*. They showed that recombinant CyaA toxin induces strong CTL responses *in vivo* without the requirement for $CD4^+$ T helper cells. High peptide-specific CTL responses were also obtained in mice immunised with recombinant CyaA toxin presenting a HIV-1 $CD8^+$ T cell epitope (Guermonprez *et al.*, 1999).

Such an approach would be a potentially efficient tool in inducing protection against intracellular microbes and tumours. Using an OVA-expressing tumour mouse model, it has also been shown that recombinant CyaA vectors carrying a $CD8^+$ OVA epitope can elicit CTL responses and protect mice against the tumour by causing a regression of established tumours in an Ag-specific manner. Therefore CyaA acts as a potentially efficient molecule to develop anti-tumour therapeutic immunity. Moreover, CyaA is able to deliver more than one $CD8^+$ T cell epitope into antigen-presenting cells and induce specific cytolytic T lymphocyte responses ($CD8^+$) *in vivo* (Fayolle *et al*, 2001). It has been demonstrated that, *in vivo*, CyaA is efficiently targeted to dendritic cells as a result of its interaction with the CD11b/CD18 intergrin that was previously identified as a specific cellular receptor for CyaA (Fayolle *et al.*, 2001). The results of Dadaglio *et al.* (2003) showed that tumoral epitopes inserted into CyaA are also efficiently processed and presented in association with human MHC molecules. They also suggested that CyaA would be capable of activating anti tumoral CTL responses in humans and highlighted the potential of CyaA for use in cancer immunotherapy.

Delivery of viral and *Mycobacterium tuberculosis* epitopes by CyaA* derivative generated both $CD4^+$ and $CD8^+$ epitope-specific T cells and the $CD4^+$ T cells were characterised as IFNγ-producing Th1 cells. Thus, induction of these T cell responses was via a mechanism other than increased intracellular cAMP production (Schlecht *et al.*, 2004; Wilkinson *et al.*, 2005; Mascarel *et al.*, 2005).

1-10 Protein-coated microcrystals (PCMCs)

One of the major problems that would be encountered in using recombinant CyaA as a vaccine component is the fact that it has to be stored in 8M urea to prevent aggregation and to maintain stability of the protein. Urea at high concentrations will have detrimental effects on mammalian cells. Thus, a new improved vaccine formulation is consequently needed to circumvent this problem. One aim of the proposed research was to formulate CyaA by a method that is applicable for large scale manufacture but would still retain the immunogenicity of the protein and promote a specific immune response.

The most attractive feature of PCMCs for pharmaceutical use is their physical robustness and resistance to damage caused by high humidity and temperature which suggests that PCMCs have great potential in the field of vaccinology (Parker *et al.*, 2003). PCMCs were reported by Kreiner *et al.* (2001) as a novel system for stabilising high-activity biocatalysts for use in organic media in polar and nonpolar solvents. Formulation of biomolecules as dry powders is an area of increasing interest because it provides stabilisation of bioactivity and serves as a route to new drug and vaccine delivery technologies. When dried by conventional routes, such as spray-drying, compounds generally contain amorphous excipients and this can limit the range of humidity and temperature over which stable powder performance may be obtained. It was demonstrated by Kreiner *et al.* (2001) that PCMCs with a crystalline core made of amino acids such as valine or salt are found to exhibit unchanged powder characteristics even when exposed to high humidity and elevated temperature, i.e. there is negligible particle agglomeration.

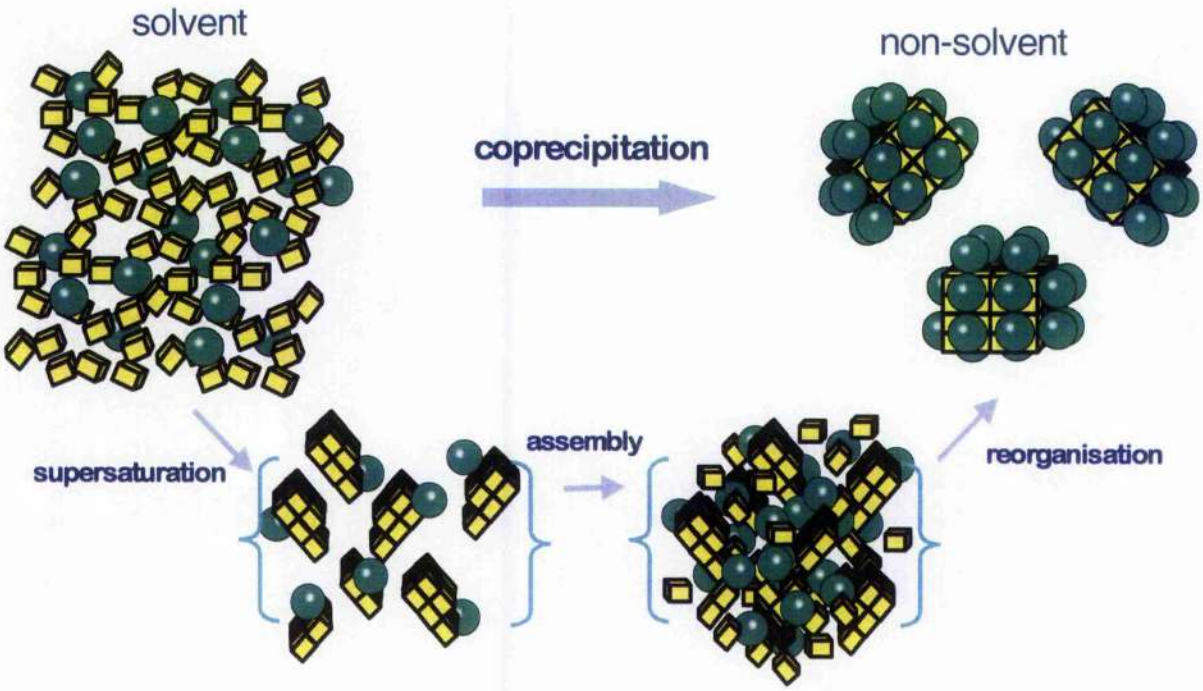
The preparation of PCMCs involves dissolution of the appropriate crystal-forming carrier together with the given biomolecule in aqueous solution. Quick dehydration of the two components is facilitated by the addition of the aqueous solution to a water-miscible organic solvent, resulting in the immediate formation of the protein-coated

microcrystals with the biomolecule immobilised on the surface of the crystalline core carrier (Ross *et al.*, 2002). The PCMCs are then filter-dried to form a free-flowing powder and the morphology, size and biomolecule content can be tuned via appropriate choice of dehydration conditions (Ross, A *et al.*, 2004).

The overall aim of using this process in the present study was to carry out the precipitation to yield CyaA-coated valine crystals. The hypothesis was that the urea would be lost during the process and consequently would not be present on the PCMCs when they were reconstituted in aqueous solution. It was also hoped that any LPS as a minor contaminant would also be lost through the process. The different procedures used to prepare the PCMCs are detailed in Materials and Methods. A generic method for organising biomolecules on microcrystals is shown in Figure 1-4. The figure attempts to explain the process of particle formation, but this mechanism remains a hypothesis. It is thought that as the aqueous solution contacts the organic solvent, super-saturation is observed, for both materials, and that due to the high lattice energies of the core materials, crystal growth is initiated, the crystal begins to grow and the protein, which is intimately associated with the growing crystal, is forced to the surface of the particles as reorganisation of the growing crystal takes place. Since the protein is insoluble in the solvent, there is nowhere else for the protein to go and it remains bound to the surface of the crystal. Two possibilites, regarding the resultant material, can be envisaged, a) both the protein and the valine precipitate separately, and the resultant precipitate is composed almost entirely of valine crystals and protein particles or b) if lattice parameters of the valine allow, the protein could become entrapped within the crystal lattice. Lattice paramaters (case b) do not permit entrapment of particles of 5 nm or larger (for protein nanoclusters) within the crystal matrix and scenario a) is also not commonly observed (M.C. Parker, personal communication).

Figure 1.4 A generic method for organising biomolecules on microcrystals

The figure attempts to explain the process of particle fomation, but this mechanism remains a hypothesis. Two possiblities, regarding the resultant material can be envisaged, both the protein and the valine precipitate separately and then reassemble or the protein and valine co-precipitae in a 1-step pocess. (Adapted from Dr. M.C.Parker, Chemistry Dept, Glasgow University)


solvent
non-solvent
coprecipitation
supersaturation
assembly
reorganisation

1-11 Aims and objectives

The overall objectives of this study were, first, to produce and purify three forms of CyaA from recombinant *E. coli* by optimised methods and then to characterise the properties of these toxins. Following characterisation of toxins, the active and invasive CyaA would be used to make protein-coated microcrystals (PCMCs). The purpose of this latter part of the research was to use the novel, patented, PCMC technology to develop highly stable vaccine formulations and also to remove urea from the toxin. Urea is normally very important for CyaA stabilisation and it was important to try to obtain conditions whereby AC enzymic activity and cell invasiveness of the CyaA protein were retained after resolubilisation of CyaA-PCMCs in aqueous buffer. The PCMCs prepared with CyaA and reconstituted in aqueous buffer would then be used as antigens in the mouse to determine if antigenicity of CyaA was retained.

Chapter 2

Materials and Methods

2-1 Bacterial strains and plasmids

The strain used for CyaA expression was *E. coli* BL21/DE3. This strain contains chromosomally-located λDNA expressing the gene encoding T7 RNA polymerase under the control of the inducible *lac* UV5 promoter. The addition of isopropyl-1-thio-β-D-galactoside (IPTG) to a growing culture induces the RNA polymerase that in turn transcribes the target DNA in the plasmid from a T7 promoter. *E.coli* BL21/DE3 lacks the *Lon* protease and outer membrane proteins (OMP) that can degrade some recombinant proteins. The enzymatically-active, acylated form of CyaA was produced from separate compatible plasmids, pGW44 and pGW54, each under the control of the inducible T7 RNA polymerase promoter (Westrop *et al.*, 1996, 1997). Plasmid pGW44 expresses the CyaA gene that encodes the CyaA protoxin. Plasmid pGW54 expresses the modifying protein, CyaC, which post-translationally acylates the CyaA protoxin. In the absence of CyaC, expressed by pGW54, a non-invasive but enzymatically-active CyaA pro-toxin is produced. A plasmid pGW44-188 encoding CyaA*, the pro-toxin lacking adenylate cyclase activity, was constructed previously in this laboratory by replacing a 1.1 kb *Nde*I/Bst*B1* fragment at the 5' end of the c*yaA* gene in pGW44 with the equivalent fragment from pACM188 (Ladant *et al.*, 1992). Expression of this plasmid produces CyaA pro-toxin with a Leu-Gln peptide insertion between codons 188 and 189 within the ATP binding site. It was used in the presence of pGW54 to produce the enzymatically-inactive, acylated CyaA. The plasmids used in this work are listed in Table 2.1.

Table 2.1 Plasmids used for producing recombinant CyaA proteins

Plasmid (s)	Relevant phenotype of CyaA	Antibiotic resistance
pGW44, pGW54	Active AC/ invasive (CyaA)	Amp/Chlor
pGW44-188, pGW54	Non-active AC/ invasive (CyaA*)	Amp/ Chlor
pGW44	Active AC / non-invasive (CyaA pro-toxin)	Amp

2-2 Growth of *E. coli*

The *E. coli* strain was grown overnight at 37°C on Luria Bertani (LB) agar (Appendix A.1) containing relevant antibiotics (ampicillin or chloramphenicol) for plasmid maintenance. The bacterial cells were subcultured into 20 ml LB broth (Appendix A.2) and incubated overnight at 37°C with shaking at approximately 200 rpm. Chloramphenicol at a final concentration of 12 µg/ml and ampicillin at a final concentration of 50 µg/ml were used in all cultures of *E. coli* containing pGW44 or pGW44-188 with pGW54, or ampicillin alone at the above concentration for *E. coli* containing pGW44 or pGW188-54 only.

2-3 Purification of plasmid DNA

Plasmid was extracted using the QIAprep® Mini prep purification system (Qiagen) according to the manufacturer's instructions. A volume of 5ml of *E. coli* culture, which had been incubated overnight, was centrifuged at 10000 x g for 10min and the bacterial pellet resuspended in 250 µl of P1 buffer (includes RNase A). The solution was transferred to a microcentrifuge tube and the cells lysed by adding 250 µl of buffer P2 and gently inverting the tube 4-5 times. 350 µl buffer of N3 were added to the lysate and mixed gently before centrifugation at 10000 x g for 10 min. The supernatant was applied to the QIA prep column followed by centrifugation at 10000 x g for 1 min, then the QIA prep column was washed with 0.5 ml buffer PB followed by centrifugation at 10000 xg for 1 min. The flow-through was discarded and the column finally washed by adding 0.75 ml of buffer PE with further centrifugation for 1 min as described previously. The QIAprep column was then placed in a clean 1.5 ml microcentrifuge tube, and the DNA was eluted with 50 µl of buffer EB (10 mM Tris. HCl, pH 8.5). This

was left to stand for 1 min and then centrifuged at 10000 x g for 1min. The flow-through was collected and stored at -20°C.

2-4 Preparation of heat shock-competent cells

0.5 ml of an overnight culture of *E. coli* was inoculated into 50 ml of pre-warmed LB and grown with good aeration at 200 rpm at 37°C until an $OD_{600\ nm}$ of 0.4 - 0.5 was obtained. The flask was chilled on ice and cells harvested by centrifugation in chilled centrifuge tubes at 6000 x g for 15 min at 4°C. The cells were suspended gently on ice in a small volume (2 ml) of ice-cold competence solution (Appendix A.3) and then made up to 25 ml with ice-cold solution and incubated for 45 min on ice. After 1 min centrifugation at 4000 x g the cell pellet was resuspended in 2.5 ml of ice-cold competence solution. Finally, ice-cold glycerol (80% v/v) was added to give a final concentration of 15% v/v (450 µl of 80% glycerol to 2.5 ml of ice cold competence solution) and then stored at -70°C.

2-5 Heat shock transformation

100 µl of the competent cells (stored at -70°C) were thawed on ice and 2 µl of plasmid DNA were added. The solution was mixed and incubated on ice for 30 min and then the tube was heated at 42°C for 90 s. One ml of pre-warmed (37° C) LB was added to the cells and incubation continued at 37° C) for 1 h. Finally 100 µl protions were spread onto the LB agar plates containing appropriate antibiotics and the plates were incubated at 37°C overnight.

2-6 Preparation of recombinant CyaA from *E.coli*

20 ml of an overnight culture of *E. coli* BL21/DE3 containing the relevant plasmids were diluted into 500 ml of LB containing appropriate antibiotics. This was incubated at 37°C with shaking at 200 rpm until an OD_{600nm} of between 0.4-0.45 was obtained (~3 h). At this point, isopropyl-1-thio-β-D-galactoside (IPTG) was added to a final concentration of 1 mM and shaking continued at 37°C for 3 h. Finally, cells were harvested at 10,000 x g for 25 min and the supernatant discarded. Cells pellets could be stored at -20°C if necessary.

2-6-1 Preparation of crude urea extract

The cell pellets were suspended in 10 ml of 20 mM histidine buffer (pH 6.0) (Appendix B.1) and were disrupted using a sonicator (Jensons Ltd, UCX-400 Watt) continuously for 2 min in ice. The cell lysate was then centrifuged at 15,000 x g for 20 min, the supernatant discarded and the pellets containing inclusion bodies of recombinant CyaA proteins re-suspended in 10 ml of 1% (v/v) N-octyl β-D glucopyranoside (Sigma) in 20 mM histidine buffer (pH 6.0). The suspension was centrifuged at 15,000 x g for 20 min and the pellet was re-suspended in 2 M urea, 20 mM histidine buffer and, after centrifugation, washed again with pyrogen free-water (LAL reagent water, Cambrex Company). These washing steps were designed to remove contaminating proteins and LPS from the inclusion bodies. The resultant pellets were solubilized in 10 ml of 8 M urea, 20 mM histidine buffer (pH 6.0) by stirring the suspension at 4°C overnight. The supernatant, obtained after centrifugation at 15000 x g for 20 min, was stored at –20°C.

2-6-1-1 Q Sepharose column chromatography

A 5 ml volume of packed Q Sepharose fast flow (Amersham) was applied to a glass Econo-Column [(1.5 x 30 cm) (Bio-Rad)] and washed with 30 ml of water to remove

excess ethanol. The column was equilibrated by washing with 30 ml of 8 M urea, 20 mM histidine buffer pH 6.0 (Appendix B.2). 2 ml of crude urea extract were applied directly to the column. After 30 min at room temperature the flow through was applied again to the column for 15 min. The column was washed with 2 M urea, 20 mM histidine buffer pH 6.0 (Appendix B.3) followed by elution of CyaA protein with increasing concentrations of NaCl (0.1-0.4 M) in 8 M urea, 20 mM histidine buffer pH 6.0 (Appendix B.4, A-C). The eluted urea extracts were analysed by SDS-PAGE for the presence of CyaA protein, and those containing significant amounts were stored at –20°C if necessary.

2-6-1-2 Butyl Sepharose chromatography

The relevant fractions from Q sepharose purification containing CyaA were pooled and diluted 1 in 5 with 4 M $(NH_4)_2SO_4$ in 20 mM histidine pH 6.0 (Appendix B.5) to give a final concentration of 0.8 M $(NH_4)_2SO_4$. A 2ml volume of packed Butyl Sepharose (Amersham) was applied to an Econo-column [(1.5 x 30 cm) (BioRad)] and washed with 30 ml of water to remove excess ethanol. The diluted CyaA sample was applied to the column after it had been equilibrated with 30 ml of 8 M urea, 20 mM histidine, and 0.8 M $(NH_4)_2SO_4$, 0.3 M NaCl, pH 6.0 (Appendix B.6, D) for 30 min. The column was washed with 2M urea, 0.8 M $(NH_4)_2SO_4$ in 20 mM histidine buffer, pH 6.0 followed by washing with 8 M urea, 20 mM histidine 0.8 M $(NH_4)_2SO4$, 0.2 M NaCl, pH 6.0 (Appendix B.7, E). Once again the column was washed with 8 M urea, 20 mM histidine, 0.6 M $(NH_4)_2SO_4$ (Appendix B.8, F). Finally the CyaA protein was eluted with 8 M urea, 20 mM histidine, 0.32 M $(NH_4)_2SO_4$, 0.08 M NaCl, pH 6.0 (Appendix B.9) and fractions containing CyaA protein pooled and stored at -70°C.

2-6-2-3 Dialysis of purified CyaA

For use in protein-coated microcrystals (PCMCs) (see section 2-15) CyaA preparations were sometimes dialysed against 8 M urea, 20 mM histidine pH 6.0 to remove $(NH_4)_2SO_4$ and NaCl. 5 ml of purified CyaA were dialysed twice against the above solution (200 ml of 8 M urea, 20 mM histidine, pH 6.0) using a 3ml capacity slide dialysis cassette (SnakeSkin® Dialysing Tubing 10000 Pierce). First, it was dialysed for 4 h at 4°C and then the dialysis solution was changed with a fresh one, and dialysis continued at 4°C overnight. The resultant preparation was used to make CyaA–PCMCs.

2-7 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was done according to the method of Laemmli (1970) in a vertical gel electrophoresis tank. Gel plates were assembled following the manufacturer's instructions. The separating gel, containing 7.5% (Appendix C.1) or 15% acrylamide (Appendix C.2), was poured between the plates to approximately 4.5 cm below the top of the plates and absolute ethanol was used as a cover. The gel was placed at room temperature for 45 min until the gel set. The stacking gel, containing 4.5% acrylamide in buffer (Appendix C.3) was poured directly onto the polymerised separating gel after washing the top of the gel with distilled water to remove traces of ethanol and drying with filter paper. Combs were placed into the gel and the gel was incubated at room temperature for 30 min. The combs were removed after the gel had set. A 10 µl aliquot of sample was added to 10 µl of solubilising buffer (Appendix C.4) and kept at 100°C for 5 min. The heated samples were loaded into wells of the gel and current applied at 120 mA until the tracking dye moved to the bottom of the separation gel. At this point the power was switched off and the gel removed. The gel was stained with coomassie

blue (Appendix C.5) for 30 min and then de-stained with de-stain solution (Appendix C.6) for several changes until protein bands were visible against a clear background.

2-8 Silver stain for lipopolysaccharide

This was done according to the method of Tsai and Frasch (1982). SDS-PAGE (15% acrylamide) was done according to the method described previously (section 2-7). A 10 µl volume of LPS control (25 µg/ml from *E. coli* BL21/DE3) and samples were added to 10 µl of solubilising buffer and boiled for 5 min. The heated samples were loaded into the gel and electrophoresis done at 120 mA until the tracking dye moved to the bottom of separation gel. The gel was removed into fixing solution (Appendix D.1) in a clean dish (20 x 20 x 5 cm) and left overnight with shaking at room temperature. The fixing solution was then replaced with oxidising solution (200 ml of 0.7% periodic acid in 40% ethanol-5% acetic acid) and left for 5 min. The gel was washed with 500-1000 ml of distilled water for 15 min, three times. The gel was placed in the staining reagent (Appendix D.2) and agitated vigorously (70 rpm) for 10 min. The gel was then placed in developing solution (Appendix D.3). LPS in the gel was stained dark brown in 2-5 min. Development was terminated when the stain reached the desired intensity or when the clear gel background showed the first signs of discolouration. The process was stopped with 10% (v/v) acetic acid solution and after washing for 30 s, with water three times, the gel was stored in water.

2-9 Estimation of protein concentration

This was done according to the method of Bradford (1976). Bovine serum albumin (BSA, Sigma) was used as standard with serial dilutions (1 in 2 to 1 in 128) in distilled water (500 to 7.8 µg/ml). Each dilution of standard and samples (25 µl) were transferred to duplicate wells in flat bottom microtitre plates followed by addition of 200 µl of protein assay reagent (Bio-Rad reagent, Coomassie brilliant blue G-250). The OD was

measured with a plate reader at 630 nm in the presence of blank (diluent) and protein concentrations determined from a standard curve derived from the OD of the BSA dilutions.

2-10 Western blotting

This was carried out according to the method of Towbin *et al.* (1979). The proteins were separated in 7.5% SDS-PAGE and the gel then soaked in transfer buffer (Appendix E.1) for 20 min. Nitrocellulose membrane (Hybond-C Amersham) and absorbent paper (Whatman 3 MM) were cut to the size of the gel and soaked in transfer buffer for 1 min. The gel was then placed between the membrane and absorbent paper with one Scotch Brite pad on each side and rolled over to make a good contact. The complete blot was placed in a tank of transfer buffer (Appendix E.1) and subjected to a voltage of 100 volts for 1 h. The membrane was then removed and washed with BSA containing Tween 20 (PBST) (Appendix E.2) and then the membrane was incubated with blocking buffer (Appendix E.3) with shaking at room temperature for approximately 1 h to prevent non-specific adsorption of the immunological reagents. A rabbit anti-CyaA serum used as primary antibody (kindly supplied by R. Parton) was diluted 1 in 1000 in blocking buffer and incubated with the blot with shaking at 4°C overnight. The membrane was washed with PBST twice for 15 min. The secondary antibody, goat-anti rabbit IgG conjugate (HRP, Scottish Antibody Production Unit), was diluted 1 in 1000 in blocking buffer and incubated with the membrane at room temperature with shaking for one hour. The membrane was then washed twice with PBST and developed with freshly prepared 3,3-diaminobenzidine (DAB) solution (Appendix F.4) for 2-10 min after which it was washed in distilled water several times to stop the reaction and the membrane was stored at room temperature in the dark.

2-11 LAL (Limulus amoebocyte lysate) assay

Limulus amoebocyte lysate is an aqueous extract of blood cells (amoebocytes) from the horseshoe crab, *Limulus polyphemus*. The test is referred to as a chromogenic assay and empolys a synthetic chromogenic substrate that, in the presence of LAL and endotoxin, produces a yellow color that is linearly related to the endotoxin concentration.The standard endotoxin reagent is a lyophilised product that comes with a certificate of analysis which states how much water should be added to the standard to give a final concentration of 50 IU/ml. This standard, once reconstituted, is suitable for use for up to a month if stored at 4°C. The diluted standards are usable within a day, otherwise they should be thrown away. Use of endotoxin-free water during the assays is very important. The Limulus amoebocyte lysate is also lyophilised and once reconstituted should be stored at -20°C where it is stable for two weeks. It is very important to use the standard endotoxin and LAL reagent as a matched standard pair. An appropriate volume of water was added to standard endotoxin and vortexed for 15 min. It also needs to be vortexed for 4-5 min prior to use again to ensure the endotoxin is not clumped. Reconstituted standard, vortexed as described above, was then used as a stock to derive a standard curve. Using endotoxin-free tubes (Sigma), 10-fold dilutions of the standard were made by mixing 900 µl of water with 100 µl of stock, mixing for 1 min between each dilution. For the standard, 5 dilutions were made from 50 IU/ml to 0.005 IU/ml. Samples were diluted 1 in 100 and 1 in 1000 as described and vortexed for 1 min. To a 96-well flat bottom microplate (Promega, Cat No: 3590), 100 µl of diluted standard and 100 µl of lysate was added in duplicate wells and 100 µl of diluted samples and lysate were added as duplicate test samples. The plate was incubated at 37°C for 15 min. It is important to add the lysate after warming. The plate was read at 405 nm, over a period of 90 min. The amount of LPS was calculated for 1 ml of sample. To calculate the

actual amount of LPS in 1 mg of CyaA, the reading was multiplied by 3.33. This is because the concentration of both CyaA and CyaA* were adjusted to 300 μg protein/ml.

2-12 Macrophage cell culture

The mouse-macrophage-like cell line (J774.2, ECAC), rat basophilic leukemia (RBL-2H3) cells (ATCC), sheep bone marrow mast cells (Dr John Huntley; Mordun Research Institute, Edinburgh), African green monkey kidney cells (Vero, ECAC) and U937, human monoblastic cells (ATCC) were used when determining cytotoxicity, apoptosis and DNA fragmentation. Each culture was passaged weekly in RPMI 1640 medium (J774.2 and U937 cells) or Dulbecco's modified eagle's medium (DMEM, Gibco) (Vero, RBL-2H3 and mast cells) containing 10% (v/v) foetal calf serum, 1% (w/v) L-glutamine 1% (w/v), amphotericin B and 1% (w/v), penicillin-streptomycin (Gibco). Cells were prepared in a sterile filter unit (Gelaire) and incubated at 37°C with 5% CO_2.

2-13 Characterisation of recombinant CyaA

2-13-1 Adenylate cyclase enzymic activity

AC enzymic activity was evaluated by measuring the change in conductance using an enzymometer (conductimetry assay) which was developed by Lawrence *et al.* (1998). The device consists of 8 glass cells (tubes) each containing platinum electrodes, which have been fused to the bottom of the tube. The sampling period is one second per cell, cycling through from one to eight glass cells. The apparatus is linked to computer software which processes the conductimetric readings and uses software to create curve slopes and total changes, and the export of standard format data files. Buffer (Appendix F.1) containing 10mM bicine, 1.5 mM magnesium acetate pH 8.0 was degassed by heating for 1-2 min >90 °C and placed under the vacuum pressure for 30 sec. After allowing the buffer to cool to room temperature, ATP was added to a final concentration

of 0.5 mM. A volume of 2 ml of buffer, 1 µl of calmodulin (1.8 mg/ml, kindly provided by Dr. A. Lawrence, Glasgow University) and 1 µl pyrophosphatase (0.5 IU, Sigma) were added to each cell. Balancing of the cells to the same base line was allowed to occur before addition of CyaA samples. The reaction was started by addition of 2 µl of CyaA sample. Curve slopes and total changes in conductance were measured on screen whilst reactions were proceeding. One especially important feature is the ability to subtract the reading of one cell from all of the others so that test samples can be standardized to a blank loading. There is no change in conductance unless the (PPi $Mg)^{2-}$ product is cleaved, freeing the Mg^{2+} ion from chelation. It is the release of the independently mobile ions, $2Pi^{2-}$ and Mg^{2+} that produce the change in conductance that is measured by the assay.

$$(ATP.Mg)^{2-} + Bicine^{-} \xrightarrow[CyaA\text{-}CaM]{} cAMP^{-} + (BicineH) + (PPi\ Mg)^{2-} \xrightarrow{pyrophosphatase} 2Pi^{2-} + Mg^{2+}$$

Finally the total conductance changes were measured by taking the gradient of the conductance curves using the equation: initial rate = y/x

µmol/min/ml for a substrate concentration of 1 mM giving a total change of x units and an initial slope of y units/min.

2-13-2 MTT Dye reduction assay (Cytotoxicity assay)

Cytotoxic activity was determined by the MTT assay as described originally by Mosmann (1983) using the CellTiter 96 ™ assay kit (Promega). This assay is based on the reduction of a yellow tetrazolium dye {3-(4,5-dimethylthiazol-2-3yl) –2-5-diphenyl tetrazoliumbromide, MTT)} into insoluble purple formazan by dehydrogenases active in mitochondria of living cells. The activity of toxin on cells inhibits this reaction. J774.2 mouse macrophage-derived cells and other cell lines were prepared in appropriate medium (Section 2-12) to a concentration of 5 x 10^5 cells/ml. A 50 µl volume of cells

was diluted in 10 µl trypan blue 0.25% and 40 µl water and transferred to a Neubauer chamber (haemocytometer). This was placed on a horizontal surface to allow the cells to settle for 1 min. Under the X 10 objective of a light microscope, the average number of cells in four squares were counted and then multiplied by two as a factor of dilution to give total cell number. Uptake of trypan blue reflects the lack of integrity of the plasma membrane and cells were examined to ensure >90% live cells. Aliquots of 50 µl of cells in duplicate were transferred to wells of a 96-well microtiter plate (Corning) and incubated at 37°C for 1 hour. A CyaA sample was diluted in cell culture medium in serial 2-fold dilutions starting at a protein concentration of 5 µg/ml. Then, volumes of 50 µl of each dilution were added in duplicate to wells of 96-well plate. The plate was incubated at 37°C for 2 hours in a humidified 5% CO_2 atmosphere before the addition of 15 µl of MTT dye (Promega) to each well and incubation continued as above for 4 h. The stop (solubilization) solution (100 µl/well, Promega) was added and incubation continued at 37°C overnight. The OD_{540nm} of the wells was measured using an ELISA reader (Life Science Int, Uk) against a positive (cells + 1% Triton X-100) and a negative (Cell + medium) control. 100% killing was taken as the mean absorbance value for the positive control. The percentage killing of cells was calculated using the following formula:

$$\text{Percentage killing} = 1 - \left(\frac{\text{test sample} - \text{positive control}}{\text{negative control} - \text{positive control}} \right) \times 100$$

2-13-3 ATP cytotoxicity assay

Cytotoxicity of CyaA for macrophges was further investigated using a bioluminescent cell viability assay (CellTiter-Glo assay, Promega). This assay determines the number of viable cells based on the quantification of light output as a measure of ATP present in the cells. ATP was measured using a bioluminescence method based on the luciferin-

luciferase reaction (Higashi *et al.*, 1985). The reaction, which results in the generation of measurable light, is given below:

$$ATP + Luciferin + O_2 \xrightarrow{Luciferase} Oxyluciferin + AMP + PP_i + CO_2 + light$$

J774.2 mouse macrophages, RBL and mast cells were cultured as described in section 2-12 and maintained at 37°C in 5% CO_2 and then diluted to 5 x 10^5 cells/ml after counting cells in the haemocytometer. A volume of 50 µl of each cell suspension was added to the wells of a 96-well flat bottom plate (Costar) and then incubated for 1 h at 37°C, 5% CO_2. CyaA samples were prepared at different concentrations in the growth medium used for cell culture. 50 µl of each concentration were added to the cells in duplicate. The plate was incubated for 6 h followed by addition of 100 µl of Cell Titer-Glo Reagent (Promega) to lyse the cells and release ATP for the luciferase reaction. Cytotoxicity was evaluated after 10 min by measuring the luminescent signal in a luminometer and comparing the relative light output to that of a negative control with no toxin added and a positive control with Triton X-100 added to a final concentration of 1% (v/v). The percentage killing of cells was calculated using the equation given in section 2.13.3.

2-13-4 Apoptosis assay

The homogeneous caspase 3/7 assay (Promega) provides a fluorescent substrate with an optimized bifunctional cell lysis/activity buffer for caspase 3/7 (DEVDase) activity as an indicator of apoptosis. Various types of mammalian cells were prepared to a cell concentration of 5 x 10^5 per ml (See section 2-12) and treated with diluted CyaA preparations as described in section 2-13-2. A volume of 50 µl of each cell dilution was added in duplicate to a 96-well flat bottom microtiter plate (Opaque) and the plate was incubated at 37°C in 5% CO_2 for 6 h. After the incubation time, 100 µl of homogenous

caspase 3/7 Reagent (Apo-ONE ^{TM} and caspase substrate Z-DEVD-Rhodamine 110 Promega) were added. These reagents were prepared by thawing the 100x substrate and buffer at room temperature, mixing by inversion or vortexing, then the substrate solution was diluted 1 in 100 with the buffer to obtain the desired volume of reagent. The microtitre plate was shaken at 200 rpm for 30 min at room temperature. Caspase activity was measured by using a fluorescence spectrometer (Perkin Elmer instrument LS 55) at an excitation wavelength of 485 ± 2.5nm and an emission wavelength of 530 ± 2.5nm. Activity was measured over a range of toxin concentrations from 2.5 to 0.010 μg/ml. The amount of fluorescent product generated is proportional to the amount of caspase 3/7 cleavage activity present in the sample.

2-13-5 DNA fragmentation as a measure of apoptosis

The J774 mouse macrophage-like cells, RBL-2H3 cells, sheep bone marrow mast cell and Vero cells were grown in 25 ml flasks as described in section 2-12 and maintained in a 5% CO_2 atmosphere at 37°C overnight. The same cells grown in appropriate media minus foetal calf serum (FCS) were included as a positive control. Urea buffer at a concentration equivalent to that used for each CyaA concentration tested was incubated as a negative control. The cells were treated with CyaA samples at the final concentration of 0.039 μg/ml. After 12 h, the cells were removed and DNA purified using a Wizard Genomic DNA purification kit (Promega) following the manufacturer's instructions. Samples (10 μl) were subjected to electrophoresis in 1% agarose in TEB buffer solution (Appendix A.4) at 100 V for 120 min and visualised by staining with ethidum bromide.

2-14 Phagocytosis procedure

The phagotest Kit (OPREGEN Pharma; BD Biosciences, Oxford, UK) allows the quantitative determination of leukocyte phagocytosis. It contains fluorescently (FITC-fluorescein isothiocyanate) labelled, opsonized bacteria (*E.coli*-FITC) and measures the overall percentage of macrophages and granulocytes showing phagocytosis in general (ingestion of one or more bacteria per cell) and the individual cellular phagocytic activity (number of bacteria per cell).

The investigation of phagocytosis can be performed either by flow cytometry or by fluorescence microscopy. Because of the quantitative analysis, very accurate work is important, especially when day to day comparisons are required. The detailed instructions result from specific experience and precise validation assays.

The ingestion activity of peripheral human granulocyte cells in whole blood, J774.2 and U937 cell lines was evaluated in the presence and absence of recombinant CyaA protein using the flow-cytometry based Phagotest kit according to the manufacturer's instructions for conjugated *E. coli.* Briefly, 100 µl of whole blood or a volume of 0.4 x 10^6 of J774.2 mouse macrophages or human monoblastic U937 cells were added at the bottom of a 5 ml Falcon tube. Samples were incubated (120 minutes, 37°C, 5% CO_2) with either CyaA or CyaA* diluted in 8 M urea, 20mM histidine (0.05, 0.1 or 0.2 µg/ml) or PBS, then incubated for another 20 minutes at 37°C in 5% CO_2 with opsonized *E. coli*-FITC cells (6 cells per leukocyte). A sample with PBS and no Bioparticle and another sample with PBS (120 min) and opsonized FITC-labeled *E. coli* cells (20 min) remained on ice for the whole period of experiment, these acted as negative controls.

At the end of the incubation time all samples were placed on ice in order to stop phagocytosis. A volume of 100 µl of ice-cold quenching solution was added and mixed

gently. Then 2ml of washing solution was added to each tube after which the tubes were centrifuged at 4°C for 5 min at 250 x g. The cell samples were incubated in washing solution containing 1% paraformaldehyde for 10 min, washed again and centrifuged as described. Finally, 200 µl of DNA staining solution were added to each tube, mixed and incubated for 10 min on ice. Cells were analysed by flow cytometry (FACSCalibur, BD Biosciences) and 5000 granulocyte events acquired to obtain the percentage and number of cells ingesting bioparticles, as well as their mean fluorescence intensity (MFI) using Cellquest Pro software (BD Biosciences). The nucleated cells were discriminated by a setting in the red fluorescence channel (DNA staining FL2-H, Fig 2.1A), detecting those events which have the DNA content of a human diploid cell (to exclude bacterial aggregates). The nucleated events were then discriminated; into lymphocytes, monocytes and polymorphonuclear cells by combined measurements of the forward angle light scatter (FSC) and side angle light scatter (SSC). The granulocyte cluster was then gated in the analysis program in the scatter diagram (FSC vs SSC) (Fig 2.1B), and its green fluorescence histogram (FL1) was analysed (Fig 2.1D and E). The SSC vs FL-1 diagram also was also set to make sure that the appropriate cell population data was collected (Fig 2.1 C).

2-15 Oxidative burst procedure

The oxidative burst component of the phagocytic process was measured in whole blood peripheral polymorphonuclear cells with the commercial flow cytometric-based BurstTest kit (OPREGEN Pharma; BD Biosciences) according to the manufacturer's instruction for *E.coli* bioparticles. This assay allows the quantitative determination of leukocyte activity without prior purification. The BurstTest assay relies on unlabelled opsonized *E.coli* bacteria as the particulate stimulus and dihydrorhodamine (DHR) 123

as a fluorogenic substrate of oxidative activity. Briefly, either heparinised whole blood, or J774.2 mouse macrophages and U937 human monocytes were incubated (120 minutes, 37°C, 5% CO_2) with either recombinant CyaA or CyaA* in 8 M urea, 20mM histidine diluted to 0.05, 0.1 or 0.2 μg protein/ml or with PBS, then incubated for another 15 minutes at 37°C in 5% CO_2 with opsonized *E. coli* cells (6 cells per leukocyte).

Fig 2.1 Data acquisition and analysis of the Phagotest and the Phago Burst by flow cytometry.

A-Gating for nucleated cells (DNA Staining), the peak shows the maximum fluorescence

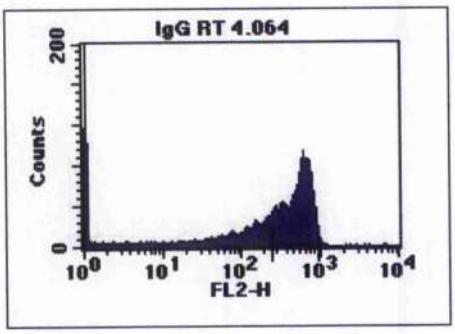
B-Gating nucleated cells, based on SSC and FSC, the cells in the circle area are viable granulocytes which are used for evaluation of phagocytosis

C-Fluorescent intensity and SSC (number of events containing fluorescence)

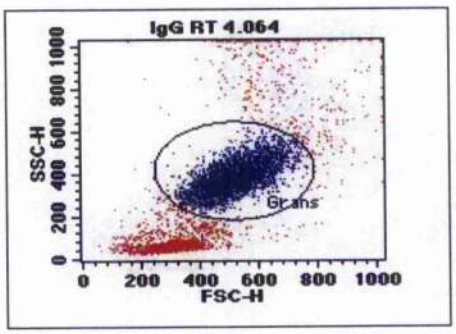
D-Histogram of fluorescent intensity of granulocytes

E-Statistical analysis of fluorescent intensity of cells. In this study, the mean of total was used which showed on part D as M2 with the highest peak of fluorescent.

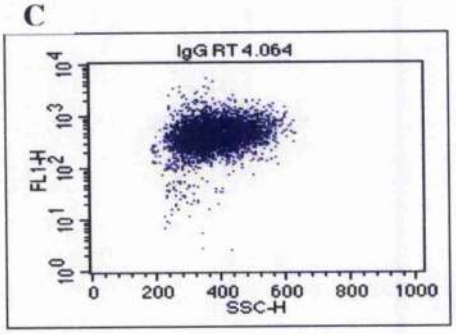
A



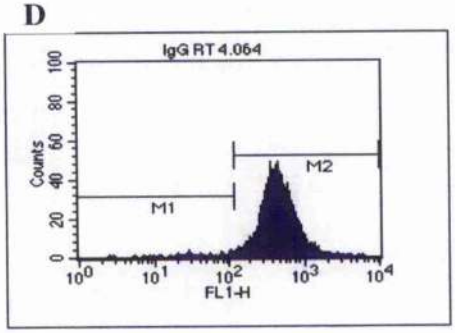
B



C



D



E

Histogram Statistics

File: IgG RT 4.064
Sample ID: IgG RT 4
Tube: Untitled
Acquisition Date: 18-Jun-05
Gated Events: 4354
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: G3
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	4354	100.00	43.54	479.84	412.94	63.64	414.18	339
M1	1, 119	82	1.88	0.82	63.28	46.53	59.77	54.74	100
M2	119, 9910	4274	98.16	42.74	487.67	430.36	62.07	417.92	339

A sample without stimulus served as negative background control. DHR 123 was then added and the cells incubated (10 minutes, 37°C, 5% CO_2). The reaction was stopped by the addition of lysing buffer, which partially fixes leukocytes and lyses erythrocytes. Finally, a DNA staining solution was used, as described for the Phagotest. Cells were analysed by flow cytometry (FACS Calibur, BD Biosciences) and 5000 granulocyte events acquired to obtain the percentage and number of cells having produced reactive oxygen radicals, as well as their mean fluorescence intensity (oxidative activity) using the Cellquest Pro software (BD Biosciences). Similar to PhagoTest, nucleated cells were discriminated by setting in the red fluorescence channel (DNA staining FL2-H, Fig 2.1 A), for those events which have DNA content as for the human diploid cell. The granulocyte cluster was then gated in the analysis program in the scatter diagram (FSC-H) vs SSC-H) (Fig 2.1B), and its green fluorescence histogram (FL1-H) was analysed (Fig 2.1 D and E). In general, in this experiment the mean total (M2) was used in statistical calculation as shown in part E.

Suspensions of J774.2 and granulocytes cells (0.5 x 10^6, 10µl) were placed on immunofluorescence slides and dried. After fixing the coverslip with fluorescent mounting medium (Citifluor, London, UK) and sealing with nail varnish, samples were viewed under a fluorescence microscope (Nikon Microphot-SA) and where necessary, images were collected with a confocal microscope (Zeiss LSM 510, Germany).

2-16 CyaA protein-coated microcrystals (PCMCs)

The procedure used to prepare PCMCs is simple, rapid and relatively inexpensive. It is a newly developed and patented process, which yields micron-sized particles in a dry formulation suitable for drug delivery to, for example, the lungs (Kreiner *et al.*, 2001). In these experiments, the crystal-forming coprecipitant (excipient) used was DL-valine. The water miscible organic solvent used was ethanol and in addition propan-2-ol was

tested on a few samples. A variety of PCMCs were prepared under different conditions. Although the mechanism of the PCMC formation process is unknown, the driving force for the organisation of the protein molecules on the surface of the excipient (DL- valine) is thought to be the high crystal lattice conformation of the core material. Essentially, the protein molecules behave as impurities in the crystallisation process and, since they are insoluble in the surrounding solvent, they remain confined to the crystal surface. Figure 2.2 shows how PCMCs were made.

2-16-1 PCMC preparation from crude CyaA in 8M urea

1 ml of crude urea extract of CyaA containing 3.4 mg protein//ml in 8 M urea, 20 mM histidine, pH 6.0 was added to 1 ml of saturated DL-valine solution (60 mg/ml in distilled H_2O) and mixed for approximately 30 s. The aqueous protein solution was then added dropwise, with rapid mixing or stirring, to 40 ml of absolute ethanol saturated with DL-valine. Stirring (1500 rpm) was continued for a set time (15-20 mins). Crystals became visible gradually. The resulting crystals were filtered onto filter paper (Millipore Ltd., UK, 0.45 μm HV) and washed with 40 ml of absolute ethanol. The filter paper was placed in a petri dish, and covered with parafilm; the parafilm was pierced to allow air to circulate in the dish and the PCMCs were left to dry overnight at room temperature.

Figure 2.2 Preparation of CyaA-protein-coated microcrystals (PCMC)

The procedure used to prepare CyaA-coated microcrysyals.

Production of 'protein coated microcrystals'

CyaA is added to concentrated solution of an inert crystal forming co-precipitant (DL-valine)

↓

co-precipitate by drop-wise addition to DL-valine -saturated ethanol

↓

decant off excess solvent and filter, then wash with 100% ethanol

↓

store as suspension or dry powder

2-16-2 PCMC preparation from crude CyaA in 2M urea

A volume of 0.5 ml of crude urea extract of CyaA containing 3.4 mg protein/ml was added to 1.5 ml of sodium phosphate buffer (Appendix B.10) to give a CyaA solution in 2M urea. The protein solution was added to 2ml of saturated DL-valine (60 mg/ml) in dH_2O and vortexed for 30 s. The solution was added dropwise to 80 ml absolute ethanol saturated with DL-valine then stirred (1500 rpm) for 15-20 min to make crystals. The solution was filtered immediately and washed with 80 ml of absolute ethanol. The filter paper was placed in a petri dish, and covered with parafilm. The PCMCs were left to dry overnight at room temprature. It should be noted that some samples were made by shaking and some were made by stirring.

2-16-3 PCMC preparation from purified CyaA

2-16-3-1 Method 1: CyaA-PCMCs

A volume of 6 ml of purified CyaA in 8 M urea, 20 mM histidine , 0.32 M $(NH_4)_2SO_4$, 0.08 M NaCl (pH. 6.0) containing 290 µg/ml of protein was added to 2ml of saturated DL-valine (60 mg/ml) and vortexed for 30 sec. The solution was added dropwise to 80 ml of absolute ethanol saturated with DL-valine then stirred (1500 rpm) for 15-20 mins to make crystals as described above (section 2-16-2).

2-16-3-2 Method 2: CyaA-BSA-PCMCs

A volume of 1 ml of purified CyaA (290 µg protein/ml) was added to 3 ml of sodium phosphate buffer pH 6.0 containing 870 µg of bovine serum albumin (CyaA: BSA 1: 10 molar ratio). The protein solution was added to 2 ml of saturated DL-valine (60 mg/ml in dH_2O) and vortexed for 30 s. All other steps were done as described in method 1.

2-16-3-3 Method 3: CyaA-CaM-PCMCs

A volume of 1 ml of purified CyaA (290 µg/ml) and 46 µl of calmodulin (1.8 mg/ml in dH_2O) were mixed to give a ratio of 1:3 molar and the mixture was added to 2 ml of saturated DL-valine (60 mg/ml in dH_2O) and vortexed for 30 s. All other steps were done as described in method 1.

2-16-3-4 Method 4: CyaA-CaM-BSA-PCMCs

A volume of 4 ml of purified CyaA (290 µg protein/ml), 200 µl of calmodulin (1.8 mg/ml) and 12 ml of sodium phosphate buffer containing 3480 µg of BSA was added to 2 ml of saturated DL-valine (60 mg/ml dH_2O) to give a molar ratio of 1: 3 and vortexed for 30 s. All other steps were done as described in method 1.

2-16-3-5 Method 5: CyaA-CaM-BSA-$CaCl_2$-PCMCs

A volume of 4 ml of purified CyaA (290 µg protein/ml), 200 µl of calmodulin (1.8 mg/ml), 20 µl 1 M $CaCl_2$ and 12 ml of sodium phosphate buffer containing 3480 µg of BSA was added to 2 ml of saturated DL-valine (60 mg/ml in dH_2O) and vortexed for 30 s. All other steps were done as described in method 1.

2-16-3-6 Method 6: CyaA-BSA-CaM-ATP-PCMCs

A volume of 4 ml of purified CyaA (290 µg protein/ml), 200 µl of 100 mM adenosine triphosphate (ATP) and 12 ml sodium phosphate buffer containing 3480 µg BSA, 200 µl of CaM (1.8 mg/ml) was added to 2ml of saturated DL-valine (60 mg/ml in dH_2O) and vortexed for 30 s. All other steps were done as described in method 1.

2-17 Determination of urea concentration of PCMCs

10 mg of PCMCs were dissolved in 10 ml of distilled water. A volume of 0.5 ml of 1 mg/ml urease (Sigma) was added to 9.5 ml of PCMC sample solution and incubated for

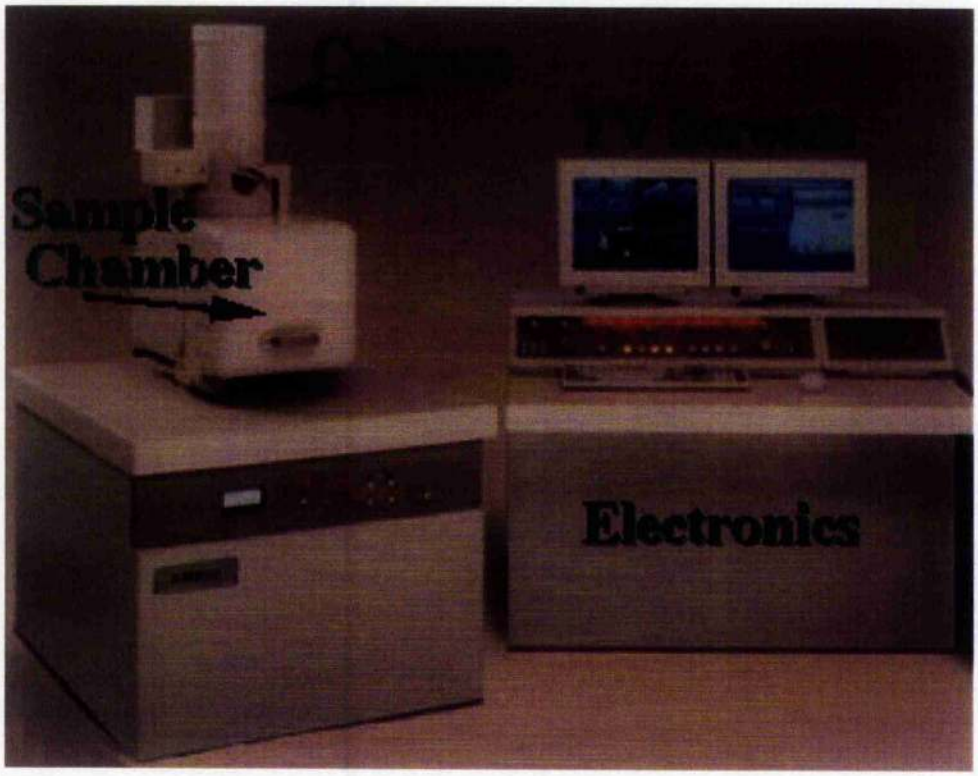
10 min. The urease converts one mole of urea to two moles of ammoina. The incubated solution was poured into a 10 ml cuvette. 4 drops of the first reagent (code HI 93715A) were added followed by 4 drops of the second reagent (code HI 93715B) and incubated for 3 min at room temperature. The amount of urea was monitored using an ammonia meter (Hana Instruments, Ion Specific Ammonia Meter, HI 93715) which measures the UV absorption of the ammonia-containing solution. The instrument directly displays concentration in mg/L of ammonia nitrogen. To convert the reading to mg/litre of urea, the display is multiplied by a factor of 1.214.

2-18 Scanning electron microscopy (SEM)

SEM was used to characterise PCMC size and morphology. The SEM is designed for direct study of the surfaces of solid objects. By scanning with an electron beam that has been generated and focused by the operation of the microscope, an image is formed in much the same way as a TV (Figure 2.3). The SEM allows a greater depth of focus than the optical microscope. For this reason the SEM can produce an image that is a good representation of the three-dimensional sample. Biological samples require coating with a metal, such as gold, prior to imaging. Each sample was loaded onto a metal stub for SEM analysis. Before the dry powder samples could be applied to the stub, the stubs had to be covered with double-sided sticky copper tape. The dry powder samples were then sprinkled lightly onto the stubs where they stuck to the copper tape. Only a fine coating of powder is required for SEM analysis and any excess powder on the stub was removed by lightly blowing over the stub surface. A thin coating of gold was then evaporated onto the stubs which were then examined using a Phillips 500 SEM over a magnification range of X800 and X1600 and images were recoverd by Imageslave Windows Software.

Figure 2.3 Scanning electron microscopy (SEM)

SEM was used to characterise PCMC size and morphology. The Scanning Electron Microscope creates the magnified images by using electrons instead of light waves. A representation of the SEM instrument is shown opposite.


Sample
Chamber
Electronics

2- 19 *In vivo* assay

2-19-1 Mice

Female BABL/c mice (Harlan, UK), aged 4 to 5 weeks, were maintained in quarantine for a week and housed in boxes of five animals each.

2-19-2 Immunisation

CyaA-CaM-BSA-PCMCs prepared as in method 4 were suspended in sterile PBS. One group of five mice was injected subcutaneously on days 0 and 28 with 0.2 ml of CyaA-CaM-BSA-PCMC solution containing 15μg of CyaA (from a preparation containing 290μg protein/ml), 67.5 μg of BSA and 5 μg of CaM per dose. A control group of 5 mice was injected with a solution containing the same amount of BSA and CaM in PBS. Mice were bled at 14 days after the second injection and serum was separated and pooled for each group and stored at -20°C.

2-19-3 Enzyme-linked immunosorbent assay (ELISA)

Antibody responses to the PCMC compounds were measured by 96-well ELISA. The ELISA plates (high bonding capacity, Greiner) were coated with 50 μl per well of antigen (purified CyaA, BSA or CaM) at final concentrations of 1μg/ml in coating buffer (Appendix G.1) and incubated overnight at 4°C. The plates were then washed with washing buffer (Appendix G.2) containing 0.05 % Tween in PBS and dried by repeated blotting on to paper towels. The plates were then blocked with 200 μl per well of 0.01% gelatin in PBS (Appendix G.3) for 1 h at 37°C. Plates were washed as before for 3 times and dried. A volume of 50 μl of PBS was added into each well from rows B to H. 100 μl of serum sample (diluted 1 in 100) were added to row A and 50 μl of the serum from row A were transferred to row B etc to make a doubling dilution series down the plate. The last 50 μl from row H were discarded and the plates were incubated

for 1 h at 37°C. The plates were washed 3 times as above and 50 µl of anti-mouse IgG peroxidase conjugate (Sigma) at 1 in 20,000 dilution were added per well and incubated for 1 h at 37°C. Before adding the OPD-FAST substrate (Appendix G.4) the plates were washed 3 times with washing buffer. After 30 min the reaction was stopped by addition of 50 µl of 3M HCl per well and the OD $_{492nm}$ was measured in an ELISA plate reader (Life Science Int, UK).

Chapter 3

Results

3-1 Production of recombinant CyaA toxin from *E. coli*

CyaA was produced in different recombinant forms and *E. coli* BL21/DE3 was used as the host strain for production of these recombinant proteins (section 2.1). The CyaA pro-toxin, which has AC enzymic activity and the CyaC protein required for posttranslational activation of cytotoxic activity, were expressed from two separate compatible plasmids, pGW44 and pGW54 respectively. Co-expression of *cyaA* and *cyaC* in the presence of 1mM IPTG in *E. coli* BL21/DE3 containing both plasmids (pGW44 and pGW54) resulted in production of biologically-active (with enzymic and invasive activity) CyaA. Expression of CyaA in the absence of CyaC from *E. coli* harbouring only pGW44 resulted in production of enzymatically-active, non-invasive (non-acylated) pro-CyaA. A plasmid pGW44-188 encoding CyaA pro-toxin lacking adenylate cyclase enzymic activity, was co-expressed with pGW54 to create catalytically-inactive, invasive CyaA (CyaA*). Both active and non-active forms of CyaA were extracted as inclusion bodies that were solubilized in 8 M urea from induced *E. coli* cultures. These extracts contained 200 kDa proteins that reacted with anti-CyaA polyclonal antibody in Western blots (Section 3.2.4). The results of cultural conditions on the yield of CyaA preparations from *E. coli* BL21/DE3 (pGW44) or *E. coli* BL21/DE3 (pGW44, pGW54) are shown in Figure 3.1.1.

Figure 3.1.1 SDS-PAGE analysis of crude CyaA preparations from *E.coli* BL21/DE3

Crude urea extracts of CyaA from *E. coli* BL21/DE3 (pGW44, pGW54) or CyaA* (pGW44/188, pGW54), before and after inducing by IPTG, were subjected to electrophoresis on a 7.5% polyacrylamide gel and stained with 1% coomassie blue solution (Section 2.7). Arrows shows the position of the molecular weight markers.

M: Molecular weight markers

Lanes 1, 4: *E.coli* whole cells without IPTG induction.

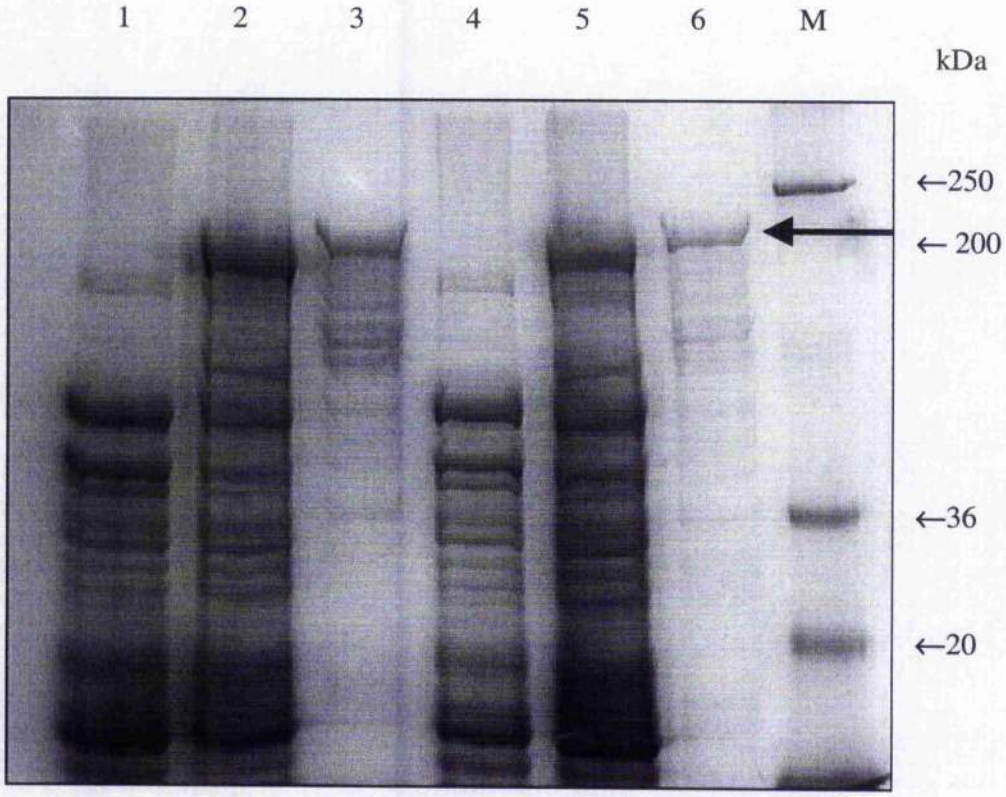
Lanes 2, 5: *E.coli* whole cells after induction with 1 mM IPTG for 3 h.

Lanes 3, 6: Crude urea extracts of CyaA preparations CyaA and CyaA*

Lines 1-3 Samples from *E. coli* BL21/DE3 (pGW44, pGW54)

Lines 4-6 Samples from *E. coli* BL21/DE3 (pGW44-188, pGW54)

The large arrow shows the position of CyaA


1
2
3
4
5
6
M
kDa
←250
← 200
←36
←20

3-2 Purification of CyaA from whole-cell urea extracts.

Crude urea extracts from *E. coli* BL21/DE3 were firstly purified by Q Sepharose column chromatography and step-wise elution with increasing concentrations of NaCl and then by Butyl Sepharose and different washing steps (section 6.1). Q Sepharose column chromatography was most effective in removing contaminating proteins and LPS. Figure 3.2.1 shows that the majority of contaminating proteins were removed in the first steps. Figure 3.2.2 shows the results of Butyl Sepharose column chromatography purification of CyaA from recombinant *E. coli* BL21/DE3. Figure 3.2.3 shows the SDS-PAGE profile after silver staining, where LPS is visualised, indicating that the crude urea extract contained a large amount of LPS, comparable to that in the control LPS sample from *E. coli* BL21/DE3. Only faint bands corresponding to this LPS band are seen in fractions 8 and 9 after Q Sepharose chromatography, indicating that most of the LPS was removed by the washing steps. Figure 3.2.4 shows the Western blotting result of the final purified CyaA preparations with anti-CyaA antibody, a rabbit polyclonal antiserum raised against the 200 kDa CyaA protein. It was clear that the combination of these two chromatographies (Q Sepharose and Butyl Sepharose) was a more suitable method for CyaA purification.

Figure 3.2.1 SDS-PAGE of crude CyaA after Q Sepharose purification

Crude urea extract of CyaA was purified by Q Sepharose. After washing and elution with different concentrations of NaCl, samples were subjected to 7.5% SDS-PAGE and staining with 1% Coomassie blue.

M:	Molecular weight markers
C:	Crude urea extract
Lanes 1, 2:	Wash with 2 M urea, 20 mM histidine
Lanes 3, 4:	Eluted with 0.1 M NaCl in 8 M urea, 20 mM histidine
Lanes 5, 6:	Eluted with 0.2 M NaCl in 8 M urea, 20 mM histidine
Lanes 7, 8	Eluted with 0.25 M NaCl in 8 M urea, 20 mM histidine
Lanes 9, 10:	Eluted with 0.3 M NaCl in 8 M urea, 20 mM histidine
Lanes 11, 12:	Eluted with 0.4 M NaCl in 8 M urea, 20 mM histidine

The large arrow shows the position of CyaA

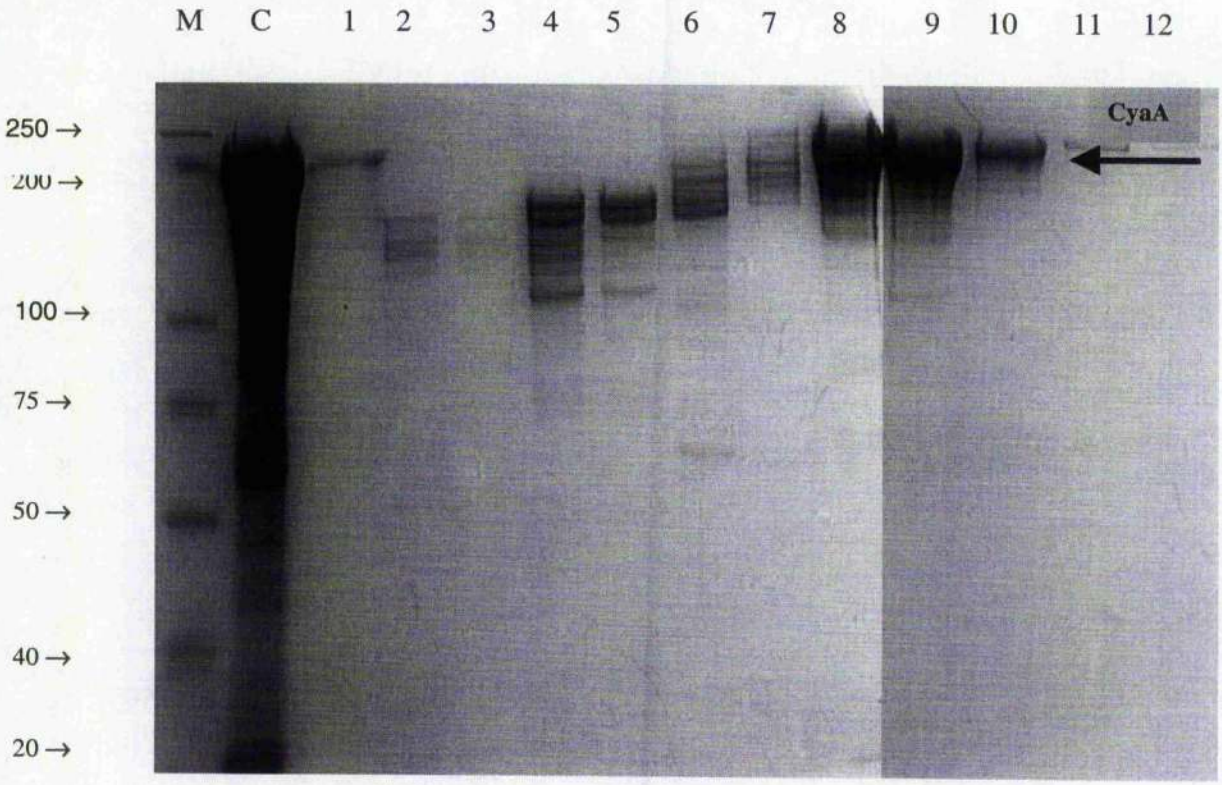

M C 1 2 3 4 5 6 7 8 9 10 11 12
250 →
200 →
100 →
75 →
50 →
40 →
20 →
CyaA

Figure 3.2.2 SDS-PAGE of CyaA after Butyl Sepharose purification

The pooled CyaA fractions from Q Sepharose were further purified by Butyl Sepharose. After washing and elution with decreasing concentrations of ammonium sulphate, fractions were subjected to 7.5% SDS-PAGE and staining with 1% coomassie blue.

M: Molecular weight markers

Lane 1: Wash with 2 M urea, 20 mM histidine, 0.8 M ammonium sulphate

Lane 2 : Wash with 8 M urea, 20 mM histidine, 0.2 M NaCl, 0.8 M ammonium sulphate

Lane 3 : Eluted with 8 M urea, 20 mM histidine, 0.6 M ammonium sulphate

Lanes 4-7 : Eluted with 8 M urea, 20 mM histidine, 0.08 M NaCl, 0.32 M ammonium sulphate

Arrow shows the position of CyaA

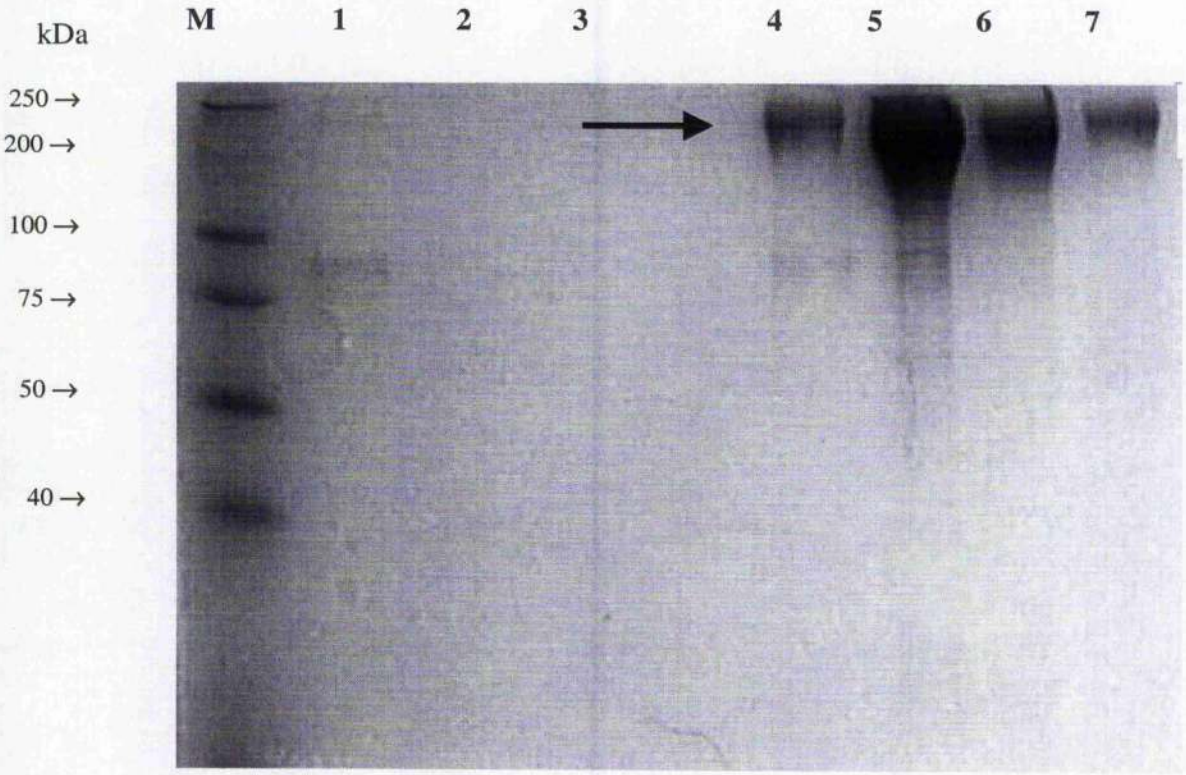

kDa
M
1
2
3
4
5
6
7
250 →
200 →
100 →
75 →
50 →
40 →

Figure: 3.2.3 LPS analysis of CyaA fractions from Q Separose purification, by silver staining

Crude urea extract of CyaA was purified by Q Sepharose. After washing and elution with different concentration of NaCl, fractions were subjected to 15 % SDS-PAGE and LPS was visualised by silver staining.

M	Molecular weight markers
Co	LPS control (from *E. coli* BL21/DE3)
Cr	Crude urea extract of CyaA from *E. coli* BL21/DE3
Lanes 1, 2:	Wash with 2 M urea, 20 mM histidine
Lanes 3, 4:	Eluted with 0.1 M NaCl in 8 M urea, 20 mM histidine
Lanes 5, 6:	Eluted with 0.2 M NaCl in 8 M urea, 20 mM histidine
Lanes 7, 8:	Eluted with 0.25 M NaCl in 8 M urea, 20 mM histidine
Lanes 9, 10:	Eluted with 0.3 M NaCl in 8 M urea, 20 mM histidine
Lanes 11, 12:	Eluted with 0.4 M NaCl in 8 M urea, 20 mM histidine

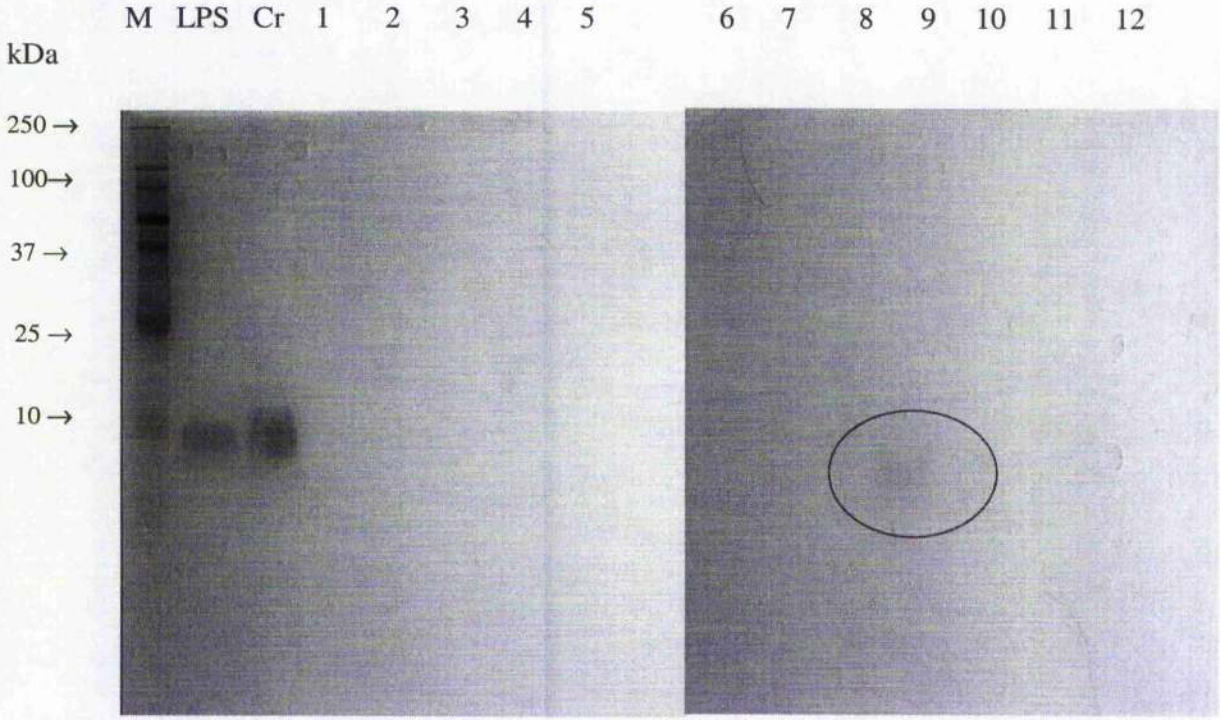

M LPS Cr 1 2 3 4 5 6 7 8 9 10 11 12
kDa
250 →
100→
37 →
25 →
10 →

Figure 3.2.4 Western blot analysis of purified CyaA preparations using anti-CyaA polyclonal antibody

Different types of purified adenylate cyclase toxin were subjected to 7.5% SDS-PAGE, transferred to nitrocellulose membrane, and probed with primary and secondary antibodies.

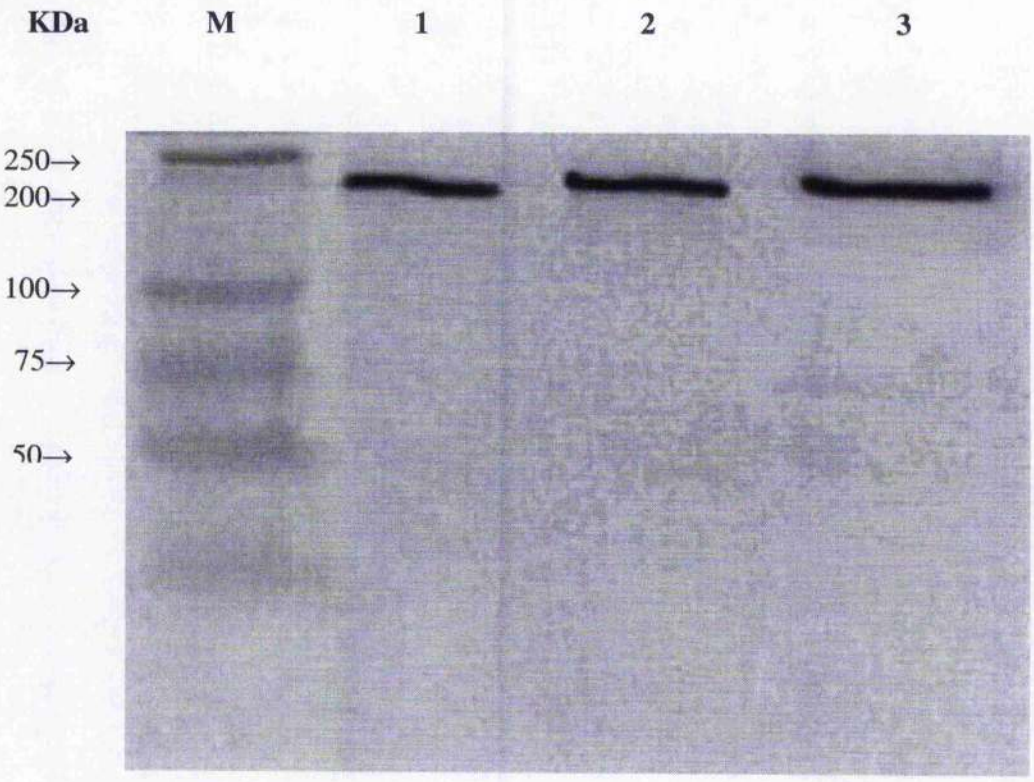
Lane M: Broad range molecular weight markers

Lane 1: Purified pro-CyaA (pGW 44)

Lane 2: Purified CyaA* (pGW44-188/pGW 54)

Lane 3: Purified CyaA (pGW 44/ pGW54)

The large arrow shows the position of CyaA


KDa
M
1
2
3
250→
200→
100→
75→
50→

3-3 Summary of SDS-PAGE analysis of CyaA preparations

The major band in the CyaA preparations migrated on a 7.5% polyacrylamide gel at around 200 kDa (Figure 3.1.1). This is the expected apparent mol. wt of CyaA (Rogel *et al.*, 1990) although the actual predicted mol. wt of the toxin is 177 kDa. Crude urea extracts of the CyaA preparation displayed many additional bands, which indicated the presence of contaminating proteins. The majority of these proteins were removed by Q Sepharose column chromatography (Figure 3.2.1). Further purification was obtained by Butyl Sepharose chromatography (Figure 3.2.2). All purified CyaA forms (CyaA, CyaA* and pro-toxin CyaA) showed very similar bands on Western blot analysis. Purified CyaA preparations probed with anti-CyaA polyclonal antibody produced a profile with a clear band at approximately 200 kDa (Figure 3.2.4). This shows that the antiserum cross-reacted with the band at a 200 kDa, the expected size of CyaA, and the same size as the band induced after IPTG treatment of *E. coli* BL21/DE3 (Figure 3.1.1).

3-4 Endotoxin estimation of purified CyaA preparations

The levels of LPS in the purified CyaA preparations were visualised by silver staining (Fig 3.2.3) and also measured by LAL assay (section 2.11). The *Limulus* amoebocyte lysate test (LAL assay) 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 horseshoe crab, *Limulus polyphemus*. The purification methods were successful in reducing the endotoxin level in each preparation. The levels of LPS were 128 and 67 IU/mg protein for CyaA and CyaA*, respectively, which is low (recommended dose <5 IU per dose for vaccine antigens, where the dose might be in the region of 10-25 μg protein). The endotoxin content of each preparation is given in Table 3.1.1 The level of LPS contamination was much lower than values reported by Mac Donald-Fyall (2002) and MacDonald-Fyall *et al.* (2004).

Table 3.1.1 Characterisation of purified CyaA preparations

Three different purified forms of CyaA were prepared and purified by Q and Butyl Sepharose chromatography. The AC enzymic and cytotoxicity activities for J774.2 macrophage-like cells were determined by conductimetry and MTT assays, respectively. LAL assay was used to measure the LPS levels.

Sample	Total volume ml	Protein µg/ml	LPS levels IU/mg protein	Cytotoxicity (50% killing of macrophages) µg protein/ml	AC enzymic activity µmol cAMP/mg protein/min
CyaA	35	290	128	0.025 ± 0.01	674 ± 9.19
Pro-CyaA	6.5	168	Not done	>2.5	815 ± 13.4
CyaA*	14	330	67	>2.5	5.5 ± 0.63

3-5 Characterisation of purified CyaA preparations

3-5-1 Adenylate cyclase assay

AC enzymic activity of purified CyaA preparations from *E. coli* BL21/DE3 [(pGW44), (pGW44, pGW54) and (pGW44-188, pGW54)] were determined by conductimetric assay (section 2. 13.1). CyaA, and pro-CyaA showed high enzymatic activity, but no significant activity was detected with CyaA* (Table 3.1.1; Figure 3.5.1.1). This is because CyaA* has a dipeptide insertion within the ATP binding site which results in a lack of AC enzymic activity. In order to calculate the AC enzymic activity in international units (IU) per mg protein, the total conductance change should be known. Total conductance changes in arbitrary units for a substrate concentration of 1 mM of ATP was 2200 and this value was used for determination of AC enzymic activity (Lawrence *et al.*, 1998). The same concentration of both CyaA, pro-CyaA and CyaA* proteins were made for use in the conductimetry assay. The conductance of CyaA preparations and level of enzymic activity of the two enzymatically-active toxins (CyaA and pro-CyaA) preparations were routinely in the range of 660-880 µmol cAMP/mg protein/min respectively.

In order to calculate the AC enzymic activity in units per mg of protein the stock preparation of CyaA (300 µg protein/ml) was used at 1 in 5 dilution and 2 µl were added to a final volume of 2 ml buffer.

Initial reaction rate = tangent of reaction (y) containing 0.06 µg protein/ml (units/min)/ total change value (x) (arbitary units).

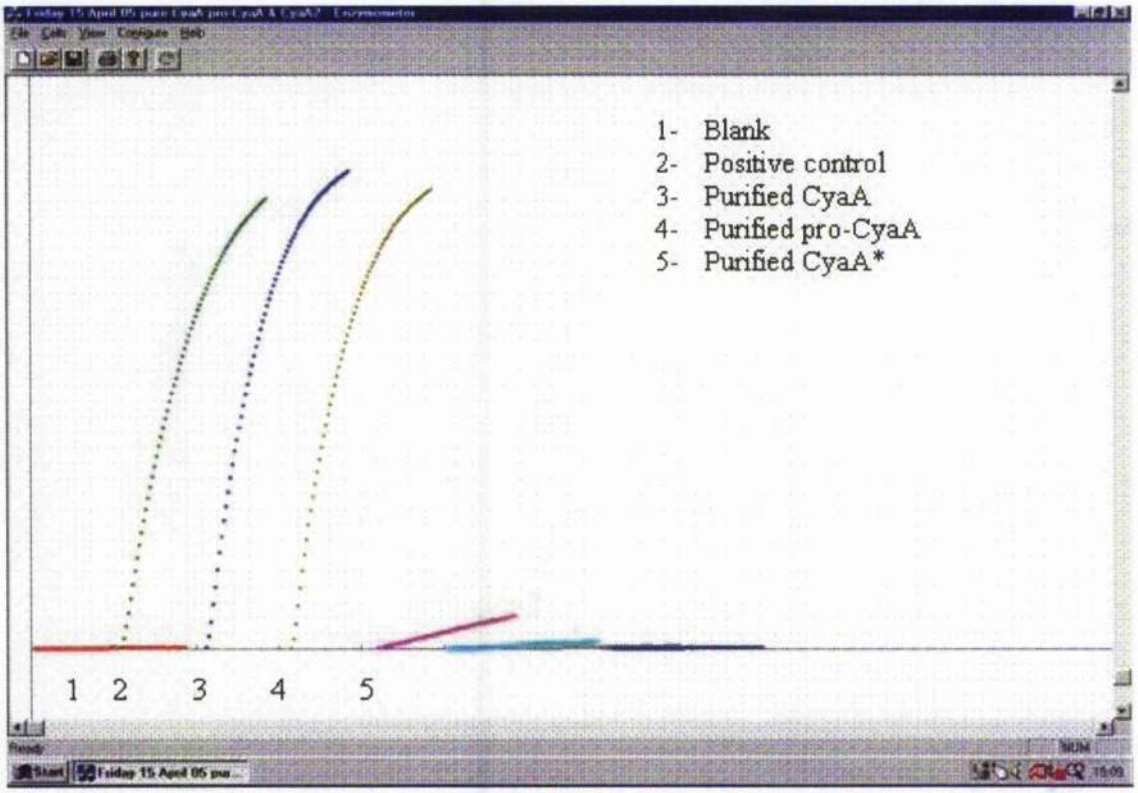
For example: 86 (y)/ 2200 (x) = 0.039 µmoles/ml/min

To convert µmoles/ml/min to µmoles/min/µg protein

= 0.039 µmoles/ml/min/ 0.06 µg protein/ml= 0.65 µmoles/min/µg protein or 650 µmoles/min/mg protein.

Figure 3.5.1.1 AC enzymic activity of purified preparations of three forms of CyaA

Three different purified forms of CyaA were diluted in 8M urea, 20mM histidine pH 6.0 to the same toxin concentration (60 µg/ml protein) . The AC enzymic activity was determined using a conductimetry assay in the presence of Bicine buffer and 1µl of inorganic pyrophosphatase, 1µl of Calmodulin and 2 µl of each sample in each cell. AC enzymic activity is indicated by the gradient of the conductance curve.The figure shows the on-screen appearance of the conductance change for each sample. Cell 1 was a blank control consisting of buffer and reagents. Cell 2 was the positive control containing a CyaA standard with defined protein, LPS and AC enzymic activity. Cell 3 was purified CyaA from a new purification. Cell 4 was purified pro-CyaA and cell 5 contained purified CyaA*.


1- Blank
2- Positive control
3- Purified CyaA
4- Purified pro-CyaA
5- Purified CyaA*
1 2 3 4 5
Conductance change
Time (s)

3-5-2 Cytotoxic effect of purified CyaA preparations

The effect of CyaA preparations on cell killing of mouse macrophage-derived (J774.2) cells grown in RPMI 1640 medium was determined by the MTT dye reaction assay using the CellTiter 96™ assay kit (Promega). This assay is based on the inhibition of the ability of active mitochondria in living cells to reduce tetrazolium dye solution. Pro-CyaA is non-acylated and only a very low cell killing effect was detected at the highest concentrations of toxin (Table 3.1.1 and Figure 3.5.2.1). CyaA demonstrated a high level of killing effect that is in line with its enzymic and invasive activities. Some cytotoxicity effect was detected with CyaA* at the higher concentrations of toxin, similar to that seen with pro-CyaA (Figure 3.5.2.1). As all preparations were prepared and solubilized in the same amount of urea buffer (starting at 8 M) it is possibile that this low level of cytotoxicity may have been due to an effect of urea at the higher concentrations of toxin (7.19, 21.2 and 5.5 μg/well for CyaA, pro-CyaA and CyaA*, respectively) (Table 3.1.1, Figure 3.5.2.1). It should be noted that all CyaA dilutions were made in RPMI 1640 medium without urea, which means that lower concentrations of toxin contained lower concentrations of urea. The results with the different CyaA preparations against J774.2 mouse macrophage cells using the MTT assay showed the importance of both the AC enzymic activity and the acylation of CyaA for cytotoxicity.

Similar experiments were performed on different mammalian cells, including undifferentiated U937 human monoblastic cells and U937 cells differentiated by treatment with phorbol myristate acetate (PMA) and IFNγ as a stimulator. However, whereas J774.2 cells were killed by CyaA at low concentrations of toxin (e.g 50% killing at 0.025 μg protein/ml), RBL-2H3 (rat basophil leukemia) cells, Vero (African green monkey kidney) cells, and mast cells (sheep bone marrow) showed less than 50% killing at all toxin concentrations tested (Figure 3.5.2.2) even though these cells were

Figure 3.5.2.1 Cytotoxic activity of CyaA preparations on J774.2 mouse macrophage cells

Cytotoxicity activity of different purified CyaA preparations was determined on the J774.2 mouse macrophage cell line by the MTT dye reduction method. The percentage of cell killing at various toxin concentrations, relative to that in RPMI 1640 medium alone was determined using the CellTitre 96^{Tm} assay (Promega) kit plate. The results represent the mean of two values (± S.D).

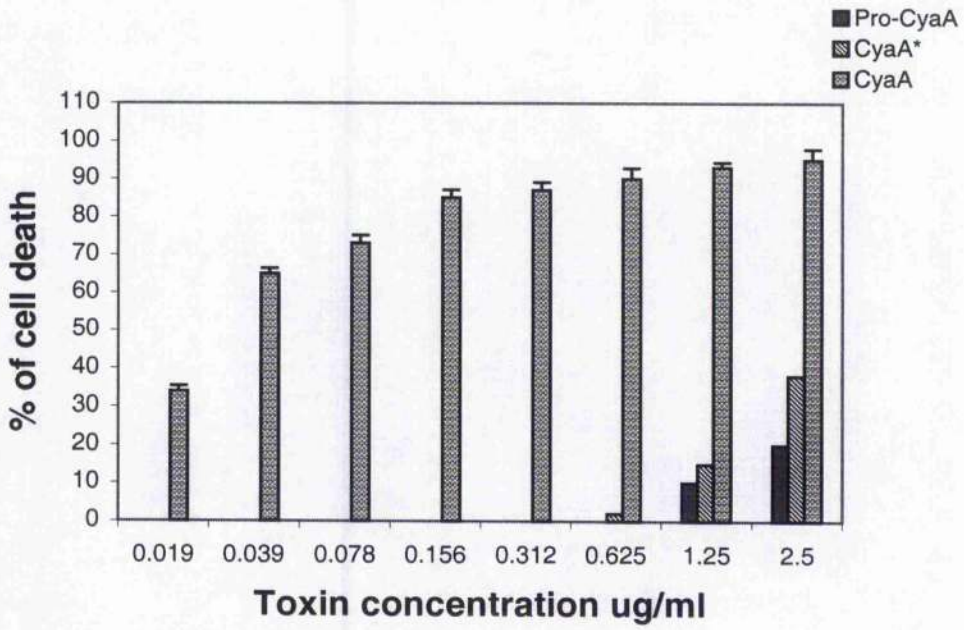
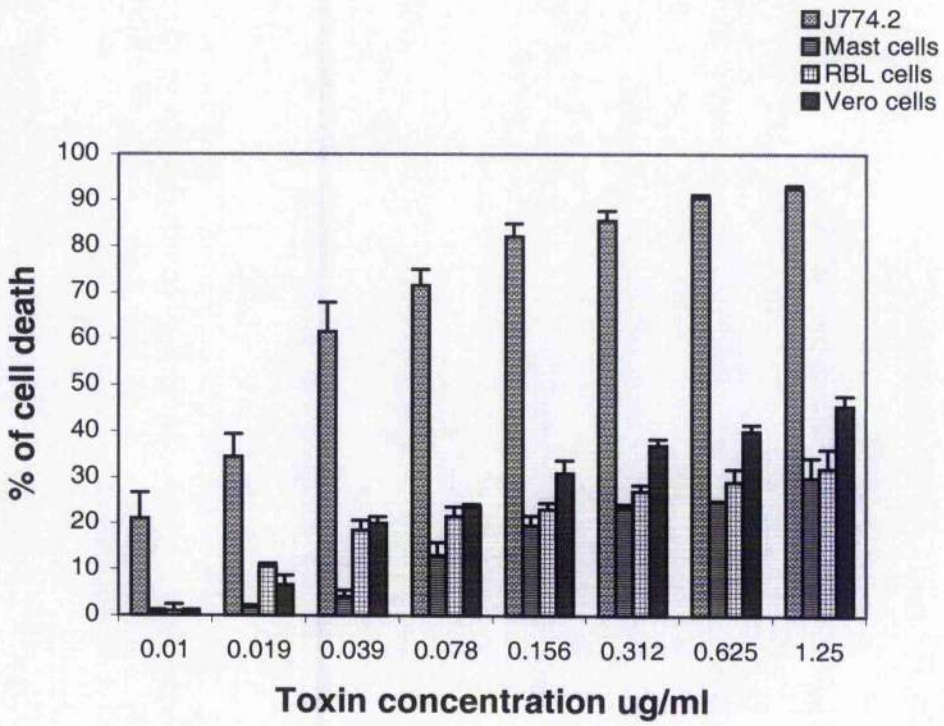

Pro-CyaA
CyaA*
CyaA
% of cell death
110
100
90
80
70
60
50
40
30
20
10
0
0.019
0.039
0.078
0.156
0.312
0.625
1.25
2.5
Toxin concentration ug/ml

Figure 3.5.2.2 Cytotoxicity of purified CyaA on different types of mammalian cells

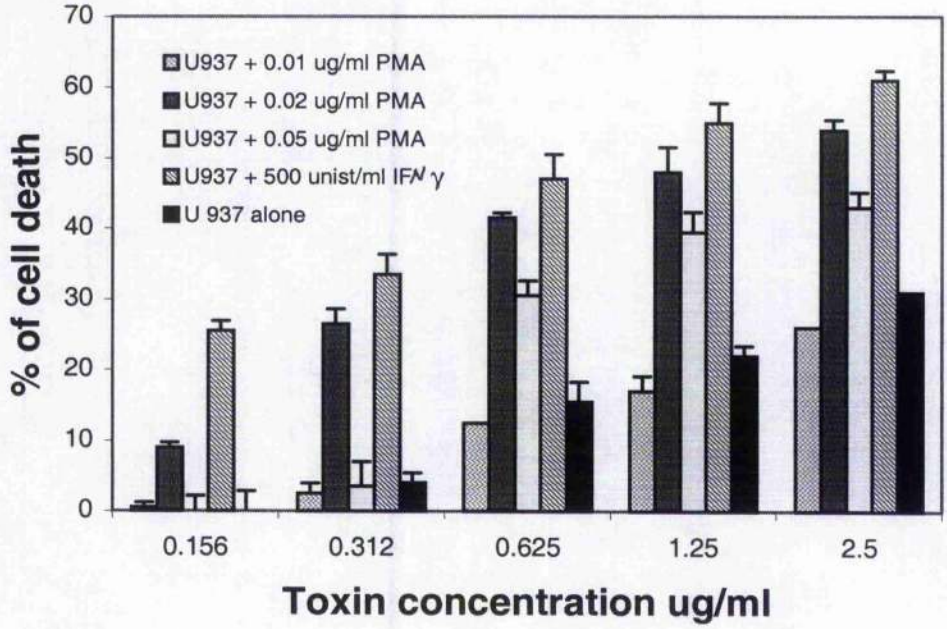
The cytotoxic activity of purified CyaA on J774.2, RB-2H3L, sheep bone marrow mast cells and Vero cells were determined by the MTT dye reduction method (CellTitre 96^{Tm} assay (Promega) kit plate). The results represent the mean of two values (± S.D)


J774.2
Mast cells
RBL cells
Vero cells
% of cell death
100
90
80
70
60
50
40
30
20
10
0
0.01
0.019
0.039
0.078
0.156
0.312
0.625
1.25
Toxin concentration ug/ml

cultured in DMEM medium which contain 5 mM of $CaCl_2$. Similarly, a maximum 30% killing was seen in undifferentiated U937 cells grown in RPMI 1640 medium but up to 60% cytotoxicity was seen with CyaA at much lower levels by differentiating the cells by adding IFNγ (500 units/ml) or 0.020 μg/ml PMA final concentration (Figure 3.5.2.3). It should be noted that U937 is an immature human monoblastic cell line. To differentiate these cells to monocytic, macrophage-like cells, they needed to be stimulated by adding PMA or IFNγ. Such cells are known to have a greater capacity to generate superoxide and express higher levels of CD11b, the receptor for CyaA (Kikuchi *et al.*, 1996).

Figure 3.5.2.3 Cytotoxicity of CyaA on U937 human monocytes with and without activator

The effect of CyaA on undifferentiated and differentiated U937 human monocytes was determined by the MTT dye reduction (CellTitre 96^{Tm} assay (Promega) kit) after incubation with IFNγ (500 units/ml) or inceasing concentrations of PMA (0.01, 0.02 and 0.05μg/ml final concentrations). The results represent the means of two values (± S.D).


70
60
50
40
30
20
10
0
% of cell death
U937 + 0.01 ug/ml PMA
U937 + 0.02 ug/ml PMA
U937 + 0.05 ug/ml PMA
U937 + 500 unist/ml IFN γ
U 937 alone
0.156
0.312
0.625
1.25
2.5
Toxin concentration ug/ml

3-5-3 The effect of added of calcium on CyaA and CyaA* cytotoxicity

The cytotoxic and haemolytic activities of the CyaA are absolutely calcium-dependent. CyaA harbours two classes of calcium binding sites of different affinities (Rose *et al.*, 1995). Binding of calcium to high affinity binding sites is thought to be required for haemolytic activity and induces major structural rearrangment of CyaA that may be involved in delivery of the AC domain into target cells. On the other hand, there are 40-45 low-affinity calcium-binding sites located in the repeat region of CyaA toxin. Binding of Ca^{2+} to these sites might be necessary for both the membrane binding capability and the hemolytic activity of the toxin and each site could bind one calcium ion. In order to test whether Ca^{2+} added to the RPMI 1640 medium could influence the cytotoxicity of CyaA and CyaA*, various concentration of Ca^{2+} ($CaCl_2$) were used. RPMI 1640 medium contains approximately 0.423 mM $CaNO_3$. The cytotoxic effect of the two forms of CyaA was assayed in the presence of added Ca^{2+} ($CaCl_2$) from 1mM to 5mM. CyaAs were diluted to concentration of 5 μg protein/ml and then $CaCl_2$ was added. Cytotoxicity was assayed by the CellTiter 96 ™ assay kit (Promega) as described the Materials and Methods (section 2.13.2). The results indicated that the cytotoxic activity of CyaA* increased with increasing calcium concentration. However, even at highest concentration of $CaCl_2$ tested (5 mM), the cytotoxic activity of CyaA* was still <1/10 th of that of CyaA preparation. No obvious effect of added Ca^{2+} on cytotoxicity was seen with CyaA toxin (Table 3.5.3.1).

Table 3.5.3.1 Effect of added Ca^{2+} on the cytotoxic activity of CyaA preparations in RPMI medium.

J774.2 mouse macrophage-like cells in RPMI medium were treated with CyaA preparations in the presence of added $CaCl_2$. Cytotoxicity (% cell killing) was measured by MTT dye reduction using the CellTiter 96 ™ assay kit (Promega). Results are the means of 2 determinations (± S.D).

$CaCl_2$ added	Concentration of CyaA (µg protein /ml) giving 50% cell killing	Concentration of CyaA* (µg protein /ml) giving 50% cell killing
0	0.030 ± 0.02	> 3
1mM	Not done	2.4
2mM	0.042 ± 0.05	1.75 ± 0.07
3mM	0.044 ± 0.01	1.2 ± 0.14
4mM	0.075 ± 0.045	1.1 ± 0.21
5mM	0.050 ± 0.03	0.75 ± 0.12

3-5-4 ATP cytotoxicity assay

The adenosine triphosphate (ATP) assay is an alternative assay for measuring cytotoxicity as it determines the number of viable cells in a culture based on quantitation of ATP, by a bioluminescence method, which signals the presence of metabolically-active cells. The amount of ATP is directly related to cell numbers. J774.2 (grown in RPMI 1640 medium), RBL-2H3 and sheep bone marrow mast cells (grown in DMEM medium) were treated with different concentrations of CyaA or CyaA* for 2 h at 37°C. The CellTiter-Glo™ reagent (section 2.13.3) was added to each sample and bioluminescence output was compared to that of a negative control (untreated) (0% cytotoxicity) preparation. As Figure 3.5.4.1 shows, according to this method 50% cytotoxicity for J774.2 cells was caused by CyaA around 0.02 μg protein/ml but was not achieved by CyaA* up to 1.25 μg protein/ml. Little cell death (<15%) was detected with CyaA* below 0.02 μg/ml. The results are similar to those recorded in the cytotoxicity (MTT) assay (Table 3.1.1 and Figure 3.5.2.1). The effect of CyaA on sheep bone marrow mast cells and RBL-2H3 cell line was also determined. CyaA was less toxic for RBL-2H3 and mast cells than J774.2 cells (Figures 3.5.4.2, 3.5.4.3) but 50 % killing was achieved at 0.2 μg/ml for mast cells and 1.0 μg/ml for RBL-2H3 cells. Thus, CyaA showed greater cytotoxicity using this assay than with the MTT assay for these cell types (see figure 3.5.2.2). There was only slight cytotoxicity by CyaA* on RBL-2H3 and sheep bone marrow mast cells, even at the highest concentrations. This effect was similar to that seen in both RBL-2H3 and mast cells by the MTT assay (Figure. 3.5.2.2). It was not determined whether addition of Ca^{2+} could enhance cytotoxicity using this assay, but DMEM medium contains 5 mM $CaCl_2$.

Figure 3.5.4.1 ATP assay for determining cytotoxicity of CyaA and CyaA* on J774.2 mouse macrophage-like cells.

Intracellular ATP was measured using the CellTiter-Glo assay kit (Promega). The graph shows the effect of CyaA and CyaA* at different protein concentrations on cell viability of J774.2 cells after 2 incubation. Each bar represents the mean of the three values (± S.D).

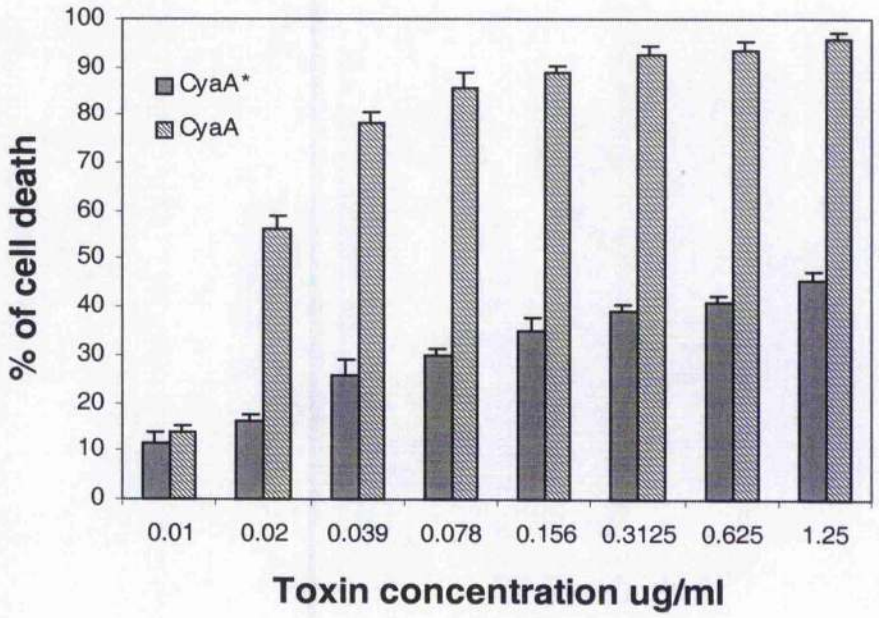

100
90
80
70
60
50
40
30
20
10
0
% of cell death
CyaA*
CyaA
0.01
0.02
0.039
0.078
0.156
0.3125
0.625
1.25
Toxin concentration ug/ml

Figure 3.5.4.2 ATP assay for determining cytotoxicity of CyaA and CyaA* on sheep bone marrow mast cells

The effect of CyaA preparations at different protein concentrations on cell survival on sheep bone marrow mast cells was determined after incubation for 2 h. Each bar represents the mean of the three values (± S.D).

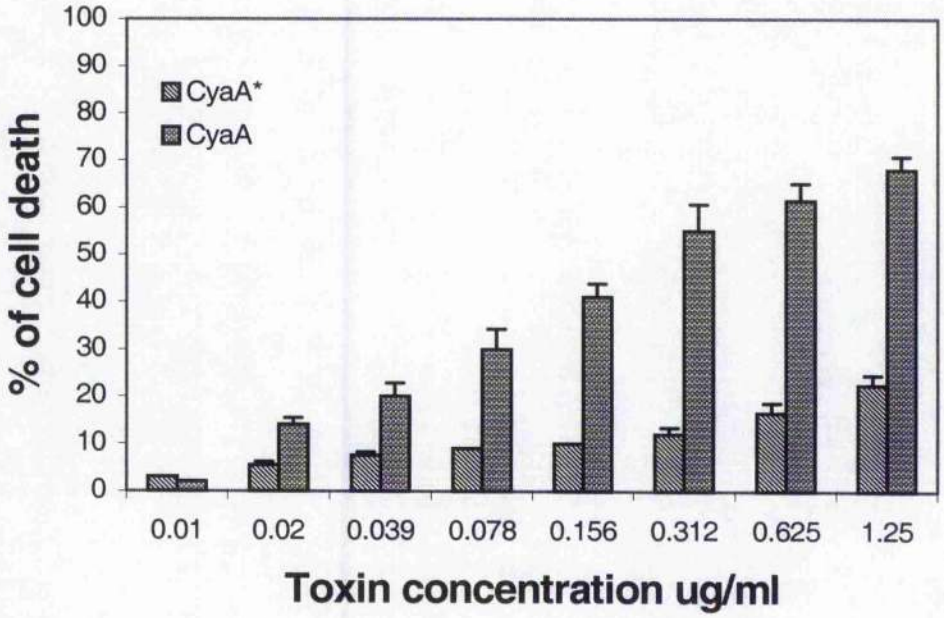
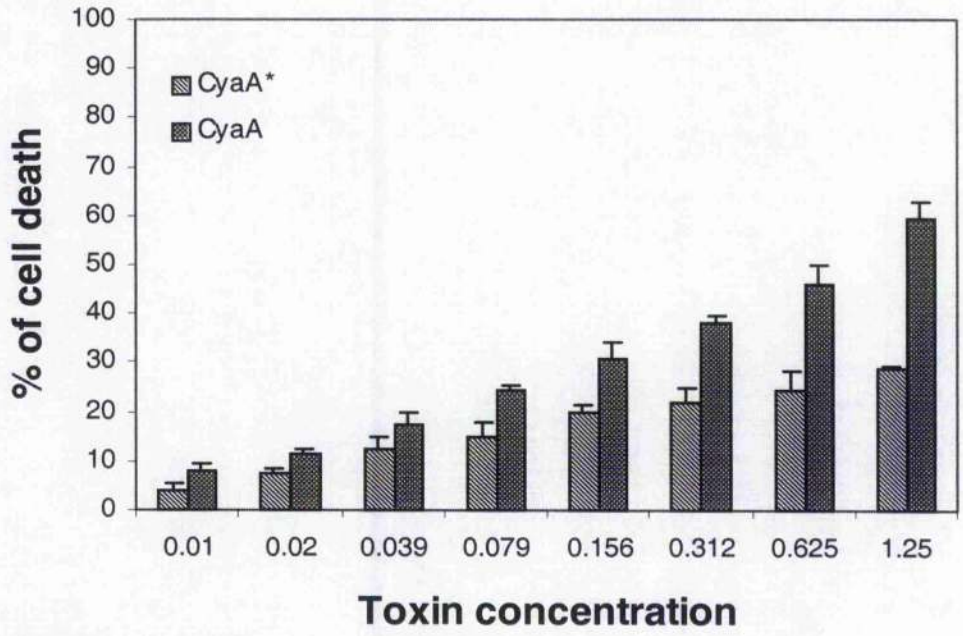

100
90
80
70
60
50
40
30
20
10
0
% of cell death
CyaA*
CyaA
0.01
0.02
0.039
0.078
0.156
0.312
0.625
1.25
Toxin concentration ug/ml

Figure 3.5.4.3 ATP assay for determining cytotoxicity of CyaA and CyaA* on RBL-2H3 cells

The effect of CyaA preparations at different protein concentrations on cell survival in RBL cells was determined after incubation of 2 h. Each bar represents the mean of the three values (± S.D).


100
90
80
70
60
50
40
30
20
10
0
CyaA*
CyaA
% of cell death
0.01
0.02
0.039
0.079
0.156
0.312
0.625
1.25
Toxin concentration

3-5-5 Caspase (apoptosis) assay

One of the main characteristics of apoptosis is the activity of caspases 3 and 7, cysteinyl aspartate-specific proteinases with key effector roles in apoptosis (Nicholoson and Thornberry, 1997). Caspases 3/7 can be used as indicators of apoptosis in macrophages and are detected by their ability to cleave a pro-fluorescent substrate to produce a fluorescent signal. The amount of fluorescent product generated is proportional to the amount of caspase 3/7 cleavage activity present in the sample and therefore reflects apoptotic activity. Figure 3.5.5.1 shows the change in caspase activity in J774.2 mouse macrophage-like cells six hours after addition of two different CyaA preparations in the presence of urea buffer. Caspase 3/7 activities were measured over a range of toxin concentrations. At these concentrations, neither urea buffer alone nor CyaA* induced any significant increase in caspase 3/7 activity from J774.2 cells. It should be noted that there was a peak of caspase 3/7 activity at 0.039 μg protein/ml with CyaA but this declined at higher toxin concentrations, possibly due to increased necrosis rather than apoptosis.

In addition, other mammalian cells were treated with CyaA under the same conditions (Fig 3.5.5.2). Measurement of caspase 3/7 activity indicated that the greatest effect of CyaA was on RBL-2H3 cells, where increasing concentration of CyaA gave increasing caspase activity. With sheep bone marrow mast cells, there was a small peak of caspase activity at 0.039 μg protein/ml, a result similar to that obtained with J774.2 cells (Figure 3.5.5.1). With Vero cells, there was no apparent effect or dose response. The same experiment was carried out with CyaA* on sheep bone marrow mast cells and RBL-2H3 cells within the same range of toxin concentrations (Figure 3.5.5.3). There was no obvious effect on mast cells even at the highest concentrations, although RBL-2H3 cells showed some response at the highest concentration of CyaA* tested (2.5 μg protein/ml).

Figure 3.5.5.1 Induction of apoptosis (caspase 3/7 activity) by CyaA, CyaA* and urea buffer in J774.2 mouse macrophages

The cells were incubated with toxin preparations or with urea at the same concentration as used in the toxin preparations, for 6 h, after which caspase 3/7 activity was determined by the Caspase-Glo™ 3/7 Assay (Promega) kit. Results are expressed as relative fluorescent light units (RFLU) and represent the mean of two values (± S.D).

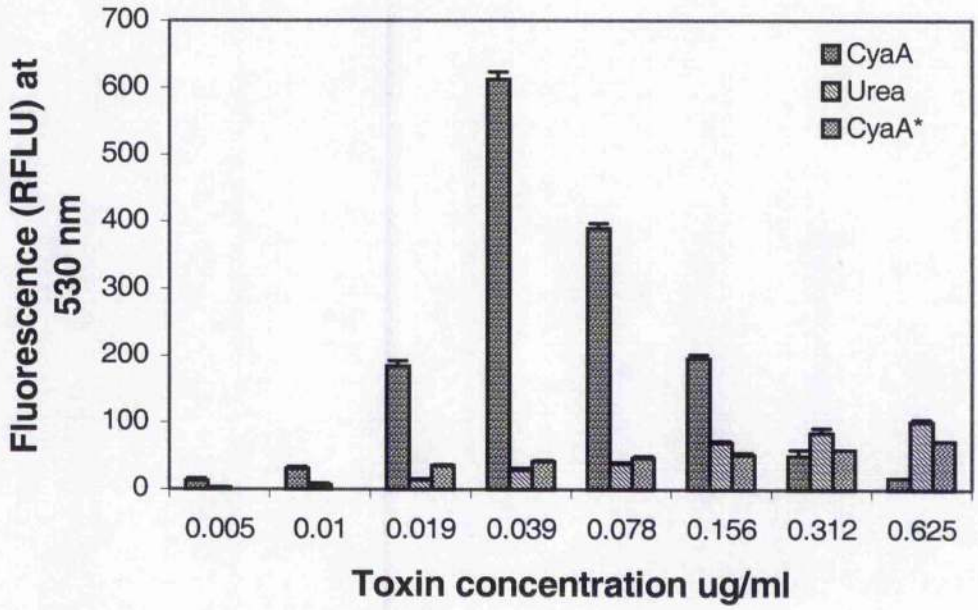

Fluorescence (RFLU) at 530 nm
700
600
500
400
300
200
100
0
CyaA
Urea
CyaA*
0.005
0.01
0.019
0.039
0.078
0.156
0.312
0.625
Toxin concentration ug/ml

Figure 3.5.5.2 Induction of apoptosis (caspase 3/7 activity) by CyaA in sheep bone marrow mast cells, RBL-2H3 cells and Vero cells.

Sheep bone marrow mast cells, RBL-2H3 cells and Vero cells were incubated with increasing doses of CyaA for 6 h. The caspase 3/7 activity was determined by the Caspase-Glo™ 3/7 Assay (Promega) kit. Results are expressed as relative fluorescent light units (RFLU) and represent the mean of two values (± S.D).

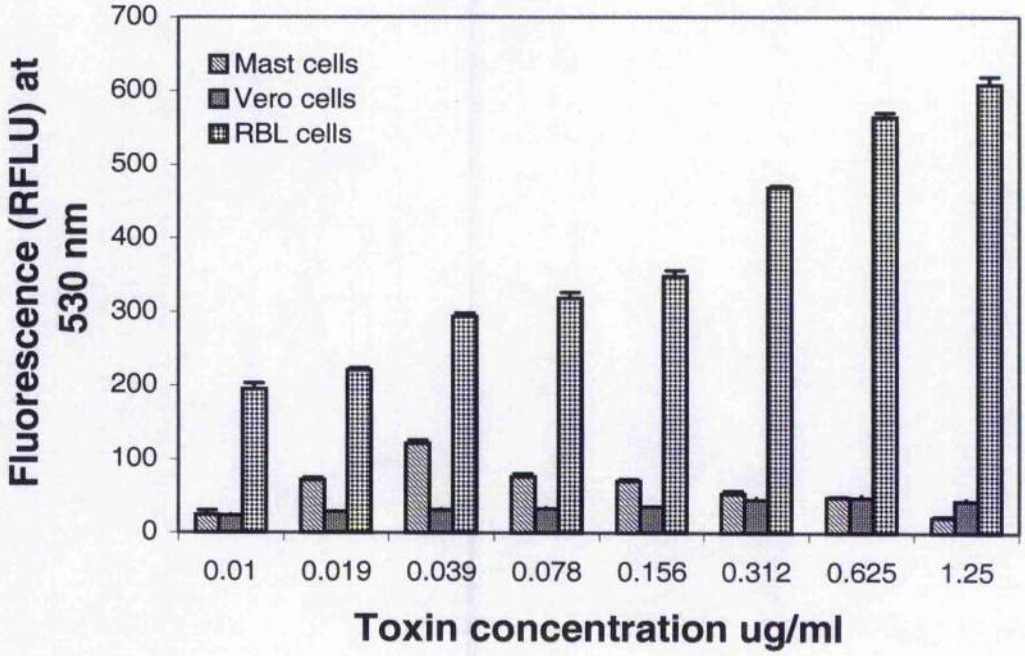
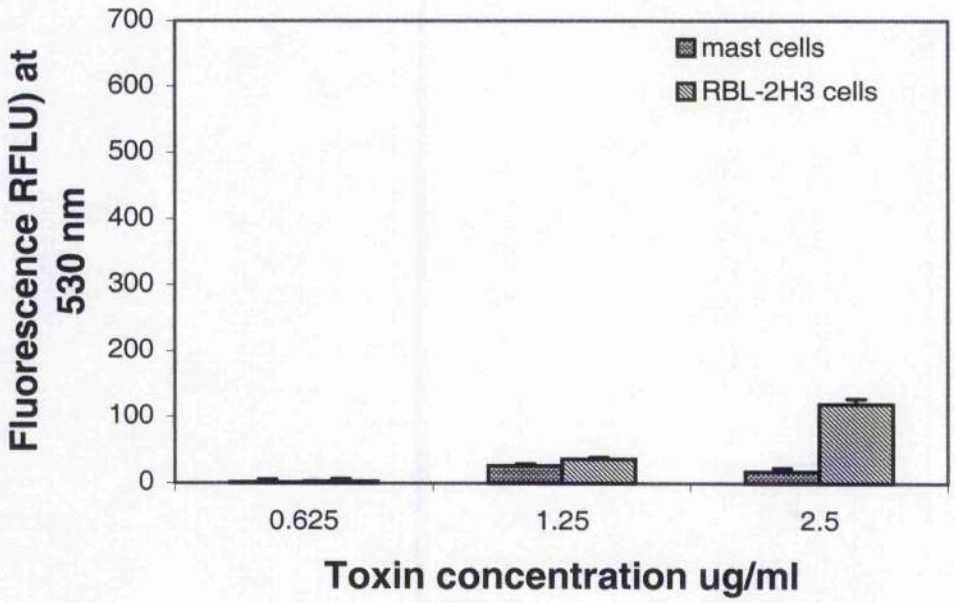

Fluorescence (RFLU) at 530 nm
700
600
500
400
300
200
100
0
Mast cells
Vero cells
RBL cells
0.01
0.019
0.039
0.078
0.156
0.312
0.625
1.25
Toxin concentration ug/ml

Figure 3.5.5.3 Induction of apoptosis (caspase 3/7 activity) by CyaA* in sheep bone marrow mast cells and RBL-2H3 cells

The change in caspase activity in sheep bone marrow mast cells, RBL-2H3 cells after incubation with increasing doses of CyaA* for 6 h. The caspase 3/7 activity was determined by the Caspase-Glo™ 3/7 Assay (Promega) kit and results are expressed as relative fluorescent light units (RFLU) and represent the mean of two values (± S.D).


700
600
500
400
300
200
100
0
Fluorescence RFLU) at 530 nm
mast cells
RBL-2H3 cells
0.625
1.25
2.5
Toxin concentration ug/ml

3-5.6 DNA fragmentation (apoptosis) assay

Fragmentation of genomic DNA into multimers of approximately 200 base pairs, a characteristic of apoptosis, was measured in J774.2 mouse macrophages-like cells and other mammalian cells (sheep bone marrow mast cells, RBL-2H3 and Vero cells). The cells were treated with either CyaA or CyaA* at 0.039 μg protein/ml final concentration for 12 h. This was chosen as the concentration that gave maximum induction of caspase 3/7 activity by CyaA in J774.2 mouse macrophage-like cells (Figure 3.5.5.1) and in mast cells by CyaA* (Figure 3.5.5.2) after incubation at 37°C with 5% CO_2 for 6 h. For DNA fragmentation, all cells were treated with the same concentration of urea as was present in the toxin samples as a negative control. As a positive control, cells were grown without foetal calf serum (FCS) for 24 h, a condition known to induce apoptosis. DNA fragmentation was evaluated by electrophoresis on a 1% agarose gel stained with ethidium bromide. Although, DNA fragmentation is not a quantitative assay, as shown in Figure 3.5.6.1, after 12 h of incubation, CyaA promoted the DNA fragmentation in mast cells and RBL 2H3 cells and showed a clear laddering of DNA fragments, a pattern characteristic of apoptosis. It should be noted that DNA from J774.2 cells treated with CyaA showed a necrotic smear possibly because of the onset of necrosis after 12 h. There was no effect seen on the DNA banding pattern from Vero cells with CyaA. Urea, at the concentration used for the toxin had no effect on DNA. The positive control, RBL cells incubated without FCS, showed a clear laddering effect. This was not apparent, however, with Vero cells incubated in the absence of FCS. As shown in Figure 3.5.6.2, the CyaA* preparation was unable to induce DNA fragmentation. Cells incubated with urea again showed no laddering pattern but RBL and J774.2 cells grown in RPMI minus FCS, showed a pronounced laddering pattern (Figure 3.5.6.2). The results indicated that AC enzymic activity is necessary for the induction of apoptosis and invasive capability alone was shown to be insufficient to cause apoptosis although

slight activity was detected with CyaA* at high concentrations after incubation with RBL-2H3 cells (Figure 3.5.5.3) but this was not apparent in the DNA fragmentation experiment (figure 3.5.6.2). The results show that there are some differences between induction of caspase 3/7 activity and DNA fragmentation among these cells. In spite of the remarkable effect of CyaA on J774.2 cells, other mammalian cells showed very low response to CyaA with the exception of RBL-2H3 cells. However, CyaA showed an obvious laddering pattern with sheep bone marrow mast cells and RBL-2H3 cells but Vero cells did not. The reason for these differences are unknown but the incubation time of treatment of toxin (6 h and 12 h for caspase 3/7 activity and DNA fragmentation, respectively) may play an important role in this matter.

Figure 3.5.6.1 Agarose gel electrophoresis of genomic DNA from different mammalian cells after incubation with CyaA

DNA fragmentation of J774.2, sheep bone marrow mast cells, RBL-2H3 cells and Vero cells treated with CyaA, was determined after incubation for 12 h. DNAs were extracted, separated by electrophoresis in a 1% agarose gel and stained with ethidium bromide (section 2.5).

Line M: Marker (1kb DNA ladder)

Lane 1: DNA from J774.2 cells + CyaA

Lane 2: DNA from mast cells + CyaA

Lane 3: DNA from RBL-2H3 cells + CyaA

Lane 4: DNA from Vero cells + CyaA

Lane 5: DNA from J774.2 cells + urea (as negative control)

Lane 6: DNA from mast cells + urea (as negative control)

Lane 7: DNA from RBL-2H3 cells + urea (as negative control)

Lane 8: DNA from RBL-2H3 cells without FCS (as positive control)

Lane 9: DNA from Vero cells + urea (as negative control)

Lane 10: DNA from Vero cells without FCS (as positive control)

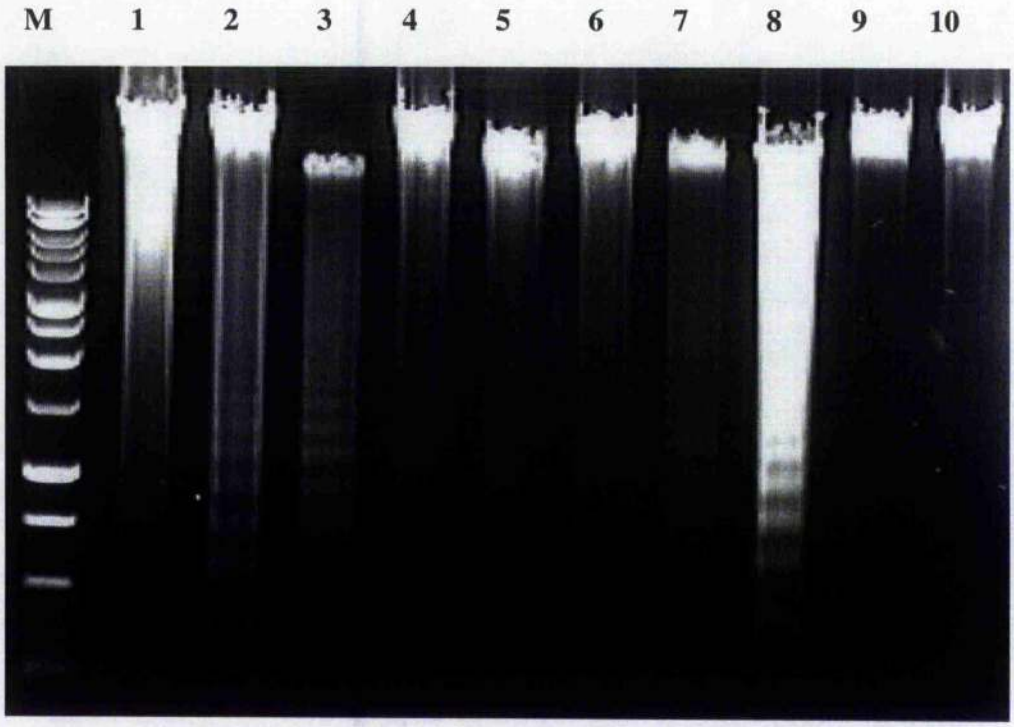

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Figure 3.5.6.2 Agarose gel electrophoresis of genomic DNA from different mammalian cells after incubation with CyaA*

DNA fragmentation of J774.2, sheep bone marrow mast cells, RBL-2H3 and Vero cells treated with CyaA* after incubation for 12 h. DNAs were extracted, separated by electrophoresis in a 1% agarose gel and stained with ethidium bromide (section 2.5).

Line M: Marker (1kb DNA ladder)

Lane 1: DNA from J774.2 cels + urea (as control negative)

Lane 2: DNA from mast cells + urea (as control negative)

Lane 3: DNA from RBL-2H3 cells + urea (as control negative)

Lane 4: DNA from Vero cells + urea (as control negative)

Lane 5: DNA from J774.2 cells + CyaA*

Lane 6: DNA from mast cells + CyaA*

Lane 7: DNA from RBL-2H3 cells + CyaA*

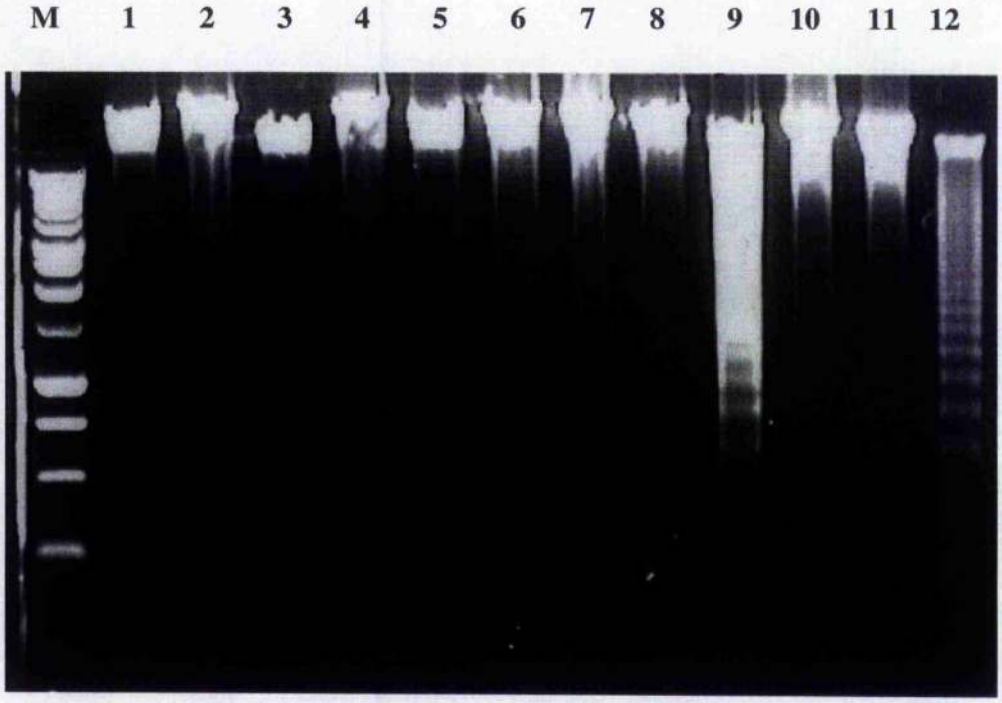
Lane 8: DNA from Vero cells + CyaA*

Lane 9: DNA from RBL-2H3 cells without FCS (as positive control)

Lane 10: DNA from J774.2 mast cells without FCS (as positive control)

Lane 11: DNA from Vero cells without FCS (as positive control)

Lane 12: DNA from J774.2 without FCS (as positive control)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3-6 Summary of characterisation of CyaA preparations

The purified CyaA toxins were characterised by several assays. The conductimetry assay was used as a novel, rapid and easy method to evaluate the level of AC enzymic activity. The results indicated that the purified forms of both CyaA and pro-CyaA were enzymatically active, whereas CyaA* was shown to have very low activity.

The MTT dye reduction cytotoxicity (CellTiter 96™ assay kit) assay with J774.2 cells showed that CyaA caused considerable cell death after incubation for 2 h at 37°C in 5% CO_2 whereas CyaA* and pro-CyaA showed more than 100-fold less activity. However, CyaA* activity was shown to be more dependent on the concentration of Ca^{2+} than CyaA. In addition, the viability of these macrophage-like cells treated with different concentrations of CyaA and CyaA* was assayed by the ATP assay using CellTiter-Glo assay, Promega. The MTT and ATP assays were compared to assess their ability to measure cytotoxicity in J774.2 cells due to CyaA toxins. The assays gave comparable results, although it was clear that the MTT assay was slightly less sensitive than the ATP assay, showing 50 % killing at 0.025 μg protein /ml compared to 0.02 μg protein/ml respectively.

Induction of apoptosis was evaluated by caspase 3/7-activity determination and DNA fragmentation assays. For the caspase assay, different mammalian cell lines were treated with two-fold serial dilutions of CyaA and CyaA* for six hours at 37°C and in 5% CO_2. The results with J774.2 cells, sheep bone marrow mast cells and RBL-2H3 cells indicated that the AC enzymic activity of CyaA was important for the induction of caspase activity or DNA fragmentation. This is presumably because CyaA is able to penetrate the host cell, bind calmodulin, and then incease the intracellular cyclic AMP concentration that can lead to apoptosis. Measurement of caspase 3/7 activity indicated

that CyaA had the greatest effect on J774.2 cells and RBL-2H3 cells, the least on Vero cells and sheep bone marrow mast cells. Interestingly, the decline in caspase activity seen with J774.2 cells at concentration higher than 0.05 μg/ml did not occur with RBL cells in which a more steady increase in caspase activity was seen with increasing concentration of CyaA.

In the DNA fragmentation assay, the greatest effect of CyaA was seen with mast cells and RBL2H3 cells. The reason for the lack of DNA laddering in J774.2 cells was not clear, but may have reflected the fact that apoptosis induction was poor above 0.039 μg/ml, as indicated by caspase activity, and the longer incubation time for DNA fragmentation (12 h) rather than 6 h for caspase activity may have resulted in a loss of DNA fragmentation due to the onset of necrosis rather than apoptosis. The onset of necrosis in J774.2 cells at CyaA concentrations above 0.039 μg/ml would explain the decline in caspase activity, in agreement with the sharp rise in cytotoxic activity of CyaA at 0.02-0.05 μg/ml (Figures 3.5.2.1 and 3.5.5.1).

3-7 Effect of CyaA preparations on phagocytosis

Using flow cytometry, the phagocytic ingestion of *E. coli* by human peripheral blood cells from healthy donors, J774.2 mouse macrophages and U937 human monoblastic cells was determined after exposure to different concentrations of CyaA and CyaA*. This technique was developed for the evaluation of phagocytosis activity in human peripheral blood (neutrophils and monocytes) and other cells. Ingestion activity was measured as the mean fluorescence intensity (MFI) produced after 20 minutes of incubation with FITC-conjugated *E. coli*. All tubes were read by flow cytometry at a wavelength of 488 nm after quenching and DNA staining (see section 2.14).

Exposure of granulocytes or J774.2 cells to different concentration of CyaA and CyaA* (0.05, 0.1, 0.2 μg/ml final concentrations) resulted in significantly lower mean neutrophil ingestion in the presence of CyaA compared to CyaA* at the same concentrations, and the unexposed controls cells (Table 3.7.1 and Figure 3.7.1). The results also showed that phagocytosis was significantly impaired by increases in CyaA concentrations for granulocytes and J774.2 cells compared to cells treated with PBS or CyaA*. With 0.2 μg protein/ml of CyaA there was almost complete (92%) inhibition of phagocytosis by J774.2 cells and 63% inhibition of phagocyotosis by human granulocyte cells. CyaA* had no obvious affect on phagocytosis except at the highest concentration of toxin used (0.2 μg/ml) where 10 % inhibition with granulocytes was seen but this was not significant. The flow cytometry histograms that represents the amount of ingested *E. coli,* also indicated the dose-dependent inhibitory effect of CyaA on J774.2 and granulocytes (Fig 3.7.1, images 2 and 4), while there was lack of inhibitory effect of CyaA* on J774.2 and granulocytes (Fig 3.7.1, images 1 and 3). In similar experiments, undifferentiated and differentiated U937 human monocytes were treated with CyaA only at 0.1μg/ml. As Table 3.7.1 shows, only differentiated cells were affected by CyaA, showing 42% inhibition of phagocytosis. Overall the results of this assay would imply that inhibition of phagocytosis by CyaA was due mainly to the AC enzymic activity of the protein.

Table 3.7.1 Suppression of phagocytosis activity of granulocytes, J774.2 and U937 cells by CyaA preparations

Toxin conc (μg/ml)	% Inhibition of J774.2 cells (mouse macrophages)	% Inhibition of granulocytes (Human peripheral blood)	% Inhibition of U937 cells (human monocytes) (without PMA)	% Inhibition of U937 cells (human monocytes) (with PMA)
CyaA (0.05)	26	28	Not done	Not done
CyaA (0.1)	79	58	0	42
CyaA (0.2)	92	63	Not done	Not done
CyaA* (0.05)	0	0		
CyaA* (0.1)	0	0		
CyaA* (0.2)	0	10		
PBS	0	0		

The statistical analysis of data by t test was as follows:

CyaA (0.0 5μg/ml) vs PBS, $P < 0.044$,, ; CyaA (0.1, μg/ml) *vs* PBS, $P < 0.004$,

CyaA (0.2 μg/ml) *vs* PBS, $p < 0.005$,

CyaA* (0.2 μg/ml) *vs* PBS, $p < 0.19$,

CyaA* (0.2 μg/ml) *vs* CyaA, $p < 0.001$

Figure 3.7.1 The effect of CyaA and CyaA* on phagocytosis of *E. coli* by J774.2 mouse macrophage-like cells and human granulocyte cells

Phagocytosis was measured in fresh human granulocyte cells and J774.2 cells by flow cytometry with the commercial BurstTEST™ assay (OPREGEN Pharma; BD Biosciences). Normalised fluorescence intensity (FL-H1) represents the number of ingested FITC-conjugated *E. coli*. The histograms show a dose-dependent reduction in phagocytosis of granulocytes treated with various concentration (0.05, 0.1, 0.2 μg/ml) of CyaA, compared with untreated cells (PBS).

Image 1: CyaA* + J774.2 cells at different concentrations

Image 2: CyaA + J774.2 cells at different concentrations

Image 3: CyaA* + human granulocyte cells at different concentrations

Image 4: CyaA + human granulocyte cells at different concentrations

Image 1

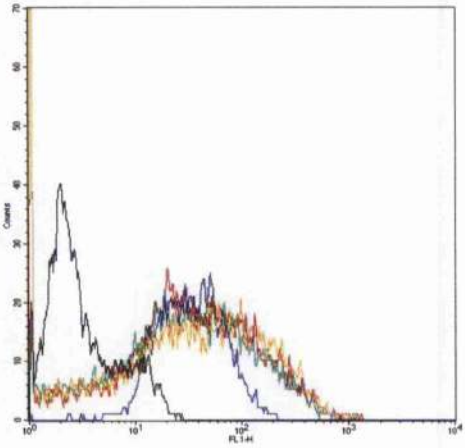


Image 2

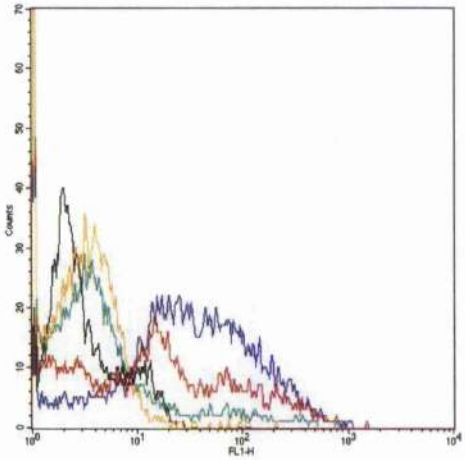


Image 3

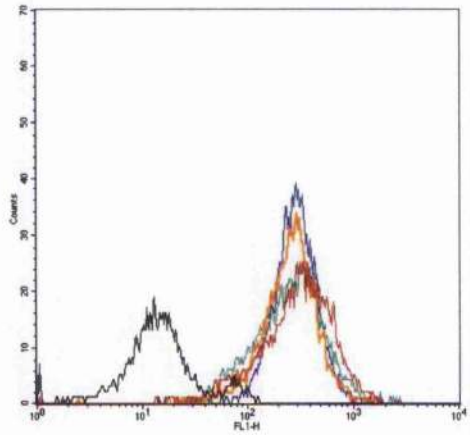
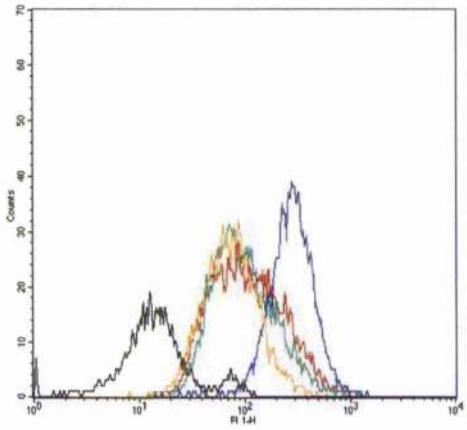


Image 4



Negative
PBS
CyaA or CyaA* 0.05 µg/ml
CyaA or CyaA* 0.1 µg/ml
CyaA or CyaA* 0.2 µg/ml

3-8 Effect of CyaA preparations on the oxidative burst

Flow cytometry assays for the measurement of the oxidative burst are based on the conversion of a non-fluorescent molecule to the fluorescent form under the influence of intracellular reactive oxygen intermediates. This conversion reflects the amount of oxidative burst induced by various stimuli. The substrate used was dihydrorhodamin-123 (DHR-123), which enters the cells as a freely permeable dye and is converted to rhodamine 123. It was used as an indicator of oxidative burst and has been found to be more sensitive than other substrates (Rothe *et al.*, 1988). The cells (human granolocytes, J774.2 and U937) were treated with either CyaA or CyaA* (0.5, 0.1 and 0.2 μg protein/ml final concentration) for two hours and then incubated with opsonised *E. coli* for 15 min. In addition, some of the cells were incubated with PBS instead of CyaA, as controls. Cells were washed and then read by flow cytometry at a wavelength of 488 nm.

Induction of the oxidative burst was significantly reduced in the presence of CyaA compared with control cells (treated with PBS). With 0.2 μg protein/ml of CyaA there was almost complete (80%) inhibition of phagocytosis by J774.2 cells and 70% inhibition of phagocotosis by human granulocyte cells. The results (Table 3.8.1 and Figure 3.8.1) showed that production of the oxidative burst was significantly impaired by increasing concentrations of CyaA compared to cells treated with PBS. CyaA* had no obvious affect on inhibition of the oxidative burst except at the highest concentration of toxin used (0.2 μg/ml protein). The statistically significant results obtained with CyaA were substantiated by the flow cytometric histograms (Figure 3.8.1 images 2 and 4). Images 1 and 3 showed that CyaA* had little effect on J774.2 and or granulocytes only at the highest concentration. Similar experiments were carried out with undifferentiated and differentiated U937 human monoblast treated with CyaA at 0.1 μg/ml. As Table 3.8.1 shows, only differentiated cells were affected by CyaA.

Table3.8.1 Inhibition of oxidative burst activity of human granolocytes, J774.2 and U937 cells by CyaA preparations

Toxin conc (μg/ml)	% Inhibition of oxidative burst in J774.2 cells (mouse macrophages)	% Inhibition of oxidative burst in granulocytes (Human peripheral blood)	% Inhibition of oxidative burst in U937 cells (human monocytes) (without PMA)	% Inhibition of oxidative burst in U937 cells (human monocytes) (with PMA)
CyaA (0.05)	18	35	Not done	Not done
CyaA (0.1)	38	55	0	40
CyaA (0.2)	80	70	Not done	Not done
CyaA* (0.05)	0	0		
CyaA* (0.1)	0	0		
CyaA* (0.2)	0	21		
PBS	0	0		

The statistical analysis of data by t test was as follows:

CyaA (0.0 5 μg/ml) vs PBS, $P < 0.003$; CyaA (0.1 μg/ml) *vs* PBS, $P < 0.024$,

CyaA (0.2 μg/ml) *vs* PBS, $p < 0.002$,

CyaA* (0.2 μg/ml) *vs* PBS, $p < 0.01$

CyaA* (0.2 μg/ml) *vs* CyaA, $p < 0.01$

Figure 3.8.1 The effect of CyaA and CyaA* on inhibition of oxidative burst in J774.2 mouse macrophage-like cells and human granulocyte cells

The oxidative burst was measured in fresh human granulocyte cells and J774.2 cells by flow cytometry with the commercial BurstTEST™ assay (OPREGEN Pharma; BD Biosciences). Normalised fluorescence intensity (FL-H1) represents the amount of the fluorogenic product, rhodamine 123, produced via reactive oxygen intermediates from the non-fluorogenic substrate, dihydrorhodamine (DHR) 123, and therefore is a measure of reactive oxidants.

Image 1: CyaA* + J774.2 at different concentrations

Image 2: CyaA + J774.2 at different concentrations

Image 3: CyaA* + human granulocyte cells at different concentrations

Image 4: CyaA + human granulocyte cells at different concentrations

Image 1

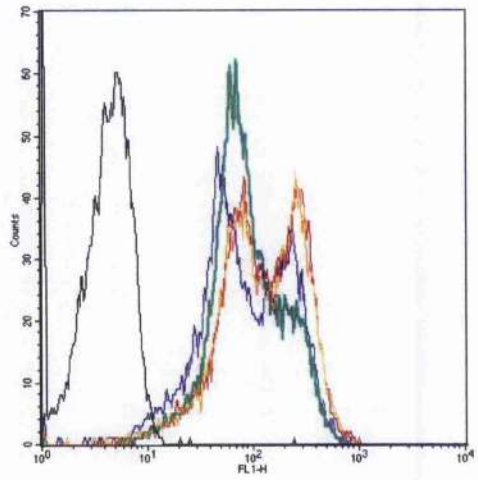


Image 2

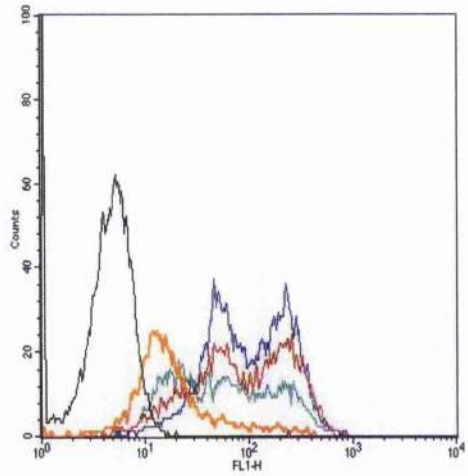


Image 3

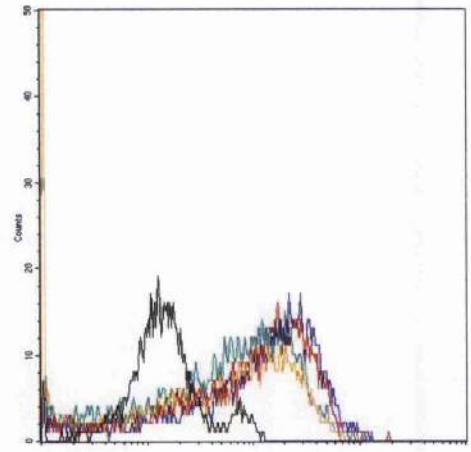
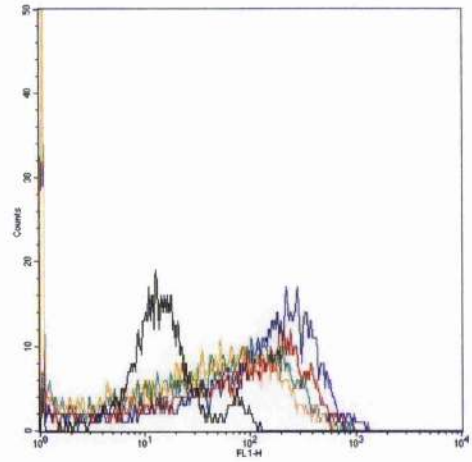


Image 4



Negative
PBS
CyaA or CyaA* 0.05 µg/ml
CyaA or CyaA* 0.1 µg/ml
CyaA or CyaA* 0.2 µg/ml

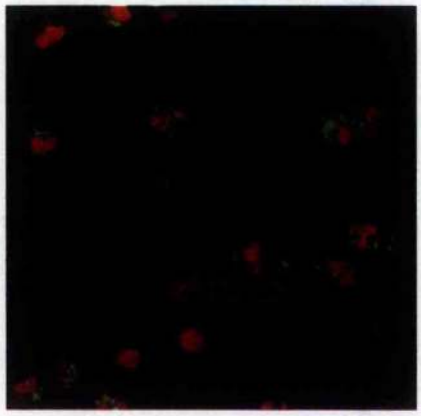
3-9 Confocal laser scanning microscopy of ingestion activity of J774.2 macrophages and human granulocytes in the presence of CyaA preparations

In this part of the study, confocal microscopy (see section 2-15) was used to determine how CyaA inhibits phagocytosis of bacteria by J774.2 cells and granulocytes. Ingestion of FITC-conjugated *E. coli* was evaluated in the presence of 0.05 μg/ml of CyaA or BSA for J774.2 cells (Fig 3.9.1) and 0.05, 0.1 and 0.2 μg/ml CyaA or BSA for granulocytes (Figs 3.9.2- 3.9.4). The green fluorescent particles representing ingested bacteria were clearly seen within the J774.2 cells in the control sample. In the presence of CyaA (0.05 μg/ml) there were possibly fewer intracellular particles and the majority were seen to be more contained to the periphery of the cells, possibly on the outside (Fig 3.9.1 and 3.9.2). With increased concentrations of CyaA, 0.1 μg/ml and 0.2 μg/ml (Fig 3.9.3), fewer fluorescent bacteria were associated with the human neutrophils compared to the control cells. The confocal laser microscopy results indicated that CyaA interfered with uptake of bacteria in support of the data obtained by flow cytometry (section. 3.7).

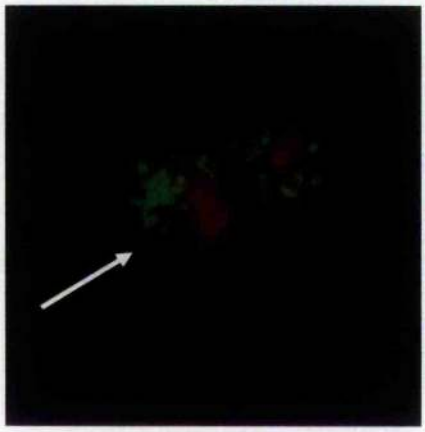
Figure 3.9.1 Morphological analysis of uptake of FITC-*E. coli* by J774.2 cells in the presence of CyaA by Confocal microscopy

Ingestion of FITC-conjugated *E. coli* was evaluated with J774.2 cells in the presence of 0.05 μg of CyaA toxin or in cell treated with PBS only. Arrowheads show intracellular bacteria.

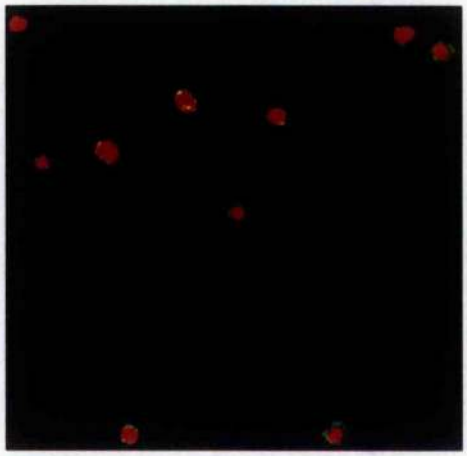
A: J774.2 + PBS (magnification of 250x)



A1: magnification of 1000x



B: J774.2 + CyaA (magnification of 250x)



B1:magnification of 4000x

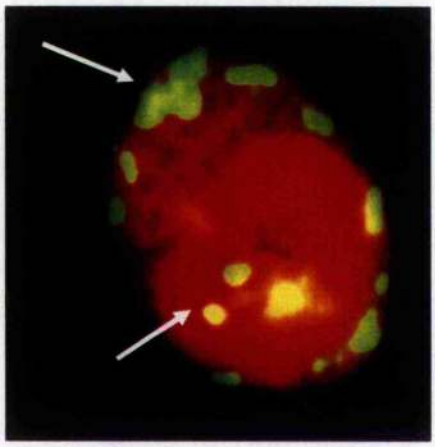
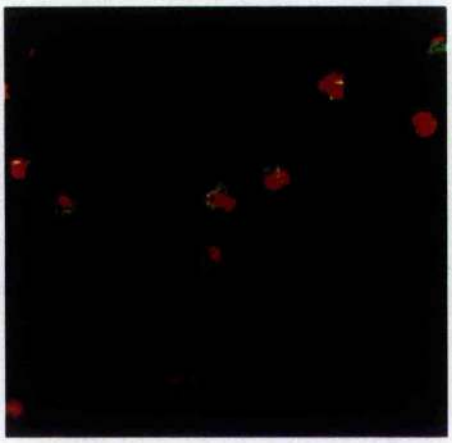


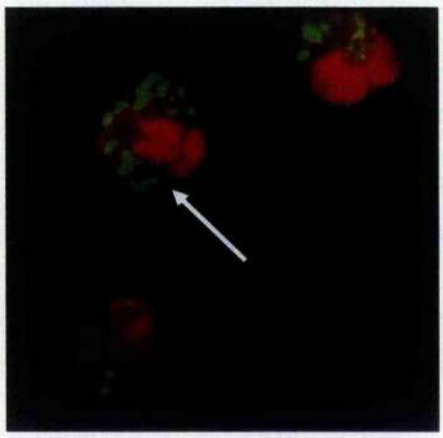
Figure 3.9.2 Morphological analysis of uptake of FITC-*E. coli* in human granulocyte cells in presence of CyaA by Confocal microscopy

Ingestion of FITC-conjugated *E. coli* was evaluated in the presence of 0.05 μg of CyaA toxin or in cells treated with PBS only in human granulocyte cells. Arrowheads show intracellular bacteria.

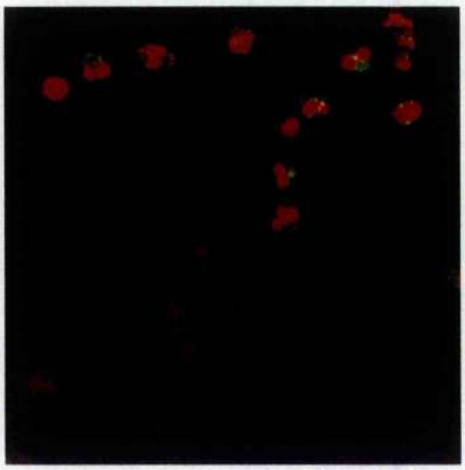
A: Granolocytes + PBS (magnification of 250x)



A1: magnification of 1000x



B: Granolocytes + CyaA (magnification of 205x)



B1: magnification of 1000x

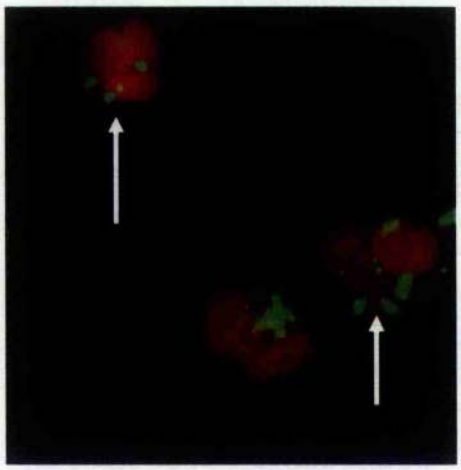
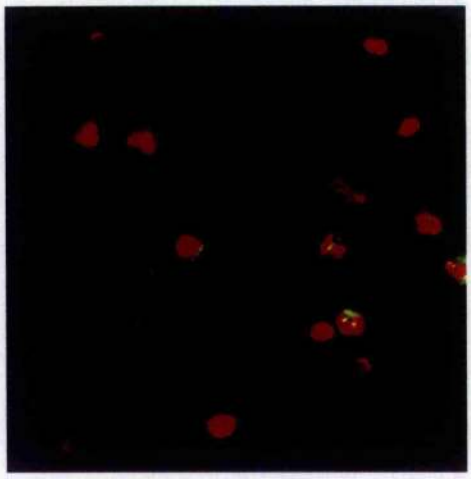


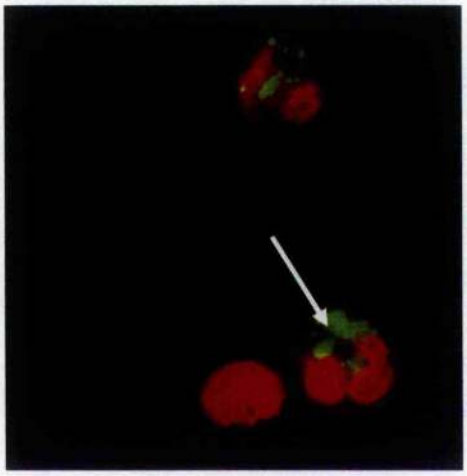
Figure 3.9.3 Morphological analysis of uptake of FITC-*E. coli* in human granolocyte cells in presence of CyaA by Confocal microscopy

Ingestion of FITC-conjugated *E.coli* was evaluated in the presence of 0.1 μg (C) and 0.2 μg (D) of CyaA toxin in human granulocyte cells . Arrowheads show intracellular bacteria.

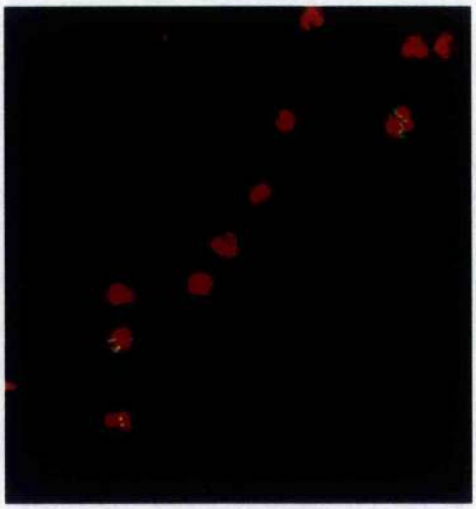
C: Granolocytes + CyaA (magnification 250x)



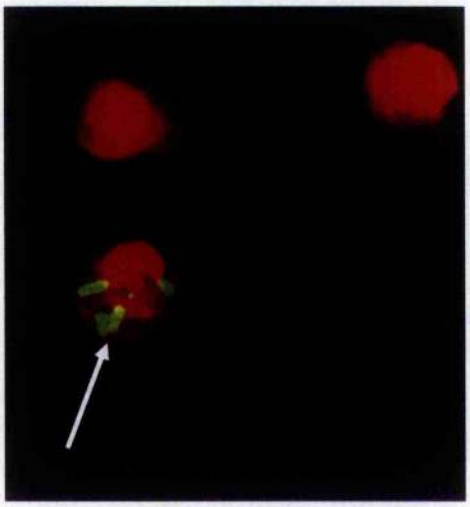
C1: magnification of 1000x



D: Granolocytes + CyaA (magnification of 250x)



D1: magnification of 1000x



3-10 Protein-coated microcrystals (PCMCs)

3-10-1 Experiments with crude CyaA

The recombinant CyaA preparations were routinely solubilized in 8M urea and such preparations would not be suitable for use as vaccine components. In consequence, it was decided to remove urea and try to create a formulation that was stable and could be solubilized in its absence whilst retaining the enzymic and cytotoxic properties of the toxin. For this purpose, the use of protein-coated microcrystals (PCMCs) was proposed. Two forms of CyaA (crude and purified) were used to make the PCMCs (section 2.13.8).

As a preliminary test, to ensure that the crystallisation process did not interfere with the structural integrity of the CyaA protein, three crude CyaA preparations (batches A, B and C) with various protein concentrations were used to make CyaA-PCMCs under the same conditions but on different occasions. To further examine the properties of PCMCs, CyaA-PCMCs were made by shaking or by stirring. After the PCMCs had been made, the next step was to determine the concentration of protein present on the PCMCs which was measured by the Bradford assay (see section 2.9). For this, 10 mg of each batch of CyaA-PCMCs were dissolved in 1 ml of 8 M urea, 20 mM histidine (pH 6.0). The AC enzymic and cytotoxicity activities of CyaA-PCMCs were determined on these urea-solubilised fractions by conductimetry and by the MTT dye reduction assay, respectively (sections 2.13.1 and 2.13.2). The results of these experiments are shown in Table 3.10.1.1.

Both AC enzymic and cytotoxic activities were somewhat diminished by PCMC formulation, but CyaA-PCMCs made by stirring displayed greater activities than those made by shaking. Indeed, a greater PCMC protein concentration was obtained by making the PCMCs using a magnetic stirrer bar then when they were made by shaking.

Table 3.10.1.1 Characterisation of CyaA-PCMCs from crude CyaA (batches A, B, C)

CyaA Sample	Buffer used to dissolve crystals	Protein concentration µg/ml	AC enzymic activity µmol/mg protein/min	Cytotoxicity 50% killing of J774.2 cells µg/ml protein	LPS content visualized on gel by silver stain
Crude urea extract A*	8 M urea, 20 mM histidine pH 6.0	2000	375	0.033	++
CyaA-PCMCs A1#	8 M urea, 20 mM histidine pH 6.0	76	159	0.069	-
CyaA-PCMCs A2♦	8 M urea, 20 mM histidine pH 6.0	97	240	0.035	-
Crude urea extract B*	8 M urea, 20 mM histidine pH 6.0	3400	379	0.049	++
CyaA-PCMCs B1#	8 M urea, 20 mM histidine pH 6.0	320	180	0.1	-
CyaA-PCMCs B2♦	8 M urea, 20 mM histidine pH 6.0	374	219	0. 034	-
Crude urea extract C*	8 M urea, 20 mM histidine pH 6.0	4000	395	0.02	++
CyaA-PCMCs C1#	8 M urea, 20 mM histidine pH 6.0	180	195	0.15	-
CyaA-PCMCs C2♦	8 M urea, 20 mM histidine pH 6.0	210	216	0.05	-

* A, B, C refer to original crude CyaA preps used for PCMCs.

\# A1, B1, C1 refer to PCMCs made by shaking (see section 2.16.1)

♦ A2, B2, C2 refer to PCMCs made by stirring (see section 2.16.2)

Using a magnetic stirrer bar, the PCMCs also formed a lot quicker compared to when they were made by shaking. A possible reason for this is that when the precipitation is carried out, the water content of solvent increases as the protein and excipient are added. This is because the excipient (DL-valine) is prepared in deionized water. Therefore, it is possible that the longer the PCMCs are left in the reaction vessel, as in the case of shaking, there is a possibility that some crystals may re-dissolve. If this was the case then the re-dissolved PCMC would be washed through the filtration stage and lead to loss in PCMCs yield and protein concentration.

A CyaA band was observed from three different batches of PCMCs (A, B and C) when they were dissolved in 8 M urea (Figures 3.10.1.1 and 3.10.1.2). To complement these assays, PCMCs prepared by shaking or stirring (batch C), were dissolved in histidine buffer alone or histidine buffer with increasing concentrations of urea, and visualised on SDS-PAGE. Results (Figure 3.10.1.3) showed that no protein band equivalent in size to CyaA was visible in urea up to 0.5 M but was present at higher concentrations. LPS is a major contaminant of crude CyaA and it was of interest to determine if it would be washed through the process. In this experiment, the LPS was visualised on SDS-PAGE gels by silver stain and showed clear evidence of LPS in the original crude CyaA but not in PCMCs dissolved in increasing concentration of urea (Fig 3.10.1.4 and Table 3.10.1.1).

Following the SDS-PAGE results another experiment was carried out with crude CyaA-PCMCs (batch C). 10 mg of each preparation (made by shaking or stirring) were dissolved in histidine buffer alone or histidine buffer with increasing concentration of urea. The AC enzymic and cytotoxicity properties were determined by conductimetry and cytotoxicity (MTT) assays. The results indicated that AC enzymic and cytotoxicity activities were only detected when CyaA-PCMCs were dissolved in at least 1 M urea

Figure 3.10.1.1 SDS-PAGE analysis of crude CyaA-PCMCs (batch A)

Crude CyaA-PCMCs were made from crude CyaA batch A by either shaking (A1) or stirring (A2), were dissolved in 8M urea, 20mM histidine buffer pH 6.0. Samples were subjected to electrophoresis on a 7.5% polyacrylamide gel and stained with 1% coomassie blue solution.

M: Molecular mass standard

Lane 1: Crude urea extract A (original)

Lane 2: Crude CyaA-PCMCs from CyaA batch A (A1)

Lane 3: Crude CyaA-PCMCs from CyaA batch A (A2)

Arrow shows the position of CyaA

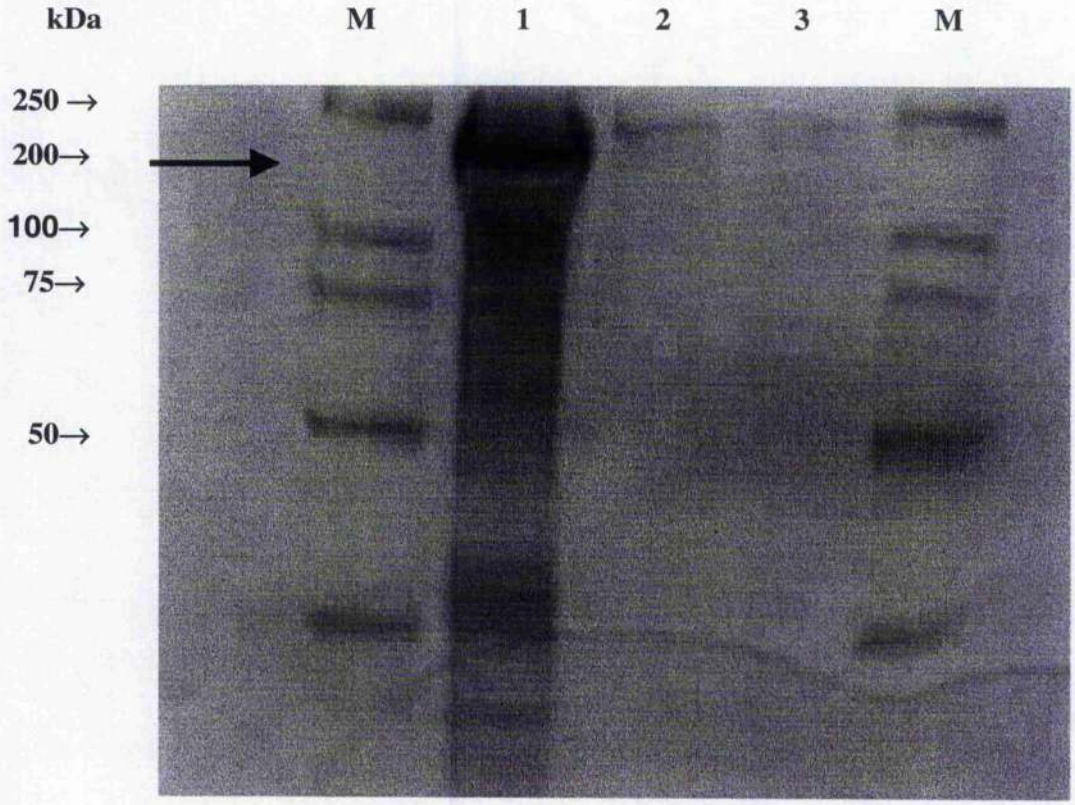

kDa
M
1
2
3
M
250 →
200→
100→
75→
50→

Figure 3.10.1.2 SDS-PAGE analysis of crude CyaA-PCMCs (batches B & C)

Crude CyaA-PCMCs, made from crude CyaA B and C, were dissolved in 8M urea, 20mM histidine buffer pH 6.0. All samples were subjected to electrophoresis on a 7.5% polyacrylamide gel and stained with 1% coomassie blue solution.

M: Molecular mass standard

Lane 1: Crude urea extract batch C (original)

Lane 2 : CyaA-PCMCs from crude C by shaking (C1)

Lane 3 : CyaA-PCMCs from crude C by stirring (C2)

Lane 4 : CyaA-PCMCs from crude B (B1)

Arrow shows the position of CyaA

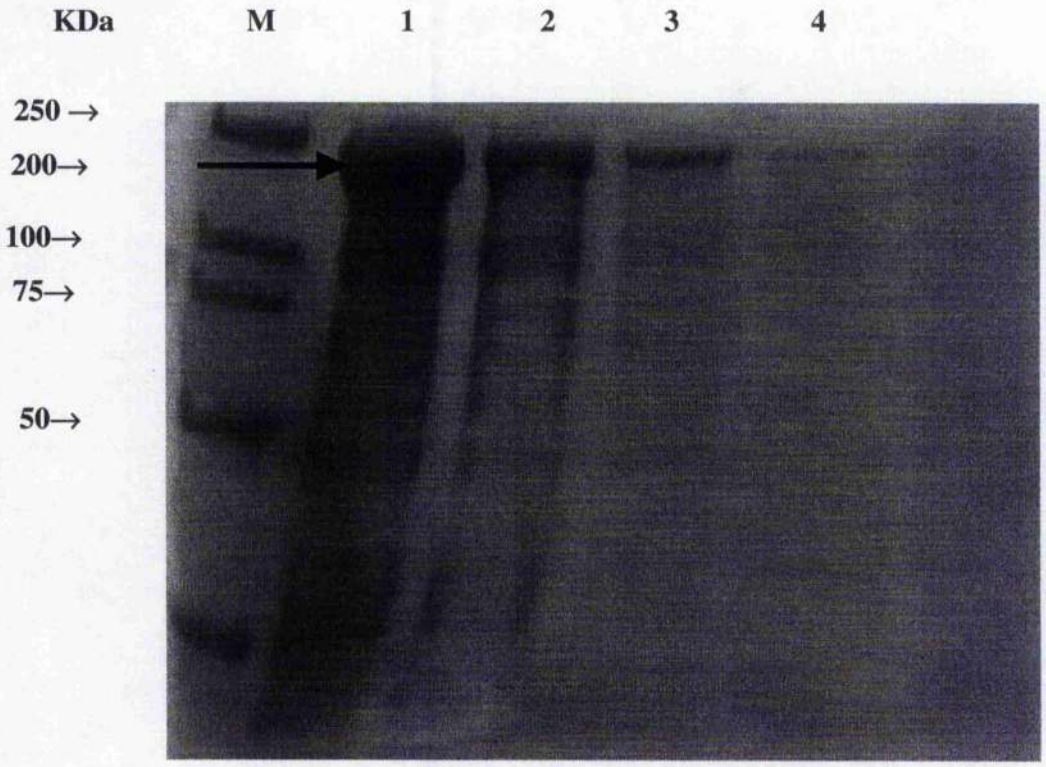

KDa
M
1
2
3
4
250 →
200→
100→
75→
50→

Figure 3.10.1.3 SDS-PAGE analysis of crude CyaA-PCMCs dissolved in different concentrations of urea (batch C)

Crude CyaA-PCMCs made from crude CyaA batch C by either shaking (A1-G1) or stirring (A2-G2) were dissolved in histidine alone buffer or increasing concentration of urea in histidine buffer (pH 6.0). All samples were centrifuged and samples of supernatant were subjected to electrophoresis on a 7.5% polyacrylamide gel and stained with 1% coomassie blue solution.

M: Molecular mass standard

Lanes A1 & A2: CyaA-PCMCs dissolved in 20mM histidine alone buffer pH 6.0

Lanes B1 & B2 : CyaA-PCMCs dissolved in 0.1 M urea, 20 mM histidine pH 6.0

Lanes C1 & C2 : CyaA-PCMCs dissolved in 0.2 M urea, 20 mM histidine pH 6.0

Lanes D1 & D2 : CyaA-PCMCs dissolved in 0.5 M urea, 20 mM histidine pH 6.0

Lanes E1 & E2 : CyaA-PCMCs dissolved in 1 M urea, 20 mM histidine pH 6.0

Lanes F1 & F2 : CyaA-PCMCs dissolved in 2 M urea, 20 mM histidine pH 6.0

Lanes G1 & G2 : CyaA-PCMCs dissolved in 5 M urea, 20 mM histidine pH 6.0

Bold arrow shows the position of the CyaA band

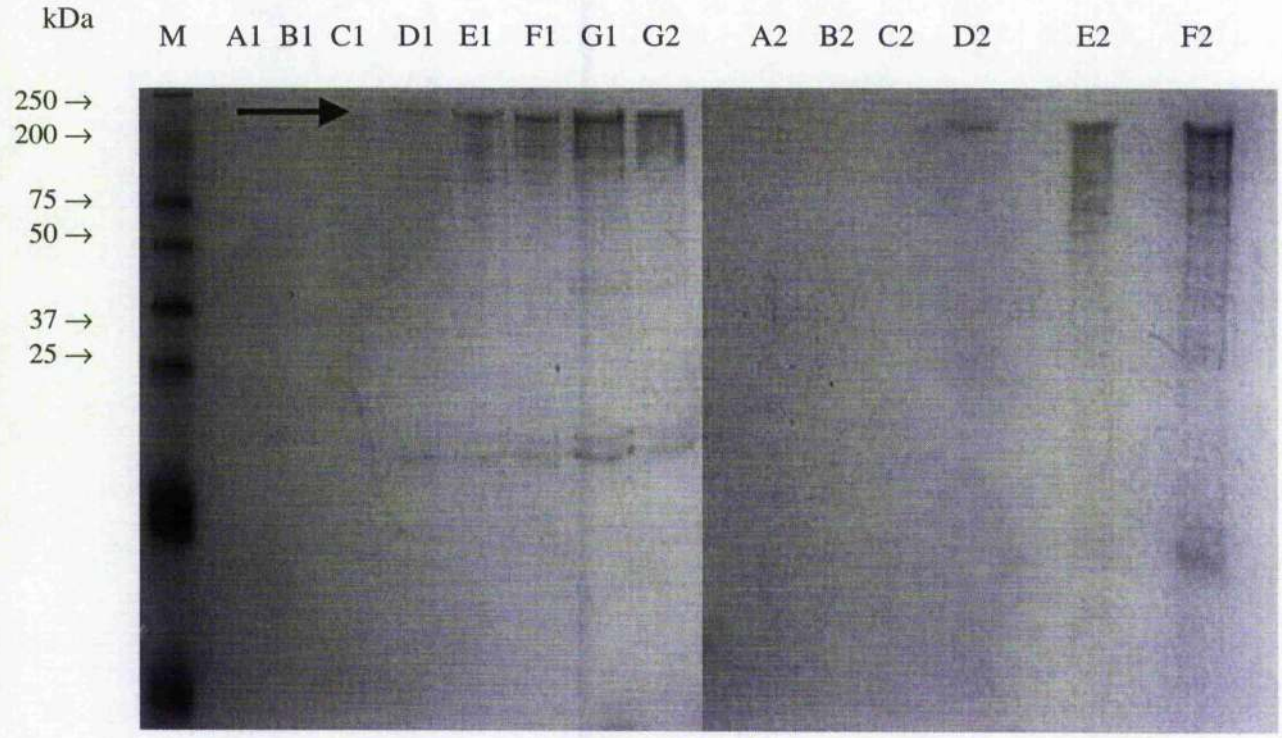

kDa
M
A1
B1
C1
D1
E1
F1
G1
G2
A2
B2
C2
D2
E2
F2
250 →
200 →
75 →
50 →
37 →
25 →

Figure 3.10.1.4 Silver stain for analysis of LPS content of crude CyaA-PCMCs dissolved in different concentrations of urea (batch C)

Crude CyaA-PCMCs were made from crude batch C and were dissolved in histidine alone buffer or increasing concentration of urea in histidine buffer (pH 6.0). All samples were centrifuged and samples subjected to electrophoresis on a 15% polyacrylamide gel and stained with silver stain to visualize the LPS.

M : Molecular weight markers

Co : LPS control

Cr: Crude urea extract batch C

Lane 1: CyaA-PCMCs dissolved in histidine alone buffer pH 6.0

Lane 2: CyaA-PCMCs dissolved in 0.1 M urea, 20 mM histidine pH 6.0

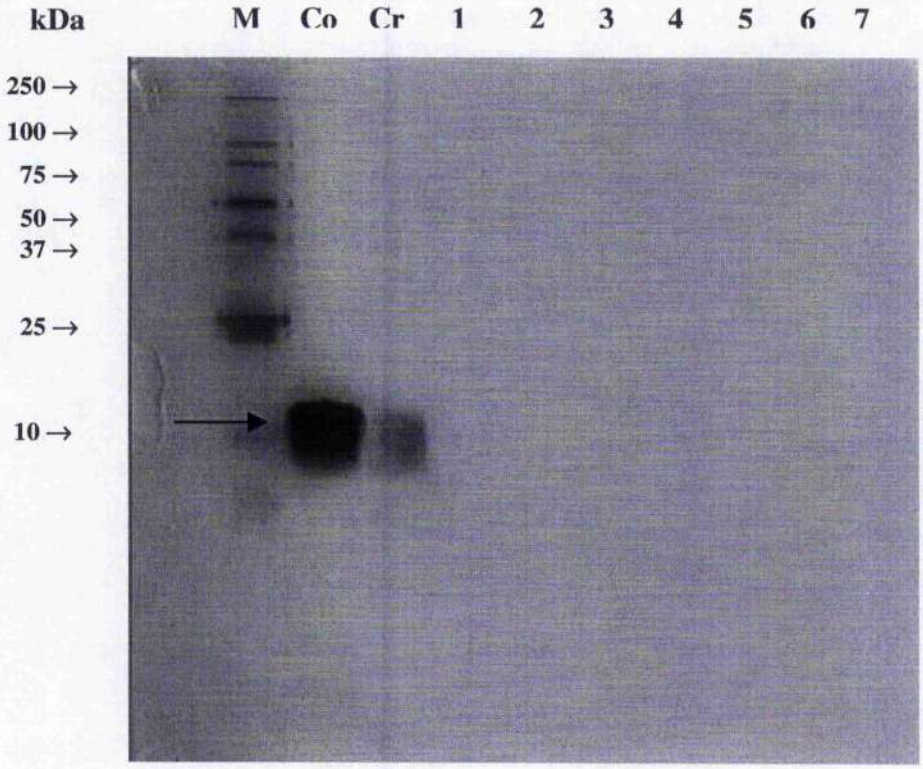
Lane 3: CyaA-PCMCs dissolved in 0.2 M urea, 20 mM histidine pH 6.0

Lane 4: CyaA-PCMCs dissolved in 0.5 M urea, 20 mM histidine pH 6.0

Lane 5: CyaA-PCMCs dissolved in 1 M urea, 20 mM histidine pH 6.0

Lane 6: CyaA-PCMCs dissolved in 2 M urea, 20 mM histidine pH 6.0

Lane 7: CyaA-PCMCs dissolved in 5 M urea, 20 mM histidine pH 6.0


kDa
M
Co
Cr
1
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7
250 →
100 →
75 →
50 →
37 →
25 →
10 →

(Tables 3.10.1.2 and 3.10.1.3). In comparison, histidine buffer alone and urea concentrations up to 0.5 M did not yield any activity. It was interesting to note that, although a CyaA protein band was visible after dissolving the PCMCd in 0.5 M urea, no enzymic or cytotoxic activities were detected with this sample. Even with 1 M urea, the enzymic and cytotoxic activity was lower than with 2 M and 5 M urea. The results of this part of study show that CyaA-PCMCs have good activities only when redissolved in buffer containing a high concentration of urea. It can be concluded that in general PCMCs have enzymic and cytoxicity activities approximately 2-fold lower than the original crude urea extract. The process of preparing PCMCs did not therefore greatly perturb the conformation of CyaA required for its enzymic and cell-invasive properties.

3.10.2 Urea concentration in CyaA-PCMCs

The removal of urea was a major aim in this project. The urea content of CyaA-PCMCs was determined by the urease assay (section 2.17), and was found to be 0.000051g/100 ml, a ratio of 1:1,000,000. The concentration of urea in the CyaA stock material in 8 M was 48 g/100ml therefore, the ratio of urea after and before PCMCs is 0.000001. The result shows that during the process the majority (> 99.99%) of urea has been removed.

Sample	Meter reading Ammonium concentration (mg/L)	Urea concentration (mg/L)	% Urea (mg/100 ml)
PCMC	0.42	0.51	0.051

Table 3.10.1.2 Characterisation of CyaA-PCMCs prepared from crude CyaA (batch C with shaking) dissolved in increasing concentrations of urea buffer

CyaA-PCMCs prepared from crude sample batch C were dissolved in 20mM histidine (A1) or in histidine buffer with increasing concentrations of urea buffer (B1-G1). The AC enzymic and cytotoxicity activities were determined by conductimetry and MTT assay on samples of supernatant after centrifugation. LPS was visualised by silver stain.

PCMCs prepared from crude CyaA batch C	Protein conc µg/ml	AC enzymic activity µmol/min/mg protein	Cytotoxicity %50 killing of J774.2 cells µg/ml protein	LPS visualized on gel by silver stain
A_1-Dissolved in 20 mM histidine pH 6.0	No protein detected	No activity detected	No cytotoxicity detected	-
B_1-Dissolved in 20 mM histidine, 0.1 M urea pH 6.0	No protein detected	No activity detected	No cytotoxicity detected	-
C_1-Dissolved in 20 mM histidine, 0.2 M urea pH 6.0	No protein detected	No activity detected	No cytotoxicity detected	-
D_1-Dissolved in 20 mM histidine, 0.5 M urea pH 6.0	No protein detected	No activity detected	No cytotoxicity detected	-
E_1-Dissolved in 20 mM histidine, 1 M urea pH 6.0	120	131	0.15	-
F_1-Dissolved in 20 mM histidine, 2 M urea pH 6.0	180	195	0.07	-
G_1- Dissolved in 20 mM histidine, 5 M urea pH 6.0	228	275	0.05	-

Table 3.11.1.3 Characterisation of CyaA-PCMCs prepared from crude CyaA (batch C with stirring) dissolved in increasing concentrations of urea buffer

CyaA-PCMCs from crude sample batch C and were dissolved in 20mM histidine (A2) or in histidine buffer with increasing concentrations of urea buffer (B2-G2). The AC enzymic and cytotoxicity activities were determined by conductimetry and MTT assay on samples of supernatant after centrifugation. LPS was visualised by silver stain.

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PCMCs prepared from crude CyaA batch C	Protein conconcentration µg/ml	AC enzymic activity µmol/min/mg protein	Cytotoxicity 50% killing of J774.2 cells µg/ml protein	LPS visualized on gel by silver stain
A_2-Dissolved in 20 mM histidine pH 6.0	No protein detected	No activity detected	No cytotoxicity directed	-
B_2-Dissolved in 20 mM hihtidine, 0.1 M urea pH 6.0	No protein detected	No activity detected	No cytotoxicity detected	-
C_2-Dissolved in 20 mM histidine, 0.2 M urea pH 6.0	No protein detected	No activity detected	No cytotoxicity detected	-
D_2-Dissolved in 20 mM histidine, 0.5 M urea pH 6.0	No protein detected	No activity detected	No cytotoxicity detected	-
E_2-Dissolved in 20 mM histidine, 1 M urea pH 6.0	165	119	0.6	-
F_2-Dissolved in 20 mM histidine, 2 M urea pH 6.0	210	216	0.15	-
G_2-Dissolved in 20 mM histidine, 5 M urea pH 6.0	295	281	0.05	-

3-10-3 Experiments with purified CyaA-PCMCs

3-10-3-1 Stability of AC enzymic activity of CyaA-PCMCs

Based on previous experiments where CyaA-PCMCs were made with crude CyaA preparations, CyaA-PCMCs were prepared with purified CyaA for further investigation (section 2.16.1 and 2.16.2). Prepared crystals were kept in different conditions for two weeks (i.e. 37, 20, 4, -20° C). 10 mg of each of two preparations (prepared from CyaA in the presence of 2 M or 8 M urea) were dissolved in 1 ml of 8 M urea, 20 mM histidine, (pH 6.0) and AC enzymic activity determined. In parallel, 100 µl of original CyaA in 8 M urea were stored at the above conditions for two weeks (Table 3.10.3.1.1). As Table 3.10.3.1.2 shows, the PCMCs prepared from CyaA in 8 M urea (batch A) displayed more stablity and higher activity than those that made from CyaA in 2 M urea (batch B) at a temperature of 4° C and above. Stability was similar at –20° C. The results from the original CyaA preparation in 8 M urea stored for 2 weeks at different temperatures showed that AC enzymic activity was retained well even at room temperature but, at 37° C, only 13.5 % of the original activity was retained (Table 3.10.3.1). It can be concluded that CyaA-PCMCs prepared from CyaA in 8 M urea pH 6.0 retained AC enzymic activity at approximately 90 % even at 37° C which is in contrast to original CyaA kept in 8 M urea at 37 ° C when 80-90 % of activity was lost. PCMCs have the potential, therefore, to be stored at ambient temperature and still retain enzymic activity.

3-10-3-2 The effect of different detergents on recovery of AC enzymic activity from CyaA-PCMCs

As mentioned previously, reasons for preparing CyaA-PCMCs was to remove the urea and possibly the LPS. The problem so far encountered was that the CyaA activities were not recoverd when CyaA-PCMCs were dissolved in histidine buffer alone. However, it

Table 3.10.3.1.1 Stability of purified original CyaA

Purified CyaA in 8M urea was examined for stability of toxin activity. 200 µl of sample were stored in different conditions (37, 20, 4 ,-20°C) for two weeks. The enzymic activity was determined by conductimetry assay. Results are the mean values ± standard deviation of 2 determinations.

Samples	AC enzymic activity (µmol/min/mg protein)
CyaA at 37 °C	109 ± 14 (13.5 %)
CyaA at room temp	600 ± 19 (84 %)
CyaA at 4 °C	750 ± 16 (92 %)
CyaA at –20 °C	812 ± 11 (100 %)

Table 3.10.3.1.1 Stability of purified CyaA-PCMCs

CyaA-PCMCs were prepared from purified CyaA in two different concentrations of urea (2 M and 8 M) to examine stability of the toxin in the PCMCs format. 10 mg of each sample were dissolved in 1 ml of 8 M urea, 20mM histidine (pH 6.0) after storage at different temperatures (37, 20, 4 ,-20°C) for two weeks. The enzymic activity was determined by conductimetry assay. Sample A (CyaA-PCMCs) prepared from purified CyaA in 8 M urea and sample B (CyaA-PCMCs) prepared from purified CyaA in 2 M urea. Results are the mean values ± standard deviation of 2 determinations.

Assay →	AC enzymic activity µmol/min/mg protein				
	Before storage	After storage two weeks			
Sample ↓		-20°C	4°C	20°C	37°C
CyaA-PCMCs (A) in 8 M urea	480 ±13 (100 %)	480 ±13 (100 %)	440 ±15 (92 %)	403 ± 4 (84 %)	402 ± 12 (84 %)
CyaA-PCMCs (B) in 2 M urea	392 ± 16	410 ± 45	272 ± 17	269 ±17	231 ±14

may be possible that addition of other reagents would help to recover the CyaA activities. To this end, a number of detergents (Hampton Research Reagent) (Appendix H) were tested. 5 mg of CyaA-PCMCs from CyaA-PCMCs batches A and B were dissolved in 100 µl of 2 % of above detergents. After 1h at room temperature the AC enzymic activity of both CyaA-PCMCs preparations was determined, and compared with that of the two preparations dissolved in 8 M urea, 20 mM histidine pH 6.0. As tables 3.10.3.2.1 and 2.10.3.2.2 showed none of the above reagents allowed good recovery of the AC enzymic activity of toxin.

Table 3.10.3. 2.1 AC enzymic activity of CyaA-PCMCs dissolved in different detergents (1-12 from Hampton Research)

CyaA-PCMCs prepared from purified CyaA (batches A and B) were dissolved in different detergents in an attempt to identify whether any detergent had an effect on the recovery of CyaA enzymic activity from CyaA-PCMCs. The AC activity was determined by conductimetry assay after 1 h at room temperature.

Sample	AC enzymic activity (μmol/min/mg protein) of PCMCs dissolved in different detergents												
	Detergent												
CyaA-PCMCs	8 M urea, 20 mM histidine	1	2	3	4	5	6	7	8	9	10	11	12
CyaA-PCMCs A in 8 M urea	480 ±13	9.5	8	4	7	11.3	15.5	6.3	9.2	8	0	4	0
CyaA-PCMCs B in 2 M urea	392 ±16	0	0	2	0	0	1	0	0	4	0	4	1

Table 3.10.3.2.2 AC activity of CyaA-PCMCs dissolved in different detergents (13-24 from Hampton Research)

CyaA-PCMCs prepared from purified CyaA (batches A and B) were dissolved in different detergents in an attempt to identify whether any detergent had an effect on the recovery of CyaA enzymic activity from CyaA-PCMCs. The AC activity was determined by conductimetry assay after 1 h at room temperature.

Sample	AC enzymic activity (μmol/min/mg protein) of PCMCs dissolved in different detergents												
	Detergent												
CyaA-PCMCs	8 M urea, 20 mM histidine	13	14	15	16	17	18	19	20	21	22	23	24
CyaA-PCMCs A from 8 M urea	480 ±13	1	1	1	2	0	0	1	1	0	0	0	0
CyaA-PCMCs B from 2 M urea	392 ±16	0	0	1	0	0	1	0	0	0	0	0	1

3-10-3-3 AC enzymic and cytotoxic properties of CyaA-PCMCs made with different components

As detergents appeared not to help with recovery of CyaA activity, it was decided to make different batches of CyaA-PCMCs which included a number of other components (e.g. CaM, BSA, ATP and $CaCl_2$) in an attempt to identify any extra component that might have an effect on CyaA-PCMCs properties (section 2.16). It is known that folding of protein in the bacterial cell is dependent on the assistance of chaperone proteins such as GroEL (Singler *et al.*, 1998) so it was thought that addition of other protein components with CyaA during PCMC formulation may aid solubility. As little AC activity was detected when CyaA-PCMCs were dissolved in histidine buffer, the CyaA may have formed aggregates on the crystal surface or aggregated when reconstituted in aqueous buffer. The toxin is known to be prone to aggregation in aqueous solution due to its hydrophobic nature and is the reason why CyaA preparations are stored in 8 M urea. Thus, introducing other biomolecules into the PCMC procedure may help to separate CyaA molecules and prevent aggregation.

CyaA-CaM-PCMCs and CyaA-CaM-BSA-PCMCs (batch number 1) were made of CyaA plus CaM and BSA in molar ratio of 1: 3 and 1: 10, respectively. 10 mg of each PCMC sample were dissolved in 1 ml of 8 M urea, 20 mM histidine (pH 6.0) to measure the total protein concentration (Bradford assay) in order to calculate the amount of protein incorporated into the PCMCs. In addition, the weight of PCMCs was obtained and this provided the theoretical amount of protein in the PCMCs by dividing the total protein added to make the PCMCs by the total weight of the PCMCs. The measured protein content of the PCMCs would allow calculation of the actual total protein in the PCMCs. In order to calculate the CyaA content of the different PCMCs, an assumption was made that each protein would coat the valine crystals equally, by weight (Table 3.10.3.3.1 and 3.10.3.3.2).

Table 3.10.3.3. 1 Properties of three types of PCMCs (batch 1)

CyaA-PCMCs were made with 2 ml of CyaA (260 μg/ml) in 8 M urea and 2 ml of DL-valine (60 mg/ml). CyaA-CaM-PCMCs were prepared by mixing 3ml of purified CyaA (260 μg/ml) with 99 μl of CaM (1.8 mg/ml). CyaA-CaM-BSA-PCMCs were prepared by mixing 4ml of purified CyaA (260 μg/ml) with 173μl of CaM (1.8 mg/ml) and 3120 μg of BSA. 10 mg of each type of PCMCs were dissolved in 8 M urea, 20 mM histidine (pH 6.0) to determine the protein concentration.

PCMCs type	Amount of protein to make PCMCs (μg)	Weight of PCMCs (mg)	Theoretical protein/mg PCMCs	Actual total protein (μg/mg, PCMCs (Bradford)	CyaA content* μg protein/mg PCMCs	Total CyaA recovered (μg) from PCMCs	% CyaA recovery
CyaA-PCMCs	520	58	8.9	6.12	6.12	355	68
CyaA-CaM-PCMCs	780 178	78	11.9	8.9	6.86	537.5	68.9
CyaA-CaM-BSA PCMCs	1040 312 3120	110	44	48	6.52	717	69.9

*assuming proteins coated equally onto PCMCs)

Table 3.10.3.3. 2 AC enzymic and cytotoxicity activities of different PCMC formulations dissolved in buffers at various pHs

CyaA-PCMCs, CyaA-CaM-PCMCs and CyaA-CaM-BSA-PCMCs were prepared according to Table 3.10.3.3.1 and 10mg of each were dissolved in Bicine buffer of three different pH values. AC enzymic and cytotoxicity activities were compared with those obtained with CyaA-PCMCs dissolved in 8 M urea, 20 mM histidine (pH 6.0) as a positive control. Results are the means of 2 determinations (± S.D).

Sample	AC enzymic activity µmol/min/mg protein and % activity	Cytotoxicity 50 % killing of J774.2 macrophages (µg protein/ml)
CyaA-PCMCs in 8 M urea, 20 mM histidine pH6.0	670 ± 14 (100)	0.12 ± 0.021 (100)
CyaA-CaM-PCMCs in Bicine pH 7.7	174 ± 6 (26)	0.51 ± 0.028 (23)
CyaA-CaM-PCMCs in Bicine pH 8.1	159 ± 5 (24)	0.38 ± 0.035 (31)
CyaA-CaM-PCMCs in Bicine pH 8.5	151 ± 9 (22)	>1
CyaA-CaM-BSA-PCMCs in Bicine pH 7.7	781 ± 12 (116)	>1
CyaA-CaM-BSA-PCMCs in Bicine pH 8.1	610 ± 11 (91)	>1
CyaA-CaM-BSA-PCMC in Bicine pH 8.0 + $CaCl_2$*	595 ± 10 (89)	>2
CyaA-CaM-BSA-PCMCs in Bicine pH 8.5	720 ± 30 (107)	0.51± 0.042 (23)
CyaA-CaM-BSA-ATP-PCMCs in Bicine pH 8.0	490 (73)	>2

* CaCl2 added to Bicine not to crystals

Table 3.10.3.3.3 AC enzymic activity of 3 types of PCMCs dissolved in different buffers

PCMCs were prepared according to tabe 3.10.3.3.1. AC enzymic activities of the different types of crystals were compared with the original CyaA preparation in 8 M urea. Results are the means of 2 determinations (± S.D).

Sample	AC enzymic activity (μmol/min/mg protein)
CyaA (original) preparation in 8 M urea, 20 mM histidine pH 6.0	734 ± 26 (100 %)
CyaA-PCMCs in 8 M urea, 20 mM histidine pH 6.0	676 ± 8 (92 %)
CyaA-CaM-BSA-PCMCs in 8 M urea, 20 mM histidine, pH6.0	692 ± 25 (94 %)
CyaA-CaM-BSA-PCMCs in 100 mM Bicine pH 8.0	720 ± 30 (98 %)
CyaA-CaM-PCMCs in 8 M urea, 20 mM histidine pH 6.0	660 ± 23 (90 %)
CyaA-CaM-PCMCs in 100 mM Bicine pH 8.0	185 ± 12 (25 %)

10 mg of each type of crystal were dissolved in 1 ml of either 8 M urea, 20 mM histidine pH 6.0 or in 100 mM Bicine buffer alone at various pHs (Bicine buffer 10 mM pH 8.0 is the normal diluent for the conductimetry assay). AC enzymic and cytotoxicity activities of these samples were assayed and compared to that of CyaA-PCMCs dissolved in 8 M urea in 20 mM histidine pH 6.0.

The results for CyaA-CaM-PCMCs showed AC enzymic and cytotoxic activities at ~ 25% of that found in 8 M urea at pH 7.7 (Table 3.10.3.3.2). Cytotoxicity improved at pH.8.1 but AC enzymic was worse at pH 8.5. In-corporation of BSA with CaM, CyaA allowed almost full recovery of AC activity at all pH values but only low recovery of cytotoxicity which was best at pH 8.5. Some of the CyaA-PCMCs from batch number 1 were dissolved in 100 mM Bicine (pH.8.0) in presence of 200 μl of 5 mM $CaCl_2$ and then assayed. The results indicated that in the presence of $CaCl_2$, most of the AC activity was retained but the cytotoxicity was decreased (Table 3.10.3.3.2). Likewise, incorporation of ATP into CyaA-CaM-BSA-PCMCs had little effect on enzymic or cytotoxic activities. In another experiment, AC enzymic activities of different types of crystals were compared in 8 M urea, 20 mM histidine (pH.6.0) and 100 mM Bicine (pH 8.0) and compared with the original CyaA preparation (Table 3.10.3.3.3). The most significant finding here is that full enzymatic activity was recovered from CyaA-CaM-BSA-PCMCs dissolved in either buffer. Again, the highest level of activity was seen in CyaA-PCMCS prepared in cooperation with CaM and BSA.

Further work involved preparation of another set of PCMCs (batch number 2). These were made using the same conditions as for batch 1 but with different concentrations of CyaA and other components (Table 3.10.3.3.4). For AC enzymic activity of these PCMCs, 10 mg of each type was dissolved in 8 M urea, 20 mM histidine pH 6.0 or 20 mM histidine buffer pH 6.0 alone. These dissolved samples were left at room

Table 3.10.3.3.4 Properties of three types of PCMCs (batch 2)

CyaA-PCMCs were made by using 2 ml of CyaA (290 μg/ml) in 8 M urea and 2 ml of DL-valine (60 mg/ml). CyaA-CaM-PCMCs were prepared by mixing 2 ml of purified CyaA (290 μg/ml) with 870 μg of BSA. CyaA-CaM-BSA-PCMCs were prepared by mixing 4ml of purified CyaA (290 μg/ml) with 200 μl of CaM (1.8 mg/ml) and 3480 μg of BSA. 10 mg of each type of PCMCs were dissolved in 8 M urea, 20 mM histidine (pH 6.0) to determine the protein concentration

PCMC type	Amount of protein to make crystals (μg)	Weight of PCMCs (mg)	Actual total protein PCMCs μg/mg (Bradford)	CyaA content* (μg/mg protein) PCMCs	Total CyaA recovered μg from PCMCs	% CyaA recovery
CyaA-PCMCs	580	82.4	6	6	494.4	85
CyaA-BSA-PCMCs	290 870	109.4	8.1	2.025	221.5	76.3
CyaA-BSA-CaM-PCMCs	1160 3480 360	115.5	27.6	6.4	739.2	63.7

* Assuming all protein coated equally onto PCMCs

temperature and enzymic activity measured at intervals for up to one week. As shown in Table 3.10.3.3.5, the AC enzymic activities derived from all the PCMCs types were retained at high level for two days in 8 M urea and then decreased noticeably. In histidine buffer alone, 20-30% of the AC activity recoved in urea was detected for both CyaA-BSA and CyaA-BSA-CaM crystals, but not for CyaA-PCMCs. This level of activity was retained up to day 7 for the CyaA-BSA crystals and up to day 3 for the CyaA-BSA-CaM-PCMCs.

Some of the CyaA-PCMCs from batch number 2 were also dissolved in histidine, HEPES or Bicine buffer at different pH values. As Table 3.10.3.3.6 shows, the highest activity, comparable to that in urea, was obtained at pH 8.0 with CyaA-CaM-BSA-PCMCs in 100 mM Bicine buffer. As was found for histidine buffer, no AC activity of CyaA-PCMCs was detected when they were dissolved in HEPES buffer whereas, in the presence of CaM or CaM, BSA some activity was seen. It can be concluded that no detectable AC activity was found when CyaA-PCMCs were dissolved in histidine or HEPES buffer whereas, with CyaA-CaM-PCMCs, 20-30% activity can be recovered in histidine buffer alone and with CyaA-CaM-BSA-PCMCs nearly complete activity is recovered (62 %). Therefore, addition of BSA or CaM during manufacture of PCMCs seemed to prevent subsequent aggregation of CyaA on removal of urea and exposure to aqueous medium. There was some indication that CyaA activity of CyaA-CaM-PCMCs and CyaA-CaM-BSA-PCMCs dissolved in histidine buffer alone was very stable at room temperature. Even greater CyaA activity was recovered when CyaA-CaM-BSA-PCMCs were dissolved in 100mM Bicine at pH.8.

Table 3.10.3.3.5 AC enzymic activity of three types PCMCs (batch 2) dissolved in different buffers and assayed over a period of seven days.

10 mg of each type of PCMCs (see Table 3.10.3.3.4) were dissolved in 200 µl of either 8 M urea, 20 mM histidine (pH 6.0) or 20 mM histidine (pH 6.0) alone. These samples were left at room temperature and enzymic activity measured at intervals for up to one week.

Sample	AC activities (µmol/min/mg protein) after 7 days						
	1 h	3 h	6 h	24 h	30 h	48 h	142 h
CyaA-PCMCs in 8M urea	432	401	424	477	379	128	76
CyaA-PCMCs in histidine	N/D	N/D	N/D	N/D	N/D	N/D	N/D
CyaA-BSA-PCMCs in 8M urea	522	522	500	545	547	375	295
CyaA-BSA-PCMCs in histidine	113	102	136	129	113	129	136
CyaA-BSA-CaM-PCMCs in 8M urea	951	781	563	980	660	N/D	21
CyaA-BSA-CaM-PCMCs in histidine	234	227	312	255	241	213	N/D

N/D = No detectable activity

Table 3.10.3.3.6 AC enzymic activity of 3 types of PCMCs (batch 2) dissolved in histidine, HEPES or Bicine buffer at different pH values

10 mg of each type of crystals were dissolved in 200 µl of histidine, HEPES or Bicine buffers at various pHs to assay AC enzymic activity. Results are the means of 2 determinations (± S.D).

Sample	AC enzymic activity (µmol/min/mg protein) (% activity)
CyaA-PCMCs in 8 M urea, 20 mM histidine (pH 6.0)	500 ± 29 (100)
CyaA-PCMCs in histidine (pH 6.0)	N/D
CyaA-PCMCs in HEPES (pH 7.0)	N/D
CyaA-BSA-PCMCs in 20mM histidine pH 6.0)	34 ± 4 (7)
CyaA- BSA-PCMCs in HEPES (pH 7.0)	45 ± 7 (9)
CyaA- BSA-PCMCs in Bicine (pH 8.0)	81 ± 5 (16)
CyaA-BSA-CaM-PCMCs in histidine H 6.0)	312 ± 9 (62)
CyaA-BSA-CaM-PCMCs in HEPES (pH 7.0)	241 ± 6 (48)
CyaA-BSA-CaM-PCMCs in Bicine (pH 8.0)	674 ± 19 (134)

N/D= No detected activity

3-10-3-4 Properties of three types of CyaA-PCMCs in the presence of different concentrations of CaM

Previous data (Table 3.10.3.3.2) had indicated that addition of CaM to CyaA (in the molar ratio of 3:1) resulted in improved recovery of cytotoxicity when CyaA-CaM-PCMCs were dissolved in Bicine buffer at pH 8.0. It was decided to increase the concentration of CaM in the PCMCs to see if this further improved cytotoxicity. Different molar ratios (3:1, 10:1, 30:1) of CaM: CyaA were used to make CyaA-CaM crystals (Table 3.10.3.4.1). The assay results indicated that, in comparison to the CyaA-CaM-PCMCs in the original formulation there was an increase in both AC enzymic and cytotoxic activities with increasing amounts of CaM incorporated into the PCMCs (Table 3.10.3.4.2).

3-10-4- 4 AC enzymic activity of purified CyaA before and after dialysis

Purified CyaA, after the final Butyl-Sepharose purification, was contained in 0.32 M ammonium sulphate, 0.08 M sodium chloride in 20 mM histidine buffer, 8 M urea pH 6.0 (section 2.6.1.2). In order to investigate the importance of these salts to the formulation and yield of PCMCs, a sample of purified CyaA was dialysed against 100 ml of 8 M urea, 20 mM histidine buffer pH 6.0 for 4 h at 4° C and then against fresh buffer overnight, to remove most of the ammonium sulphate and sodium chloride. The dialysed CyaA and original toxin preparation were then used to make PCMCs under the same condition. The results indicated that, in the absence of ammonium sulphate and sodium chloride, only a small amount of crystals formed which were very amorphous and difficult to manipulate, for example, for weighing out. However, the AC enzymic activities of both samples were assayed after they were dissolved in 8 M urea, 20 mM histidine, pH 6.0. There was little difference in AC activity between the two types of

PCMCs when reconstituted (699 and 536 μmol/min/mg protein for non-dialysed and dialysed preparations, respectively).

Table 3.10.3.4.1 Properties of CyaA-PCMCs prepared in the presence of different concentrations of CaM (batch 3)

CyaA-CaM-PCMCs were made by using 3 ml of CyaA (260 μg/ml) in 8 M urea and 2 ml of DL-valine (60 mg/ml). CyaA-CaM-PCMCs was prepared by mixing 3 ml of purified CyaA (260 μg/ml) with increasing amounts of CaM. 10 mg of each type of PCMCs were dissolved in 8 M urea, 20 mM histidine (pH 6.0) to determine the protein concentration

PCMCs type	Amount of protein to make PCMCs (μg)	Weight of PCMCs (mg)	Theoretical μg total protein/mg PCMCs	Actual total protein (μg/mg PCMCs (Bradford)	CyaA content* (μg protein/mg crystal)	Total CyaA recovered (μg)
CyaA-CaM 3x PCMCs	780 248	95	10.82	4.7	3.6	339
CyaA-CaM-10x PCMCs	780 827	71.2	22.6	6.42	3.15	224
CyaA-CaM-30x PCMCs	780 2481	158.3	20.6	5.12	1.23	194.5

* Assuming all proteins coated equally onto PCMCs

Table 3.10.3.4.2 AC enzymic and cytotoxic activities of three types of CyaA-CaM-PCMCs in the presence of different concentrations of CaM (batch 3)

The AC enzymic and cytotoxic activities of three types of CyaA-CaM-PCMCs in 100 mM Bicine were compared to those of CyaA-CaM 3x-PCMCs dissolved in 8 M urea buffer.

Sample	AC enzymic activity µmol/min/mg protein (% activity)	Cytotoxicity 50 % killing of J774.2 cells µg protein/ml (% activity)
CyaA-CaM3x-PCMCs in 8 M urea, 20 mM histidine pH 8.0	770 ± 14 (100)	0.12 ± 0.04 (100)
CyaA-CaM 3x-PCMCs in 100 mM Bicine pH 8.0	86 ± 9 (11)	2.2 ± 0.014 (5)
CyaA-CaM 10x-PCMCs in 100 mM Bicine pH 8.0	160 ± 8 (21)	1.7 ± 0. 021 (7)
CyaA-CaM 30x-PCMCs in 100 mM Bicine pH 8.0	422 ± 23 (55)	1.47 ± 0.11 (8)

3-10-3-5 AC enzymic and cytotoxicity activities of CyaA in the presence of components used to prepare PCMCs

Experiments had indicated that, to achieve good AC and cytotoxicity activities from CyaA-PCMCs when they were redissolved in aqueous solutions, CyaA needed to be co-crystallised not only with valine, which was part of the normal procedure, but also with CaM or BSA or both. In order to investigate whether valine, CaM or BSA had any effect on the properties of CyaA in the absence of PCMC formulation, all of the above substances were included with CyaA in AC enzymic and cytotoxicity assays. The results of the assays indicated that these additions had little effect on AC activity but that CaM interfered with cytotoxic activity when added with CyaA. However, this apparent inhibition was overcome if BSA and valine were added in addition to CaM (Table 3.10.3.5.1).

In a similar experiment, CyaA-BSA-CaM-PCMCs (batch 2) were dissolved in PBS at pH 7.3 and the AC enzymic and cytotoxic activities of the solution were determined. Interestingly, the results showed almost complete recovery of enzymic activity and reasonable cytotoxic activity (559 ± 29 μmol/min/mg protein and 50 % killing at 0.4 μg protein/ml, respectively).

Table 3.10.3.5.1 AC enzymic and cytotoxicity activities of CyaA in the presence of components used to prepare PCMCs

The activities of the original form of CyaA alone in 8 M urea, 20 mM histidine pH 6.0 or when mixed with components of PCMCs were determined by conductimetry and cytotoxicity assays and then compared. Valine was used at 60 mg/ml final concentration, CaM and BSA was used at molar ratio 3:1 and 10:1 respectively. Results are the mean values ± standard deviation of 2 determination

Sample	AC enzymic activity µmol/min/mg protein (% activity)	Cytotoxicity µg/ml protein required for 50 % killing of J774.2 cells (% activity)
CyaA alone	880 ± 3.5 (100)	0.021± 0.001 (100)
CyaA+Valine	770 ± 2.82 (87)	0.024 ± 0.003 (87)
CyaA+CaM	976 ± 4.24 (111)	0.062 ± 0.01 (34)
CyaA+BSA+CaM+Valine	928 ± 3.5 (105)	0.03 ± 0.009 (70)

3-11 Scanning electron microscopy (SEM) images

The SEM is designed for directly studying the surfaces of solid objects. By scanning with an electron beam that has been generated and focused by the operation of the microscope, an image is formed in much the same way as a TV. The SEM allows a greater depth of focus than the optical microscope. For this reason the SEM can produce an image that is a good representation of the three-dimensional sample.

SEM gave a good indication of particle size and morphology of the valine crystals and PCMC powders. It was seen that by slightly changing the experimental parameters, SEM images showed an enormous change in the size and morphology of the resulting PCMCs. SEM was carried out on 10 different samples. Some of these samples were with DL-valine alone; DL-valine BSA plus urea buffer without CyaA (images 1-4) and others were CyaA-PCMCs with CaM, or BSA-PCMCs (images 5-10).

From the images 1-3 it can be concluded that the DL-valine crystals are relatively large and regular but of various sizes (approximately 3-10μm). However, sample 4 has more regular crystals of similar morphology with approximate size of 4-15 μm. Note that in the images 2 and 3 some salts were seen and the crystals were more irregular. It is quite difficult to pinpoint an accurate size of each crystal type, as there seemed to be a variety of different sized crystals in all samples. Crystals in image 1 has a fairly regular rectangular shape, which is typical of valine crystals (Dr. M. C. Parker, personal comunication).

The crystals in sample 5 made from crude CyaA are large and rectangular with average size 20-40 μm in diameter and some salts were seen on the crystals. It is of interest to compare the morphology of these crystals with those of image 2 prepared from the buffer in which crude CyaA was solubilized (8 M urea, 20 mM histidine). The presence

Figure 3.11.1 Scanning electron microscopy

1: Valine crystals

These were made with DL-valine precipitated with saturated ethanol in the absence of any protein. 60 mg of DL-valine was dissolved in 2 ml deionised water then used to make crystals as in the PCMC process.

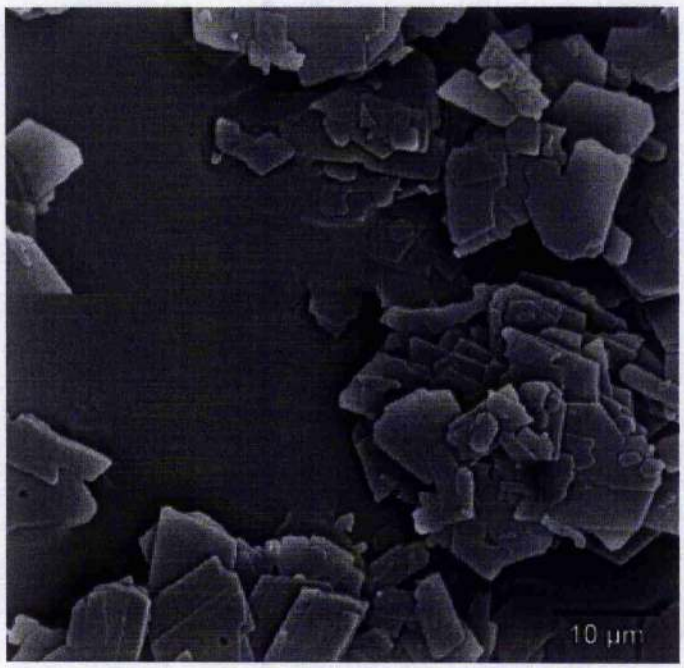
2: Valine-urea buffer 1 crystals

These were made with the buffer used in final Butyl Sepharose purification. 8 M urea, 0.32 M ammonium sulphate, 0.08 M sodium chloride in 20 mM histidine (pH 6.0) were precipitated with saturated ethanol.

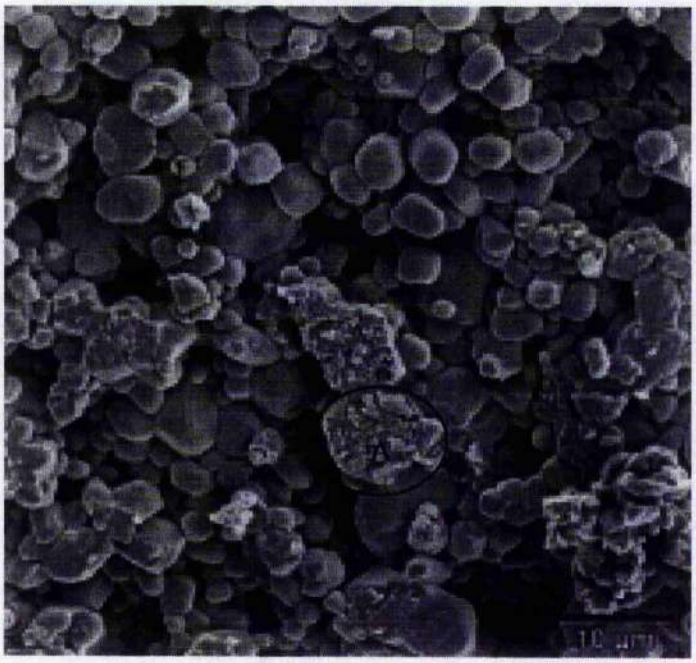
A= Salt

.

1



2



of CyaA has created rectangular, regular crystals. Similarly, crystals samples 6 to 10 made with purified CyaA have a well-defined shape, but are smaller than CyaA-PCMC crystals. The samples however, have an approximately 1-5 μm diameter size, smaller than those prepared from crude CyaA (image 5). The shape and size of crystals derived from CyaA-BSA-CaM-ATP (image 10) seem to be different. They are comprised of small and large particles of different morphology, some pyramidial, fused rods and flat plates. Small, regular in size crystals remain attached to the large crystals.

3: Valine-urea buffer 2 crystals

These were made with DL-valine in 8 M urea, 20 mM histidine. 60 mg of valine was dissolved in 2 ml of de-ionised water and 2 ml of 8 M urea in 20 mM histidine (pH 6.0) and precipitated with saturated ethanol.

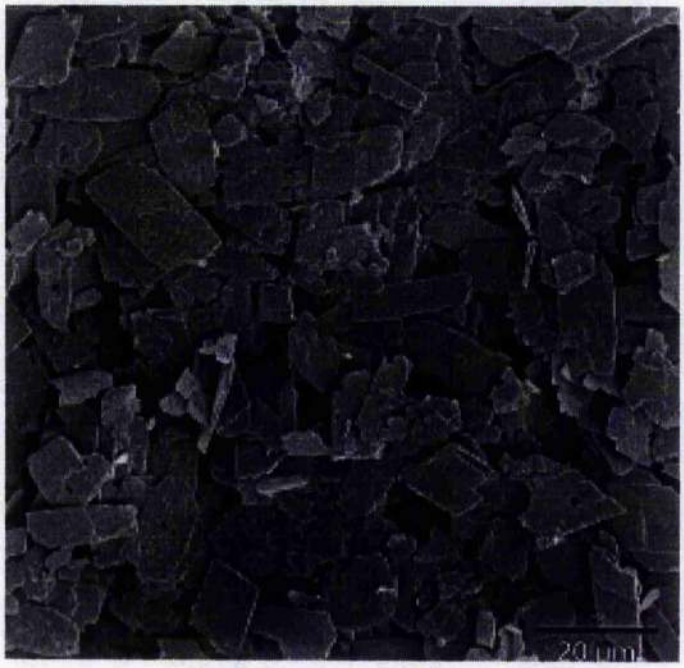
A= Salts

4: Valine-BSA-PCMCs

These were made with DL-valine and BSA. 60 mg of valine was dissolved in 2 ml of de-ionised water together with 1150 μg of BSA and precipitated with saturated ethanol to make valine-BSA-PCMCs

3

4



5: CyaA-PCMCs from crude CyaA

These were made with crude urea extract of CyaA and DL-valine. 60 mg of valine was dissolved in 2 ml of deionised water and then 2 ml of CyaA was added and precipitated with saturated ethanol to make crude CyaA-PCMCs

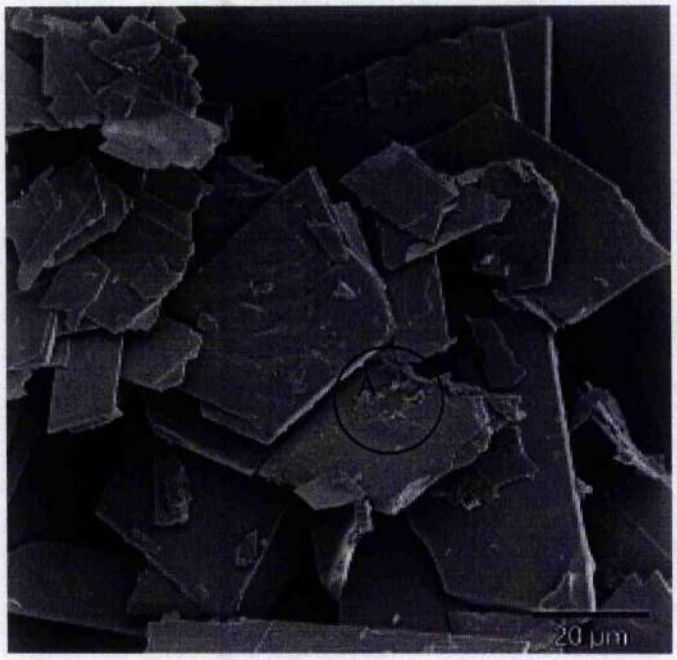
A= Salts

6: CyaA-PCMCs from crude CyaA

These were made with purified CyaA and DL-valine. 60 mg of valine was dissolved in 2 ml of deionised water and then 2 ml of purified CyaA was added and precipitated with saturated ethanol to make a purified CyaA-PCMCs

A= Flat plates

5



6

7: CyaA-CaM- PCMCs from purified CyaA

These were made with purified CyaA, CaM, and DL-valine. 60 mg of valine was dissolved in 2 ml of deionised water, then 2 ml of purified CyaA and 0.2ml CaM (1.8 mg/ml) was added and then precipitated with saturated ethanol to make purified CyaA-PCMCs

Image 8: CyaA-BSA-PCMCs from purified CyaA

These were made with purified CyaA, BSA and DL-valine. 60 mg of valine was dissolved in 2 ml of deionised water, then 2 ml of purified CyaA and BSA(1150 µg) were added and then precipitated with saturated ethanol to make a purified CyaA-BSA-PCMCs

7

Image 9: CyaA-BSA-CaM-PCMCs from purified CyaA

These were made with purified CyaA, CaM, BSA and DL-valine. 60 mg of valine was dissolved in 2 ml of deionised water, then 2 ml of purified CyaA and 0.2ml CaM (1.8 mg/ml) and BSA (1150µg) was added and then precipitated with saturated ethanol to make purified CyaA-M- PCMCs

A= Flat plates
B= All small particles thought to be salts

Image 10: CyaA-BSA-CaM-ATP-PCMCs from purified CyaA

These were made with purified CyaA, BSA and DL-valine. 60 mg of valine was dissolved in 2 ml of deionised water, and then 2 ml of purified CyaA, CaM (1.8 mg/ml) and BSA (1150µg) and ATP (100mM) were added and then precipitated with saturated ethanol to make a purified CyaA-CaM-BSA-ATP-PCMCs

A= Pyramidal

B= Flat plates

C= Fused rods

D= Small and regular in size

9

10

3-12 Humoral response to CyaA-BSA-CaM-PCMCs

To investigate whether CyaA was still immunogenic after formulation as PCMCs, BALB/c mice were immunised with CyaA-CaM-BSA-PCMCs that had been dissolved in PBS pH 7.3. As a control, BSA and CaM were dissolved in PBS at the same concentration as used in the CyaA-BSA-CaM-PCMCs and also used to immunised mice. The mice received two (0.2 ml) doses of CyaA-CaM-BSA-PCMCs (containing 15 μg of CyaA, 67.5 μg BSA and 5 μg CaM per dose) or BSA plus CaM at days 0 and 28 (section 2.18.3). Heart bleeds were taken at 2 weeks after the second dose and sera were pooled for each group. ELISA was used to determine the humoral responses to CyaA, BSA and CaM. Anti-CyaA IgG levels were compared with those of a mouse anti-CyaA hyperimmune serum. This was supplied by Dr. R. Parton.

Figure 3.12.1 shows the results of the ELISA with CyaA as the coating antigen. It can be seen that there was a different dose response for each sample. CyaA-CaM-BSA-PCMC generated a good anti-CyaA IgG response in mice whereas BSA plus CaM which was used as a negative control did not. The highest anti-CyaA IgG response was seen in the control hyperimmune serum.

When BSA was used as the coating antigen for ELISA there were good responses to sera from mice which had received BSA as immunogen (Figure 3.12.2). There was no obvious response seen against CyaA hyperimmune serum.

Little antibody response was seen when mouse anti-CyaA-CaM-BSA-PCMC or anti-BSA-CaM sera were tested against CaM as coating antigen (Figure 3.12.3).

It can be concluded that CyaA-CaM-BSA-PCMCs were able to raise CyaA-specific antibodies when used as an immunogen, indicating that important epitopes were conserved on CyaA in the PCMCs.

Figure 3.12.1 ELISA responses to CyaA in mice immunised with CyaA-CaM-BSA-PCMCs or BSA-CaM

Anti-CyaA IgG levels in sera from mice immunized either with two doses of CyaA-BSA-CaM-PCMCs or BSA plus CaM at days 0 and 28. Terminal bleeds were taken at two weeks after the second immunization. CyaA was use of as the coating antigen for ELISA. The results are the means of two values at each serum dilution (± S.D).

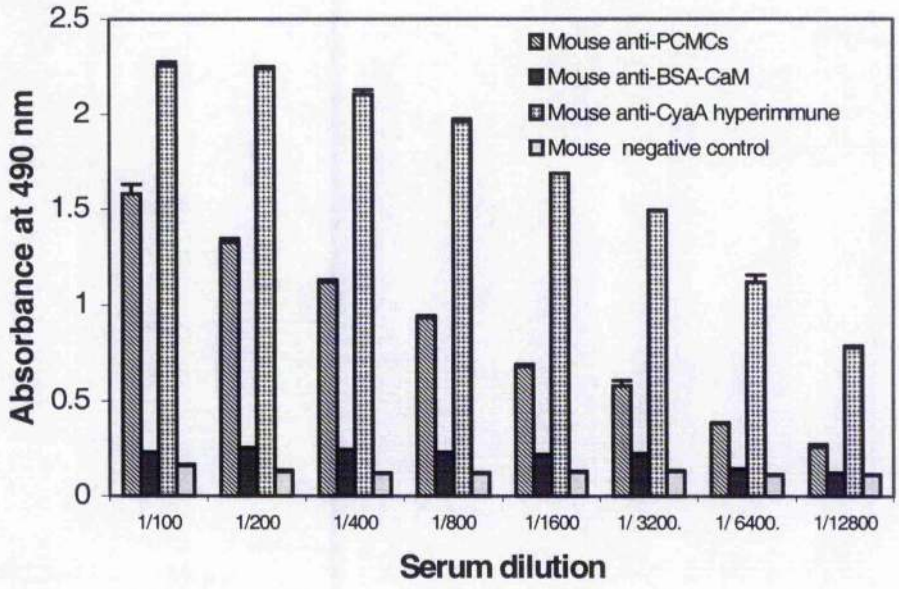

2.5
2
1.5
1
0.5
0
Absorbance at 490 nm
Mouse anti-PCMCs
Mouse anti-BSA-CaM
Mouse anti-CyaA hyperimmune
Mouse negative control
1/100
1/200
1/400
1/800
1/1600
1/ 3200.
1/ 6400.
1/12800
Serum dilution

Figure 3.12.2 ELISA responses to BSA in mice immunised with CyaA-CaM-BSA-PCMCs or BSA-CaM

Anti-BSA IgG levels in sera from mice immunized either with two doses of CyaA-BSA-CaM-PCMCs or BSA plus CaM at days 0 and 28. Terminal bleeds were taken at two weeks after the second immunization. BSA was use of as the coating antigen for ELISA. The results are the means of two values at each serum dilution (± S.D).

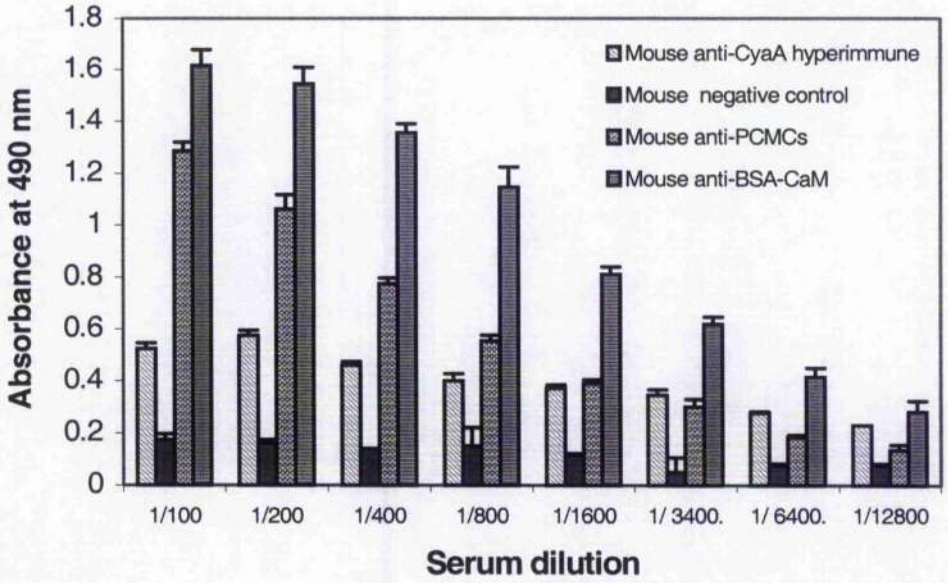
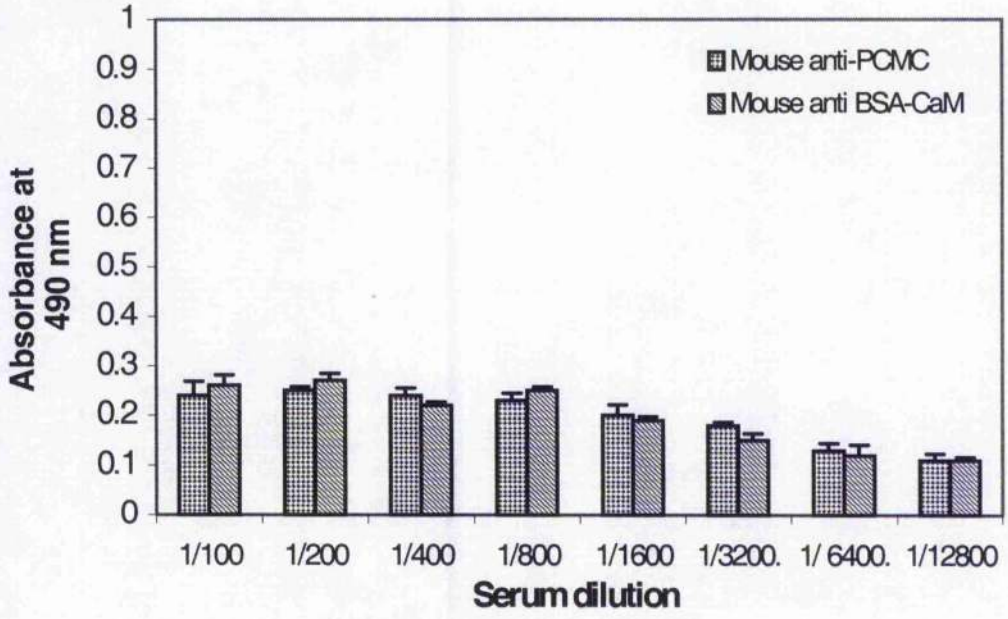

Absorbance at 490 nm
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Mouse anti-CyaA hyperimmune
Mouse negative control
Mouse anti-PCMCs
Mouse anti-BSA-CaM
1/100
1/200
1/400
1/800
1/1600
1/ 3400.
1/ 6400.
1/12800
Serum dilution

Figure 3.12.3 Anti-CaM responses in mice immunised CyaA-CaM-BSA-PCMCs or BSA-CaM

Anti-CaM IgG levels in sera from mice immunized either with two doses of CyaA-BSA-CaM-PCMCs or BSA plus CaM at days 0 and 28. Terminal bleeds were taken at two weeks after the second immunization. CaM was use of as the coating antigen for ELISA. The results are the means of two values at each serum dilution (± S.D).


Mouse anti-PCMC
Mouse anti BSA-CaM
Absorbance at 490 nm
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1/100
1/200
1/400
1/800
1/1600
1/3200.
1/ 6400.
1/12800
Serum dilution

3-13 Effect of mouse anti-CyaA-BSA-CaM-PCMC sera on the AC enzymic and cytotoxic activities of CyaA

The serum samples described in section 3-12 were diluted in PBS or RPMI to investigate any neutralisation effect on AC enzymic and cytotoxicity activities, respectively. A preparation of purified CyaA toxin was diluted in PBS to a final concentration of 60 μg protein/ml. A volume of 50 μl of CyaA and an equal volume of doubling dilutions of sera, 1/4 down to 1/128, were mixed and incubated at 37°C for 1 h. The AC enzymic activity of these samples was then determined by conductimetry assay. The same dilutions of sera and CyaA at concentration of 0.16 μg protein per well (previously shown to kill 85% of J774.2 cells in 2 h as measured by MTT assay) were prepared and incubated as above. A volume of 50 μl of each of these samples was added to 50 μl of J774.2 macrophage-like cell suspension (25000 cells per well) in 96-well plate in duplicate. Cytotoxicity was then evaluated by the MTT dye reduction assay (section 2.13.2) and compared to that of CyaA toxin alone.

The results of the conductimetry assay showed that the serum from mice immunised with CyaA-BSA-CaM-PCMCs, particularly at lower dilutions, was able to inhibit the enzymic activity (Table 3.12.1). The MTT assay on these samples indicated that no cells were killed at lower dilutions (1 in 8), but when CyaA was incubated with sera at higher dilutions, cell killing gradually increased (Table 3.12.2). The results showed that the antibody produced from mice immunised with CyaA-CaM-BSA-PCMCs, was able to neutralise both the AC enzymic and cytotoxic activities of CyaA *in vitro*.

Table 3.12.1 AC enzymic activity of CyaA alone or CyaA with different dilutions of serum from mice immunised with CyaA-BSA-CaM-PCMCs

Sample	AC enzymic activity of serum concentrations (μmol/min/mg protein)						
	Serum dilution						
	Control no serum	1/2	1/4	1/8	1/16	1/32	1/64
CyaA plus Control serum from mice immunised with BSA plus CaM		567	N/D	N/D	N/D	N/D	N/D
CyaA plus serum from mice immunised with CyaA-BSA-CaM-PCMC	618	N/D	32	45	68	90	136

N/D: not done

Table 3.12.2 Cytotoxicity of CyaA alone or CyaA with different dilutions of sera from mice immunised with CyaA-BSA-CaM-PCMCs

Sample	Percentage of killing of J774.2 cells					
	Serum dilutions					
	Control no serum	1/8	1/16	1/32	1/64	1/128
CyaA plus serum from mice immunised with CyaA-BSA-CaM-PCMC	85	0	14	18	21	28

CHAPTER 4

Discussion

4-1 Production of CyaA from recombinant *E. coli* strains

A 200kDa form of CyaA was produced from urea extracts of recombinant *E. coli* BL21 by Sebo *et al* (1991), where the *cyaA* and *cyaC* genes were co-expressed in pCACT3 from the *lac* promoter to produce fully-active CyaA (enzymatically-active and invasive). In the present work it was demonstrated that *E. coli* BL21/DE3 (pGW44 and pGW54) produced CyaA protein that was enzymatically-active and invasive. In our system it was also possible to produce non-acylated, non-invasive but enzymatically-active 200kDa CyaA from a urea extract of *E. coli* BL21/DE3 (pGW44). From *E. coli* BL21/DE3 (pGW44-188) a CyaA pro-toxin can be produced that lacks AC enzymatic activity due to a dipeptide insertion in the ATP binding site at the N-terminal end of the toxin. When pGW44/188 was co-expressed with pGW54, a 200 kDa invasive CyaA with very low AC activity was produced. SDS-PAGE analysis demonstrated the same sized bands for all of these three CyaA forms (Figure 3.1.1).

4-2 Purification of CyaA from recombinant *E. coli* strains

The active and non-active forms of recombinant CyaA were extracted as crude urea extracts from inclusion bodies formed in *E. coli* BL21/DE3 and purified by a combination of two different methods. The aim of purification was to remove contaminating proteins and LPS. Q Sepharose column chromatography was used as an ion exchange method followed by Butyl Sepharose chromatography as a hydrophobic interaction method. Large amounts of CyaA proteins were obtained by these two

methods of purification, with only a few lower molecular weight proteins present. The majority of contaminating proteins and lipopolysaccharide (LPS) were removed through Q Sepharose purification (Figure 3.2.1 and Table 3.1).

Recombinant proteins from *E. coli* BL21/DE3 are frequently contaminated with endotoxin due to the high quantities of LPS in the cell envelope of this gram-negative bacterium. In the present study, crude CyaA and CyaA* toxins were found to be contaminated with some LPS, which was visualised by silver staining (Figure 3.2.3). Using the two different techniques (Q Sepharose and Butyl Sepharose column chromatography) the majority of LPS was removed (Figure 3.2.1 and Table 3.2.2). The endotoxin content of each CyaA preparation is given in Table 3.1.

Ross, P *et al.* (2004) found that dialysis of pure CyaA against Dulbecco's phosphate-buffered saline (PBS), 1mM EDTA and 1 M urea (pH 4.6) and then against Dulbecco's-PBS, 1 mM $CaCl_2$ and 2 M urea (pH 8.0) reduced the amount of contaminating endotoxin in samples to 0.220 μg/mg of protein. According to Petsch *et al.* (1998), chromatography with an endotoxin adsorbant such as polymyxin B was ineffective in reducing endotoxins to an acceptable level. Other studies showed that use of Triton-114 was not suitable for removing LPS (Morrison and Jacobs, 1976; Aida and Pabst, 1990; Petsch *et al.*, 1998). Czuprynski and Welch (1995) demonstrated that 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 a biologically-active moiety.

In the present study, although a complete removal of LPS from CyaA preparations was not possible, a very low amount of LPS contamination (0.012 μg/mg protein) was obtained. As LPS is a very stable molecule at high temperatures (180-250°C), the

destruction of CyaA toxins by heating the CyaA preparations at 100°C would reveal any effect due to endotoxin alone. Such experiments would not eliminate possible synergistic interaction between LPS and CyaA, but adding different amounts of LPS to pure preparations of CyaA could show any such effect. The advantage of using Q Sepharose and Butyl Sepharose purification methods was the capacity to obtain very pure proteins with acceptable LPS levels. The level of LPS contamination was low enough for CyaA to be acceptable as a vaccine component, as the maximum amount allowed is 5 IU per vaccine dose. If a vaccine dose of 25 µg CyaA were to be used, this would contain 3 IU of endotoxin vaccine dose where one IU=100 pg endotoxin (Poole *et al.*, 1997).

4-3 Characterization of purified CyaA preparations

4-3-1 Adenylate cyclase enzymic activity

Lawrence *et al.* (2002) developed a conductimetric assay for determination of adenylate cyclase activity of CyaA. This method offers a large number of practical advantages in comparison with the more usual radiochemical method. The benefits of the new conductimetry method are in terms of cost, ease of managing data and in particular, the procedure is rapid. In this study, the conductimetric assay was used to measure the specific enzymatic activity of purified CyaA preparations and after different treatments, such as PCMC preparation. Both CyaA pro-toxin and CyaA were enzymatically-active, but the CyaA pro-toxin displayed a higher specific activity than the acylated form (Table 3.1.1). These results are similar to the results reported by Westrop *et al.* (1996) and Hormozi *et al.* (1999), who, using the conventional ^{32} αP-ATP assay, found the pro-toxin to have greater enzymatic activity. In the present investigation, the specific activities of CyaA and CyaA-pro toxin were 660 and 880 µmol/min/mg protein

respectively, compared with 626, 674 and 148 and 217 μmol/min/mgprotein reported by Hormozi *et al.* and Westrop *et al* , respectively. These differences may be a reflection of the accuracy of the method used, or measurement of the protein concentrations or differences in the methods of preparation. CyaA* demonstrated very low enzymic activity as expected. The specific activity of 5 μmol/min/mg protein obtained for CyaA* was in agreement with the study of Ladant *et al.* (1992).

4-3-2 Cytotoxicity assays

The acylated CyaA toxin was shown to be cytotoxic against J774.2 mouse macrophage-like cells. The primary biological function of the toxin is presumably to deliver its enzymic activity into the mammalian cells, eventually resulting in their intoxication by the production of cyclic AMP. This occurs at low concentration of toxin, insufficient to cause formation of membrane channels that would cause cell lysis, as the toxin is only weakly cytolytic. CyaA pro-toxin was shown to be much less toxic at the same concentrations as the acylated CyaA but, at higher concentrations, some cytotoxicity was observed. This agrees with previous findings by Rogel *et al.* (1989) who showed that the toxin must be acylated by CyaC modification to present maximum toxicity. The small degree of cytotoxicity displayed by the acylated but non-enzymatic CyaA* form may have been due to a small amount of enzymatic activity remaining or to its pore-forming activity.

In addition to J774.2 mouse macrophage-like cells, different mammalian cells (sheep bone marrow mast cells, RBL-2H3 cells and U937 human monocytes) were treated with different concentrations of CyaA and CyaA* and their effects were compared. The results showed that the effects of the CyaA toxins on J774.2 and the other mammalian cells were different. J774.2 cells were affected by CyaA at very low concentration (50% killing occurred at 0.025 μg protein/ml) but activity against the other cells tested was

much less. The reasons for these differences are not clear, but may be due to the absence of CyaA receptor (CD11b/CD18) on the surface of the other mammalian cells, as CD11b/CD18 is mainly found on the surface of monocytes, neutrophils and dendritic cells (Guermonprez *et al.*, 2001).

It can be concluded from the difference between the cytotoxicity profiles of the CyaA and CyaA* toxin preparations suggests that the cell killing by CyaA is due mainly to the intrinsic AC enzymatic activity of the protein after it has entered into the target cell. Without this activity, only low toxicity was observed. However, it was noted that addition of high concentrations of Ca^{2+} in the assay with CyaA* increased to some extent the cytotoxic activity of this non-enzymatically-active derivative. The Ca^{2+} may encourage a conformation of CyaA* that is more suited to pore formation.

The MTT dye reduction assay and the adenosine triphosphate (ATP) assay were both used to assess the cytotoxic effects of CyaA and CyaA* on different cell lines (J774.2, sheep bone marow mast cells and RBL-2H3 cells). When compared, both assays produced similar results, but the ATP assay apparently detected 50% cell death at lower concentrations of both toxins. There are a number of possible reasons for these results. The MTT assay assumes that the capability of cells to reduce MTT remains constant throughout the cell culture period. Since mitochondria are more robust than the cells that contain them, it is possible for injured cells to continue to reduce MTT (Petty *et al.*, 1995). Several researchers have shown the ATP assay to be a sensitive and reliable method of quantifying viable cell numbers from eukaryotic and prokaryotic populations (Squatrito *et al* 1995; Stollenwerk *et al.*, 1998). Based on these data, it could be concluded that it is a more sensitive assay for the measurement of cytotoxicity by CyaA toxins. However, CyaA will deplete intracellular ATP by formation of cAMP and, as this assay measures ATP, this may give a false indication of the extent of cell killing.

4-3-3 Apoptosis assays

Apoptosis is an active process of cellular suicide that is triggered by a variety of physiological and stress stimuli (Zychlinsky and Sansonetti 1997; Gao and Abu Kwaik, 2000). It is now thought that interference with programmed host cell death (apoptosis) is a common consequence of interaction with bacterial pathogens, as a number of bacterial pathogens appear to be capable of manipulating host cell apoptotic pathways. At least three possible roles for programmed cell death in bacterial diseases have been reported: activation of apoptosis to destroy cells, utilisation of apoptosis to initiate inflammation and inhibition of host cell apoptosis. In addition, host cell apoptosis might represent a host defense reaction (Zychlinsky and Sansonetti, 1997). In the context of virus infection, apoptosis undoubtedly results in the destruction of the viral genome and serves as an innate defence mechanism to reduce virus replication. However, some viruses express anti-apoptotic proteins that efficiently block apoptosis induced by death receptors or in response to stress signalled through mitochondria. Viral interference with host cell apoptosis leads to enhanced viral replication and may promote cancer (Tschopp *et al.*, 1998; Benedict *et al.*, 2003). In the case of pertussis, CyaA induces apoptosis which, expressed early during the course of colonisation, may allow bacteria to survive in the initial stages of the infection. When the bacteria have successfully colonised the tissue *Bordetella* ceases to produce the toxin (Khelef *et al.*, 1993). *B. pertussis* is able to kill macrophages through the induction of apoptosis and CyaA expression is essential for the bacterium to promote cell death. It has been proposed that apoptosis by CyaA is induced by the increase in cAMP production, which has been shown to be responsible for changes in protein phosphorylation (Duprez ***et al.***, 1993; Khelef *et al.*, 1993) but the mechanism for this has not yet been elucidated. As McConkey and Orrenius (1994) noted, apoptosis could also be the consequence of the depletion of ATP due to the high AC enzymic activity deregulating the protein cascade.

Khelef and Guiso (1995) suggested that apoptosis was not induced by the pore-forming properties of CyaA. The reason for their suggestion was that a mutant CyaA that lacks AC activity but retained haemolytic activity was not lethal for murine macrophages. However, the haemolytic property of CyaA may also be responsible for apoptosis, since pore formation can result in changes in intracellular calcium levels, which in turn can activate programmed cell death (McConkey and Orrenius 1994).

In this study, the effect of CyaA and CyaA* were investigated by two related assays, induction of caspase 3/7 activity and DNA fragmentation, as measures of apoptosis. Interestingly, among those cells treated with enzymatically-active, acylated CyaA, J774.2 macrophage-like cells and RBL-2H3 cells showed the greatest caspase induction whereas no obvious induction of caspase activity was observed in mast cells and Vero cells. As shown in Figs 3.5.5.1 and 3.5.5.3 none of these cells were induced by CyaA* to present any caspase activity. It should be noted that urea was tested at the concentration used in the CyaA preparations and showed no ability to induce caspase activity. Three possibilities for the lack of activity of CyaA on mast cells and Vero cells could be; 1) the lack of CyaA receptors on the surface of these cells; 2) the incubation time of CyaA with these cells (6 h) may not have been sufficient to induce the process; 3) these cells may not produce caspase 3/7.

In contrast, the results obtained with the DNA fragmentation (apoptosis) assay showed that the CyaA induced programmed cell death in J774.2 macrophage-like cells, mast cells and RBL-2H3 cells. Thus, in this assay mast cells showed clear evidence of apoptosis after 12 h treatment with CyaA. However, although a clear laddering effect was seen in mast cells and RBL-2H3 cells, only a smear of DNA fragments was seen in J774.2 cells. One possibility for the differences seen in caspase induction and DNA laddering with J774.2 macrophage-like cells could be related to differences in the

incubation time. During prolonged incubation, cell lysis or necrosis may set in with J774.2 cells as suggested by the decline in caspase activity observed with higher concentrations of CyaA (Figure 3.5.5.1). It was demonstrated by Khelef and Guiso (1995) that DNA fragmentation was induced by CyaA in J774.2 macrophage-like cells and that a mutant CyaA lacking AC activity but retaining haemolytic activity did not induce this feature of apoptosis. No apoptosis effect was seen on J774.2 macrophage-like cells, sheep bone marrow mast cells, RBL-2H3 cells and Vero cells in the present study when they were treated with CyaA*. Khelef *et al*, (1993) reported that purified CyaA was the only *B. pertussis* virulence factor able to induce macrophage death through apoptosis. They also demonstrated that mutant or non-enzymatically-active CyaA was ineffective in promoting programmed cell death, indicating that the AC activity of CyaA was required for its action. These results and the data presented here also suggested that DNA fragmentation or induction of caspase 3/7 activity is due to adenylate cyclase enzymic activity provided by CyaA. It can be concluded that cells infected with CyaA show a decrease in intracellular ATP, and a rise in cAMP, which may contribute to mitochondrial dysfunction and result in apoptosis.

4-3-4 Inhibition of phagocytosis

Phagocytosis provides a specialised mechanism for regulated ingestion and intracellular destruction of microbial pathogens as well as of apoptotic host cells and debris. In general, professional phagocytes, including neutrophils (which migrate from the blood to a site of infection) and macrophages (which constitutively reside in tissues and are less motile than neutrophils), accomplish most phagocytosis. By confining the mechanisms of microbial killing and digestion to distinct intracellular compartments (lysozomes) of these cells, damage to host cells and tissues is minimised during the process of killing offending microbes. In addition to disposing of microbial pathogens,

phagocytosis (especially by macrophages and dendritic cells) initiates the process of antigen processing and presentation for development of adaptive immune responses (Ramachandra *et al.*, 1999).

In order to better understand the importance of this defence mechanism, a flow cytometry assay was developed to study phagocytosis and oxidative burst of neutrophils stimulated by bacteria (Lehmann *et al.*, 2000). A flow cytometry investigation was made in order to choose appropriate target cells, opsonisation conditions, and fluorochromes. These authors used FITC-*E. coli* for phagocytosis experiments and the extracellular fluorescence was quenched by trypan blue to differentiate it from that due to intracellular bacteria.

Host defence against *B. pertussis* relies to some extent upon phagocytosis and killing of the bacterial cells. The adenylate cyclase toxin appears to be an important virulence factor against phagocytosis, as evidenced by the efficient phagocytosis of mutants that fail to express this toxin (Weingart and Weiss, 2000). These authors also showed that CyaA, added separately, blocks phagocytosis even when bacteria are not expressing the toxin.

In this study, different concentrations of CyaA and CyaA* were used to evaluate their modulatory effects on phagocytosis. The results of the ingestion activity experiment suggested that CyaA inhibits the phagocytosis of *E. coli* by J774.2 macrophage-like cells and by human granulocytes as it caused a decrease in the mean fluorescent intensity (MFI) measured by flow cytometry (Figures 3.8.5 and 3.8.6). Although the concentration of CyaA used in the ssay caused appreciable cell killing of J774.2 cells as determined by the MTT assay, it should be emphasised that the effect of CyaA on phagocytosis was measured only in viable cells, by the flow cytometric method here. In contrast, no significant decrease of phagocytosis activity was noted in response to

treatment of J774.2 macrophage-like cells or human granulocytes with various concentrations of CyaA*. The poor AC enzymic activity of CyaA* is presumably responsible for the lack of effect on phagocytosis activity in these cells, when compared with CyaA.

An additional experiment was performed under the same conditions on the U937 human monoblastic cell line in the absence or in the presence of PMA. PMA is used as a stimulator to differentiate this cell-line into monocyte-like cells. The effect of PMA shown in this study was that it potentiated the phagocytosis activity of U937 cells. However, the cytotoxic effect of CyaA was greater on PMA-differentiated U937 cells than on undifferentiated cells (see Fig 3.6.2.3). Thus, it can be suggested that PMA at 0.020μg at final concentration could stimulate the immature cells to differentiate, which CyaA could, then affect. Further investigation is recommended to show how differentiation of U937 cells might influence their susceptibility to CyaA, for example by increasing the number of receptors for the toxin.

It is very clear that phagocytic functions, such as migration, ingestion and release of reactive oxygen intermediates (ROI), which appear to be responsible for the killing activity, are important host defence mechanisms against *Bordetella*. Evasion of these functions may be the principal tools by which the bacteria can persist in the sites of colonization and proliferate further. One obvious strategy in defence against phagocytosis is direct attack by the bacteria upon the professional phagocytes using extracellular enzymes or toxins that kill or at least block the activity of phagocytes. Extracellular proteins that inhibit phagocytosis include the exotoxin A of *Pseudomonas aeruginosa*, which kills macrophages, and the bacterial exotoxins that are adenylate cyclases e.g. anthrax toxin EF and *B. pertussis* CyaA which decrease phagocytic activity. Thus, the reduction of ingestion and oxygen-dependent killing functions is a

predominant activity *of B. pertussis* CyaA. However, the results of this study show that the full activity of CyaA is necessary for phagocytic inhibitory activity as CyaA* had little effect on phagocytosis. These results are keeping with the results reported by Galgiani *et al.* (1988). They showed that an adenylate cyclase toxin-containing extract blocked neutrophil-mediated inhibition of *N*-acetylglucosamine incorporation by arthroconidia of *Coccioides immitis* in a dose-dependent manner. The authors indicated that CyaA, rather than other virulence factors of *B. pertussis*, was responsible for the inhibitory effects on PMN. In the present study, the effect of CyaA preparations were examined and the interactions of human granulocytes and J774.2 mouse phagocyte-like cells with *E. coli in vitro* were compared. The results suggest that CyaA impairs the functions of human granulocytes and J774.2 cells phagocytosis in a dose dependent manner and that this activity is dependent on the AC activity of the toxin.

4-3-5 Inhibition of oxidative burst

The first line of defence against microbial infection is provided by neutrophils. Upon phagocytosis of bacteria, a respiratory burst occurs, initially reducing oxygen to form superoxide, with secondary formation of H_2O_2, OH^- and other oxygen-derived molecules that participate in bacterial killing (Uhlinger *et al.*, 1993). An early report showed that culture medium and bacterial extract from *B. pertussis*, containing adenylate cyclase of high specific activity, were able to inhibit the chemiluminescence (CL) response of human alveolar macrophages and neutrophils to zymosan (Confer and Eaton, 1982). Others have reported the role of CyaA in the inhibition of this response. CyaA toxin produces high levels of intracellular cAMP in human monocytes and inhibits the oxidative response to a variety of particulate or soluble stimuli (Pearson *et al.*, 1987). Other researchers demonstrated that *B. pertussis* can survive intracellularly within various leukocyte cell types, including human macrophages and

polymorphonuclear cells (Saukkonen ***et al.***, 1991; Steed *et al.*, 1991). It is possible that the inhibition of oxidative burst in these cells would benefit intracellular survival of the bacteria and may be an important evasion strategy for *B. pertussis*.

The results of oxidative burst experiments in the current study suggested that CyaA inhibits the oxidative burst of J774.2 macrophage-like cells and human granulocytes as it induced a decrease in mean fluorescent intensity (MFI) as measured by flow cytometry. Although the concentration of CyaA used in the ssay caused appreciable cell killing of J774.2 cells as determined by the MTT assay, it should be emphasised that the effect of CyaA on oxidative burst was measured only in viable cells, by the flow cytometric method here In contrast, CyaA* did not appear to inhibit the oxidative burst when it was compared with CyaA at the same concentrations. The results indicated that the AC enzymic activity of CyaA toxin was necessary for the inhibition of oxidative burst in these cells. This result is in line with Steed *et al.* (1991) who indicated that CyaA inhibited (the phagocyte) respiratory burst activity in human PMNL.

A similar experiment, preformed on U937 human monocytes in the presence and in the absence of PMA, showed that CyaA inhibited the oxidative burst by approximately 40% when tested at 0.1 µg/ml final concentration on PMA-activated monocytes, but was ineffective on non-differentiated cells. Thus, PMA could stimulate the immature cells to differentiate, which CyaA then affected. These data suggest that the activity of CyaA on these cells is best seen when they have differentiated, possibly to express high level of receptor for CyaA.

Interestingly, it has been demonstrated that the leukotoxin of *Mannheimia haemolytica,* a RTX toxin with structural homology to CyaA, but without an adenylate cyclase domain, can stimulate the respiratory burst activity of neutrophils (Maheswaran *et al.,* 1992). On the other hand, pertussis toxin (PT), another virulence factor of *Bordetella*

pertussis that, like CyaA, also increases intracellular cAMP levels, could inhibit the respiratory burst activity of human neutrophils (Burnette, 1993). This suggests that an increase in cAMP levels might be responsible for the inhibition of respiratory burst activity by CyaA, which is borne out by the lack of activity exhibited by CyaA*.

4-4 CyaA protein- coated microcrystals (PCMCs)

PCMCs represent a novel method for the formulation of a wide range of biomolecules, including proteins, peptides, DNA, and vaccines. PCMCs consist of a core crystalline material, such as a sugar, amino acid or salt on which the biomolecules are immobilised (Kreiner *et al.,* 2001). The preparation of PCMCs is straightforward and applicable in any laboratory. The process involves dissolution of the appropriate crystal-forming carrier together with the given biomolecule in aqueous solution. Immediately, dehydration of the two components is facilitated by the addition of the aqueous solution to a water-miscible organic solvent, resulting in the rapid formation of the PCMCs with the biomolecule immobilised on the surface of the crystalline core carrier (via a crystal-lattice mediated self-assembly process). These PCMCs may be stored as a suspension or filter-dried to form a free-flowing powder (Ross *et al.*, 2002).

CyaA is a toxin and would not be used as vaccine component. However, there is a functional assay for the detoxified form CyaA* which is the antigen form that would possibly be used in a vaccine. The reason for using CyaA in the present study was that available functionalassays e.g. AC activity and ctototoxic activity could be used as tools to examine its activity and stability before and after formolation as PCMCs.The aim of current work was to prepare CyaA-coated microcrystals free of urea and to improve stability of CyaA with a view to preparing a novel vaccine formulation. This would provide, on redissolving in an appropriate aqueous solution, a sample of CyaA, in the absence of urea, as a model antigen to be used *in vitro* and *in vivo*. Purified CyaA

preparations are routinely stored in 8 M urea because, in aqueous solution at high concentration, CyaA tends to aggregate due to the hydrophobic nature of the protein. The crystalline core carrier material DL-valine was chosen with CyaA at an initial concentration of 300 μg protein/ml added to a saturated solution of 60 mg/ml DL-valine. This aqueous solution was slowly added into 40 ml of valine-saturated ethanol solution under vigorous stirring. This resulted in the formation of CyaA-protein coated microcrystals. The solvent was then removed from the crystals using a Millipore filtration system (Millipore Ltd., UK) to yield upon drying, a free-flowing crystalline powder. Control samples of valine-PCMCs or other PCMCs without the addition of CyaA were also produced by this method to determine the effect of CyaA inclusion on crystal size and morphology. The yield of CyaA-PCMC crystals recovered varied (50-90%) from the theoretical amount. The actual loading of protein on the PCMC was determined by Bradford assay. The recovered yield and actual protein loading of CyaA onto the DL-valine crystals could probably be improved by optimisation of the formulation strategy. The CyaA-PCMCs properties were then determined by dissolving PCMCs in 8 M urea buffer or other buffers to assess activities by conductimetry and cytotoxicity assay.

Scanning electron microscopy (SEM) of CyaA-PCMCs and DL-valine PCMCs, revealed that immobilisation of CyaA onto the valine crystal surface generally reduced the overall crystal size (on average to less than 10 μm), as well as the relative dimensions of the crystal faces particularly if CyaA was used in association with other proteins such as BSA or CaM. In addition, the crystal shape was less clearly defined due to the immobilisation of CyaA. It was clear, however, that this process could be used to simultaneously dehydrate and immobilise CyaA onto the surface of water-soluble microcrystals. However, there were differences in AC enzymic activity recovery in different buffers and different types of CyaA-PCMC preparations. Improvements to the

process were designed to obtain a good cytotoxicity and AC activity, suggesting that the antigenicity of the CyaA molecule would be preserved and would be suitable for vaccine applications.

4-4-1 Formulation and stability of CyaA as different types of PCMCs

Different CyaA-PCMCs (prepared from crude and pure CyaA) were made in various conditions e.g from 2M urea or from 8M urea for crude CyaA and with different ratios of CaM, BSA, $CaCl_2$, and ATP for pure CyaA. Then, AC enzymic activity, cytotoxicity and the stability of PCMCs were determined. The stability of CyaA-PCMCs was evaluated at different temperatures after two weeks. As shown in Table 3.11.2.1.2, CyaA-PCMCs retained high AC activities when they were stored at -20°C, 4°C, 20°C, or 37°C, indicating that the CyaA-PCMCs are stable at ambient temperatures. This feature would have useful applications for vaccine formulations to be used in developing countries where cold storage may prove difficult.

4-4-2 AC enzymic and cytotoxicity activities of different types of PCMCs

It was shown that all of the CyaA-PCMC preparations tested had both cytotoxic and enzymatic activities when crystals made from crude or pure CyaA were dissolved in 2-8M urea. This is evidence that the process used to make the PCMCs was working well to preserve the toxin activities. The problem that was encountered early on was that the the CyaA could not be recoverd when crystals were dissolved in aqueous buffer (20 mM histidine alone). A urea concentration more than 1 M was required for the complete dissolution of the crystals and recovery of CyaA, which indicated that further investigation into methods of solubilising the PCMCs was required. Purified CyaA toxin was used for these experiments. A large range of different detergents (non-ionic and zwitterionic) was used in attempts to solubilise the CyaA-PCMCs, but little AC

activity was detected. At this stage in the investigation, urea was still the best reagent for solubilisation of CyaA-PCMC preparations.

Following the encouraging results on CyaA-PCMCs allowing for recovery of CyaA activities when PCMCs were dissolved in 2-5 M urea buffer, further investigation into methods of CyaA-PCMCs preparation were carried out. A range of compounds (BSA, CaM, $CaCl_2$ and ATP) which might act as chaperones to prevent aggregation of CyaA, either on the PCMCs as they formed or upon resolubilation, were selected. CaM and BSA were added with CyaA to DL-valine to prepare different types of CyaA-PCMCs for testing the CyaA properties in different aqueous buffers and at various pH values. BSA had been used previously as a stabiliser for CyaA. Bellalou *et al.* (1990) reported that when *B. pertussis* was grown in a medium containing high levels of BSA, both the adenylate cyclase and haemolysin functions were maintained in the toxin whereas, without these additions to the growth medium, the CyaA toxin was cleaved to produce a 45 kDa protein with AC activity only. Calmodulin is a key calcium ion sensor and versatile intracellular second messenger that can interact with targets via N- and C-terminal domains. Calcium binding to CaM induces conformational changes, from a mainly hydrophobic closed state to an open conformation, exposing a large hydrophobic binding pocket (Bhattacharya *et al.*, 2004). Previous work showed that the AC enzymic activity of CyaA is stimulated up to 1000-fold by binding of CaM to the AC domain. ATP and Ca^{2+} also bind to CyaA. The results obtained in this study showed that incorporating BSA with CyaA in the PCMCs allowed retention of AC enzymic and cytotoxic activities, particularly when combined with CaM, when the PCMCs were subsequently dissolved in aqueous buffer (Table 3.11.2.2.2). The AC enzymic activity in histidine buffer was stable for at least 7 days (Table 3.11.2.2.5). The data indicated that incorporating CaM into PCMC increased AC enzymic activity of CyaA but not its

cytotoxicity activity, upon resolubilisation. It has been reported previously that CaM interfered with the invasive properties of CyaA (Hervekr and Ladant, 1997). The results showed that incorporation of ATP and Ca^{2+} had no obvious effect on the properties of CyaA-PCMCs.

Based on work carried out in this study it can be concluded that encouraging results have been obtained. The process by which the PCMCs are made has proven to be successful in coating the CyaA onto the valine crystals and, in addition, it seems as though most of the urea is removed when the CyaA coats the crystals. This is necessary if the PCMC preparations are to be used as a vaccine and hence the fact that there seems to be very little urea present in PCMC preparations is encouraging. Further work needs to be conducted with a range of CyaA-PCMCs, prepared with other components such as the inclusion of vaccine adjuvants, and a more detailed parallel investigation should be made into their immunological properties so as to provide a deeper understanding of this promising technique.

4-5 Humoral responses to CyaA-CaM-BSA-PCMCs

The aim of this part of work was to show that CyaA, after incorporation into PCMCs, was still immunogenic. Accordingly, BALB/c were immunised with CyaA-CaM-BSA-PCMCs or BSA plus CaM. Purified CyaA was used which showed in SDS-PAGE as a 200-kDa protein with a high level of AC enzymic and cytotoxicity activities. Immunisation with two doses of CyaA-CaM-BSA-PCMCs gave rise to high anti-CyaA IgG titres (Figure 3.13.1). These results are consistent with previous studies presented by Hormozi *et al.* (1999) and MacDonald-Fyall *et al.* (2004) who reported that CyaA was highly immunogenic in mice and was able to stimulate serum anti-CyaA IgG antibody responses. The serum from PCMC-immunised mice was also able to neutralise

the enzymic and cytotoxic activities of CyaA, which indicated that the structural integrity of CyaA was retained in the PCMCs such that the antibodies that were produced recognised the native toxin. A similar study was done by Murdan *et al.* (2005) with diphtheria toxoid (DT) as a model antigen. DT was used to make DT-coated microcrystals with L-glutamine and the PCMCs were then dissolved in buffer and administered intramuscularly to mice following by a booster dose of the same material after four weeks. The DT-specific antibody in serum on day 42 was determined by ELISA, which showed a strong IgG response. However, neutralisation studies of toxin activity were not performed.

PCMCs offer a number of advantages for the preparation of biomolecules for vaccine or drug delivery as the process is straightforward to apply and does not require specialist laboratory equipment and, importanly, particles show good stability towards stress conditions of high temperature and humidity. The overall conclusion from the present study was that PCMCs of CyaA co-precipitated with CaM and BSA could be used for immunisation to induce specific antibody responses to the toxin. Further work needs to be conducted with the inclusion of vaccine adjuvants and more detailed investigation onto immunological responses after storage of PCMCs at ambient temperature. In addition, time did not allow an assessment as to whether immunisation of mice with CyaA-PCMCs allowed protection against challenge with virulent *B. pertussis*.

Appendix A: Media composition

A.1 Luria Bertani agar (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 Luria Bertani broth (LB)

Tryptone (BDH Laboratory supplies)	10 g
Yeast extract (BDH)	5 g
sodium chloride	
Made up to 1 litre in distilled water	10 g

A.3 Competence solution

$CaCl_2$	1.46 g
$MnCl_2$	1.384 g
sodium acetate	0.756 g

Made up to 100 ml in distilled water

A.4 TEB solution

Tris-Base	54 g
boric acid	27.5 g
EDTA (0.5M)	20 ml

Made up to 1000 ml in distilled water

Appendix B: CyaA expression and purification buffers

B.1 Histidine buffer

histidine (20 mM)	0.0314 g

Made up to 100 ml in distilled water and adjusted to pH 6.0

B.2 Urea in histidine buffer (Equilibration buffer)

urea (8 M)	48 g
histidine (20 mM)	0.0314g

Made up to 100 ml in distilled water and adjusted to pH 6.0

B.3 Washing buffer

urea (2 M)	12 g
histidine	0.0314 g
sodium chloride (0.3 M)	1.75 g
ammonium sulphate (0.8 M)	10.57 g

Made up to 100 ml in distilled water and adjusted to pH 6.0

B.4 Eluting buffer (A)

urea (8 M)	48 g
sodium chloride	0.584 g
histidine (20 mM).	0.0314 g

Made up to 100 ml in distilled water and adjusted to pH 6.0

B.4 Eluting buffer (B)

urea (8 M)	48 g
sodium chloride	1.168 g
histidine (20 mM).	0.0314 g

Made up to 100 ml in distilled water and adjusted to pH 6.0

B.4 Eluting buffer (C)

urea (8 M)	48 g
sodium chloride	1.75 g
histidine (20 mM).	0.0314 g

Made up to 100 ml in distilled water and adjusted to pH 6.0

B.5 Dilution solution

histidine (20 mM)	0.0314

ammonium sulphate (4 M)	52.9 g

Made up to 100 ml in distilled water and adjusted to pH 6.0

B.6 Washing buffer (D)

urea (8 M)	48 g
histidine (20 mM)	0.0314
ammonium sulphate (0.8 M)	10.57 g
sodium chloride (0.3 M)	1.75 g

Made up to 100 ml in distilled water and adjusted to pH 6.0

B.7 Washing buffer (E)

urea (8 M)	48 g
ammonium sulphate (0.8 M)	10.57 g
sodium chloride (0.2 M)	1.168 g
histidine (20 mM)	0.0314 g

Made up to 100 ml in distilled water and adjusted to pH 6.0

B.8 Washing buffer (F)

urea (2 M)	12 g
ammonium sulphate (0.6 M)	7.92 g
histidine (20 mM)	0.0314 g

Made up to 100 ml in distilled water and adjusted to pH 6.0

B.9 Eluting buffer

urea (8 M)	48 g
ammonium sulphate (0.32 M)	4.3 g
sodium chloride (0.08 M)	0.446 g
histidine (20 mM).	0.0314 g

Made up to 100 ml in distilled water and adjusted to pH 6.0

B.10 PCMCs buffer for immunisation

$Na_2 H PO_4$ (1 M)	57.7 ml
$Na H_2 PO_4$ (1 M)	42.3 ml

Dilute the combined stock solution to 1000 ml with distilled water adjusted to pH 7.3

Appendix C: Protein analysis solutions

C.1 Separating gel (7.5%)

acrylamide/bis solution	2.5 ml
dH_2O	3.8 ml
Tris-HCl (1.5 M, pH 8)	3.75 ml
20% (w/v) SDS	100 μl
TEMED	10 μl
10% (w/v) ammonium persulphate (APS)	100 μl

C.2 Separating gel (15%)

acrylamide/bis solution	5 ml
dH_2O	1.3 ml
Tris-HCl (1.5 M, pH 8)	7.6 ml
20% (w/v) SDS	100 μl
TEMED	10 μl
10% (w/v) Ammonium persulphate (APS)	100 μl

C.3 Stacking gel

acrylamide/Bis solution	157 μl
dH_2O	714 μl
Tris-HCl (0.5 M, pH 6.8)	300 μl
20% (w/v) SDS	11.9 μl
TEMED	1.18 μl
10% (w/v) Ammonium persulphate (APS)	6 μl

C.4 Protein loading buffer

glycerol	5 ml
20% (w/v) SDS	2.5 ml

2-mercaptoethanol	0.5 ml
Tris (0.5 M, pH 6.8)	2.5 ml
bromophenol blue	0.25% (w/v)

C.5 Coomassie gel stain

coomassie blue	0.5 g
methanol	50 ml
acetic acid	100 ml
Made up to 1000 ml in distilled water	

C.6 Destain buffer

methanol	50 ml
acetic acid	100 ml
Made up to 1000 ml in distilled water	

Appendix D: Silver stain buffer

D.1 Fixing buffer (40% ethanol-5% acetic acid)

absolute ethanol	400 ml
acetic acid	50 ml
Made up to 1000 ml in distilled water	

D.2 Staining buffer

sodium hydroxide (0.1M)	28 ml
ammonium hydroxide	2 ml
silver nitrate 20% (w/v) e.g. (1 g silver nitrate in 5 ml of water)	5 ml
Distilled water	115 ml

D.3 Developing buffer

formaldehyde (37%)	0.5 ml
citric acid	2ml
Distilled water	200 ml

Appendix E: Western blotting solution

E.1 Electroblotting buffer

Tris base	7.2 g
glycine	33.4 g
dH_2O	2000 ml
methanol	600 ml

Make up to 3000 ml with dH_2O and adjusted to pH to 8.0

E.2 Blocking buffer

Marvel skimmed milk	6 g
Tween 20	400 μl
dH_2O	200 ml

E.3 DAB solution

3-3' diaminobenzidine (DAB, Sigma))	0.05 g
PBS	98 ml
CoCl2 (1%) w/v	2 ml
H_2O_2 (30%)	100 μl

Appendix F: CyaA enzymic assay

F.1 Bicine buffer

Bicine	10 mM
magnesium acetate	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 and adjusted to pH 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 (Gibco)	10%

G.4. Acetate Buffer (X 10)

sodium acetate	68.04 g
glacial acetic acid	1632 μl

Made up to 1 litre in distilled water

Appendix H: Detergent Screen 1 Formulation-Hampton Research

Reagent number	Detergent	MW	Type
1	C12E9	583.10	N
2	C12E8	539.10	N
3	n-Dodecyl-ß-D-maltoside	510.60	N
4	Sucrose monolaurate	524.60	N
5	CYMAL®-6	508.50	N
6	TRITON® X-100	631.00	N
7	CTAB	364.50	I
8	Deoxy BigChap	862.10	N
9	n-Decyl-ß-D-maltoside	482.60	N
10	LDAO	229.40	N
11	CYMAL®-5	494.50	N
12	ZWITTERGENT® 3-12	335.60	Z
13	Nonyl-ß-D-glucoside	306.40	N
14	1 -s-Octyl-ß-D-thioglucoside	308.40	N
15	DDAO	201.40	N
16	HECAMEG	335.40	N
17	n-Octanoylsucrose	468.50	N
18	Heptyl-ß-D-thioglucoside	274.30	N
19	n-Octyl-ß-D-glucoside	292.40	N
20	CYMAL®-3	466.50	N
21	C-HEGA-10	377.50	N
22	ZWITTERGENT® 3-10	307.60	Z
23	MEGA-8	321.40	N
24	n-Hexyl-ß-D-glucoside	264.30	N

N=Non Ionic

Z= Zwitterionic

References:

Abbas, A. K., Lichtman, A. H. and Pober, J. S (2000). Cellular and Molecular Immunology. Philadelphia: Saunders.

AD HOC group for the study of *pertussis* vaccine. (1988). Placebo-controlled trial of two acellular vaccines in Sweden, protective efficacy and adverse events. Lancet. **1,** 955-960.

Ahuja, N., Kumar, P. and Bhatnagar, R. (2004). The adenylate cyclase toxins. Crit. Rev. Microbiol. 30, 187-196.

Aida, Y. and Pabst, M.J. (1990). Removal of endotoxin from protein solutions by phaseseparationusingTritonX-114. J. Immunol. Methods. **132,** 191-1955

Akerley, B.J. and Miller, J.F. (1993) Flagellin gene transcription in *Bordetella bronchiseptica* is regulated by the BvgAS virulence control system. J. Bacteriol. **175,** 3468-3479.

Allen, A.G., Thomas, R.M., Cadisch, J.T. and Maskell, D.T. (1998). Molecular and functional analysis of the lipopolysaccharide biosynthesis locus wlb from *Bordetella pertussis, Bordetella parapertussis* and *Bordetella bronchiseptica.*Mol. Microbiol. **29,** 27-38.

Alonso, S., Pethe, K., Meilcarek, N., Raze, D. and Locht, C. (2001) Role of ADP-ribosyltransferase activity of pertussis toxin in toxin-adhesin redundancy with filamentous hemagglutinin during *Bordetella pertussis* infection. Infect. Immun. **69,** 6038-6043.

Amano, K., Fukushi, K. and Watanabe, M. (1990). Biochemical and immunological comparison of lipopolysaccharide from *Bordetella* species. J. Gen. Microbiol. **136,** 481-487.

Amsbaugh, D. F., Li, Z. M. and Shahin, R. D. (1993). Long-lived respiratory immune response to filamentous haemagglutinin following *Bordetella pertussis* infection. Infect. Immun. **61,** 1447-52.

Andrews, R., Herceg, A. and Roberts, C. (1997). *Pertussis* notifications in Australia. 1991 to 1997. Commun. Dis. Intell. **29,** 145-149.

Aoyama, T., Sunakowa, K., Iwata, S., Takeuchi, Y. and Fugii, R. (1996). Efficacy of short-term treatment of pertussis with clarithromycin and azithromycin. J.Pediatr. **129,** 761-764.

Arciniega, J.L,. Hewlett, E.L., Jonson, F.D., Deforest, A., Wassilak, S., Onorato, I.M., Manclark, C.R and Burns, D.L. (1991). Human serological response to envelope associated protein and adenylate cyclase toxin of *Bordetella pertussis.* J. Infect. Dis. **163,** 135-142.

Aseeva, L. E., Shevchenko, L. A., Shimaniuk, N. I., Rubler, B. D. and Mishankin, B. N. (1987).. Assessment of the modulating effect of adenylate cyclase of the plague

microbe on guina pig peritoneal leukocytes with the aid of chemiluminescence. Microb. Epidemiol. Immunobiol. 7, 59-63.

Bagley, K.C., Abdelwahab, S.F., Tuskan, R.G., Fouts, T.R. and Lewis, G.K. (2002). Pertussis toxin and the adenylate cyclase toxin from *Bordetella pertussis* activate human monocyte-derived dendritic cells and dominantly inhibit cytokine production through a cAMP-dependent pathway. J. Leukoc. Biol. **72**, 962-969.

Bamberger, E., Lahat, N., Gershtein, R., Benilevi, D., Shapiro, S . Kassis, I., Rubin, I., and Srugo, I. (2005). Diagnosing pertussis: the role of polymerase chain reaction. Isr. Med. Assoc. J. **7**, 351-354.

Barnard A., Mahon B.P., Watkins J., Redhead K.,Mills K.H.G. (1996). Th1/Th2 cell dichotomy in acquired immunity o *Bordetella pertussis*: variables in the *in vivo* priming and *in vitro* cytokine detection techniques affect the classification of T-cell subsets as Th1, Th2 or Th0, Immunol. **87,** 372–380.

Bassinet, L., Fitting, C., Housset, B., Gounon, P,. Cavvaillon, Jean-Marc and Guiso, N. (2004). *Bordetella pertussis* adenylate cyclase-hemolysin induces interkeukin-6 secretion by human tracheal epithelial cells. Infect. Immun. **Sep**, 5530-5533.

Bassinet, L., Gueirard, P., Maitre, B., Housset, B., Gounon, P. and Guiso, N. (2000). Role of adhesins and toxins in invasion of human tracheal epithelial cells by *Bordetella pertussis*. Infect. Immun. **68,** 1934-1941.

Bauer, M.E. and Welch, R.A. (1996). Characterization of an RTX toxin from enterohemorrhagic *Escherichia coli* O157:H7. Infect Immun. **64,** 167-75.

Bellalou, J., Ladant, D. and Sakamoto, H. (1990). Synthesis and secretion of *Bordetella pertussis* adenylate cyclase toxin as a 200-kDa protein. Infect. Immun. **58,** 1195-1200.

Benedict, C.A., Banks, T.A. and Ware, C.F. (2003). Death and survival: viral regulation of TNF signalling pathways. Curr. Opin. Immunol. **15,** 59-65.

Betsou, F., Sebo, P. and Guiso, N. (1993). CyaC mediated activation is important not only for toxic but also for protective activities of *Bordetella pertussis* adenylate cyclase hemolysin. Infect. Immun. **61**, 3583-3589.

Betsou, F., Sebo, P. and Guiso, N. (1995). The C-terminal domain is an essential for protective activity of the *Bordetella pertussis* adenylate cyclase-haemolysin. Infec. Immun. **63**, 3309-3315.

Bhakdi, S., Greulich, S., Muhly, M., eberspacher, B., Becker, H., Thiele, A. and hugo, F. (1989). Potent leukocidal action of *Escherichia coli* hemolysin mediated by permeabilisation of target cell membranes. J. Exp. Med. **169**, 737-754

Bhakdi, S., Muhly, M., Korom, S. and Schmidt, G. (1990). Effects of *Escherichia coli* hemolysin on human monocytes. Cytocidal action and stimulation of interleukin 1 release. J. Clin. Invest. 85, 1746-1753.

Biron, C.A., Nguyen, K.B., Pien, G.C., Cousens, L.P. and Salazar-Mather, T.P. (1999). Natural killer cells in antiviral defence. Function and regulation by innate cytokines. Annu.Rev. Immunol. **17**, 189-220

Black, S. (1997). Epidemiology of pertussis. Pediatr. Dis. J. **16**, S85-S89.

Blackburn P.E. (2000). Characterisation of the virulence-related, automembrane proteins. PhD thesis, University of Glasgow.

Bokhari S. H. (2002). Characterisation and secretion of *Bordetella pertussis* autotransporter proteins. PhD thesis, University of Glasgow.

Bordet J., Gengou, O. (1906). Le microbe de la coqueluche. Ann. Inst. Pasteur (Paris) **20**, 731-741.

Boschwitz, J.S., van der Heide, H.G., Mooi, F.R. and Relman, D.A. (1997). *Bordetella bronchiseptica* expresses the fimbrial structural subunit gene *fimA*. J. Bacterial. **179**, 7882-7885.

Boucher, P., Murakami,K., Ishimama, A. And Stibitz, S. (1997). Nature of DNA binding and RNA polymerase interaction of the *Bordetella pertussis* BvgA transcriptional activator at the fha promoter. J Bacteriol. **179,** 1755-63.

Boucher, P., Menozzi, F.D., Locht, C. (1994). The modular architecture of bacterial response regulators: Insights into the activation mechanism of the BvgA transactivator of *Bordetella .pertussis*. J. Mol. Biol, **241**: 363-377.

Boucher, P.E. and Stibitz, S. (1995). Synergistic binding of RNA polymerase and BvgA phosphate to the pertussis toxin promoter of *Bordetella pertussis*. J. Bacteriol. **177**, 6486-6491.

Bradford, M.N. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Brennan, M. J., Hannah, J. H. and Leininger, E (1991). Adhesion of *Bordetella pertussis* to sulfatides and to the Gal Nac beta 4 Gal sequence found in glycosphingolipids. J. Biol. Chem. **266**, 18872-18881.

Brodeur, B.R., Martin, D., Hamel, J., Shahin, R. D. and Leferriere, C. (1993). Antigenic analysis of the saccharide moiety of the lipooligosaccharide of *Bordetella pertussis*. Springer. Semin. Immunopathol. **15**, 205-215.

Bromberg, K., Tannis, G. and Steine, P. (1991). Detection of *Bordetella pertussis* associated with the alveolar macrophages of children with human immunodeficiency virus infection. Infect. Immun. **59**, 4715-4719.

Brown, J.F., Leite, F., Czuprynski, C.J. (1997). Binding of *Pasteurella haemolytica* leukotoxin to bovine leukocytes. Infect. Immun. **65**, 3719-3724

Burnette, W. N. (1992). Perspectives in recombinant pertussis toxoid development, In: Koff, W., Six H.R. (Eds), Vaccine research and development, Marcel Dekker, New York. 143-193.

Cahill, E. S., Hagan, D. T., Ilum, L. and Redhead, K. (1993). Mice are protected against *Bordetella pertussis* infection by intra-nasal immunisation with filamentous haemagglutinin. FEMS. Microbial. Lett. **107**, 211-216.

Carbonetti, N.H., Artamonova, G.V., Dudley, E., Mays, R.M. and Worthington, Z.E. (2003). Pertussis toxin plays an early role in respiratory tract colonisation by *Bordetella pertussis*. Infect. Immun. **71**, 6358-6366.

Caroff, M., Chaby, R., Karibian, D., Perry, J., Deprun, C. and Szabo, L. (1990). Variation in the carbohydrate regions of *Bordetella pertussis* lipopolysaccharide: electrophoretic, serological, structural features. J. Bacteriol. **172**, 1121-1128.

Caroff, M., Tacken, A. and Szabo, L. (1988). Detergent- accelerated hydrolysis of bacterial endotoxin and determination of the anomeric configuration of glycosyl phosphate present in the isolated lipidA fragment of *Bordetella pertussis* endotoxin. Carbohydr. **15,** 273-282.

Castro, M. G., McNamara, U. and Carbonetli, N. H. (2001). Expression, activity and cytotoxicity of pertussis toxin S1 subunit in transfected mammalian cells. Cell. Microbiol. **3**, 45-54.

Cattaneo, L.A. (1996). The seroepidemiology of *Bordetella pertussis* infections; a study of persons ages 1-65 years. J. Infect. Dis. **173,** 1257-1262.

Cavalieri, S.J., Bohch, G.A. and Snyder, I. S. (1984). *Escherichia coli* α-hemolysin: characteristics and probable role in pathogenicity. Microb. Rev. **48**, 326-343.

Cheers, C., and. Gray, D. F. (1969). Macrophage behavior during the complaisant phase of murine pertussis. Immunology **17**, 875-887.

Cherry, J. D. (1996). Historical review of pertussis and the classical vaccine .J. Infect. Dis. **174**, 259-264.

Cherry, J.D. (1997). Comparative efficacy of acellular pertussis vaccines: an analysis of recent trials. Pediatr. Infect Dis J. **16**, 90-96.

Cherry, J. D. (1999). Epidemiological, clinical, laboratory aspects of pertussis in adults. Clin. Infect. Dis. **28,** 112-117.

Cherry, J.D., Brunell, P.A., Golden, G.S. and Karzon, D.T. (1988). Report the task force on pertussis and pertussis immunization. Pediatrics. **81**, 939-984.

Cherry, J.D., Gornbein, J., Heininger, U. and Stehr, K. (1998). A search for serologic correlates of immunity to *Bordetella pertussis* cough illness. Vaccine. **16,** 1901-1906.

Clinkenbeard, K.D. and Upton, M.L. (1991). Lysis of bovine platelets by *Pasteurella haemolytica*. Am.J.Vet.Res. **52,** 453-457.

Confer, D. L. and Eaton, J. W. (1982). Phagocyte impotence caused by an invasive bacterial adenylate cyclase. Nature. **217**, 948-950.

Cookson, B. T., Tyler, A. N. and Goldman, W. E. (1989). Primary structure of the peptidoglycan-derived tracheal cytotoxin of *Bordetella pertussis*. Biochem. **28**, 1744-1749.

Coote, J. G. (1992). Structural and functional relationships among the RTX toxin determinants of Gram-negative bacteria. FEMS. Microbiol, **88**, 137-161.

Coote, J. G. (1996). The RTX toxin of Gram-negative bacterial pathogens. Med. Biol. **7**, 53-62

Cotter, P.A. and Jones, A.M. (2003). Phosphorelay control of virulence factor gene expression in *Bordetella*. Trends. Microbiol. **11**, 367-373.

Cotter, P.A. and Miller, J.F. (1997). A mutant in the *Bordetella. bronchiseptica* bvgS gene results in reduced virulence and increased resistance to starvation, identifies a new class of Bvg-regulated antigens. Mol. Microbiol. **24**, 671-685.

Cotter, P.A. and Miller, J.F. (2001). Bordetella. In E.A. Groisman (ed) . Principles of bacterial pathogenesis. Academic Press. Ltd., London. UK. 619-674.

Cowell, J. L., Hewlett, E. L. and Manclark, C. R. (1979). Intracellular localization of the demonenecrotic toxin of *Bordetella pertussis*. Infect. Immun. **25**, 896-901.

Croinin, T.O., Grippe, V.K. and Merkel, T.J (2005). Activation of the vrg6 promoter of *Bordetella pertussis* by RisA. J. Bacteriol. **187**, 1648-1658.

Crowcroft, N., Andrews, N., Rooney, C., Brisson, M. and Miller, E. (2002). Deaths from pertussis are underestimated in England. Arch. Dis. Child. **86**, 336-338.

Cullinane, L.C., Alley, M.R., Marshall, R.B. and Manktelow, BW. (1987). Bordetella parapertussis from lambs. New Zealand Veterinary Jurnal. **35,** 175

Cundell, D.R., Kanathakumar, K., Taylor, G.W et al (1994). Effect of tracheal cytotoxin from *Bordetella pertussis* on human neutrophil function in vitro. Infect. Immun. **62**, 639-643.

Czuprynski, C.J., Neol, E.J., Ortiz-Carranza, O. and Srikumaran, S. (1991). Activation of bovine neutrophils by partially purified Pasteurella haemolytica leukotoxin. Infect. Immun. **59**, 3126-3133.

Czuprynski, C.J. and Welch, R.A. (1995). Biological effects of RTX toxins: the possible role of lipopolysaccharide. Trends. Microbiol. **3,** 480-483.

Dadgalio, G., Morel, S., Bauche, C., Moukrim, Z., Lemonnier, F. A., Van den Eynde., B.J., Ladant, D. and Leclerc, C. (2003). Recombinant adenylate cyclase toxin

of *Bordetella pertussis* induces cytotoxic T lymphocyte responses against HLA*0201-restricted melonoma epitopes. Inter. Immunol. **15**, 11423-1430

Dalya, G., Peter, M., Strebel, B. B., Muireann, B., Raffi, T., Evelyn, F., Melinda, W. and John, R. (1999). Changing epidemiology of *pertussis* in the United States: Increasing reported incidence among adolescents and adults. Clin. Infect. Dis. **28**, 1230-1237.

Decker, M. D. and Edwards, K. M. (1996). The multicenter acellular pertussis trial: an overview. J. Infect. Dis. **174,** 270-275.

Decker, M. D. and Edwards, K. M. (2000). Acellular pertussis vaccines. Pediatr. Clin. North. Am. 47, 309-335.

Denoel, P., Godfroid, F., Guiso, N., Hallander, H. and Poolman, J. (2005). Comparison of acellular pertussis vaccines-induced immunity against infection due to *Bordetella pertussis* variant isolates in a mouse model. Vaccine. **26**, 1-9

Deora, R. (2004). Multiple mechanisms of bipA gene regulation by the *Bordetella* BvgAS phosphorelay system. Trends. Microbiol. **12,** 63-65.

Deora, R., Bootsma, H.J., Miller, J.F. and Cotter, P.A. (2001). Diversity in the *Bordetella* virulence regulon: transcriptional control of a Bvg⁻ intermediate phase gene. Mol. Microbiol. **40**, 669-683.

Department of Health (1990). Whooping cough (*pertussis*) In: Department of Health (eds) Immunization against Infectious Disease. London. HMSO. 20-27.

Di Fabio, J.L., Caroff, M., Karibian, D., Richards, J.C. and Perry, M.B. (1992). Characterization of the common antigenic lipopolysaccharide O-chain produced by *Bordetella pertussis* and *Bordetella parapertussis*. FEMS. Microbiol. Lett. **76**, 275-281

Dinh, T., Paulsen, I.T. and Saier, M.H.J.R. (1994). A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of gram-negative bacteria. J. Bacteriol. **176**, 3825-2832.

Donato, G.M., Hsia, H.L., Green, C.S. and Hewlett, E.L. (2005). Adenylate cyclase toxin (ACT) from *Bordetella hinzii*: characterization and differences from ACT of *Bordetella pertussis*. J Bacteriol. **187,** 7579-7588.

Dorittke, C., Vandamme, P., Hinz, K.H., Schemken-Brik, E.M., Wirsingvon. And Konig, C.H. (1995). Isolation of a *Bordetella avium*-like organism from a human specimen. Eur. J. Clin. Microbiol. Infect. Dis. **14**, 451-4.

Drum, C.L., Yan, S.Z., Bard, J., Shen, Y.Q., Lu, D., Soelaiman, S., Grabarek, Z., Bohm, A. and Tang, W.J. (2002). Structural basis for the activation of anthrax adenylyl cyclase exotoxin by calmodulin. Nature. **415**, 396-402

Duprez, E., Gjertsen, B.J., Bernard, O., Lantte, M. and Doskeland, S.O. (1993). Antiapoptotic effect of heterozygously expressed mutant RI (Ala336-->Asp) subunit of cAMP kinase I in a rat leukemia cell line. J.Biol. Chem. **268**, 8332-8340.

Edwards, K. M., Decker, M. D. and Mortimer, E. A. (1999). Pertussis vaccine. In: Plotkin, B.J., Orrenstein, W.A. (eds) Vaccines. Philadelphia. W.B. Saunders and Co, 393-344.

El-Azami-El-Idrissi, M., C. Bauche, J. Loucka, R. Osicka, P. Sebo, D.Ladant, and C. Leclerc. (2003). Interaction of *Bordetella pertussis* adenylate cyclase with CD11b/CD18: role of toxin acylation and identification of the main integrin interaction domain. J. Biol. Chem. **278,** 38514–38521.

Endoh, M., Amitani, M. and Nakase, Y. (1986). Purification and characterization of heat labile toxin from *Bordetella bronchiseptica*. Microbiol. Immun. **30**, 659-673.

Endoh, M., Nagai, M., Burns, D.L., Manclark, C.R. and Nakase, Y. (1990). Effect of exogenous agents of the action of *Bordetella parapertussis* heat-labile toxin on guina pig skin. Infect. Immun. **58**, 1456-1460.

Ennis, D.P., Cassidy, J. P. and Mahon, B.P. (2005). Whole cell pertussis vaccine protects against *Bordetella pertussis* exacerbation of allergic asthma. Immunology. letter. **97**, 91-100.

Fayolle, C., Osickova, A., Osicka, R., Henry, T., Rojas, M. J., Saron, M. F., Sebo, P. and Leclerc, C. (2001). Delivery of multiple epitopes by recombinant detoxified adenylate cyclase of *Bordetella pertussis* induces protective antiviral immunity. J. Virol 7330-7338.

Fayolle, C., Sebo, P., Ullmann, A. and Leclerc. (1996). In vivo induction of CTL responses by recombinant adenylate cyclase of *Bordetella pertussis* carring viral $CD8^+$ T cell epitopes. J. Immunol. **165**, 4697-4706.

Fernandez, R. C and Weiss, A. A. (1994). Cloning and sequencing of a *Bordetella pertussis* serum resistance locus. Infect. Immun. **62**, 4727-4738.

Fery, J. and Kuhnert, P. (2002). RTX toxins in Pasteurellaceae. Int. J. Med. Microbiol. **292**, 149-158.

Finn, T.M. and Amsbaugh, D.F (1998). Vag8, a *Bordetella pertussis* bvg-regulated protein. Infect. Immun. **66,** 3985-3989.

Finn, T.M. and Stevens, L.A. (1995). Tracheal colonization factor: a *Bordetella pertussis* secreted virulence determinant. Mol. Microbiol. **16**, 625-634.

Flak , T.A. and Goldman, W.E. (1996). Autotoxicity of nitric oxide in airway disease. Am. J. Respir. Crit Care. Med. **154,** 202-206.

Flak, T. A. and Goldman, W. E. (1999). Signaling and cellular specificity of airway nitric oxid production in pertussis. Cell. Microbiol. **1,** 51-60.

Flak, T. A., Heiss, L. N., Engle, J. T. and Goldman, W. E. (2000). Synergistic epithelial responses to endotoxin aand a naturally occuring muramyl peptide. Infect. Immun. **68,** 1235-1242.

Forestier, C. and Welch, R.A. (1990). Nonreciprocal complementation of the hlyC and lktC genes of the *Escherichia coli* hemolysin and *Pasteurella haemolytica* leukotoxin determinants. Infect Immun. **58**, 828-832.

Friedman, R. L. (1988). *Pertussis*: The disease and new diagnosis methods. Clin. Microbiol. **1**, 365.

Galaiani, J.N., Hewlett, E.L. and Friedman, R.L. (1988). Effects of adenylate cyclase toxin from *Bordetella pertussis* on human neutrophil interactions with Coccidioides immitisandStaphylococcusaureus.Infect Immun. **56**, 751-755.

Gale, J. L., Thapa, P. B., Wassilak, S. G., Bobo, J. K., Mendelman, P. M. and Foy, H. M. (1994). Risk of serious acute neurogical illness after immunization with diphtheria-tetanus-pertussis vaccine. A population-based case control study. JAMA. **271**, 37-41.

Gao, L.Y. and Abo Kwaik, Y. (2000). Hijacking of apoptotic pathways by bacterial pathogens. Microb. Infect. **2**, 1705-1719.

Gentile, F., Knipling, L.G., Sackett, D.L and Wolff. J. (1990). Invasive adenylate cyclase of *Bordetella pertussis*. Physical, catalytic and toxic properties. J. Biol. Chem. **265**, 10686-10692.

Geuijen, C. A., Willems, R. J. and Mooi, F. R. (1996). The major fimbrial subunit,of *Bordetella pertussis* binds to sulfated sugars. Infect. Immun. **64**, 2657-2665.

Geuijen, C. A., Willems, R. J., Bongaerts, M., Top, J., Gielen, H. and Mooi, F. R. (1997). Role of the *Bordetella pertussis* minor fimbrial subunit, FimD, in colonization of the mouse repiratory tract. Infect. Immun. **65**, 4222-4228.

Glaser, P., Latant, D., Sezer, O., Pichot, F., Ullmann, A. and Danchin, A. (1988). The calmodulin sensitive adenylate cyclase of *Bordetella pertussis*-cloning and expression in *Escherichia coli*. Mol. Microbiol. 19-30.

Goldman, W. E. (1988). Tracheal cytotoxin of *Bordetella pertussis*. Wardlaw, A.C., Parton, R. (eds) Pathogenesis and Immunity in pertussis. Chichester: John Wiley and Sons, 231-246.

Golovkina, T.V., Shlomchi, M., Hannum, L. and Chervonsky, A. (1999). Organogenic role of B lymphocytes in mucosal immunity. Science. **286,** 1956-1968

Goodnow, R.A. (1980). Biology of *Bordetella bronchiseptica*. Microbiol. **44**, 722-738.

Goodwin, M. S., and A. A. Weiss. (1990). Adenylate cyclase toxin is critical for colonization and pertussis toxin is critical for lethal infection by *Bordetella pertussis* in infant mice. Infect. Immun. **58,** 3445-3447.

Gotto, J.W., Eckhardt, T., Reilly, P.A., Scott, J.V., Cowell, J.L., Metalf, T.N 3rd, Mountzouros, K., Gibbons, J.J Jr. and Siegel, M. (1993). Biochemical and immunological properties of two forms of pertactin, the 69,000-molecular-weight outer membrane protein of *Bordetella pertussis*. Infect Immun. **61**, 2211-5.

Gray, M. C., Donato, G. M., Hones, F.R., Kim, T and Hewlett, E.L. (2004). Newly secreted adenylate cyclase toxin is responsible for intoxication of target cells by *Bordetella pertussis*. Mol. Microbiol. **53**, 1709-1719.

Greenlee, D. V., Andreasen, T. J. and Storm, D. R. (1982). Calcium-independent stimulation of *Bordetella pertussis* adenylate cyclase by calmodulin. Biochem. **21**, 2759-2764.

Grimprel, E. and Begue, P. (1998). What uses for the cellular pertussis vaccine? Arch.Pediatr. **5**, 557-560.

Gross, R. and Rappuoli, R. (1988). Pertussis toxin promoter sequences involved in modulation. J. Bacteriol. **171**, 4026-4030.

Gueirard, P. and Guiso, N. (1993). Virulence of *Bordetella bronchiseptica*: role of adenylate cyclase hemolysin. Infect. Immun. **61**, 4072-4078.

Gueirard, P. G., Minoprio, P. and Guiso, N. (1996). Intranasal inoculation of *Bordetella bronchiseptica* in mice induces long- lasting antibody and T-cell mediated immune response. Scand. J. Immunol. **43** (2), 181-192.

Gueirard, P., Leblay, K., Lecoustumier, A., Chaby, R. and Guison, N (1998). Variation in *Bordetella bronchiseptica* lipopolysaccharide during human infection. FEMS. Microbiol. Lett. **162**, 331-337.

Guermonprez, P., Khelef, N., Blouin, E., Rieu, P., Ricciardi-Castagnoli, P., Guiso, N., Ladant, D. and Leclerc, C. (2001). The adenylate cyclase toxin of *Bordetella pertussis* binds to target cells via the $\alpha_m\beta_2$ integrin (CD11b/CD18). J. Exp. Med. **193**, 1035-1044.

Guermonprez, P., Ladant, D., Karimova, G., Ullmann, A. and Leclerc, C. (1999). Direct delivery of the *Bordetella pertussis* adenylate cyclase toxin to the MHC class I antigen presentation pathway. J. Immunol. **162**, 1910-1916.

Guiso, N., Grimprel, E., Anjak, I. and Begue, P. (1993). Western blot analysis of antibody responses of young infants to pertussis infection. Eur. J. Clin. Microbiol. Infect. Dis. **12**, 596-600.

Guiso, N., Szaanik. M and Rocancourt, M. (1991). Protective activity of *Bordetella pertussis* adenylate cyclse and haemolysin against bacterial colonization. Mirobiol. Pathogenesis. **11**, 423-431.

Guris, D., Strebel, P.M., Barhenheier, B., Brennan, M., Tachdjian, R., Finch, E., Wharton, M. and Livengood, J.R. (1999). Changing epidemiology of pertussis in the United States: increasing reported incidence among adolescents and adults, 1990-1996. Clin. Infect. Dis.**28**, 1230-7.

Gustafsson, L., Hallander, H.O., Olin, P., Reizenstein, E. and Storseater, J. (1996) .A controlled trail of a two component acellular, a five component acellular, a whole cell *pertussis* vaccine. New. Eng. J. Med. **334,** 349-355.

Hallander, H. O., Gnarpe, J. and Olin, P. (1999) *Bordetella pertussis, Bordetella parapertussis, Mycoplasma pneumoniae, Chlamydia pneumoniae* and persistent cough in children. Scand. J. Infect. Dis. 31, 281-286.

Hanski, E. and Coote, J. G (1991). *Bordetella pertussis* adenylate cyclase toxin. Sourcebook of bacterial protein toxins. 349-366.

Hanski, E. and Farfel, Z. (1985). *Bordetella pertussis* invasive adenylate cyclase. Partial resolution and properties of its cellular penetration. J.Biol. Chem. **260**, 5526-5532.

Hazenbos WL, Geuijen CA, van den Berg BM, Mooi FR, van Furth R. (1995) *Bordetella pertussis* fimbriae bind to human monocytes via the minor fimbrial subunit FimD. J. Infect. Dis. **171**, 924-929.

Hazenbos, W.L., van den Berg, B.M. and van Furth, R. (1993). Very late antigen-5 and complement receptor type 3 cooperatively mediate the intraction between *Bordetella pertussis* and human monocytes. J. Immuniol. **151**, 6274-6282.

Hazes, B. and Read, R.T. (1997). Accumulating evidence suggests that several AB-toxins subvert the endoplasmic reticulum-associated protein degradation pathway to entertargetcells. Biochemistry. **36**, 11051-11054.

Heiss, L. N., Lacaster, J.R.J., Corbett, J.A. and Goldman, W.E. (1994). Epithelial autotoxicity of nitric oxide: role in the respiratory cytopathtology of pertussis. Proc. Natl. Acad. Sci. USA. **91**, 267-270.

Heiss, L. N., Moser, S.A., Unanue, E.R. and Goldman, W.E. (1993). Interleukin-1 is linked to the respiratory epithelial cytopathology of pertussis. Infect. Immun. **61,** 3123-3128.

Henderson, I. and Nataro, J.P. (2001). Virulence functions of autotransporter proteins. Infect. Immun. **69**, 1231-1243.

Henricks, P.A., Binkhorst, G.J., Drijrer, A.A. and Nijkamp, F.P. (1992). Pasteurella haemolytica leukotoxin enhances production of leukotriene B4 and 5-ydroxyeicosatetraenoic acidbybovinepolymorphonuclearleukocytes. Infect Immun. **60,** 3238-3243.

Heveker, N. and Ladant, D. (1997). Characterization of mutant Bordetella pertussis adenylate cyclase toxins with reduced affinity for calmodulin. Implications for the mechanism of toxin entry into target cells. Eur. J. Biochem. **243,** 643-649.

Hewlett, E. l. (2000). *Bordetella* species . In; Mandell, G.L., Bennett, E.L., Dolin, R. (eds) Principle and Practice of Infectious Disease. Philadelphia. Churchill Livingstone. 2414-2422.

Hewlett, E. L. and Cherry, J. D. (1997). New and improved vaccines against pertussis. In: Levine, M. M., Woodrow, G.C., Kaper, J.B., Cobon, G.S. (eds) New Generation Vaccines. New York.Marcel Dekker. 367-410.

Hewlett, E. L. and Halperin, S. A. (1998) Serological correlates of immunity to *Bordetella pertussis. Vaccine.* **16,** 1899-1900.

Hewlett, E. L., Gray, L., Allitta, M., Ehramann, I., Gordon, V. M. and Gray, M.C. (1991). Adenylate cyclase toxin from *Bordetella pertussis.* Conformational change associated with toxin activity. J. Biol. Chem. **266**, 17503-17508.

Higashi, T., Isomoto,A., Tyuma, I., Kakishita, E., Uomoto, M. and Nagai, R. (1985). Quantitative and continuous analysis of ATP release from blood platelets with firefly luciferase luminescence.Thromb. Haemost. **53,** 65-69.

Hoeflich, K.P. and Ikura, M. (2002). Calmodulin in action: diversity in target recognition and activation mechanisms. Cell. **108**, 739-742.

Hoppe, J.E. (1996). Update on epidemiology, diagnosis, treatment of pertussis. J. Clin. Microbiol. Infect. Dis. **15**, 189-193.

Hoppe, J.E. (2000). Neonatal pertussis. J. Pediatr. Infect. Dis. **19,** 244-247.

Horiguchi, Y., Inoue, N., Mausda, M., Kashimoto, T., Katahira, J., Sugimoto, N. and Matsuda, M. (1997). *Bordetella bronchiseptica* dermonecrotizing toxin induces reorganization of actin stress fibers through deamination of Gln-63 of the binding protein Rho. *Proc. Natl. Acad. Sci. USA.* 16294-16302.

Horiguchi, Y., Nakai, T. and Kume, K. (1989). Purification and characterization of *Bordetella bronchiseptica* dermonecritic toxin. Microb. Pathog. **6**, 361-368.

Hormozi, K., Parton, R. and Coote, J.G (1999). Adjuvant and protective *properties* of native and recombinant *Bordetella perussis* adenylate cyclase toxin preparations in mice. FEMS. Immunol. Med. Microbiol. **23,** 273-282.

Hozbor, D., Rodriguez, M.E. and Yantorno, O. (1994). Use of cyclodextrin as an agent to induce excretion of *Bordetella pertussis* antigens. FEMS. Immunol. Med. Microbiol. **9**, 117-124.

Hurwitz, E.L. and Morgenstern, H. (2000) Effects of diphtheria-tetanus-pertussis or tetanus vaccination on allergies and allergy-related respiratory symptoms among children and adolescents in the United States. J. Manipulat. Physiol. Ther. **23,** 81-90.

Ide, M. (1974). Adenylate cyclase toxin of bacteria. Arch. Biochem. Biophys. **144,** 262-268. Infect. Immun. **72**, 1568-1579.

Isacson, J.B., Trollfors, J. Taranger, Ackrisson. and lagergard. (1993). How common is whooping cough in a non vaccinating country? Pediater. Infect. Did. J. **12,** 284-288.

Issartel L, J. P., Koronakis, V. and Hughes, C. (1991) Activation of *Escherichia coli* prohaemolysin to the mature toxin by acyl carrier protein-dependent fatty acylation. Nature. **351**, 759-761.

Ivanoff, B. and Robertson, S. E. (1997) Pertussis: a wordwide problem. Dev. Biol. Stand. **89**, 3-13.

Iwaki, M., Ullmann, A. and Sebo, P. (1995). Oligomerization of the adenylate cyclase toxin of *Bordetella pertussis*: evidence from in vitro complementation of individually inactive mutants. Mol. Microbiol.

Jacob-Dubisson, F., Buisine, C., Mielcarek, N., Clement, E., Menozzi, F. D. and Iocht, C. (1999). Channel formation by FhaC, the outer membrane protein is involved in the secretion of the filamentous haemagglutinin. J. Biol. Chem. **274**, 37731-37735.

Jones, D.H., Mcbride, B.W., Jeffery, H.O., Hagan, D.T., Robinson, A. and Farrar, G.H. (1995). Protection of mice from *Bordetella pertussis* respiratory infection using microencapsulated pertussis fimbriae. Vaccine. **13,** 675-681.

Jungnitz, H., West, N.P., Walker, M.J., Chhatwal, G.S. and Guzman, C.A. (1998). A second two-component regulatory system of *Bordetella bronchiseptica* required for bacterial resistance to oxidative stress, production of acid phosphatase, and in vivo persistence. Infect. Immun. **66**, 4640-4650.

Kachlany, S.C., Fine, D.H. and Figurski, H.H. (2000). Secretion of RTX leukotoxin by *Actinobacillus actinomycetemco mitans*. Infect. Immun. **68,** 6094-6100.

Kania, S.A., Rajeev, S., Burns, E.H., Odom, T.F., Holloway, S.M. and Bemis, D.A. (2000). Characterisation of fimN, a new *Bordetella bronchiseptica* major fimbrial subunit gene. Gene. **256,** 149-155.

Karakelian, D., Lear, J.D., Lally, E.T. and Tanaka, J.C. (1998). Characterization of *Actinobacillus actinomycetemcomitans* leukotoxin pore formation in HL60 cells. Biochim. Biophys. Acta. **1406**, 175-87.

Kashimato, T., Katahira, J., Cornejo, W. R., Masuda, M., Fukuoh, a., Matsuzawa, T., Ohnishi, T. and Horiguchi, Y. (1999). Identification of functional domains of *Bordetella* dermonecrotizing toxin. Infect. Immun. **67**, 3727-3732.

Katada, T., Tamura, M. and Ui, M. (1983). The A protomer of islet-activating protein, *pertussis* toxin, as an active peptide catalysing ADP-ribosylation of a membran protein. Arch. Biochem. Biophys. **224**, 290-298.

Kattar, M.M., Chavez, J.F., Limaye, A.P., Rassoulian-Barrett, S.L., Yarfitz, S.L., Carlson, L.C., Houze, Y., Swanzy, S., Wood, B.L. and Kookson, B.T. (2000). Application of 16S rRNA gene sequencing to identify *Bordetella hinzii* as causative agent of fatal septicemia. J. Clin Microbiol. **38,** 789-794.

Kerr, J. R. and Malthews, R. C. (2000). *Bordetella pertussis* infection: pathogenesis management, and the role of protective immunity. Eur. J. Clin. Microbiol. Infect. Dis. **19,** 77-78.

Khelef, N. and Guiso. (1995). Induction of macrophage apoptosis by *Bordetella pertussis* adenylate cyclase-hemolysin. FEMS. Microbiol. Lett.**134**, 27-32.

Khelef, N., Bachelet, C.M. and Guoso, N. (1994). Characterisation of murine lung inflammation after infection with parental *Bordetella pertussis* and mutants deficient in adhesins or toxin. Infect. Immun. **62**, 2893-2900.

Khelef, N., Sakomato, H and Guiso, N. (1992). Both adenylate cyclase and hemolytic activities are required by *Bordetella pertussis* to initiate infection. Microb. Pathog. **12,** 227-235.

Khelef, N., Zychlinsky, A. and Guoso, N. (1993). *Bordetella pertussis* induce apoptosis in macrophage: role of ACT hemolysin. Infect. Immun. **61**, 4064-4071.

Kikuchi, H., Lizuka, R., Sugiyama, S., Gon, G., Mori, H., Arai, M., Mizumoto, K., Imajoh-Ohmi, S. (1996). Monocytic differentiation modulates apoptotic response to cytotoxic anti-Fas antibody and tumor necrosis factor alpha in human monoblast U937 cells. J. Leuko. Biol. **60**, 778-783.

Kimura, M. and Kuno-Sakai, H. (1988). Acellular pertussis vaccines and fatal infections. Lancet. **16,** 881-882

Knapp, O., Maier, E., Polleichtner, G., Masin, J., Sebo, P. and Benz, R. (2003). Channel formation in model membranes by the adenylate cyclase toxin of *Bordetella pertussis*: effect of calcium. Biochemistry. **8,** 8077-84

Ko, K.S., Peck, K.R., Oh, W.S., Lee. N.Y., Lee, J.H. and Song, J.H. (2005). New species of *Bordetella, Bordetella ansorpii* sp. nov., isolated from the purulent exudate of an epidermal cyst. J. Clin. Microbiol. **43**, 2516-2519.

Kobisch, M. and Novotny, P. (1990). Identification of a 68-kilodalton outer membrane protein as the major protective antigen of *Bordetella bronchiseptica* by using specific-pathogen-free.piglets. Infect. Immun. **58**, 352-357.

Kotob, S.I., Husman, S.Z. and Burns, D.L. (1995). Localization of the promoter for the ptl genes of *Bordetella pertussis*, which encode proteins essential for secretion of pertussis toxin. Infect. Immun. **63**, 3227-3230.

Kreiner, M., Moore, B.D. and Parker, M.C. (2001) Enzyme-coated micro-crystals: a 1-step method for high activity biocatalyst preparation. Chem. Common. 1096-1097.

Ladant, D. and Ullmann, A. (1999). *Bordetella pertussis* adenylate cyclase: a toxin with multiple talents. Trend. Microbiol. **7**, 172-176.

Ladant, D., Glaser, P. and Ullmann, A. (1992). Insertional mutagenesis of pertussis adenylate cyclase. J. Biol. Chem. **267**, 2244-2250.

Ladant, E. and Hewlett, A. (1999). Commentary on the pathogenesis of pertussis. Clin. Infect. Dis. **28,** 94-98.

Lally, E.T., Hill, R.B, Kieba, I.R.and Korostoff, J. (1999). The interaction between RTX toxins and target cells. Trend. Microbiol. **7**, 356-361.

Lally, E.T., Golub, E.E., Kieba, T.R., Taichman, N.S., decker, S., Berthold, P., Gibson, C.W., Demuth, D.R. and Rosenbloom, J. (1991). Structure and function of the *LtxB* and *Ltx D* genes of the *Actinobacillus actinomycetemcomitans* leukotoxin complex. Microbiol. Pathog. **11**, 111-121

Lambert-Buisine, C., Willerg, E., Locht, C. and Jacob-Dubuisson, F. (1998). The N-terminal characterization of *Bordetella pertussis* filamentous haemagglutinin. Mol. Microbiol. **178**, 1053-1060.

Langley, J.M., Halperin, S.A., Boucher, F.D. and Smith, B. (2004). Azithromycin is as effective as and better tolerated than erythromycin estolate for the treatment of pertussis. Pediatrics. **114**, 96-101.

Lasfarguse, A., Caroff, M. and Chaby, R. (1993). Structural features involved in the mitogenic activiting of *Bordetella prtussis* LPSs for spleen cells of C3H/He mice. FEMS. Immunol. Med. Microbiol. **7**, 119-129.

Lawrence, A.J., Coote, J.G., Kazi, Y.F., Lawrence, P.D., MacDonald-Fyall, J., Orr, B.D., Parton, R., Riehle..., M., Sinclair, J., Young, J. and Price, N.C. (2002). A direct pyrophosphatase-coupled assay provides new insights into the activation of the secreted adenylate cyclase from *Bordetella pertussis* bycalmodulin. J. Biol. Chem. **277**, 22289-96.

Lawrence, A.J., Coote, J.G., Maclean, A.G., Parton, R. and Young, J.D. (1998). Conductimetryforenzymeteaching. Biochem. Soc. Trans. **26**, 197.

Le, T., Cherry, J.D., Chang, M. D., Knoll, M.D., Lee, M. L. and Barenkamp, S. (2004). Immune responses and antibody decay after immunization of adolescents and adults with an acellular *pertussis* vaccine; the APERT Study. J. Infect. Dis. **190**, 535-544.

Lear, J.D., Furblur, U.G., Lally, E.T. and Tanaka, J.C. (1995). *Actinobacillus actinomycetemcomitans* leukotoxin forms large conductance, voltage-gated channels when incorporated into planer lipid bilayers. Biochim. Biophys. Acta. **1238**, 34-41.

Leef, M., Elkins, K. L., Barbic, J. and Shahin, R. D. (2000). Protective immunity to *Bordetella pertussis* requires both B-cells and $CD4^+$ T-cells for key functions other than specific antibody production. J. Exp. Med. **191**, 1841-1852.

Lehman, A.K., Sornes, S. and Halstensen, A. (2000). Phagocytosis: measurment by flow cytometry. J. Immunol. Methods. **21**, 229-242

Leininger, E., Roberts, M., Kenimer, J.G et al (1991). Pertactin, an Arg-Gly-Asp cotaining *Bordetella perussis* surface protein that promotes adherence of mammalian cells. Proc. Natl. Acad. Sci. USA. **88**, 345-349.

Livey, I. and Wardlaw, A. C. (1984). Production and properties of *Bordetella pertussis* heat-lable toxin. J. Med. Microbiol. **17**, 91-103.

Livey, I., Duggleby, C.J. and Robinson, A. (1987). Cloning and nucleotide sequence analysis of the serotype 2 fimbial subunit gene of *Bordetella pertussis*. Mol. Microbiol. **1**, 203-209.

Locht, C. and Antoine, R. (1999). *Bordetella pertussis* protein toxin. The Comprehensive Sourcebook of Bacterial Protein Toxins. 130-146.

Locht, C., Bertin, P., Menozzi, F. D. and Renauld. G. (1993). The filamentous hemagglutinin a multifaceted adhesin producede by virulent *Bordetella* species. Mol. Microbiol. **9**, 653-660.

Locht, C., Geoffroy, M. C. and Renauld, G. (1992). Common accessory genes for the *Bordetella pertussis* filamentous hemagglotinin and fimbriae share sequence similarities with the *pap*C and *pap*D gene families. EMBO. J. **11**, 3175-3183.

Loetscher, P., Seitz, M., Clark-Lewis, I., Baggiolinini, M. and Moser, B. (1994). Monocyte chemotactic proteins MCP-1, MCP-2, MCP3 are major attractives for human $CD4^+$ and $CD8^+$ T lymphocytes. FASEB. J. **8**, 1055-1060.

Lopez-Moreno, M. E. *Bordetella* (1952) Microbial. Esp. **5**, 177-181.

Lydyard, P.M., Whelan, A. and Fanger, M.W. (2000). Instant notes in immunology. Guilford. UK.

MacDonald-Fyall, J. (2002). The protective and immunomodulatory properties of *Bordetella pertussis* adenylate cyclase toxin. Ph.D thesis.

MacDonald-Fyall, J., Xing, D., Corbel, M., Baillie, S., Parton, R. and Coote, J. (2004). Adjuvanticity of native and detoxified adenylate cyclase toxin of *Bordetella pertussis* towards co-administered antigens. Vaccine. **22,** 4270-81.

Maheswaran, S.K., Weiss, D.J., Kannan, M.S., Townsend, E.L., Reddy, K.R., Whiteley, L.O. and Srikumaran. S. (1992). Effects of *Pasteurella haemolytica* A1 leukotoxin on bovine neutrophils: degranulation and generation of oxygen-derived free radicals.Vet. Immunol. Immunopathol. **33,** 51-68.

Mahon, P.B., Brady, M.T. and Mills, K.H. (2000). Protection against *Bordetella pertussis* in mice in the absence of detectable circulating antibody: implication for long-term immunity in children. J. Infect. Dis. **181,** 2087-2091.

Mahon, P.B., Sheahan B. J., Griflin,F., Murphy, G. and Mills, K. H. G. (1997). Atypical disease after *Bordetella pertussis* respiratory infection of mice with targeted disruptions of interferon-γ receptor or immnoglobulin π chain genes. J. Exp. Med. **186**, 1843-1851.

Mangan, D.F., Taichman, N. S., Lally, E.T. and Wahl, S.H. (1991). Lethal effect of *Actinobacillus actinomycetemcomitans* leukotoxin on human T lymphocytes. Infect. Immun. **59**, 3267-3272.

Masmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: applicationtoproliferationandcytotoxicityassays.J. Immunol .Methods. **65,** 55-63.

Marcuse, E.K. and Wentz, K.R. (1990). The NCES reconsidered: summary of a 1989 workshop. National Childhood Encephalopathy Study. Vaccine. **8,** 531-535.

Marques, P.R. and Carbonetti, N.H. (1997). Genetic analysis of pertussis toxin promoter activation in *Bordetella pertussis*. Mol. Micribiol. **24,** 1215-1224.

Mascarell, L., Fayolle, C., Bauche, C. and Ladant, D. (2005). Induction of neutralizing antibodies and Th1-polarised and CD4-independent CD8+ T cell responses following delivery of human immunodefiency virus type 1 Tat preotein by recombinant adenylate cyclase of *Bordetella pertussis*. J. Virol. **79,** 9872-9884.

Mastrantonio, P., Spigaglia, P., van Oirschot, H., ven der Heide, H.G., Heuvelman, K., Stefanlli, P. and Mooi, F.R. (1999). Antigen variants in *Bordetella pertussis* strains isolated from vaccinated and unvaccinated children. Microbiology. **145,** 2069-2075.

Masure, H. R. (1992). Modulation of adenylate cyclase toxin production as *Bordetella pertussis* enters human macrophages.Proc. Natl. Acad. Sci. U S A. **15,** 6521-5.

Mattoo, S,. Foreman-Wykert, A.K., Cotter, P.A. and Miller, J.F. (2001). Mechanism of *Bordetella* pathogenesis. Front.Biosci. **6,** E168-E186.

Mattoo, S. and Cherry, J.D. (2005). Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infection due to *Bordetella pertussis* and other *Bordetella* subspecies. Clin. Microbiol. Rev. **18,** 326-382.

Mattoo, S., Mills, J. F. and Cotter, P. A. (2000). Role of *Bordetella bronchiseptica* fimbriae in tracheal colonization and development of a humoral immune response. Infect. Immun. **68,** 2042-2033.

Mazengia, E., Silva, E. A., Peppe, J. A., Timperi, R. and George, H. (2000). Recovery of *Bordetella holmesii* from patients with pertussis-like symptoms: use of pulsed-field gel electrophoresis to characterise circulating strains. J. Clin. Microbiol. **38,** 2330-2333).

McConkey, D.J. and Orrenius, S. (1994). Signal transduction pathways to apoptosis. Trend. Cell. Biol. **4,** 370-374.

McGuirk, P,. Mahon, B.P., Griffin, F. and Mills, K.H. (1998). Compartmentalization of T cell responses following repiratory infection with *Bordetella pertussis*: hypore sponsiveness of lung T cells is associated with modulated expression of the co-stimulatory molecule CD 28. Eur. J. Immunol. **28,** 153-163.

McGuirk, P. and Mills, K. H. G. (2000). A regulatory role for interleukin 4 in differential inflamatory responses in the lung following infection of mice primed with Th1-or Th2-inducing *pertussis* vaccines. Infect. Immun. **68,** 1383-1390.

Medical Research Council (1954). Acellular and defined component pertussis vaccine against pertussis . In: Wardlaw, A.C., Parton, R. (eds) Pathogenesis and Immunity in Pertussis. Chichester. John Wiley and Sons, 399-417.

Melker, H. E., de Conyn-van S, Sprenger, M. A. F. Schellekens, M. J. W. and Kinkhoest, J. F. P. (1995). Pertussis in Nederland 1989-1994. Nederlands Tijdschrift Geneeskunde. **139**, 1280-1286.

Menozzi, F.D., Boucher, P. E., Riveau, G., Gantiez, C. and locht, C. (1994). Surface-associated FHA induces autoagglutination of *Bordetella pertussis*. Infect. Immun. **62,** 4261-4269.

Menozzi, F.D., Gantiez, C. and Locht, C. (1991). Interaction of the *Bordetella pertussis* FHA with heparin. FEMS. Micrbiol. Lett. **62**, 59-64.

Menozzi, F.D., Mutombo, R., Renauld, G., Gantiez, C., Hannah, J.H., Leininger, E., Brennan, M. J. and Locht, C. (1994). Heparin-inhibitable lectin activity of the FHA adhesion of *Bordetella pertussis*. Infect. Immun. **62**, 769-778.

Merkel, T.J. and Stibitz, S. (1995). Identification of a locus required for the regulation of bvg-repressed genes in *Bordetella pertussis*. J.Bacteriol. **177**, 2727-2736.

Merkel, T.J., Boucher, P.E., Stibitz, S. and Grippe. V.K. (2003). Analysis of bvgR expression in *Bordetella pertussis*. J. Bacteriol. 2902-2912.

Merkel, T.J., Barros, C. and Stibitz, S. (1998). Characterisation of the bvgR locus of *Bordetella pertussis* . J.Bacteriol. **180,** 1682-1690.

Mills, K. H. G. (2000). Immunity to *Bordetella pertussis*. Microb. Infect. **3,** 655-677.

Mills, K. H. G., Ryan, E. and Mahon, B. P. (1998). A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral amd cell- mediated immunity in protection against *Bordetella pertussis*. Infect. Immun. **66**, 594-602.

Mills, K.H. (2001). Immunity to *Bordetella pertussis*. Microbes. Infect. **3**, 655-77.

Mills, K.H., Barnard, A., Watkins, J. and Redhead, K. (1993). Cell mediated immunity to *Bordetella pertussis*: role of Th1 cells inbacterial clearance in a murine respiratory infection model. Infect. Immun. **61**, 399-410.

Mobberely-Schuman, P. S., Connelly, B. and Weiss, A. (2003). Phagocytosis of *Bordetella pertussis* induced with convalescent serum. J. Infect. Dis. **187**, 1646-1653.

Mock, M. and Ullmann, A. (1993). Calmodulin-activated bacterial adenylate cyclases as virulence factors. Trend. Microbiol. **1**, 187-192.

Montaraz, J.A., Novotny, P. and Ivanyi, J. (1985). Identification of a 68 kDa protective protein antigen from *Bordetella bronchiseptica*. Infect. Immun. **47**, 744-751.

Mooi, F.R., Jansen, W.H., Brunings, H., Gielen, H., van der Heide, H.G., Walvoort, H.C. and Guinee, P.A. (1992). Construction and analysis *of Bordetella pertussis* mutants defective in the production of fimbriae. Microb. Pathog. **12**, 127-135.

Mooi, F.R., van Oirschot, H., Heuvelman, K., van der Heide,H.G., Gaastra, W. and Willems, R.J. (1998). Polymorphism in the *Bordetella pertussis* virulence factors p.69/pertactin and pertussis toxin in the Netherlands: temporal trends and evidance for vaccine-driven evolution. Infect. Immun. **66,** 670-675.d

Moreno Lopez, M. (1952). The genus Bordetella. By Manuel Moreno Lopez, 1952] Enferm Infecc Microbiol Clin. **8,** 480-5.

Morrison, D.C. and Jacobs, D.M. (1976). Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides.Immunochemistry. **13,** 813-818.

Muller, F. M., Hoppe, J. E. and Wirsing vK. C (1997). Laboratory diagnosis of pertussis. State of the art in 1997. J. Clin. Microbiol. **35**, 2435-2443.

Murdan, S., Somavarapu, S., Ross, A.C., Alpar, H.O. and Parker, M.C. (2005). Immobilisation of vaccines onto micro-crystals for enhanced thermal stability. Int. J. Pharm. **30**, 117-121.

Murphy, K.M., Murphy, T.L., Szabo, S.J., Jacson, N.G., Guler, M.L., Gorham, J.D. and Gubler, U. (1997). Regulation of IL-12 receptor expression in early T-helper responses implies two phases of Th1 differentiation: capacitance and development. Chem Immunol. **68**, 54-69

Murphy, K.M., Ouyang, W., Farrar, J.D., Yang, J., Ranganuth, S., Asnagli, H., Afkarian, M. and Murphy, T.L. (2000). Signaling and transcription in T helper development. Annu. Rev. Immunol. **18**, 451-494.

Nennig, M.E., Shinefield, H.R., Edward, K.M., Black, S.B. and Fierman, B.H. (1996). Prevalence and incidence of adult pertussis in an urban population. JAMA. **5,** 1672-1674.

Nicholson, D.W. and Thornberry, N.A. (1997). Caspase: Killer proteases. Trend. Biol. Sci. **22**, 299-306.

Njamkepo, E., Delisle, F., Hagege, I., Geerbaud, G. and Guiso, N. (2000). *Bordetella holmesii* isolated from a patient with sickle cell anemia: analysis and comparison with other *Borderella holmesii* isolates. Clin. Microbiol. Infec. **6,** 131-136.

Olin, P., Rasmussen, F., Gustafsson, L., Hallander, H.O., Heeijbel, H. and the Ad Hoc Group for the study of pertussis vaccines. (1997). Randomised controlled trial of two-component, three- component, and five componenet acellular pertussis vaccines compared with whole cell pertussis vaccine. Lancet. **350,** 1569-1577.

Oliver, D. C. and Fernandez, R. C. (2001). Antibodies to BrkA augment killing of *Bordetella. pertussis.* Vaccine. **20**, 235-241.

Oliver, D. C., Huang, G and Fernandez, R. C. (2003). Identification of secretion determinants of the *Bordetella pertussis* BrkA autotransporter. J. Bacteriol. 489-495.

Parker, M.C., Partridge, J., Ross, A.C. and Moore, B.D. (2003). Protein-coated microcrystals: particle engineering for the advanced delivery of biomolecules. Abstr. Pap. Am. Chem. Soc. 2269, 475-PMSE.

Parkhill, J., Sebaihia, M., Preston, A., *et al* (2003). Comparative analysis of the genome sequences of *Bordetella perussis, Bordetella parapertussis* and *Bordetella bronchiseptica*. Nat. Genet. **35**, 32-40.

Parton, R (1999). Changing perspectives on pertussis and pertussis vaccination. Rev.Med. Microbiol. **2,** 121-128.

Parton, R. (1999). Review of the biology of *Bordetella pertussis*. Biolgicals. **27**, 71-76.

Passerini de Rossi, B. N., Frriedman, L. E., Gonzalez, F. L., Castello, P. R., Franco, M. A and Rossi, J. P. (1999). Identification of *Bordetella pertussis* virulence-associated outer membrane proteins. FEMS. Microbiol. Lett. 1172, 9-13.

Paul, W.E. and Seder, R.A. (1994). Lymphocyte responses and oytokine. Cell. **28**, 241-251.

Pearson, R.D., Symes, P., Conboy, M., Weiss, A.A. and Hewlett, E.L. (1987). Inhibition of monocyte oxidative responses by *Bordetella pertussis* adenylate cyclase toxin. J. Immunol. **139**, 2749-2754.

Peppler, M.S. (1984). Two physically and serologically distinct LPS profiles in strains of *Bordetella pertussis* and their phenotype varians. Infect. Immun. **43,** 224-232.

Petsch, D., Rantze, F. and Anspach, F.B. (1998). Selective adsorption of endotoxin inside a polycationic network of flat-sheet microfiltration membranes. J. Mol. Recognite. **11**, 222-230.

Peterson, A. and Maskell, D. (2001) The molecular genetics and role in infection of lipopolysaccharide biosynthesis in the *Bordetellae*. J Endotoxin. Res.7. 251-61.

Petty, R.D., Sutherland, L.A., Hunter, E.M. and Cree, I.A. (1995). Comparison of MTT and ATP-based assays for the measurement of viable cel number.Biolumin hemilumin. **10**, 29-34.

Pigott, N., Novelli, V., Pooboni, S., Firmin, R. and Goldman, A. (2002). The importance of herd immunity against infection. Lancet. 360, 645.

Pittman, M. (1991) History of the development of pertussis vaccine. Dev. Biol. Stand. **73,** 13-29.

Pollard, R. (1983). Whooping cough in Fiji. Lancet. **18**, 1381

Poole, S., Dawson, P. and Gaines, R.E (1997). Second intyernational standard for endotoxin: calibration in an international collaboration study. J. Endotoxin. Res. **4**, 221-231.

Porter, J. F., Connor, K. and Donachie,W. (1994). Isolation and characterization of *Bordetell apara pertussis* Like bactria from ovine lungs. Microbiol. **140**, 261-266.

Preston, A., Thomas, R. and Maskell, D. (2002). Mutational analysis of the *Bordetella pertussis* wlb LPS biosynthesis locus. Microb. Pathog. **33:**91–95.

Preston, N.W. (1987). Eradication of pertussis by vaccination. Lancet. **6,** 1312.

Preziosi, P.M., Yam, A., Wassilak, S.G., Chabirand,L Ndiaye, M., Dia, M1., Dabis, F. and Simondon1, F. (2002). Epidemiology of pertussis in a West African ommunity before and after Introduction of a Widespread Vaccination Program. Am. J. Epidemiol. **15**, 897-898.

Pullinger, G. D., Adams, T.E., Mullan, P. B., Garrod, T. I. and Lax, A. J. (1996). Cloning, expression and molecular characterization of the dermonecrotic toxin gene of *Bordetella spp*. Infect. Immun. **64**, 4163-4171.

Ramachandra, L Chu, R.S., Askew, D., Noss, E.H., Canaday, D. H., Potter, N.S., Jojnsen, A., Krieg, A.M., Nedrud, J.G., Boom, WH. And Hardin, C.V. (1999). Phagocytic antigen processing and effects of microbial products on antigen processing and T-cell responses. Immunol. Rev. **168**, 217-239.

Rappuoli, R. (1994). Pathogenicity mechanism of *Bordetella.* Curr. Top. Microbiol. Immunol. **34,** 1563-1569.

Rappuoli, R., Pizza, M., Podda, A., De Magistris, M. T. and Nencioni, L. (1991). Towards third- generation whooping cough vaccines. Trend. Biotechnol. **9,** 232-238.

Rawbow, A.A., Fernandez, R.C. and Weiss, A.A. (1998). Characterization of BrkA expression in *Bordetella bronchiseptica*. Infect. Immun. **66**, 3978-80.

Redhead, K. (1995). Pertussis vaccines- old and new, In; Alden, D.A.A., Hormeache, C. (eds), Molecular and Clinical Aspect of Vaccine Development, John Wiley and Sons, Chichester. 259-322.

Redhead, K., Watkins, J., Barnard, A. and Mills, K. H. G. (1993). Effective immunization against *Bordetella pertussis* respiratory infection in mice is dependent on induction of cell mediated-immunity. Infect. Immun. **61**, 3190-3198.

Reina, J., Bassa, A., Lionpart, I., Borrell, N., Gomez, J. and Serra, A. (1991). Pneumonia caused by *Bordetella bronchiseptica* in a patient with a thoracic trauma. Infect. **19**, 46-48.

Relman, D., Tuomanen, E., Falkow. S., Golenbock, D. T., saukonen, K andWright, S. D. (1990). Recognition of a bacterial adhesion by an integrin: macrophage CR3 (alpha M beta 2, CD11b/CD18) binds filamentous hemaglutinin of *Bordetella pertussis*. Cell. **61**, 1375-1382.

Renauld-Mongenie, G., Cornette, J., Mielcarek, N., Menozzi, F.D. and Locht, C. (1996). Distict roles of the N-terminal and C-terminal precursor domains in the biogenesis of the pertussis filamentous hemagglutinin. J. Bacteriol. **178**, 1053-1060.

Roberts, M. and Parton, R. (2001). *Bordetella pertussis.* In: M. Sussmann (ed) Med. Med. Microbiol. 1565-1617.

Roberts, M., Cropley, I., Chatfield, S. and Dougan, G. (1993). Protection of mice against respiratory *Bordetella pertussis* infection by intranasal immunization with P.68 and FHA. Vaccine. **11**, 866-872.

Roberts, M., Tite, J.P., Fairweather, N.F., Dougan, G. and Chales, I.G. (1992). Recombinant P.68 pertactin: immunogenesity and protection of mice against *Bordetella pertussis* infection. Vaccine. **10**, 43-48.

Rogel, A., Meller, R. and Hanski, E. (1991). Adenylate cyclase toxin from *Bordetella pertussis*. The relationship between induction of cAMP and hemolysin. J. Biol. Chem. **266**, 3154-3161.

Rogel, R., Schultz, J.E., Brownlie, R.M., Coote, J.G., Parton, R. and Hanski, E. (1989). *Bordetella pertussis* adenylate cyclase: purification and characterization of the toxic form of the enzyme. EMBO J. **8**, 2755-2760.

Roop, R. M. ., Veit, H. P., Sinsky, R. L., Veit, S. P., Hewlett, E. L. and Kornegay, E. T. (1987) Virulence factors of *Bordetella bronchiseptica* associated with the production of infectious atrophic rhinitis and pneumonia in experimentally infected neonatal swine. Infect. Immun. **55**, 217-222.

Rose, T., Sebo, P., Bellalou, J. and Ladant, D. (1995). Interaction of calcium with ACT of *Bordetella pertussis*. Characterization of multiple calcium-binding site and calcium-induce conformation change. J. Biol. Chem. **70**, 26370-26376.

Ross, A. C., Partridge, J., Vos, J., Ganeasan, A., Stevens, H.N.E., Moore, B.D. and Parker, M.C. (2004). Engineering the morphology of protein coated micro-crystals. J. Pharm. Pharmacol. **56**, 45

Ross, A. C., Partridge, J., Flores, M.V., Moore,B.D., Parker, M.C. and Stevens, H.N.E. (2002). Peptide and protein drug delivery using protein coated micro-crystals. In: Proc. 29th Int. Symp. Control. Rel. Bioact. Mater. 53.

Ross, P. J., Lavelle, E.C., Mills, K.H. and Boyd, A.P. (2004). Adenylate cyclase toxin from *Bordetella pertussis* synergizes with lipopolysaccharide to promote innate interleukin-10 production and enhances the induction of Th2 and regulatory T cells.

Rothe G, Oser A & G. Valet. (1988). Dihydrorhodamin 123: a new flow cytometric indicator for respiratory burst activity in neutrophil granulocytes. Naturwissenschaften 75: 354 - 355.

Rowatt, E. (1957). The growth of *Bordetella Pertussis*: J. Gen. Microbiol. **17**, 297-326.

Roy, C.R. and Falkow, S. (199I). Identification of *Bordetella pertussis* regulatory sequences required for transcriptional activation of the fhaB gene and autoregulation of the bvgAS operon. J. Bacteriol. **173**, 2385-2392.

Roy, C.R., Miller, J.F. and Falkow, S. (1990). Autogenous regulation of the *Bordetella pertussis* bvg ABC operon. Proc. Natl. Acad. Sci. U S A. **87**, 3763-7.

Ryan, M., Murphy, G., Ryan, E., Nilsson, L., Shackley, F., Gothfors, L., Qymar, K., Miller, E., Storsaeter, J. and Mills, K.H. (1998). Distinct T-cell subtypes induced with whole cell and acellular pertussis vaccines in children. Immunology. **93**, 1-10.

Sakamoto, H., Bellalou, J., Sebo, P. and Ladant, D. (1992). *Bordetella pertussis* adenylate cyclase toxin. Structural and functional independence of the catalytic and hemolytic activities. J. Biol. Chem. **267**, 13598-13602.

Sato, H. and Sato, Y. (1985). Protective antigens of *Bordetella pertussis* mouse protection test against intracerebral and aerosol challenge of *Bordetella pertussis*. Dev. Biol. Stand. **61**, 461-467.

Sato, H. and Sato, Y. (1990). Protective activities in mice of monoclonal antibodies against pertussis toxin. Infect. Immun. **58**, 3369-337.

Sato, Y. and Sato, H. (1988). Animal models of *pertussis*. In Wardlaw, A.C., Parton, R. (eds) Pathogenesis and Immunity in Pertussis. Chichester: John, Wiley and Sons, 309-325.

Sato, Y., Kimura, M. and Fukumi, H. (1984). Development of a pertussiscomponent vaccine in Japan. Lancet. **1**, 122-126.

Saukkonen, K., Cabellos, C., Burroughs, M., Prasad, S. and Tuomanen, E. (1991). Integrin-mediated localization of *Bordetella pertussis* within macrophages: role in pulmonary colonization. J. Exp. Med. **173**, 1143-1149.

Saukkonen, K., Burnette, W.N., Masure, H.R. and Tuomanen, E. (1992). Pertussis toxin has eukaryotic-like carbohydrate recognition domains.Proc Natl Acad Sci U S A. 89, 118-122.

Scarlato, V., Prugnola, A., Arico,B. and Rappuoli, R. (1990). Positive transcriptional feedback at the bvg locus controls expression of virulence factors in *Bordetella pertussis*. Proc. Natl. Acad. Sci. USA. **87**, 6753-6757.

Schlecht, G., Loucka, J., Najar, Hossain., Sebo, P. and Leclerc, C. (2004). Antigen targeting to CD11b allows efficient presentation of Cd4+ and CD8+ T cell epitopes and in vivo Th1-polarised T cell priming. J. Immunol. **173**, 6089-6097.

Schmitt-Grohe, S., Cherry, J.D., Heininger, u. and Uberall, M.A., Pineda, E. and Stehr, K. (1995). Pertussis in German adults. Clin. Infect. Dis. **21**, 860-866.

Schoop, J., Thome, M., Hofmann, K. and Meinl, E. (1998). The fight of viruses agains apoptosis. Curr. Opin. Genet. Dev. **8,** 82-87.

Sebo, P. and Ladant, D. (1993). Repeat sequences in the *Bordetella pertussis* adenylate cyclase toxin can be recognized as alternative carboxy-proximal secretion signal by the *Escherichia coli* alpha-haemolysin translocator. Mol. Microbiol. **9**, 999-1009.

Sebo, P., Glaser, P., Sakamoto, H. and Ullmann, A.(1991). High-level synthesis of active CyaA of *Bordetella pertussis* in a recombinant *Escherichia coli* system. Gen, **104,** 19-24.

Shahin, R. D., Amsbaugh, D. F. and Leef, M. F. (1992). Mucosal immunization with FHA protects against *Bordetella pertussis* respiratory. Infect. Immun. **60**, 1482-1488.

Shahin, R. D., Hamel, J., Leef, M.F. and Brodeur, B.R. (1994). Analysis of protective and non protective monoclonal antibodies specific for *Bordetella pertussis* lipooligosaccharide. Infect. Immun. **62,** 722-725.

Shahin, R.D., Brennan, M.J., Li, Z.M., Meade, B.D. and Manclark, C.R. (1990). Characterization of the protective capacity and immunogenicity of the 69-kD outer membrane protein of *Bordetella pertussis*. J. Exp. Med. **1**, 63-73.

Shen, Y., Lee, Y.S., Soelaiman, S., Beergson, P., Lu, D., Chen, A., Beckingham, K., Grabarek, Z., Mrksich, M. and Tang, W.J. (2002). Physiological calcium concentrations regulate calmodulin binding and catalysis of adenylyl cyclase exotoxins. EMBO. J. **16**, 6721-6732.

Sheu, G. C., Wo, Y. Y. and Lu, C. H. (1997). Preparation and characterisation of pertussis toxin subunit. J. Microbiol. Immunol. Infect. **30**, 182-193.

Shewen, P.E. and Wilke, B.N. (1982). Cytotoxin of *Pasteurella haemolytica* acting on bovine leukocytes Infect. Immun. **35**, 91-94.

Sidey, F. M., Furman, B. L. and Wardlaw, A. C. (1989). Effect of hyperreactivity to endotoxin onto the toxicity of pertussis vaccine and pertussis toxin in mice. Vaccine. **7**, 237-241.

Singler, P.B., Xu, Z., Rye, H.S., Burston, S.G., Fenton, W.A. and Hoewich, A.L. (1998). Structure and function in GroEL-mediated protein folding. Annu.Rev.Bioch. 67, 581-608.

Skowronski, D.M., Remple, V. P., Macnabb, J., Pielak, K., Patrick, M.D., Halperin, S. A. and Scheiffele, D. (2003). Injection-site reactions to booster doses of acellular pertussis vaccine: rate, severity, and anticipated impact. Pediatrics. **112,** 453.

Sloan, L. M., Hopkins, M.k., Mitchell, P.s., Vetter, E.A., Rosenblatt, J. E., Harmsen, W. S., Cockerill, F. R. and Patel, R. (2002). Multiplex light Cycler PCR assay for detection and differentiation of *Bordetella pertussis* and *Bordetella parapertussis* in nasopharyngeal specimens. J. Clin. Microbiol. **40**, 96-100.

Smith, A.M., Guzman, C.A. and Walker, M.J. (2001). The virulence factors of *Bordetella pertussis*: a matter of control. FEMS. Microbiol. Rev. **25**, 309-333. Review

Squatrito, R.C., Connor, J.P. and Buller, R.E. (1995). Comparison of a novel redox dye cell growth assayto theATP bioluminescence assay. Gynecol.Oncol. 58, 101-105.

Stainer, D. W. (1988). Growth of *Bordetella pertussis* in Wardlaw A.C, Parton, R.

Steed, L. L., M. Setareh, and R. L. Friedman. (1991). Hostparasite interactions between *Bordetella pertussis* and human polymorphonuclear leukocytes. J. Leukocyte Biol. **50**, 321-330.

Steffen, P., Goyard, S. and Ullmann, A. (1996). Phosphorylated BvgA is sufficient for transcriptional activation of virulence regulated gene in *Bordetella pertussis*. EMBO. J.**15**, 102-109.

Stenson, T.H., Allen, A.G., Al-Meer, J.A., Marshall, D. and Peppler, M.S. (2005). Bordetella pertussis risA, but not risS, is required for maximal expression of Bvg-repressed genes. Infect. Immun. **73**, 5995-6004.

Stevens, P. and Czuprynski. C. (1996). *Pasteurella haemolytica* leukotoxin induces bovine leukocytes to undergo morphologic changes consistent with apoptosis in vitro. Infect. Immun. **64**, 2687-2694.

Stollenwerk, M., Fallgen, C., Lundberg, F., Tegenfeldt, J.O., Montelius, L. and Ljungj, A. (1998). Quantitation of bacterial adhesion to polymer surfaces by bioluminescence. Zentralbl. Bakteriol. **287**, 7-18.

Storsaeter, J., Hallander, H.O., Gustasfsson, L. and Olin, P. (1998). Levels of anti-pertussis antibodies related to protection after household exposure to *Bordetella pertussis*. Vaccine. **16**, 1901-1906.

Storsaeter,J., Hallander, S.A., Farrington, C.P., Olin, P., Mollby, R. and Miller, E. (1990). Secondary analysis of the efficacy of two acellular pertussis vaccines evaluated in a Swedish phase III trial. Vaccine. **8**, 457-461.

Tanaka, M., Vitek, C.R., Pascual, F. B., Bisgard, K.M., Tate, J.M. amd Murphy, T.V. (2003). Trends in pertussis among infants in the United States, 1980-1999. JAMA. **290**, 2968-2975.

Tang, Y. W. Hapkins, M.K., Kolbert, C.P., Hartley, P.A., Severance,P.J. and Persing, D.H. (1998). *Bordetella holmesii*-like organisms assciated with septicemia, endocarditis and respiratory failure. Clin .Infec .Dis, **26**, 389-392.

Taranger, J., trollfors, B., Lagergard, T., Sundh, V., Bryla, D.A., Schneerson, R. and Robbins, J.B. (2000). Correlation between pertussis toxin IgG antibodies in postvaccination sera and subseqent protection against pertussis. J. Infect. Dis. **181**, 1010-1013.

Tasi, C.M. and Frasch, C.E. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**, 115-119.

Torre, D., Ferrario, G., Bonetta, G., Perversi,. L Tambini, R. and Speranza, F. (1994). Effects of recombinant human gamma interferon on intracellular survival of *Bordetella pertussis* in human phagocytic cells. FEMS. Immunol. Med. Micrbiol. **9,** 183-188.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Biotechnology. **24,** 145-149

Trent, M.S., Worsham, L.M. and Ernst-Fonberg, M.L. (1998). The biochemistry of hemolysin toxin activation: characterization of HlyC, an internal protein acyltransferase. Biochemistry. **31**, 4644-4652.

Tschoop, J., Thome, M., Hofmann, K. and Meinl, E. (1998). The fight of viruses agains apoptosis. Curr. Opin. Genet. Dev. **8,** 82-87.

Tuomanen, E. and Weiss, A. (1985). Characterisation of two adhesions of B*ordetella pertussis* for human respiratory epithelial cells. J.Infect. Dis. **152**, 118-125.

Tuomanen, E.L., Prasad, S.M., George, J.S., Hoepelman, A.L., Ibsen, P., Heron, I. and Starzyk, R.M. (1993) Reversible opening of the blood brain barrier by anti bacterial antibodies. Proc.Nat. Acad. Sci. USA. **90**, 7824-7828.

Tuomannen, E.L., Zapiain, L.A., Galvan, P. and Helwett, E.L. (1984). Characterization of antibody inhibiting adherence of *Bordetella pertussis* to human respiratory epithelial cells. J. Clin. Microbiol. **20**, 167-170.

Uhlinger, D.J., Tyagi, S.R., Inge, k.l. and Lambeth, J.D. (1993). The respiratory burst oxidase of human neutrophils. Guanine nucleotides and arachidonate regulate the assembly of a multicomponent complex in a semirecombinant cell-free system. J. Bio.l Chem. **25**, 8624-8631.

Ui, M. (1988). The multiple biological activities of pertussis toxin. In: Pathogenesis and Immunity in Pertussis (ends A.C. Wardlaw and R.Parton), 121-145. John Wiley, Chichester.

Ui, M. and Miller, J.F. (1995). BvgAS is sufficient for activation of the *Bordetella pertussis* ptx locus in *Escherichia coli*. J. Bactriol. **177**, 6477-6485.

Uphoff, T.S and Welch, R.A. (1990). Nucleotide sequencing of the *Proteus mirabilis* calcium-independenthemolysin genes (*hpmA* and *hpmB*) reveal sequence similarity with the *Seratia marcescens* hemolysin genes (*shlA* and *shlB*. J. Bacteriol. **172**, 1206-1216.

Van den Akker, W. M. R. (1998). LPS expression within the genus *Bordetella.* Influence of temperature and phase variation. Microbiol. **144**, 1527-1535.

Van den Berg, B. M., Beekhuizen, H., Willems, R. J. L., Mooi, F. R. and van Furth, R. (1999). Role of *Bordetella pertussis* factors in adherence to epithelial cell lines derived from the human respiratory tract. Infect. Immun. **67**, 1056-1062.

Van Wintzingerode, F., Schattke, A., Siddiqui, R.A., Roscik, U., Gobel, U.B. and Gross, R. (2001). *Bordetella petrii* sp. nov, isolated from an anaerobic bioreactor, and emended description of the genuse *Bordetella*. Int.J. Syst. Evol. Microbiol. **51**, 1257-1265.

Vandamme, P., Heyndrickx, M., Vancanneyt, M *et al.* (1996). *Bordetella trematum* sp. nov, isolated from wounds and ear infections in humans, reassessment of *Alcaligenes denitrificans* Ruger and Tan 1983. Int. J. Syst. Bacteriol. **46**, 849-858.

Vandamme, P., Hommez, J., Vancanneyt, M., Monsieurs, M., Hoste, B., Cookson, C. H., Wirsing Von konig, K. and Blackall, P. J. (1995). *Bordetella hinzii* sp.nov, isolated from poultry and human. Int. J. Syst. Bacteriol. **43**, 37-45.

Walker, K. E. and Weiss, A. A. (1994). Characterization of the dermonecrotic toxin in member of genus *Bordetella*. Infect. Immun. **62,** 3817-3828.

Wang, Z., Clarke, C. and Clinkenbeard, K.D. (1998). *Pasteurella haemolytica* leukotoxin-induced increase in phopholipase A2 activity in bovine neutrophils. Infect. Immun. **66**, 1885-1890.

Wardlaw, A.C. and Patrton ,R. (1988). The host parasite relationship in pertussis. In: Pathologenicity and Immunity in pertussis, (Eds Wardlaw, A.C. and Parton, R.). John Wiley and Sons, Chichester. pp. 327-347.

Watanabe, M., Komatsu, E., Abe, K., Iyama, S., Sato, T. and Nagai, M. (2002). Efficacy of pertussis components in an acellular vaccines, as a murine model of respiratory infections and a murine intracerebral challenge model. Vaccine. **20**, 1429-1434.

Watanabe, M., Takimoto, H., Kumazawa, Y. and Amano, K. (1990). Biological properties of LPSs from *Bordetella* species. J.Gen. Microbiol. **136**, 489-493.

Weingart, C.L. and Weiss, A.A. (2000). *Bordetella pertussis* virulence factors affect phagocytosis by human neutrophils. Infect. Immun. **68**, 1735-1739.

Weingart, C.L., Mobberley-Schuman, P., Hewlett, E., Gray, M. C. and Weiss, A.A. (2000). Neutralizing antibodies to adenylate cyclase toxin promote phagocytosis of *Bordetella pertussis* by human neutrophils. Infect. Immun. Dis. 7152-55.

Weingart, G. L., Keitel, W. A., Edwards, K.M. and Weiss, A.A. (2000). Characterisation of bacterial immune response following vaccination with acellular pertussis vaccine in adults. Infect. Immun. **68**, 7175-7179.

Weiss, A. A. (1997). Mucosal immune defences and response of *Bordetella pertussis*. ASM News. **63**, 22-27.

Weiss, A. A. and Goodwin, M. S. (1989). Lethal infection by *Bordetella pertussis* mutants in the infant mouse model. Infect.Immun. **57**, 3757-64.

Welch, R.A. (1991). Pore forming cytolysins of gram negative bacteria. Mol. Microbiol. **5,** 521-528.

Welch, R.A., Bauer, M., Kent, A.D., Leeds, A.J., Moayeri, M., Regassa, L.B. and Swenson, D.L. (1995). Battling against host phagocytes: the wherefore of the RTX family of toxins. Infect. Agents. Dis. **4,** 254-272.

Welch, R.A., Forestier, C., Lobo, A., Pellett, S., Thomas, W. and Rowe, G. (1992). The synthesis and function of the Escherichia coli hemolysin and related RTX exotoxins. FEMS. Microbiol. Immunol. **5,** 29-36

Westrop, G. D., Hormozi, E. K., Dacosta, N. A., Parton, R. and Coote, J.G (1997). Structure-function studies of the adenylate cyclase toxin of *Bordetella pertussis* and the leukotoxin of *Pasteurella haemolytica* by heterologous C protein activation and constructionofhybridproteins. J. Bacteriol. 179, 871-789.

Westrop, G. D., Hormozi, E. K., Dacosta, N. A., Parton, R. and Coote, J.G (1996). *Bordetella pertussis* Adenylate cyclase toxin: proCyaA and CyaC protein synthesised separately in *E. coli* produce active toxin in vitro. Gene. **21**, 91-99.

Wilkinson, K.A., Simsova, M., Scholvinck, E., Sebo, P., Leclerc, C., Vordermeier, H.M., Dickson, S.J., Brown, J.R., Davidson, R.N., Pasvol, G., Levin, M. and Wilkinson, R.J. (2005). Efficient Ex vivo stimulation of *Mycobacterium tuberculosis*-specific T cells by genetically detoxified *Bordetella pertussis* adenylate cyclase antigen toxoids. Infect. Immun. **73,** 2991-2998

Willems, R. J. and Mooi, F.R. (1996). From whole cell to acellular vaccines. Reviews in Medical Microbiology. **7,** 13-21.

Willems, R. J., vander Heide, H. G. and Mooi, F.R. (1992). Characterisation of *Bordetella pertussis* fimbrial gene cluster which is located directly downstream of the filamentous haemagglutinin gene. Mol. Microbiol. **6,** 2661-2671.

Willems, R.G., Kamerbeek, J. and Geuijjen, C.A., Top, J., Gielen, H., Gaastra, W. and Mooi, F.R. (1998). The efficacy of a whole cell vaccine and fimbriae against *Bordetella pertussis* and *Bordetella parapertussis* in a respiratory mouse model. Vaccine. **16,** 410-416.

Wolf, A.A. and Cook, G.H. (1973). Activation of thyroid membrane adenylate cyclase by purine nucleotides. J. Biol. Chem. **248,** 350-355.

Wolf, J., Cook, G. H., Goldhammer, A. R. and Berkowitz, S. A. (1980). Calmodulin activates prokaryotic adenylate cyclase. Proc. Natl. Acad. Sci. U.S.A. **77,** 3840-3844.

Woolfrey, B.F. and and Moody, J.A. (1991). Human infections associated with *Bordetella bronchiseptica*. Clinical microbiology Reviews. **4,** 243-255.

Workman, M.L. (2003). Cellular basis of bacterial infection. Crit. Care. Nurs. Clin. North. Am. **15,** 1-11.

World Health Organization (1999). Pertussis vaccines: Position paper, Wkly. Epidemiol. Rec. **74,** 137-144.

Yeh, S.II. (2003). Pertussis: persistent pathogen, imperfect vaccine. Expert. Rev. Vaccines. **2**, 113-127.

Zaretzky, F.R., Gray, M.C. and Hewlett, E.L. (2002). Mechanism of association of adenylate cyclase toxin with the surface of *Bordetella pertussis*: A role for toxin-filamentous haemagglutinin intraction. Molecular. Microbiology. **45**, 1589-1598.

Zychlinsky, A. and Sansonetti, P. (1997). Apoptosis as a proinflammatory event: what can we learn from bacteria-inducedc elldeath? Trends. Microbiol. **5**, 201-204.

Zychlinsky, A. and Sansonetti, P. (1997). Perspectives series: host/pathogen interactions. Apoptosis in bacterial pathogenesis. J. Clin. Invest. 100, 493-495.

Petty, R.D., Sutherland, L.A., Hunter, E.M. and Cree, I.A. (1995). Comparison of MTTand ATP-based assays for the measurement of viable cel number.Biolumin hemilumin. 10, 29-34.

Department of Pathology, University of Dundee, Ninewells Hospital and Medical School, Scotland.

Cell viability assays are widely used to assess the effect chemotherapeutic drugs and other agents on cell lines and have shown promise for the prediction of tumour chemosensitivity. In this study we have compared two viability assays using Daudi and CCRF-CEM cell lines over a range of 1500-100,000 cells/well of a microplate. The ATP assay was able to detect the lower limit of 1563 cells/well with luminescence values at least 100 x background readings, while the MTT assay could not detect less than 25,000 cells/well above background readings. The ATP assay also showed better reproducibility and sensitivity when cells were grown in microtitre plates over several days, and is particularly useful for the measurement of viability with low cell numbers.

Towbin, H., Staehelin, T. and Gordon, J. (1979).
Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Biotechnology. 24, 145-149

Lawrence, A.J., Coote, J.G., Maclean, A.G., Parton, R. and Young, J.D. (1998).
Conductimetry for enzymeteaching. Biochem. Soc. Trans. **26**, 197.

Sons, Chichester, 1995, pp. 259–322.
Sons, Chichester, 1995, pp. 259–322.

Drum, C.L., Yan, S.Z., Bard, J., Shen, Y.Q., Lu, D., Soelaiman, S., Grabarek, Z., Bohm, A. and Tang, W.J. (2002). Structural basis for the activation of anthrax adenylyl cyclase exotoxin by calmodulin. Nature. **415**, 396-402

Rothe G, Oser A & G. Valet. (1988). Dihydrorhodamin 123: a new flow cytometric indicator for respiratory burst activity in neutrophil granulocytes. Naturwissenschaften 75: 354 - 355.