The immunomodulatory and cytotoxic effects of different forms of recombinant adenylate cyclase toxin

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Declaration

I hereby declare that the work presented in this thesis is my own, except where otherwise cited or acknowledged. No part of this thesis has been presented for any other degree.

Yiu Chong Gordon Cheung

2006

In loving memory of Allan Cheng (1979 – 2005)

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Abstract

This thesis describes the small- and large-scale purification of four different recombinant forms of CyaA, namely: fully functional enzymically-active, acylated and invasive CyaA; an acylated and invasive CyaA form lacking adenylate cyclase (AC) enzymic activity (CyaA*); and the non-acylated, poorly-invasive forms of these toxins, proCyaA and proCyaA*, respectively. Only proCyaA and CyaA showed AC enzymic activity. Only CyaA and CyaA* were haemolytic towards sheep erythrocytes and cytotoxic towards mouse J774.2 macrophage-like cells. Both haemolysis and cytotoxicity by CyaA and CyaA* only occurred in the presence of calcium. CyaA was cytotoxic towards J774.2 cells at low concentrations (50% killing at 0.1 μg protein/ml) and this paralleled low levels of caspase 3/7 activity, a measure of apoptotic activity. At higher toxin concentrations of CyaA (> 0.5 μg protein/ml), caspase 3/7 activity declined despite high levels of cell killing measured by the cytotoxicity assay. This may have been due to the onset of necrotic killing at higher concentrations. CyaA* could only kill J774.2 cells at a toxin concentration greater than 0.5 μg protein/ml and the effect was more calcium-dependent than that of CyaA. Cell killing by CyaA* also occurred in the absence of apoptosis. These data suggested that CyaA killed J774.2 cells by two different mechanisms, involving apoptosis at low concentrations (which was cAMP-dependent) and necrosis by pore-formation at higher concentrations. ProCyaA and proCyaA* showed no apoptotic, cytotoxic or haemolytic activities. CyaA inhibited the zymosan-stimulated oxidative burst of J774.2 cells. ProCyaA and CyaA* were ~500-fold less active at inhibiting the zymosan-stimulated oxidative burst of J774.2 cells compared with native CyaA. Thus, inhibition of the zymosan-stimulated oxidative burst by CyaA was a result of both invasive and AC enzymic activities. This also indicated that proCyaA was able to invade cells, albeit at a very low level, but that any increase in intracellular cAMP levels created by intracellular proCyaA was unable to induce apoptosis.

Circular dichroism (CD) was used to assess any differences in the secondary structure of the four CyaA forms. There were similar changes in tertiary structure of all the CyaA forms as shown by intrinsic tryptophan fluorescence and near UV CD studies in the absence and presence of 1 mM $CaCl_2$. Similar conformational changes in protein secondary structure of all four CyaA forms, at 0.5 mg protein/ml, were observed by far UV CD in the absence and presence of 1 mM $CaCl_2$. However, at 0.05 mg protein/ml, the spectral amplitude of CyaA was decreased by 2-fold suggesting that CyaA was able to adopt two stochiometric forms. Preliminary analytical ultracentrifugation (AUC)

studies indicated that CyaA did not exist as a single molecular species in solution. At 3 mg protein/ml, different oligomeric forms of CyaA (monomers, dimers and trimers) as well as the presence of degradation products (20 – 80 kDa) were present. Further optimisation of AUC studies is required to overcome the problem of non-ideality (caused by either a repulsion between charged molecules (proteins) that is not shielded by solvent counter-ions or by size-exclusion effects arising from extended/elongated conformations) which may have interfered with the analyses.

None of the CyaA forms alone conferred significant ($P > 0.05$) protection against *B. pertussis* in a murine intranasal challenge model. Mice immunised with ACV alone showed significantly ($P < 0.05$) reduced bacterial numbers in the lungs at 7 days after intranasal challenge, compared with control mice. Further reduction in bacterial numbers was observed when mice were immunised with ACV + CyaA or ACV + CyaA*, but the enhanced protection was only significant ($P < 0.05$) with CyaA*. Co-administration of CyaA* with ACV caused a significant ($P < 0.05$) increase in IgG2a antibody levels against pertactin, compared with mice immunised with ACV alone. Spleen cells from mice immunised with ACV + CyaA* secreted higher amounts of IL-5, IL-6, IFNγ and GM-CSF than cells from mice immunised with ACV + CyaA or ACV alone, after stimulation *in vitro* with a mixture of *B. pertussis* antigens. Spleen cells from mice immunised with ACV + CyaA* also secreted higher amounts of IFNγ and GM-CSF than cells from mice immunised with CyaA* alone, after stimulation *in vitro* with CyaA*. Macrophages from mice immunised with ACV + CyaA* produced significantly ($P < 0.05$) higher levels of nitric oxide than macrophages from mice immunised with CyaA* alone, ACV alone or ACV + CyaA after stimulation *in vitro* with a mixture of *B. pertussis* antigens or heat-killed *B. pertussis* cells. These data suggested that the enhancement of protection provided by CyaA* was due to an augmentation of both Th1 and Th2 immune responses to *B. pertussis* antigens. Mice immunised intraperitoneally with the different CyaA forms alone were protected against *B. pertussis* challenge in an aerosol model. This may have been due to a lower challenge dose compared to that used in the intranasal challenge model. Further experiments are required to understand the nature of protection afforded by the different CyaA forms alone.

Microarray analysis was used to investigate the effects on gene expression in mouse bone marrow-derived macrophages (BMMs) after treatment with three different CyaA forms (CyaA, CyaA* and proCyaA*). Preliminary experiments showed greater changes of transcription in BMMs after treatment with 20 ng protein/ml of CyaA for 24 h compared with 2 h. This toxin concentration induced low levels of cell killing (5%

killing after incubation for 2 h, 20% killing after incubation for 24 h). In a further experiment, BMM gene responses to the same concentration of CyaA, CyaA* and proCyaA* were assessed after 24 h treatment of BMMs. Over 1,600 genes were up- or down-regulated greater than 2-fold ($P < 0.05$) by CyaA and ~1,000 genes were up- or down-regulated greater than 3-fold ($P < 0.01$). A majority (32.5%) of the latter up-regulated genes were associated with inflammation. In addition, genes which coded for proteins found associated with several cell-signalling cascades were found to be up-regulated, as well as several pro-apoptotic genes. No anti-apoptotic gene expression was detected. Approximately 40% of genes down-regulated greater than 3-fold were associated with cell growth, division and differentiation, compared with only ~3% of the up-regulated genes. CyaA* altered the transcription of only two genes greater than 2-fold ($P < 0.05$) whereas no genes were altered to this extent by proCyaA*. These results indicate that the great majority of gene changes induced by CyaA, at 20 ng protein/ml, were mediated by increases in intracellular cAMP and not by binding of CyaA to cell receptors.

List of contents

List of figures

List of tables

Abbreviations

αα	= amino acid
AC	= adenylate cyclase
ACV	= acellular (pertussis) vaccine
ANOVA	= analysis of variance
ATP	= adenosine 5'-triphosphate
BMM	= bone marrow-derived macrophage
bp	= base pair
BSA	= bovine serum albumin
°C	= degrees Celsius
cAMP	= cyclic adenosine monophosphate
CD	= circular dichroism
CFU	= colony-forming units
CHAPS	= 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate
CI	= confidence interval
cm/mm	= centimetre/millimetre
CMI	= cell-mediated immunity
CO_2	= carbon dioxide
CV	= column volume
CyaA	= adenylate cyclase toxin
Da/kDa	= dalton/kilodalton
DC	= dendritic cell
DEAE	= diethylaminoethyl
DMEM	= Dulbecco's Modified Eagle Medium
DNA	= deoxyribonucleic acid
dPT	= detoxified pertussis toxin
DTaP	= diphtheria tetanus acellular (pertussis) vaccine
EDTA	= ethylenediaminetetraacetic acid
ELISA	= enzyme-linked immunosorbent assay
ERK	= extracellular signal-regulated kinase
EU	= ELISA units or Endotoxin units
FACS	= fluorescence activated cell sorter
FBS	= foetal bovine serum
FHA	= filamentous haemagglutinin
Fim	= fimbriae
g/mg/μg/ng	= grams/milligrams/micrograms/nanograms
GFP	= green fluorescent protein
(G)M-CSF	= (granulocyte)/macrophage – colony stimulating factor
h	= hour(s)
HBSS	= Hank's balanced salt solution
HH	= Hank's HEPES (buffer)
kb	= kilobase
IFNγ	= interferon gamma
Ig	= immunoglobulin
IL-(X)	= interleukin-(number)
IM	= inner membrane
IPTG	= isopropyl-1-thio-β-D-galactoside
IU	= international units
l/ml/μl	= litres/millilitres/microlitres
LA	= Luria-Bertani agar
LAL	= *Limulus* amoebocyte lysate
LB	= Luria-Bertani broth

LDH	= lactate dehydrogenase
LPS	= lipopolysaccharide (endotoxin)
M/mM/μM/nM	= molar/millimolar/micromolar/nanomolar
MFI	= median fluorescence intensity
min	= minute(s)
MM6	= monomac 6
mRNA	= messenger RNA
MTT	= 3-(4,5-di-methylthiazol-2-3-yl)-2,5-diphenol tetrazolium bromide
M_w	= molecular weight
NaCl	= sodium chloride
NO	= nitric oxide
$OD_{x\ nm}$	= optical density at wavelength X nm
o/n	= overnight
ORFs	= open reading frames
PRN	= pertactin
PAGE	= polyacrylamide gel electrophoresis
PBS	= phosphate-buffered saline
PCR	= polymerase chain reaction
PMNL	= polymorphonuclear leukocyte
PT	= pertussis toxin
RNA	= ribonucleic acid
RPE	= R-phycoerythrin
rpm	= revolutions per minute
RPMI	= Roswell Park Memorial Institute
RT	= room temperature
RTX	= Repeats in ToXin
sec	= second(s)
SDS	= sodium dodecyl sulphate
SHD	= single human dose
TCR	= T-cell receptor
Th (1/2)	= helper T-cell (1/2)
TNFα	= tumor necrosis factor alpha
Tris	= Tris(hydroxylmethyl)aminoethane
(f/n) UV	= (far/near) ultraviolet
v/v	= volume by volume
w/v	= weight by volume
WCV	= whole-cell (pertussis) vaccine

Chapter 1

Introduction

1.1 The genus *Bordetella*

Bordetella spp. are small Gram-negative, non-sporing coccobacilli and are approximately 0.35 × 1.5 μm in size. Bordet and Gengou (1906) first described *Bordetella pertussis* as the aetiological agent of pertussis or whooping cough and, since then, there have been nine named species assigned to the genus *Bordetella*. The other species are *B. parapertussis* (human and ovine strains), *B. bronchiseptica*, *B. avium*, *B. holmesii*, *B. hinzii*, *B. trematum*, *B. petrii* and *B. ansorpii*. Originally, *B. pertussis* was classified as *Haemophilus pertussis* but in 1952, Moreno-Lopez created a new genus *Bordetella* in honour of the work performed by Jules Bordet (Moreno-Lopez, 1990). *B. bronchiseptica* and *B. parapertussis* were described by Ferry (1910) and Bradford and Slavin (1937), respectively. *B. bronchisceptica* is recognised as a respiratory tract pathogen in pigs, dogs, cats, rodents and, occasionally, humans whereas *B. parapertussis* can also cause a mild form of pertussis in humans but strains have also been isolated from sheep (Cullinane *et al.*, 1987). *B. avium* causes coryza and rhinotracheitis in poultry (Kersters *et al.*, 1984). *B. hinzii* can be found in the respiratory tracts of chickens and turkeys but has been associated with rare cases of fatal human septicaemia (Kattar *et al.*, 2000) and cholangitis of the liver in humans (Arvand *et al.*, 2004). *B. holmesii* was first described in human blood cultures (Weyant *et al.*, 1995). It has also been detected from the sputum of patients with pertussis-like symptoms (Tang *et al.*, 1998) and from asplenic patients (Shepard *et al.*, 2004). *B. trematum* has been isolated from wounds and ear infections in humans and from leg ulcers in diabetics (Daxboeck *et al.*, 2004). *B. petrii* was isolated from a mixed anaerobic bioreactor (von Wintzingerode *et al.*, 2001) and, since then, only one clinical isolate has been detected from a human patient with mandibular osteomyelitis (Fry *et al.*, 2005). *B. ansorpii* is the newest described member of the *Bordetellae* and was isolated from the purulent exudate of an epidermal cyst (Ko *et al.*, 2005). Sequences of the 16S rRNA genes and analyses of cellular fatty acid composition and differences in biochemical tests revealed that *B. ansorpii* belongs to the genus *Bordetella* but is phylogenetically distinct from previously-described *Bordetella* species.

1.1.1 Genetic relationship between the *Bordetella* species

The genus *Bordetella* forms a distinct cluster separated from members of the genera *Achromobacter* and *Alcaligenes* within the β2 subdivision of *Proteobacteria* as deduced by comparison of their 16S rRNA sequences (von Wintzingerode *et al.*, 2001). The genomes

of three of the most studied species within the *Bordetellae*, *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, were sequenced to gain new insights into the genetic differences that could account for host specificities and pathogeneses (Parkhill *et al.*, 2003). Sequence comparisons of the three genomes suggest that *B. pertussis* and *B. parapertussis* recently and independently evolved from *B. bronchiseptica*-like ancestors. However, both *B. bronchiseptica* and *B. parapertussis* encode significantly more functional genes (5007 and 4404 predicted ORFs, respectively) than *B. pertussis* (3816 predicted ORFs). *B. pertussis* has 114 ORFs found only in its genome but not in *B. parapertussis* or *B. bronchiseptica*. Interestingly, a large portion of the DNA found within the *B. bronchiseptica* genome, but not in *B. pertussis* or *B. parapertussis*, is attributed to prophages. The loss of genes by *B. pertussis* and *B. parapertussis* include loci involved with metabolism, membrane transport and the biosynthesis of membrane structures. In addition to the lower number of genes, *B. pertussis* and *B. parapertussis* contain 358 and 200 pseudogenes, respectively. Pseudogenes (non-functional genes) are formed when the gene has been inactivated by in-frame stop codons, frameshift mutations or if an insertion sequence (IS) element has been introduced into the gene. IS elements are simple mobile genetic elements that do not carry any information other than that required for their mobility and are involved in large-scale re-ordering of genomes. *B. pertussis* contains 261 copies of three IS elements and *B. parapertussis* has 112 copies of two IS elements. Interestingly, none of the IS elements found in the *B. pertussis* or *B. parapertussis* genomes were found in the *B. bronchiseptica* genome used for sequencing, although, IS elements have been found in other *B. bronchiseptica* isolates (Gerlach *et al.*, 2001). The loss of so many functional genes in the genome of *B. pertussis* may explain the restricted host range of *B. pertussis* compared with *B. bronchiseptica* and *B. parapertussis*.

1.2 The disease pertussis

The transmission of whooping cough is thought to be mediated through the inhalation of droplets produced from infected patients, principally through coughing. *B. pertussis* adheres to ciliated epithelial cells of the bronchial tree and then proliferates and colonises the upper respiratory tract whilst producing several types of virulence factors (Section 1.5) including toxins that damage surrounding ciliated cells. Generally, there is a 7 - 14 day incubation period before clinical manifestations appear. The symptoms of whooping cough can be separated into three stages: the catarrhal, paroxysmal and convalescent stages. The catarrhal stage is characterised by mildly elevated coughing and the production of mucus which can be accompanied by a mild fever. The paroxysmal stage occurs roughly 7 - 10 days after the catarrhal stage and is characterised by increased severity in coughing. Typically, 5 - 10 or more forceful coughs are made during a single

expiration (a paroxysm) followed by a massive inspiratory effort during which the classic whoop occurs. Uncontrollable bouts of coughing can occur for up to 20 h or more. Vomiting, bulging eyes, protrusion of the tongue and hypoxia may follow. The paraxysomal stage can last between 1 - 6 weeks or sometimes longer. The continual bouts of coughing can cause complications such as subconjunctival haemorrhage, pneumothorax, rupturing of the diaphragm, rectal prolapse, tetanic seizures and dehydration. The convalescent stage is when the bouts of coughing become less common and viable *B. pertussis* organisms cannot be detected from the respiratory tract. In spite of well-implemented infant vaccination programmes, pertussis has remained endemic in many parts of the world, especially amongst young infants (de Melker *et al.*, 2000; Crowcroft and Britto, 2002; Skowronski *et al.*, 2002; Crowcroft *et al.*, 2003b). Unfortunately, most cases of pertussis are in developing countries. A recent estimate by Crowcroft *et al.* (2003b) suggested that there could be up to 48 million cases annually of pertussis worldwide, with ~300,000 deaths. Figure 1 shows the number of cases in the England and Wales from 1940 – 1998. There was a dramatic decline of deaths caused by pertussis from the late 1940s to the early 1960s due to the increase in vaccine coverage. However, due to a controversy over the safety of whole-cell pertussis vaccines in the mid 1970s (Section 1.7.2), there was a drop in vaccine uptake and the increase in notifications of disease in England and Wales (Fig. 1), until the early 1980s when vaccine uptake recovered. Over the past 2 decades, the numbers of cases have slowly increased (Fig. 1), but this could be due to increased surveillance or greater awareness of the disease.

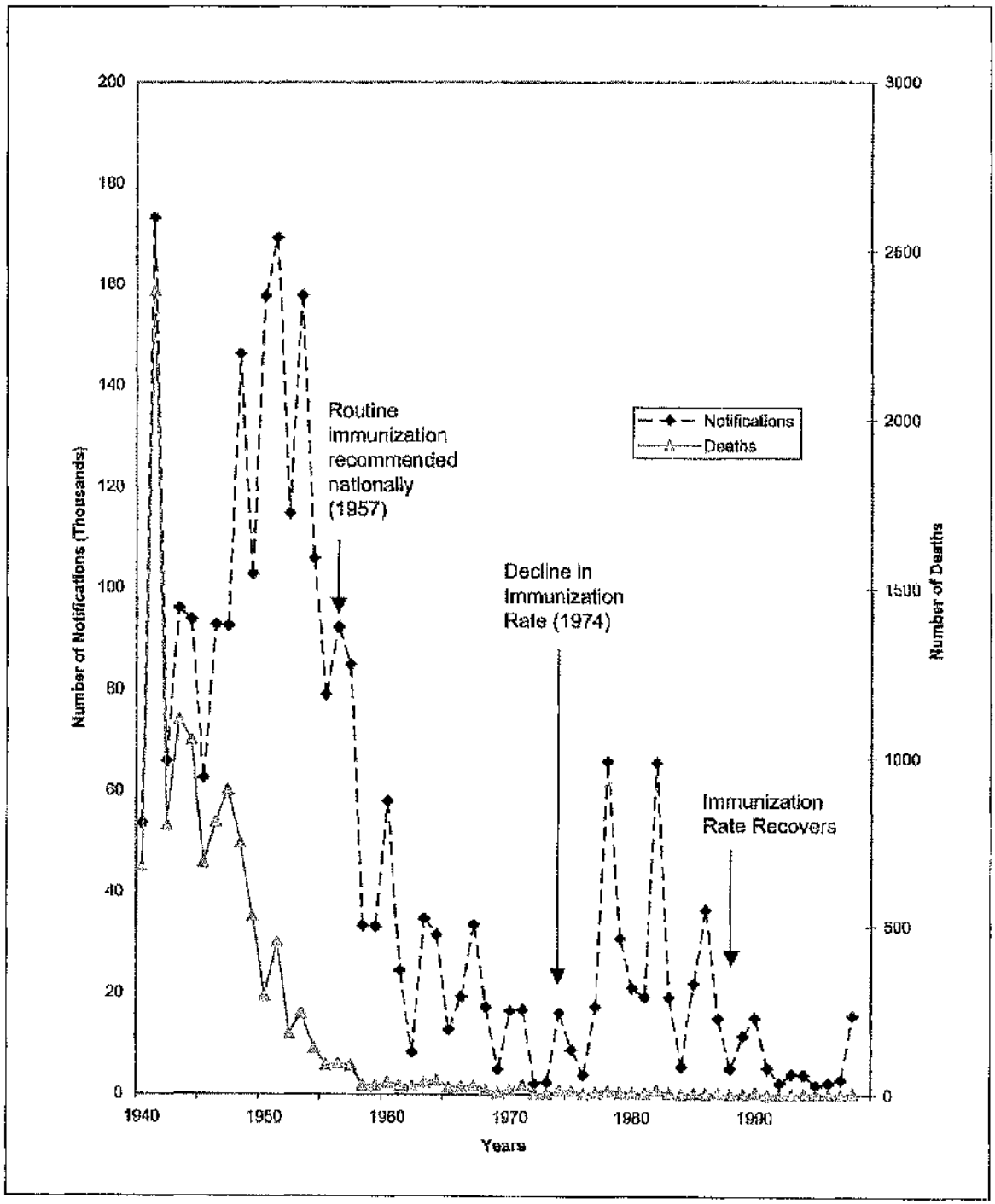
1.3 Diagnosis and treatment

There are several ways to detect pertussis. Traditionally, specimens are collected from the upper respiratory tract by nasopharyngeal aspiration or by using nasopharyngeal swabs for the positive identification of *B. pertussis*. Specimens are cultured on freshly prepared (< 8 weeks old) Bordet Gengou (BG) agar or charcoal agar supplemented with 10% horse blood and 40 mg/L cephalexin and incubated at 37 °C in a humidified atmosphere for 5 - 7 days (Muller *et al.*, 1997). Serological detection tests, such as agglutination, complement fixation and ELISA can be used. Alternatively, the polymerase chain reaction (PCR) has made the detection of *B. pertussis* and diagnosis of infectious disease faster compared with the more conventional methods of detection (Cloud *et al.*, 2003). The use of specific primers also makes it possible to distinguish between species of *Bordetella*.

Generally, erythromycin is used to treat pertussis infection (Hoppe, 1992). In most cases, erythromycin administered during the catarrhal stage of illness is sufficient to

Figure 1 Cases of pertussis in England and Wales, UK

Whooping cough notifications and mortality—England and Wales, 1940 – 1998.



Adapted from Baker (2003).

shorten the duration of illness (Bergquist *et al.*, 1987). For patients who are allergic to erythromycin, trimethoprim-sulphamethoxazole, azithromycin and claithromycin, are also suitable alternatives for the effective treatment of pertussis (Hoppe *et al.*, 1996; Lebel *et al.*, 2001; Langley *et al.*, 2004).

1.4 *Bordetella* virulence regulon

The regulation of virulence by *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. avium* is controlled for the most part by a two-component signal transduction system encoded by the *bvgAS* locus. BvgA is a 23 kDa cytoplasmic protein which has a N-terminus receiver domain and a helix-turn-helix motif at the C-terminus (Boucher and Stibitz, 1995) whereas BvgS is a 135 kDa protein composed of a transmembrane receptor linked to a periplasmic domain, a transmitter, a receiver and a histidine phosphotransfer domain (HPD) (Stibitz and Yang, 1991). When *Bordetella* are grown at 37 °C, the BvgAS phosphorelay system is activated and induces the transcription of *vags* [vir-activated genes] (BvgAS was originally called vir (Weiss and Falkow, 1984) to create the Bvg^+ phase). The periplasmic domain of BvgS autophosphorylates at His729 in the transmitter domain. A phosphate group is then transferred from His729 to Asp1023 of the receiver domain and subsequently onto His1172 of the HPD. Finally, the phosphate can then be transferred to Asp54 on BvgA (Uhl and Miller, 1994; 1996). Phosphorylated homodimers of BvgA bind to regions of DNA containing direct or inverted heptameric consensus repeats, TTTC[C/G]TA, located upstream of the RNA polymerase binding site of Bvg-regulated genes (Boucher *et al.*, 2003). The Bvg^+ phase of *B. pertussis* is characterised by the expression of adhesins such as filamentous haemaggluutinin, fimbriae and pertactin and toxins including pertussis toxin and adenylate cyclase toxin. The genes encoding the virulence factors are temporally regulated with filamentous haemagglutinin being the first to appear upon positive environmental cues in Bvg^+ phenotypes.

In the laboratory, the BvgAS system can be deactivated by changing the incubation temperature from 37 °C to 25 °C or by growing cells in the presence of millimolar concentrations of $MgSO_4$ or nicotinic acid (Melton and Weiss, 1993) which causes the *Bordetella* species to become avirulent (the Bvg^- phase). The Bvg^- phase is characterised by the absence of *vag* expression but a number of *vrgs* (vir-repressed genes) are expressed. The BvgAS phosphorelay system also induces the transcription of a gene, *bvgR*, located downstream of the *bvgAS* operon. BvgR, a 32 kDa protein, negatively regulates the virulence repressed genes (*vrgs*) (Merkel *et al.*, 1998a). Under Bvg^- conditions, *bvgR* transcription ceases, relieving the inhibition of *vrg* transcription.

For *B. bronchiseptica*, the Bvg^- phase is characterised by the expression of several proteins associated with metabolism and αα transport and flagella, responsible for

motility. In contrast, *B. pertussis* and *B. parapertussis* are always non-motile, due to inactivation by multiple insertions of IS elements within the flagellar operons (Parkhill *et al.*, 2003). The Bvg^- phase of *B. pertussis* is characterised by the expression of several outer-membrane proteins of unknown function (Graeff-Wohlleben *et al.*, 1995), although a role for the Bvg^- phase in *B. pertussis* has yet to be identified. A third phase, the Bvg intermediate (Bvg^i) phase, has been identified when *B. bronchiseptica* is grown in sub-modulating conditions, such as growth in the presence of 0.4 - 0.2 mM nicotinic acid. The Bvg^i phase is characterised by the absence of the Bvg-repressed phenotype, the presence of a subset of Bvg-activated virulence factors and the expression of several proteins that are expressed maximally or exclusively in this phase, such as BipA (Deora *et al.*, 2001), a 1576 $\alpha\alpha$ transmembrane protein (Stockbauer *et al.*, 2001). The Bvg^+ phase of *B. pertussis* is necessary for respiratory infection (Merkel *et al.*, 1998b) whereas the Bvg^- phase of *B. bronchiseptica* is better adapted to survive under conditions of extreme nutrient deprivation (Cotter and Miller, 1994; Martinez de Tejada *et al.*, 1998) suggesting that the Bvg^i phase of *B. bronchiseptica* is important for transmission between hosts. The Bvg^i phase of *B. pertussis* appears to be similar to that of *B. bronchiseptica* suggesting that *B. pertussis* may also use this phase for transmission by the aerosol route (Fuchslocher *et al.*, 2003).

In addition to BvgAS, a second two-component regulatory system called RisAS has been described for *B. pertussis* (Stenson and Peppler, 1995) and *B. bronchiseptica* (Jungitz *et al.*, 1998). This consists of a response regulator (RisA) and a sensor kinase (RisS). It appears that only RisA is functionally important in *B. pertussis* as RisA was responsible for the expression of *vrgs*, such as surface-exposed proteins Vra-a and Vra-b, whereas no effect on gene expression was observed in a *B. pertussis risS* mutant (Stenson *et al.*, 2005). In addition, *B. pertussis risA* mutants were less able to invade HeLa cells. In *B. bronchiseptica*, the *ris* locus is essential for bacterial resistance to oxidative stress and the production of acid phosphatase, as well as *in vivo* persistence (Jungitz *et al.*, 1998).

1.5 The virulence factors of *B. pertussis*

B. pertussis secretes a range of adhesins and toxins which are required for adherence and evasion of the immune system, respectively. Filamentous haemagglutinin, pertactin and fimbriae (adhesins), and pertussis toxin and the adenylate cyclase toxin (toxins) are the most studied virulence factors involved in the pathogenicity of *B. pertussis*.

1.5.1 Filamentous haemagglutinin (FHA)

FHA is expressed as a 367 kDa precursor, FhaB, which has a N-terminal signal sequence and a C-terminal region which are both proteolytically-processed to form the

mature 220 kDa FHA form (Jacob-Dubuisson *et al.*, 1996; Renauld-Mongenie *et al.*, 1996). FhaB is transported through the bacterial inner membrane (IM) by the sec-dependent pathway and then channelled through a β-barrel porin structure located on the outer membrane consisting of FhaC, during which 8 – 9 kDa of the N-terminus is cleaved. A subtilisin-like autotransporter/protease, SphB1, has been shown to cleave ~130 kDa of the C-terminus of FhaB (Coutte *et al.*, 2003) which is thought to occur on the cell surface. Mature FHA is monomeric and shaped like a hairpin with head, shaft and tail regions and is roughly 2 nm × 45 nm (Arai and Sato, 1976; Makhov *et al.*, 1994). Along the shaft region and on the hairpin end of FHA are Arg-Gly-Asp (RGD) sequence motifs. *In vitro* studies have shown that these motifs can facilitate adherence to monocytes/macrophages and other leukocytes expressing the CD11b/CD18 (CR3) receptor or via the leukocyte response integrin/integrin-associated protein complex (Ishibashi *et al.*, 1994). Bronchial epithelial cells expressing the very late antigen 5 receptor are also susceptible to *B. pertussis* invasion which is dependent on the presence of RGD motifs on FHA (Ishibashi *et al.*, 2001). A carbohydrate recognition domain and a heparin-binding site have also been identified in FHA (Menozzi *et al.*, 1991; Prasad *et al.*, 1993). Biological effects of FHA include facilitating the attachment *B. pertussis* to the ciliated epithelium of the respiratory tract of host cells (Tuomanen and Weiss, 1985) and induction of phagocytosis of *B. pertussis* by engaging with CR3 (Relman *et al.*, 1990). Interestingly, FHA also increases the surface expression of CR3 on human neutrophil PMNLs *in vitro* (Mobberley-Schuman and Weiss, 2005). However, FHA can inhibit T cell proliferation as a response to antigen stimulation *in vitro* (Boschwitz *et al.*, 1997). It can also stimulate production of cytokines, such as IL-10 in murine macrophages and induce apoptosis of human monocyte-like cells and bronchial epithelial cells (McGuirk and Mills, 2000a; Abramson *et al.*, 2001). In summary, FHA has an important role in adhesion but has an ability to manipulate the immune system which in turn may create more favourable conditions for *B. pertussis* proliferation. An immune response to this antigen could prevent the efficient colonisation of *B. pertussis*. For this reason, FHA is included as part of commercially-available acellular pertussis vaccines (Section 1.7.3).

1.5.2 Pertactin (PRN)

PRN is synthesised as a 95.5 kDa protein encoded by the *prn* gene. PRN consists of a N-terminal signal peptide, a 69 kDa outer-membrane protein segment and a 30 kDa transmembrane C-terminal domain. The N-terminal sequence is recognised by the sec-dependent pathway for passage through the IM and the immature PRN form is navigated to the outer cell membrane where the P30 region acts as an autotransporter by spanning the bacterial membrane to allow the passage of the 69 kDa domain. PRN may remain

membrane bound, extending into the environment or it may be released, by either autoproteolytic activity or cleaved by another outer membrane protease (Henderson *et al.*, 1998). PRN contains a number of RGD motifs as well as several proline-rich regions and leucine-rich repeats indicating that PRN has a role in adhesion (Emsley *et al.*, 1996). PRN, used in subunit vaccines, can enhance protection of mice against respiratory challenge with virulent *B. pertussis* (Novotny *et al.*, 1991; Romanos *et al.*, 1991; Roberts *et al.*, 1993). Thus, PRN is a protective antigen and is now included in many commonly-used acellular pertussis vaccines (Section 1.7.3).

1.5.3 Pertussis toxin (PT)

Of the *Bordetella* species, only *B. pertussis* expresses PT. *B. parapertussis* and *B. bronchiseptica* contain the genes to express PT but due to mutations in the promoter regions, they are transcriptionally silent (Arico *et al.*, 1987). PT, at 106 kDa, is composed of 6 polypeptides, named S1 to S5, encoded by the *ptxA* to *ptxE* genes, respectively. S1 is the A (enzymic) part of the toxin whereas S2 – S5 make up the pentameric B (binding) part of the toxin. The S2, S3, S4 and S5 polypeptides are assembled in a 1:1:2:1 ratio and held together by non-covalent interactions. The combined structure of PT categorises it into the AB_5 family of toxins, like that of cholera toxin of *Vibrio cholerae*. Each polypeptide has a N-terminal signal sequence, suggesting that they are all transported into the periplasmic space by the general export pathway. In the periplasm, the PT subunits are assembled and the holotoxin is then secreted in an active and assembled form (Pizza *et al.*, 1990; Nencioni *et al.*, 1991). The S1 subunit of PT, in its reduced form, can catalyse the transfer of ADP-ribose from nicotinamide adenine dinucleotide (NAD) to the α subunit of guanine nucleotide-binding proteins (G proteins) in eukaryotic cells (Tamura *et al.*, 1982; Katada *et al.*, 1983). When G proteins, such as G_i (inhibitory), G_t (transducing), and G_o, are ADP-ribosylated by PT, they become inactive. G_i normally inhibits adenylyl cyclase and activates K^+ channels, G_t activates cGMP phosphodiesterase and G_o activates K^+ channels, inactivates Ca^{2+} channels and activates phospholipase C-β (Ui, 1990). Disruption of G protein activity by PT leads to increased activity of adenylyl cyclase resulting in increased cAMP levels and to several biological effects *in vivo*, including histamine sensitisation and enhancement of insulin secretion (Munoz, 1981; Pittman, 1984). In addition, PT has been shown to inhibit immune cell functions such as chemotaxis and migration of neutrophil PMNLs, macrophages and lymphocytes (Meade *et al.*, 1984; Brito *et al.*, 1997; Lyons, 1997). PT is also mitogenic and has immunosuppressive (Carbonetti *et al.*, 2004) and adjuvant properties (Section 1.9.3). Chemically-treated or genetically-detoxified PT (dPT) derivatives now form part of all commercially-available acellular pertussis vaccines (Section 1.7.3) as it has been shown that dPT is protective in mice against intracerebral and

acrosol *B. pertussis* challenges (Sato *et al.*, 1984; Sato and Sato, 1988, 1990). Moreover, monocomponent ACVs containing dPT alone have been shown to be efficacious in human field trials (Sato *et al.*, 1984; Trollfors *et al.*, 1997, 1998).

1.5.4 Lipopolysaccharide (LPS)

LPS or endotoxin of *B. pertussis* is structurally and chemically different from well-documented smooth-types of LPS expressed by members of the *Enterobacteriaceae* family or other members of the genus *Bordetella*. Smooth LPS consists of three regions: lipid A, core oligosaccharide and a long polysaccharide O-antigen chain. *B. pertussis* expresses two types of LPS, LPS-A and LPS-B, which are structurally distinct (Peppler, 1984). LPS-B is smaller than LPS-A and consists of lipid A linked via a single ketodeoxyoctulosonic acid (KDO) residue to a branched oligosaccharide core structure containing various sugars including heptose and glucose. By contrast, LPS-A consists of LPS-B plus three different sugar moieties; *N*-acetyl-*N*-methylfucosamine, 2,3-deoxy-di-*N*-actylmannuronic acid and *N*-acetylglucosamine (Caroff *et al.*, 1990; Lasfargues *et al.*, 1993). The structure of *B. pertussis* LPS is different from that of *B. parapertussis* and *B. bronchiseptica* in that it lacks a repetitive O-antigenic structure, a feature similar to that of rough LPS or lipooligosaccharide (LOS) of *Enterobacteriaceae* (Martin *et al.*, 1992; Preston *et al.*, 1996). The O-antigen biosynthesis locus in *B. pertussis* has been disrupted by an IS element (Preston *et al.*, 1999, Parkhill *et al.*, 2003). The genes which code for LPS are found in the *wlb* locus of *Bordetella* species (Allen and Maskell, 1996; Preston *et al.*, 2002).

LPSs from *Bordetella* species are pyrogenic, mitogenic and toxic (Ayme *et al.*, 1980; Watanabe *et al.*, 1990). The role of LPS in pathogenicity has been investigated using *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* LPS mutants (Harvill *et al.*, 2000). In a mouse model of respiratory infection, *B. pertussis*, *B. bronchiseptica* and human *B. parapertussis* strains expressing only LPS-B showed decreased colonisation. The importance of O-antigen was also investigated, as the O-antigen chains are thought to prevent complement binding to the surface of the bacterial cell. Moreover, of these three species, only *B. pertussis* does not express it. Most *B. pertussis* strains, when grown *in vitro*, are somewhat sensitive to killing by naïve serum but serum sensitive strains recovered from mice showed increased resistance to serum killing (Pishko *et al.*, 2003). This is possibly mediated by BrkA, a virulence factor which has serum resistance properties (Fernandez and Weiss, 1994) or it may be due to changes in *B. pertussis* LPS itself (Schaeffer *et al.*, 2004).

1.5.5 Adenylate cyclase toxin (CyaA)

CyaA (also known as: ACT [adenylate cyclase toxin]/AC-hly [adenylate cyclase haemolysin]) of *B. pertussis* belongs to the Repeats in ToXin (RTX) family. RTX toxins have a series of C-terminal nonapeptide Gly/Asp rich repeats which vary from six to 40 repeats between members. The RTX family includes the prototype α-haemolysin of pathogenic *E. coli* (HlyA) and the leukotoxins of *Pasteurella haemolytica* (LtxA) and *Actinobacillus* spp. (Welch, 1991; Coote, 1992; Stanley *et al.*, 1998). As with all RTX members, the pro-toxin is post-translationally modified by an acyl transferase in order to facilitate interaction with cells to exert their toxic effects (Welch, 1991; Coote, 1992).

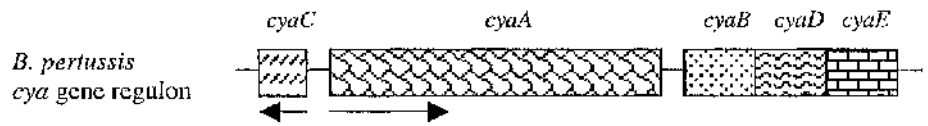
CyaA was first described as a constituent of *B. pertussis* commercial WCVs in the early 1970s (Wolff and Cook, 1973) and was later found associated with the cell surface of *B. pertussis* (Hewlett and Wolff, 1976). Subsequently, two sizes of CyaA, at 45 kDa and 220 kDa, were described (Rogel *et al.*, 1988; Hewlett *et al.*, 1989; Masure and Storm, 1989). However, it was shown that the toxic form of CyaA was approximately 220 kDa (Hewlett *et al.*, 1989; Rogel *et al.*, 1989) which could be cleaved proteolytically to produce a smaller protein with a M_w between 45 – 47 kDa (Ladant *et al.*, 1986; Rogel *et al.*, 1988; Bellalou *et al.*, 1990a). This smaller fragment was shown to have adenylate cyclase (AC) enzymic activity (Gilboa-Ron *et al.*, 1989). The 220 kDa protein was later cloned and expressed from *E. coli* (Brownlie *et al.*, 1988; Glaser *et al.*, 1988b).

The structural gene, *cyaA*, was found to encode a 1706 residue bi-functional protein that contained a N-terminal AC domain (αα 1 - 400) and a C-terminal haemolytic/pore-forming domain (αα 401 - 1706) (Glaser *et al.*, 1988a): no other member of the RTX toxin family has an enzymic domain. CyaA is also produced by *B. bronchiseptica*, *B. parapertussis* and *B. hinzii* (Endoh *et al.*, 1980; Betsou *et al.*, 1995b; Donato *et al.*, 2005). Moreover, the CyaAs of *B. pertussis* and *B. bronchiseptica* share 98% αα homology and contain identical domains for AC activity, calmodulin binding, pore-forming activity and Ca^{2+} binding. The CyaAs of *B. parapertussis* and *B. bronchiseptica* have similar activities to that of CyaA of *B. pertussis* but the toxins are antigenically distinct (Gueirard and Guiso, 1993; Khelef *et al.*, 1993) which is likely due to amino acid differences in the second and third group of Gly/Asp rich motifs (Betsou *et al.*, 1995b). Interestingly, CyaA expressed from *B. hinzii* is non-haemolytic and cannot increase intracellular cAMP in mouse macrophages, despite the presence of AC activity as determined by the conversion of radiolabelled ATP to cAMP in a cell-free system (Donato *et al.*, 2005). This may be attributed by the lack of *cyaC* expression (Donato *et al.*, 2005) which codes for an acyl transferase required to palmitoylate CyaA (Section 1.5.5.3.1).

1.5.5.1 Gene organisation

The *cyaA* operon consists of an '*A*' pro-toxin gene followed by the '*B*', '*D*' and '*E*' genes (Fig. 2) and has a common layout like that of other RTX members (Welch, 1991; Coote, 1992).

Figure 2 Schematic diagram of the *cya* gene operon in *B. pertussis*



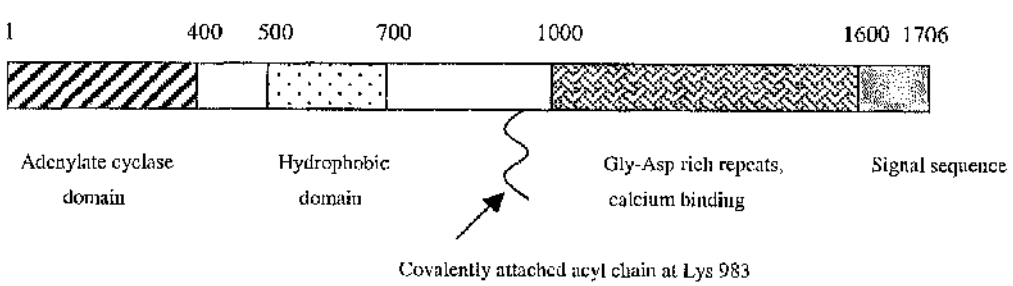
Arrows indicate direction of transcription.

cyaC is found upstream with an ORF on the opposite strand to the *cyaA*, *cyaB*, *cyaD* and *cyaE* genes and is transcribed in the opposite direction, under the control of its own promoter (Barry *et al.*, 1991). A Bvg promoter is located upstream of *cyaA* which means that, under suitable environmental conditions, CyaA production is switched on (Goyard and Ullman, 1993) by the BvgAS phosphorelay system (Karimova *et al.*, 1996). The *cyaB*, *cyaD* and *cyaE* genes encode a 712 αα putative transmembrane protein (located on the inner bacterial envelope), a 440 αα anchored cytoplasmic membrane protein, and a 474 αα outer membrane protein, respectively. The proteins all assemble across the bacterial envelope and excrete CyaA directly into the external medium without a periplasmic intermediate (Glaser *et al.*, 1988b), analogous to the *E. coli* α-HlyA Type I secretion system (Hartlein *et al.*, 1983). However, 90% of adenylate cyclase activity is located on the bacterial cell surface and it has been suggested that FHA has a role in retaining CyaA on the bacterial surface (Zaretzky *et al.*, 2002). A recent study by Gray *et al.* (2004) demonstrated that only newly secreted CyaA was responsible for intoxication of cells and not surface bound CyaA.

1.5.5.2 N-terminal adenylate cyclase domain

The AC domain is located on the N-terminus of the toxin comprising the first 400 αα (Fig. 3).

Figure 3 Schematic diagram of the *B. pertussis* CyaA protein



Tryptic digestion of the 400 αα AC domain generates two fragments: T25 (αα 1 - 224) and T18 (αα 225 - 399) (Glaser *et al.*, 1991). T25 possesses the catalytic domain and T18 contains the calmodulin binding site (Ladant, 1988; Munier *et al.*, 1991). Several helical structural elements of the AC domain interact with calmodulin (Guo *et al.*, 2005). A separate study of the AC domain described three important regions which have significant roles in catalysing the cAMP reaction (Gordon *et al.*, 1989). These domains consist of 13 - 24 αα residues, with structural homology (66 - 80%) to the AC domain of the *Bacillus anthracis* oedema factor (Gordon *et al.*, 1989). Lysines 58 and 65, within the first domain (αα 54 - 77), form the catalytic substrate-binding site (Glaser *et al.*, 1991). Asp 188 and Asp 190, in between αα 184 - 196, are thought to be involved in a putative stabilising complex with ATP-Mg^{2+} to allow efficient catalysis to occur (Oldenberg and Storm, 1993). αα 184 - 196 and αα 294 - 314, bracket a helical structure around Trp242, shown to interact with calmodulin (Glaser *et al.*, 1989; Ladant *et al.*, 1989; Glaser *et al.*, 1991).

1.5.5.3 C-terminal haemolytic/pore-forming domain

Depicted as from αα 401 - 1706 (Glaser *et al.*, 1988a) (Fig. 3), the haemolytic/pore-forming domain consists of a post-translational modification site(s), 42 Gly/Asp rich repeats, hydrophobic domains, an unprocessed secretion signal and a putative AC translocating domain.

1.5.5.3.1 Acylation for activation

The *cyaC* gene product is an acyl transferase which covalently attaches fatty acyl chains onto the pro-toxin (Barry *et al.*, 1991; Hackett *et al.*, 1994) and this process is also required for the haemolytic and cytotoxic activities of CyaA (Barry *et al.*, 1991; Hewlett *et al.*, 1993; Heveker *et al.*, 1994). *B. pertussis* CyaA is solely palmitoylated by an ε-amide linkage at Lys983 (Hackett *et al.*, 1994). However, with the advent of recombinant forms of CyaA (rCyaA) expressed in *E. coli*, it was shown that rCyaA is acylated on both Lys983 and Lys860 (Hackett *et al.*, 1995) by a mixture of fatty acids: 87% palmitoylation (C_{16}) occurs at Lys983 but 67% of CyaA molecules are palmitoylated at Lys860 and the rest is myristoylated (C_{14}) (Hackett *et al.*, 1995). Acylation on both Lys983 and Lys860 is thought to be the reason for the reduced haemolytic activity of rCyaA compared with native CyaA expressed from *B. pertussis* which is only acylated on Lys983 (Sebo *et al.*, 1991; Hackett *et al.*, 1994; Basar *et al.*, 1999).

1.5.5.3.2 Gly/Asp rich nonapeptide repeats and calcium binding

CyaA has 42 Gly/Asp rich nonapeptide repeats located between αα 1007 - 1706 (Glaser *et al.*, 1988a). Ca^{2+} ions bind to these repeats which have a consensus sequence

GGXGXDXLX, arranged in 5 groups separated by 20 - 30 residue-long α-helix forming αα (Rose *et al.*, 1995). A small number of nonapeptide Gly/Asp rich repeats are also found in the alkaline protease of *Pseudomonas aeruginosa*, of which the 3D structure has been solved (Baumann *et al.*, 1993). Data from this structure indicate that the repeats are organised in a β-roll consisting of 2 opposing sheets of parallel β-strands comprised of the last 5 αα of each nonapeptide repeat (XDXLX) connected by loops derived from the first 4 αα of the repeated sequence (GGXG). Ca^{2+} is hexacoordinated between two adjacent loops of the β-roll, and as a whole, each repeat binds one Ca^{2+} ion (Rose *et al.*, 1995). CyaA has 3 - 5 high and ~40 low affinity Ca^{2+} binding sites (Rose *et al.*, 1995). In the presence of Ca^{2+}, CyaA undergoes a conformational change (Hewlett *et al.*, 1991; Rose *et al.*, 1995; Rhodes *et al.*, 2001; Bauche *et al.*, 2006) perhaps by the formation of β-sheet helices within the repeat domain of CyaA (Rhodes *et al.*, 2001) which enables interaction with target cells (Knapp *et al.*, 2003). HlyA and CyaA both require calcium to cause haemolysis in erythrocytes, and this is inhibited in the presence of ion chelators, such as EGTA and EDTA (Hanski and Farfel, 1985; Gentile *et al.*, 1988; Ludwig *et al.*, 1988; Boehm *et al.*, 1990a and b). Some studies have shown HlyA and CyaA to be haemolytic in the presence of these ion chelators (Bhakdi *et al.*, 1986; Rogel *et al.*, 1991). However, it may be that Ca^{2+} ions, from growth media or through purification procedures, can bind tightly to the high affinity Ca^{2+} binding sites and cannot be removed by chelation, resulting in the changes in structure required for membrane interaction and haemolysis.

1.5.5.3.3 Secretion of CyaA

An unprocessed secretion signal is encoded at the 3' end of *cyaA* (Sebo and Ladant, 1993). *In vitro* complementation studies, with truncated CyaA forms, determined that residues contained within the last 217 residues of CyaA, were essential for toxin secretion (Iwaki *et al.*, 1995; Bejerano *et al.*, 1999).

1.5.5.4 Haemolytic and cytotoxic functions of CyaA

Acylated CyaA preferentially interacts with and invades mammalian target cells that express the $\alpha_M\beta_2$ integrin receptor CD11b/CD18 (Guermonprez *et al.*, 2001) which is found on granulocytes, neutrophil PMNLs, macrophages, dendritic cells (DCs), myeloid cells, natural killer (NK) cells, $CD8^+$ T-cells and on B-cells (Bell *et al.*, 1999). Several authors (Szabo *et al.*, 1994; Hackett *et al.*, 1995; Gray *et al.*, 1998; Basler *et al.*, 2006a; Hewlett *et al.*, 2006) suggest that, in the presence of calcium, CyaA acts in two parallel and distinct ways after membrane insertion. One results in the insertion of CyaA monomers into the target membrane followed by the formation of oligomeric channels or pores (Osickova *et al.*, 1999; Knapp *et al.*, 2003; Section 1.5.5.4.1). The second, after CyaA

membrane insertion by toxin monomers, is the direct translocation of the AC domain into cells to cause a supraphysiological increase in cAMP after activation of the AC enzymic moiety by host calmodulin (a process referred to as intoxication) (Gray *et al.*, 1998; Basler *et al.*, 2006a; Hewlett *et al.*, 2006). The consequences of increased cAMP levels within phagocytes are discussed in Section 1.5.5.4.2.

1.5.5.4.1 Haemolysis and pore-formation by CyaA

CyaA has also been shown to form small (0.6 - 0.8 nm diameter) transient, ion-permeable channels in target membranes (Bellalou *et al.*, 1990b; Ehrmann *et al.*, 1991; Rogel *et al.*, 1991; Benz *et al.*, 1994). No erythrocyte receptor has been found for CyaA although recent work by Vojtova *et al.* (2006) suggested that clusters of ~20 CyaA toxin molecules co-localise in erythrocyte membrane microdomains (rafts), that are typically enriched in gangliosides, sphingomyelin and cholesterol (Simons and Ikonen, 1997), further supporting the role of an oligomeric structure which produces small pores or channels. However, other authors suggest that CyaA oligomers consist of only 2 - 4 toxin monomers (Iwaki *et al.*, 1995; Lee *et al.*, 2005). Certainly, pores have not been visualised by electron microscopy.

Haemolysis of sheep erythrocytes requires greater concentrations of CyaA and longer incubation periods compared with intoxication (Hewlett *et al.*, 1989; Bellalou *et al.*, 1990b; Ehrmann *et al.*, 1991; Rogel *et al.*, 1991; Szabo *et al.*, 1994; Gray *et al.*, 1998). Haemolysis occurs only very slowly at 0 – 2 °C and is significantly reduced compared with incubation at 37 °C (Gray *et al.*, 1998). A hydrophobic segment, from αα 500 - 700, is suggested to play a critical role in the penetration of cells by the predicted formation of four membrane-spanning domains (Hanski and Coote, 1991). Mutant CyaAs that lack Gly/Asp rich repeats show a reduced haemolytic capacity (Knapp *et al.*, 2003). In addition to haemolysis, it was shown by scanning electron microscopy, that CyaA could induce morphological changes of erythrocytes, such as shrinkage, formation of membrane projections, blebbing and swelling (Vojtova *et al.*, 2006).

1.5.5.4.2 Intoxication

Once CyaA has bound onto the target cell, it is able to translocate the AC domain across the membrane. The translocation of the enzymic domain of *B. anthracis* oedema factor into target cells is calcium-dependent and happens after 10 min, suggesting receptor-mediated endocytosis (Gordon *et al.*, 1989). However, CyaA is believed not to enter cells by this route (Donovan and Storm, 1990) since intracellular AC activity is detected within seconds (Gordon *et al.*, 1989). This implies that the AC domain is translocated directly across the plasma membrane (Otero *et al.*, 1995; Khelef *et al.*, 2001). Residues around and

within αα 400 - 500 of CyaA are reported to play an important role in the translocation of the AC moiety into target cells (Gray *et al.*, 2001). Point mutations at αα 509 and αα 516 reduce the translocation capacity of the AC domain and monoclonal antibodies which bind to αα 373 - 399 of CyaA inhibit intoxication of target cells. Residues 373 - 489 are thought to undergo a conformational change, potentially forming an amphipathic α-helical segment, allowing the intracellular delivery of the AC domain (Osickova *et al.*, 1999) which can be hindered by random insertional mutagenesis of CyaA (Osicka *et al.*, 2000). Generally, such a dramatic increase of intracellular cAMP levels can impair and inhibit immune effector cell functions (Bourne *et al.*, 1971; Bourne *et al.*, 1974). Indeed, the increase of cAMP by CyaA in phagocytes impairs chemotaxis (Confer and Eaton, 1982), inhibits the oxidative burst (Pearson *et al.*, 1987), inhibits phagocytosis (Bassinet *et al.*, 2000) and induces apoptosis (Khelef and Guiso, 1995; Gueirard *et al.*, 1998; Bachelet *et al.*, 2002) leading to cell death. Intracellular cAMP levels are proportional to the amount of CyaA being introduced into cells as CyaA is subject to intracellular degradation (Friedman *et al.*, 1987; Gilboa-Ron *et al.*, 1989). Furthermore, it was generally thought that acylation was mandatory for interaction with target cells for intoxication (Barry *et al.*, 1991; Hewlett *et al.*, 1993). However, recent studies have shown that high concentrations of non-acylated CyaA can intoxicate macrophages by delivery of the catalytic domain (Boyd *et al.*, 2005; Hewlett *et al.*, 2006) but such toxins have a reduced propensity to oligomerise and form pores (Lee *et al.*, 2005). Intoxication by non-acylated CyaA may be explained by the ability of non-acylated CyaA to bind, with less affinity, to cells expressing the CR3 receptor (El-Azami-El-Idrissi *et al.*, 2003).

1.5.5.5 CyaA as a virulence factor

The generation of a *B. pertussis* Tn5 transposon mutant, defective in the production of CyaA, provided evidence that CyaA was an important virulence factor (Weiss *et al.*, 1984). The *B. pertussis* CyaA⁻ mutant was considerably less virulent than the wild-type *B. pertussis* parent strain following murine intranasal challenge because it was rapidly cleared from the lungs with no viable bacteria remaining at 10 days post challenge (Weiss and Goodwin, 1989; Goodwin and Weiss, 1990).

Other studies showed that *B. pertussis* CyaA mutants were severely compromised in their ability to adhere and to multiply in mouse lungs (Khelef *et al.*, 1992; Carbonetti *et al.*, 2005). These data indicate that CyaA has an important role during *B. pertussis* infection. Mice infected with these CyaA mutants caused little cellular influx into bronchoalveolar lavage fluids compared with parental virulent strains (Khelef *et al.*, 1994; Carbonetti *et al.*, 2005). However, Carbonetti *et al.* (2005) suggested that CyaA may have a role later on during *B. pertussis* infection by intoxicating arriving phagocytes.

1.5.6 Other virulence factors of *B. pertussis*

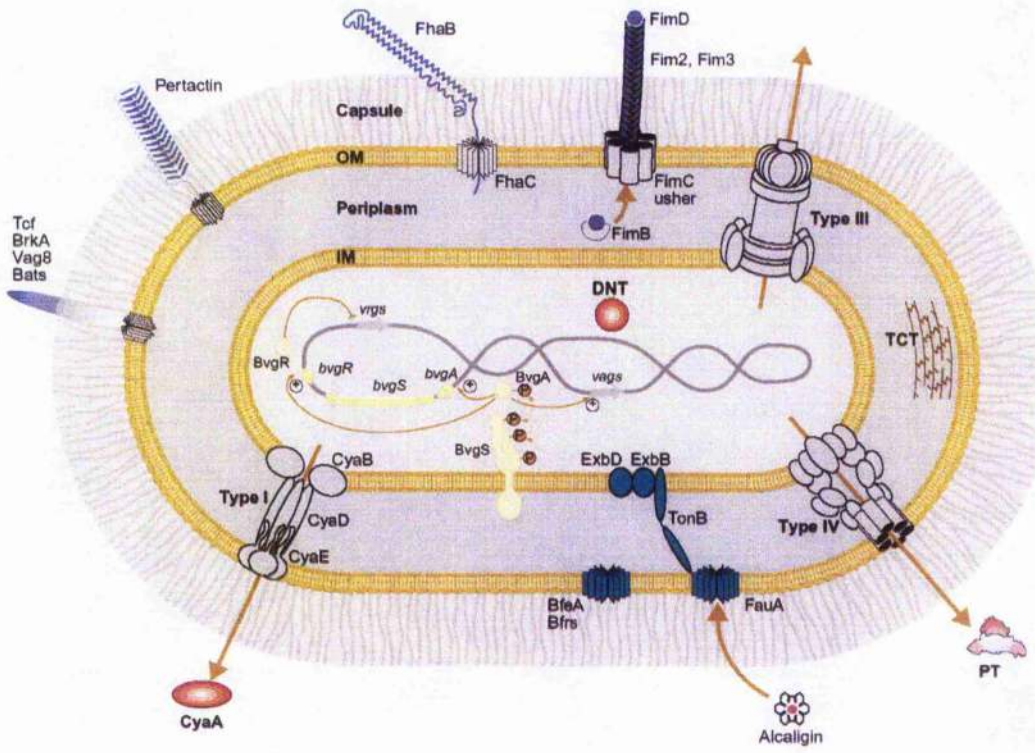
B. pertussis secretes a wide range of other virulence factors (Fig. 4). These include serotype-specific fimbriae 2 and 3 and tracheal colonisation factor (TCF) (involved in adhesion), dermonecrotic toxin (DNT) and tracheal cytotoxin (TCT) (toxins) and autotransporter proteins, such as BrkA and Vag8. A study by Hot *et al.* (2003) confirmed that these genes were up-regulated in *B. pertussis* in the Bvg^+ phase using microarray technology. With the available data from the *Bordetella* sequencing project, the identification of new *vags* and *vrgs* is likely in the near future.

1.6 Immunity to infection

The immune system is an organisation of cells and molecules with specialised roles in the defence against infection. An immune response involves recognition of the pathogen or foreign material (antigen), such as bacterial lipopolysaccharide (LPS), lipoteichoic acids, bacterial DNA and double–stranded RNA, and then a reaction to eliminate them. The immune system has evolved two types of responses to infection; innate (non-adaptive) and adaptive immunity. Innate immunity consists of physiological barriers and cellular defences which include phagocytic cells (neutrophil PMNLs, monocytes and macrophages), cells that release inflammatory mediators (basophils, mast cells and eosinophils) and natural killer cells. The innate immune responses also use molecular components such as chemokines, cytokines and complement. Cytokines have a direct role in defence by acting as messengers both within the immune system and other systems of the body, forming an integrated network that is highly involved in the regulation of immune responses (Roitt *et al.*, 1998). Chemokines are a superfamily of more than 40 members and are important for cell activation, differentiation and trafficking (Nickel *et al.*, 1999). Chemokines are also involved in many biological processes which include cell proliferation, apoptosis and in host defences (Gerard and Rollins, 2001; Murakami *et al.*, 2004). Complement is group of about 20 serum proteins which interact with each other or with cells of the immune system, such as phagocytes. Complement can activate phagocytes, induce pores in the surface of Gram-negative bacteria, enveloped viruses and other organisms non-specifically or by opsonising (coating) the surface of micro-organisms and immune complexes, so that they can be recognised by cells expressing complement receptors (Roitt *et al.*, 1998). The effector mechanisms of innate immunity, are activated immediately after infection and rapidly control the replication of the infecting pathogen. Microorganisms coated with antibodies and/or complement are engulfed by phagocytes and then subjected to a wide range of toxic intracellular molecules, including superoxide anions, nitric oxide, antimicrobial proteins and lysozyme.

Figure 4 The virulence factors of *B. pertussis*

B. pertussis is depicted as a Gram-negative organism with inner and outer membranes (IM and OM), a periplasm and a capsule. The adhesins, such as Fim, FhaB, pertactin, TCF (tracheal colonisation factor), BrkA (conveys resistance to killing by complement) and Vag8 (adhesin) are shown in blue; the toxins, such as PT, CyaA and DNT (dermonecrotic toxin) are in red; the accessory proteins, such as FhaC, FimB and FimC are in grey; the iron uptake systems are in green; and the regulatory systems BvgA, BvgS and BvgR are in beige. The large brown arrows represent the orientation of export and import of virulence factors and siderophores, respectively. The thinner brown arrows show the phosphorelay and the regulation circuit. The type III secretion system, resembling a needle-like projection, allows *B. pertussis* to translocate effector proteins directly into the plasma membrane or cytoplasm of eukaryotic cells.



Adapted from Locht *et al.* (2001).

Most pathogens are encountered after they are inhaled or ingested. Responses to inhaled antigens occur in the lingual, palatine and pharyngeal adenoids. The respiratory mucosa contains dendritic cells (DCs) for uptake, processing and transport of antigens to the draining lymph nodes. T-lymphocyte receptors (TLRs), such as those expressed on phagocytes, recognise antigens and activate signal-transduction pathways that induce the expression of a variety of immune-response genes, including inflammatory cytokines. The signals induced on recognition of foreign antigens by the innate immune system, in turn, control the activation of adaptive immune responses. For this reason, containing the infection until the lymphocytes can begin to deal with it has long been considered the main function of innate immunity.

In adaptive immunity, the proliferation of naïve immune cells (e.g. T cells (or T lymphocytes) and B cells) during the first encounter with an antigen generates both effector immune cells and antibody-secreting plasma cells as well as 'memory cells'. The memory cells enable a superior secondary immune response to be mounted after a subsequent encounter with the same antigen. Because memory cells are increased in number relative to naïve cells and also more readily triggered, the secondary response is more rapid than the primary immune response. It produces a larger number of T cells and, in the case of B cells, induces greater levels of antibody with an affinity for the antigen than the levels of antibody achieved by the primary response.

T cells form an important part of the adaptive immune response. Approximately 5 - 10% of T cells express the γ/δ T-cell receptor (TCR) but this receptor does not recognise antigen in the form of peptide-MHC complexes (discussed below). γ/δ T cells can be found in mucosal epithelia and may be important in protecting the mucosal surfaces of the body (Roitt *et al.*, 1998). The remaining population of T cells express the α/β TCR which can recognise a complex that is formed by a peptide seated within the groove of a MHC molecule expressed by antigen-presenting cells (APCs). Mature α/β T cells express either CD4 or CD8 molecules and, together with CD3, they form an essential part of the TCR complex. $CD8^+$ T-cells are usually cytotoxic and recognise antigen-derived peptide presented by MHC class I molecules expressed by most somatic cells. By contrast, $CD4^+$ T cells usually act as helper T-cells and recognise antigen presented by MHC class II molecules normally expressed by a subgroup of immune cells that includes B cells, macrophages and DCs. Activated DCs are a key cellular component of the innate immunity and are particularly efficient at initiating (priming) naïve T cells because they express large amounts of co-stimulatory B7-1 and B7-2 (also known as CD80 and CD86, respectively) and CD40 molecules. B7 and CD40 molecules bind with CD28 and CD154, respectively and help to activate the cells.

Cross-linking of the TCR, which occurs when it binds to peptide-MHC complexes on cell surfaces of an APC, initiates phosphorylation of tyrosines in the cytoplasmic tails of the CD3 complex. The presence of other surface protein interactions between the T cell and APC facilitate this process, e.g. B7 and CD40 interaction. The transduction of the downstream signal to the nucleus initiates the transcriptional activation of various genes. Thus, APCs can activate helper $CD4^+$ T cells by presenting peptide-MHC class II complexes. $CD4^+$ T cells can be divided into two major sub-populations characterised by their cytokine secretion profile. Generally, type 1 helper (Th1) T-cells secrete IL-2 and IFNγ but not IL-4, IL-5, or IL-6. Type 2 helper (Th2) T-cells secrete IL-4, IL-5, IL-6, and IL-10 but not IL-2 or IFNγ. Table 1 lists a few commonly described cytokines, their sources of production and their effects. Cytokines have a central role in influencing the type of immune response needed for optimal protection against particular types of infectious agents. For example, the release of IL-12 by APCs stimulates the production of IFNγ by Th1 cells. IFNγ also efficiently activates macrophages, enabling them to kill intracellular organisms. However, not all signals from cytokines and cell-surface molecules are stimulatory. For example, IL-10 often antagonises Th1 responses. T cells expressing cytokines associated with both Th1 and Th2 cells are termed Th0 cells (Mosmann *et al.*, 1989).

The B cell also mirrors this process of signal transduction. B cells can produce different subclasses of IgA and IgG, some of which have different roles in immunity. For example, IgG2a subclasses (in the mouse) are considered to be responsible for opsonisation and complement fixation whereas IgG1 may be more important in neutralising toxins and inhibiting bacterial adherence. The production of cytokines by Th1 cells facilitates cell-mediated immunity, including the activation of macrophages and T-cell-mediated cytotoxicity; on the other hand, Th2 cells help B cells produce antibodies. However, it is now no longer reasonable to consider cell-mediated and antibody-mediated responses separately, as no cell-mediated response is likely to occur in the total absence of antibodies.

1.7 Immunity to pertussis

In recent years, an increasing number of cases of pertussis have been reported in more mature age groups, specifically adolescents and adults (Howlett and Edwards, 2005). Although vaccination has markedly reduced the incidence of pertussis in developed countries, vaccine-induced immunity to pertussis weakens considerably from young adulthood, about 5 years after vaccination (Fine and Clarkson, 1982).

Table 1 A limited sample of cytokines, listed with their principal activities, sources and targets

Cytokine	Immune system source	Principal targets	Principal effects
IL-1β	Macrophages and other antigen-presenting cells (APCs)	T cells, B cells, macrophages, endothelium, tissue cells	Enhancement of T cell activation in response to antigen, macrophage stimulation
IL-2	Activated Th1 cells	T cells	Proliferation of B cells and activated T cells, activation of cytotoxic T-lymphocytes (CTLs) and macrophages
IL-4	Th2 cells and mast cells	T cells, B cells	B-cell growth factor, isotype selection, IgE, IgG1. Induction of MHC class II expression on B cells. Inhibition of chemokine production
IL-5	Th2 cells and mast cells	B cells	B-cell growth and differentiation, IgA selection
IL-6	Activated Th2 cells, APCs and other somatic cells	B cells	B cell differentiation, synergistic with IL-1 and TNF on T cells
IL-8	Macrophages other somatic cells	Neutrophil PMNLs, T cells	Chemoattractant for neutrophil PMNLs and T cells, superoxide release
IL-10	Activated Th2 cells, $CD8^+$ T and B cells, macrophages	Th1 cells	Inhibition of cytokine synthesis, promotes B cell proliferation and antibody production, suppresses cellular immunity
IL-12	Macrophages and B cells	T cells	Induction of Th1 cells, IFNγ production
TNFα	Activated macrophages	Macrophages, tissue cells	Activation of macrophages and CTLs, enhanced MHC class I production, induces signalling pathways that lead to proliferation
IFNγ	Activated Th1 and natural killer cells	Leukocytes, tissue cells, Th2 cells	MHC class I and II induction, neutrophils PMNLs and macrophage activation, macrophage cytokine synthesis, promotes Th1 responses
GM-CSF	Activated T cells and macrophages	Macrophage precursors	Proliferation of granulocyte and macrophage precursors and activators

Adapted from Roitt *et al.* (1998) and from

http://www.biosource.com/content/literatureContent/methodPDFs/CytokineMethods.pdf

These data support the conclusion that vaccination in infancy does not afford long-term protection and indicates a need for booster immunisation in older age groups (Jenkinson, 1988; Ruuskanen *et al.*, 1991; Tran Minh *et al.*, 1998; Dagan *et al.*, 1999; Miller *et al.*, 2000; Edmunds *et al.*, 2002; Greenberg, 2005). There is increasing recognition that infected adolescents and adults can transmit pertussis to infants too young to be vaccinated (He *et al.*, 1994a; Gilberg *et al.*, 2002; Crowcroft *et al.*, 2003a). In many developing countries, where vaccine coverage is low, whooping cough still remains a major problem, especially among young infants less than 6 months of age. Vaccines to pertussis have been in circulation since the 1940s. However, as will be discussed, a difference is seen in the immune response after natural infection and the types of vaccines used to immunise individuals.

1.7.1 Immune responses to pertussis infection

Several studies in humans infected with pertussis have shown high IgG and IgA antibody titres against several *B. pertussis* antigens, such as lipooligosaccharide (LOS), FHA, PRN, and fimbriae (Ashworth *et al.*, 1983; He *et al.*, 1994b; Mink *et al.*, 1994; Trollfors *et al.*, 1999) suggesting a role of antibodies against pertussis infection. Indeed, Munoz *et al.* (1981) showed that serum which contained antibodies to PT were able to passively protect mice against intracerebral challenge with *B. pertussis*. In contrast, serum containing only antibodies to FHA were unable to protect mice against intracerebral challenge with *B. pertussis*. $Ig^{-/-}$ mice, which are defective in B cells and antibody production, developed a chronic infection after aerosol challenge with *B. pertussis* (Mahon *et al.*, 1997). Full protection in $Ig^{-/-}$ mice immunised intranasally three times with formalin fixed *B. pertussis* was only observed after transfer of *B. pertussis*-immune B cells (Leef *et al.*, 2000).

B cells can act as APCs for T cells, facilitating immune responses to foreign antigens. Thus, it was not surprising to find that T cells also play a role in immunity against *B. pertussis* infection. T and B cell-deficient (*scid*) mice succumbed to death at 3 weeks post-aerosol infection with *B. pertussis* (Barbic *et al.*, 1997). In addition, Mills *et al.* (1993) showed that adoptive transfer of *B. pertussis* specific $CD4^+$ T cells, but not immune $CD8^+$ T cells, from mice primed by *B. pertussis* infection, were capable of conferring protection to athymic mice. Another mouse model of infection demonstrated that $Ig^{-/-}$ mice could be partially protected by intranasal immunisation with formalin fixed *B. pertussis* in the absence of antibodies (Leef *et al.*, 2000).

Several studies have investigated the type of immune response as a result of *B. pertussis* infection in humans. *B. pertussis* antigen-specific T-cell clones from human adults and children have been shown to produce IFNγ and IL-2 (both Th1-associated

cytokines) in response to antigen-stimulation *in vitro* (Pepploni *et al.*, 1991; Hafler *et al.*, 1998; Mascart *et al.*, 2003). Moreover, *B. pertussis* antigen-specific peripheral blood mononuclear cells from *B. pertussis*-infected or convalescent children also produced produce IFNγ and IL-2, but undetectable IL-4 or IL-5 (Ryan *et al.*, 1997b) following antigen-stimulation *in vitro*. IFNγ (a Th1-inducing cytokine) has also been shown to enhance the ability of macrophages to kill *B. pertussis in vitro* (Torre *et al.*, 1994; Mahon *et al.*, 1999). These studies provide evidence that strong cell-mediated responses are induced during and after natural infection with *B. pertussis*. In addition, strong cell-mediated responses are required to protect against natural infection with *B. pertussis*.

1.7.2 Immune responses to whole-cell pertussis vaccines

The high mortality rates due to pertussis infection prompted the production of preventative methods for pertussis. Soon after *B. pertussis* was first isolated, initial vaccines were made and consisted of killed whole *B. pertussis* cells. During the 1940s, whole-cell vaccines (WCVs), made from formaldehyde-treated *B. pertussis* cells, were introduced to reduce the global incidence of pertussis (Sato and Sato, 1999; Mattoo and Cherry, 2005). These vaccines were extremely effective in protection against *B. pertussis* infection. By the late 1940s, combined WCVs were introduced which included diphtheria and tetanus toxoids (DTP). Despite the high efficacy of these vaccines, numerous side-effects were reported after immunisation. These included redness, swelling and pain at the site of immunisation. Other types of symptoms such as drowsiness, fever, vomiting and persistent crying were observed after repeated immunisation doses. Finally, there has been much concern with the association of neurological disease and death with WCVs. For example, in the UK, extensive media coverage of the potential side effects attributed to WCVs led to a decline of vaccine uptake during the mid 1970s (Roberts and Parton, 2001). As a consequence, there were large pertussis epidemics until 1982 when vaccine uptake recovered (Section 1.2).

Mice immunised subcutaneously with WCVs showed pertussis-specific antibodies in serum samples which exceeded levels found in non-immunised mice (Willems *et al.*, 1998). In addition, mice immunised intranasally with WCVs showed high levels of pertussis-specific IgA antibodies in lung lavages compared with non-immunised mice (Berstad *et al.*, 1997). WCVs also induce high levels of antibodies to PT, FHA, PRN and fimbriae in humans, although there was no direct evidence to link antibody levels and the levels of protection (Gustafsson *et al.*, 1996; Greco *et al.*, 1996; Simondon *et al.*, 1997; Olin *et al.*, 1997). It is becoming clear, however, from studies in mice (Redhead *et al.*, 1993; Barnard *et al.*, 1996; Mahon *et al.*, 1996) and in humans (Peppoloni *et al.*, 1991; Ausiello *et al.*, 1997; Ryan *et al.*, 1998a), that effective immunisation against *B. pertussis*

is also dependent on the induction of cell-mediated immunity which is similar to the type of immunity induced by natural infection with *B. pertussis* (Section 1.7.1). T cells from mice and from human infants immunised with WCVs, were shown to secrete high levels of IL-2 and IFNγ; but not IL-4 or IL-5, after *in vitro* stimulation with *B. pertussis* antigens (Redhead *et al.*, 1993; Barnard *et al.*, 1996; Ausiello *et al.*, 1997; Ryan *et al.*, 1998a; van den Berg *et al.*, 2000). In addition, macrophages from mice immunised with a WCV secreted high levels of nitric oxide in response to *in vitro* stimulation with heat-killed *B. pertussis* cells (Xing *et al.*, 1998). Naïve murine macrophages secreted high levels of IL-12 in response to *in vitro* stimulation with heat-killed *B. pertussis* cells (Mahon *et al.*, 1996). It has been shown that IFNγ can augment IL-12 production by macrophages in response to LPS (Skeen *et al.*, 1996). Thus, the high LPS levels in WCVs (Robinson *et al.*, 1985) may contribute to the documented detection of high levels of IL-12 (Mahon *et al.*, 1996). Furthermore, high levels of anti-*B. pertussis* Ig2a antibodies (indicative of a cell-mediated response) were detected in mouse sera after two immunisations with WCV (van den Berg *et al.*, 2000). In summary, these data indicate that WCVs induce cell-mediated responses in mice and in humans.

B. pertussis can invade and survive in many different types of cells, such as epithelial cells (Bassinet *et al.*, 2000) and macrophages (Friedman *et al.*, 1992; Hazenbos *et al.*, 1994). However, intracellular survival of *B. pertussis* does not appear to be required for the induction of strong Th1 cell-mediated immune responses as similar immune responses are induced by the subcutaneous administration of WCVs containing dead bacteria (Redhead *et al.*, 1993; Barnard *et al.*,1996; Mahon *et al.*, 1996; Leef *et al.*, 2000; van den Berg *et al.*, 2000).

1.7.3 Immune responses to acellular pertussis vaccines

After the observations that WCVs could be severely reactogenic, new generation ACVs were developed and are steadily replacing WCVs. Numerous reports have demonstrated that ACVs are less reactogenic than WCVs (Roberts and Parton, 2001; Mattoo and Cherry, 2005). However, there has been much debate as to whether all ACVs have the same efficacies as WCVs (Garcia-Sanz *et al.*, 1985; Trollfors *et al.*, 1995; Simondon *et al.*, 1997). Many studies have shown that ACVs are highly effective at preventing the severe manifestation of pertussis in both humans and mice (Barnard *et al.*, 1996; Olin *et al.*, 1997; Cherry *et al.*, 1998; Storsaeter *et al.*, 1998) although one study suggested that mice immunised with ACVs were poorer at inducing significant neutrophil PMNL infiltration in the lungs following aerosol challenge compared with WCVs (McGuirk and Mills, 2000b).

ACVs contain a combination of purified immunogenic protein antigens from *B. pertussis*, such as genetically or chemically detoxified PT (dPT), PRN, FHA or serotype 2 and 3 fimbriae. Studies have shown that ACVs which contain more purified *B. pertussis* antigens show increased efficacy against mild disease (Greco *et al.*, 1996; Gustafsson *et al.*, 1996). In early clinical trials of ACVs, it was difficult to define quantitative correlations between specific anti-pertussis antibody levels and protection against disease (Blumberg *et al.*, 1992; Greco *et al.*, 1996; Gustafsson *et al.*, 1996). However, efficacy trials with ACVs completed later in Sweden and in Germany showed that high antibody titres against PRN and to a lesser extent against fimbriae and PT, in the sera of children, correlated with protection (Cherry *et al.*, 1998; Storsaeter *et al.*, 1998). ACVs invoke a strong Th2 cytokine profile (IL-4 and IL-5) in mice (Redhead *et al.*, 1993; Barnard *et al.*, 1996; van den Berg *et al.*, 2000) but, in humans, a mixed Th1 and Th2 cytokine response is induced as T cells secrete IFNγ in addition to IL-4 and IL-5 (Ausiello *et al.*, 1997; Ryan *et al.*, 1998a). Interestingly, Cassone *et al.* (1997) concluded that ACVs were better inducers of Th1 responses than the WCV but this conclusion could have been due to differences in efficacies of the particular vaccines used in that study (Canthaboo *et al.*, 2001). Moreover, inclusion of IL-12 (a Th1-inducing cytokine) with an ACV, increased its efficacy to a level similar to that of a WCV in mice (Mahon *et al.*, 1996).

In summary, investigations into the types of immune responses induced by WCV, ACVs or by natural infection have been truly informative. With these data, the potential to create more efficacious vaccines is possible now that key elements of the immune system have been identified for protection against *B. pertussis*.

1.8 CyaA as a candidate protective antigen

CyaA is currently not included in any of the commercial ACV preparations, despite the observation of anti-CyaA antibodies in convalescent sera of human adults and in neonates after *B. pertussis* infection (Farfel *et al.*, 1990; Arciniega *et al.*, 1991; Arciniega *et al.*, 1993; Betsou *et al.*, 1993; Cherry *et al.*, 2004). In addition, there have been several studies which have shown CyaA from *B. bronchiseptica*, *B. pertussis* and *B. parapertussis* to be protective in mice. Guiso *et al.* (1991) showed that mice immunised subcutaneously with 2×25 μg of purified full length CyaA or 2×4 μg of AC fragments from *B. pertussis* protected mice against intranasal challenge with *B. pertussis* Tohama or 18.323. The protective efficacies of purified CyaA or AC fragments were lower than the protective efficacy of a WCV indicating that CyaA was not the only factor involved in bacterial colonisation in *B. pertussis*. Interestingly, purified CyaA or AC fragments from *B. parapertussis* protected against bacterial colonisation with *B. parapertussis* but did not protect against *B. pertussis* colonisation (Khelef *et al.*, 1993a). In a separate study by

Gueirard and Guiso (1993), mice immunised subcutaneously with 2 × 15 μg of purified CyaA from *B. bronchiseptica* were protected against colonisation of *B. bronchiseptica* by the intranasal route and had protective activity similar to that of a *B. bronchiseptica* WCV suggesting that *B. bronchiseptica* CyaA is a major protective antigen against *B. bronchiseptica* infection. Mice immunised subcutaneously with 2 × 15 μg of purified CyaA from *B. pertussis* were protected against *B. pertussis* infection but not against *B. bronchiseptica* indicating that the CyaAs expressed from these three *Bordetella* species are immunologically distinct. It may also imply that if CyaA were included in an ACV, it would not make the vaccine cross protective.

The ability of recombinant CyaA expressed from *E. coli* to protect against *B. pertussis* has also been investigated. Mice immunised subcutaneously with 2 × 15 μg of purified recombinant CyaA from *E. coli*, in alum, were protected against *B. pertussis* intranasal challenge (Hormozi *et al.*, 1999). In a separate study, mice immunised intraperitoneally with 1 × 25 μg of purified recombinant CyaA from *E. coli*, without alum, were protected against *B. pertussis* aerosol challenge (MacDonald-Fyall, 2002). However, in both studies, protection was not observed with a recombinant non-acylated form of CyaA, indicating that acylation of CyaA by CyaC is important for protection in mice (Betsou *et al.*, 1993; Hormozi *et al.*, 1999). MacDonald-Fyall (2002) also showed that intraperitoneal immunisation with one dose of CyaA (25 μg) lacking AC enzymic activity (CyaA*, which has a di-peptide insertion in the AC domain between residues 188 and 189 in the catalytic site of domain) protected mice against *B. pertussis* aerosol challenge but not against intranasal challenge. The level of protection afforded by CyaA*, in the aerosol challenge model, was similar to native CyaA, suggesting that the AC enzymic activity does not contribute to protection. Therefore, if CyaA was included in any future ACVs, then an enzymically-inactivated form would be preferred. However, Monneron *et al.* (1988) showed that polyclonal antibodies raised against *B. pertussis* CyaA and mammalian AC were cross-reactive. Therefore, further investigations are needed if CyaA were to be used in a vaccine.

1.9 Bacterial toxins as immunomodulatory agents

Most purified antigens are poorly immunogenic when ingested or inhaled (McGhee *et al.*, 1992). However, bacterial toxins, such as *B. pertussis* pertussis toxin (PT), *V. cholerae* cholera toxin (CT) and the heat-labile toxin (LT) of enterotoxigenic *E. coli*, have been shown to act as powerful mucosal adjuvants for nasal or oral delivery of protein antigens. CT, PT and LT have also been used as adjuvants by other routes (see below). CT, LT and PT are all AB_5 toxins which consist of a pentameric binding 'B' domain, and an

ADP-ribosylating enzymic 'A' moiety. Like PT, CT and LT bind NAD and transfer the ADP-ribose moiety to a target G protein which results in an intracellular accumulation of cAMP and cell intoxication (Spangler, 1992). CT and LT have high homology (80% identity) in their primary structure (Dallas and Falkow, 1980; Spicer *et al.*, 1981) and bind to GM1 mono-sialogangliosides receptors on the surface of eukaryotic cells. GM1 receptors are found in abundance on the luminal surface of intestinal epithelial cells (Spangler, 1992). All 3 bacterial toxins are powerful mucosal adjuvants but are too toxic for general use (Levine *et al.*, 1983). Therefore, enzymically-inactive or partially enzymically-inactive toxin mutants have been generated to deal with the problem of toxicity and, as a result, the mechanisms of adjuvanticity to foreign antigens, as well as immunomodulatory properties of these toxins are slowly being uncovered.

1.9.1 Cholera toxin (CT) as an adjuvant

CT is a potent mucosal adjuvant that can induce mucosal antibody responses to co-administered antigens through the nasal or oral routes (Elson and Ealding, 1984; Yamamoto *et al.*, 1997a; Imaoka *et al.*, 1998). CT has also been shown to induce systemic adjuvanticity in mice immunised intraperitoneally with CT plus bovine serum albumin (BSA) (Park *et al.*, 2003). This was demonstrated by 10-fold higher levels of serum anti-BSA total IgG and mucosal IgA antibodies compared with mice immunised with BSA alone. The disadvantage of using native CT as an adjuvant is its ability to induce oedema at the site of injection (Kay and Ferguson, 1989). The toxicity of CT has been overcome by site-directed mutageneses in the enzymic domain. Lomada *et al.* (2004) showed that non-toxic CT-2*, which has Arg7-Lys and Glu112-Gln substitutions and therefore lacks ADP-ribosylating activity, was very efficient at acting as a musocal adjuvant towards peptide antigens by inducing IFNγ production from helper T cells and CTLs in mice. Another study showed that CT with a Glu29-His substitution, which showed no ADP-ribosylating activity but 1.2% of wild-type CT toxicity, acted as an adjuvant in mice towards a recombinant Norwalk virus-virus like particle vaccine when co-administered by the oral route, by inducing virus-specific IgG and IgA production as well as inducing a stronger Th2 response compared with control mice given vaccine only (Periwal *et al.*, 2003). However, not all CT mutants, such as CTK63, which contains a Ser63-Lys substitution in the enzymic subunit and has negligible enzymic activity, are efficient adjuvants towards co-administered antigens (Douce *et al.*, 1997). Similarly, the non-toxic CT B pentamer (CTB) is generally not regarded as an efficient adjuvant for co-administered antigens (Lycke *et al.*, 1992; Holmgren *et al.*, 1993) although mice immunised intranasally and subcutaneously with CTB non-conjugated to antigen have been shown to effectively at raise antigen-specific serum IgG antibody titres (Isaka *et al.*, 1999). CTB but can be a

highly efficient mucosal carrier molecule for antigens linked by chemical or genetic conjugation by inducing mucosal antibody responses to the linked antigen in mice (Lipscombe *et al.*, 1991; Bergerot *et al.*, 1997). CTB conjugated with antigen has been shown to act as an adjuvant by other immunisation routes, such as intranasally and subcutaneously (Price *et al.*, 2005). It has been suggested that coupling of protein to CTB promotes strong binding to GM1 receptors and hence antigen uptake, enhancing the induction of antibody responses to the linked antigen (McKenzie and Halsey, 1984; Czerkinsky *et al.*, 1989). When native CT is administered mucosally as an adjuvant with bystander antigens, in mice, mixed Th1-Th2 responses (Hörnquist and Lycke, 1993; Kjerrulf *et al.*, 1998) and MHC class I-restricted CTL responses (Bowen *et al.*, 1994; Simmons *et al.*, 1999) have been reported. The latter could be explained by the selective presentation of antigen by DCs to $CD8^+$ cells (Porgador *et al.*, 1998). Clearly, site-directed mutants of CT and the B subunits have great potential to be used as therapeutic agents although the full adjuvant activity requires an intact CT molecule covalently linked to the ADP-ribosylating enzymic protein unit (Lycke *et al.*, 1992). However, after intranasal administration, GM1-ganglioside binding CT or CTB and co-administered antigens have been shown to accumulate in the olfactory epithelium and bulb (Fujihashi *et al.*, 2002). These sites allow access to the central nervous system which, may be a potential problem for the use of CT as a mucosal adjuvant.

1.9.2 Heat-labile toxin (LT) as an adjuvant

LT has been shown to have adjuvant properties. Mice immunised subcutaneously three times with LT (10 ng) and the *Helicobacter pylori* urease antigen showed enhanced protection against *H. pylori* challenge and increased serum anti-urease total IgG antibody levels compared with mice immunised subcutaneously with urease alone (Weltzin *et al.*, 2000). A parallel study with the LT binding domain (LTB) also showed that it could act as an adjuvant towards *H. pylori* urease antigen, although a greater toxin concentration (50 μg) was required to see this effect compared with LT (10 ng) (Weltzin *et al.*, 2000). Other studies have shown that LTB alone can act as an adjuvant towards other antigens (Elson, 1984; Pizza *et al.*, 2001). Like that of CT, enzymically-inactive mutants have been constructured and evaluated as adjuvants. These include LTK63, which contains a Ser63-Lys substitution, and LTR72, which has a Ala72-Arg substitution, are two of the most extensively studied LT mutants (Pizza *et al.*, 1994; Guiliani *et al.*, 1998). LTK63 has no ADP-ribosylating activity but LTR72 has 1% of wild-type ADP-ribosylating activity. Both LTK63 and LTR72 are better adjuvants than LTB (Douce *et al.*, 1995; de Haan *et al.*, 1996) suggesting an important role for the enzymically inactive A subunit in the modulation of the immune response. LTK63 and LTR72 have no adjuvanticity at very low

doses but their adjuvanticities are enhanced by increasing their doses (Pizza *et al.*, 2001). The mucosal delivery of LTK63 and LTR72 with an acellular pertussis vaccine can confer protection against *B. pertussis* infection in mice (Ryan *et al.*, 1999). LTK63 and LTR72 can also enhance immune responses to a wide variety of antigens after mucosal immunisation, including ovalbumin (Giuliani *et al.*, 1998), keyhole limpet haemocyanin (KLH) (Douce *et al.*, 1999), FHA, PRN and PT of *B. pertussis* (Ryan *et al.*, 1999) and ricin (Kende *et al.*, 2006). A LT mutant LT192G (Arg192-Gly substitution), which has a 10-fold reduction in toxicity *in vitro*, has been shown to elicit a mixed Th1/Th2 response towards KLH (Douce *et al.*, 1999) whereas wild-type LT suppresses Th1 responses but induces Th2 responses (Petrovska *et al.*, 2003).

1.9.3 Pertussis toxin (PT) as an adjuvant

PT is a well known adjuvant which can enhance IL-4 and IgE production to co-administered antigens in mice (Mu and Sewell, 1993; Samore and Siber, 1996). However, due to the toxic nature of PT, genetically-detoxified PT derivatives have been created by introducing mutations into the enzymic domain (Nencioni *et al.*, 1990; Podda *et al.*, 1990). Mice immunised intranasally with 3 μg of PT-9K/129G, which has Arg9-Lys and Glu129-Gly substitutions, and has no ADP-ribosylating activity, was shown to behave as a better adjuvant than native PT towards the 50 kDa non-toxic fragment C of tetanus toxin (FrgC), by enhancing anti-FrgC total IgG levels (Roberts *et al.*, 1995). This suggested that the enzymic toxic activity might hinder the adjuvant activities of PT. Spleen cells from mice immunised intraperitoneally once with 5 μg of PT or with 5 μg of PT-9K/129G in combination with co-administered antigens were shown to produce high levels of IFNγ and IL-5 after *in vitro* antigen-stimulation indicating the induction of a combined Th1 and Th2 response (Ryan *et al.*, 1998b). It is thought that PT mediates its adjuvanticity by through a cAMP-dependent pathway (Bagley *et al.*, 2002) or by inhibiting G_i protein-signalling (He *et al.*, 2000; Hou *et al.*, 2003). However, since PT-9K/129G has no enzymic activity, the mechanism of adjuvanticity may be mediated by the ability of PT to bind to cell receptors to induce cell-signalling cascades.

In summary, CT, LT and PT and their detoxified counterparts can behave as adjuvants towards co-administered antigens. These studies have shown the potential to use detoxified versions of the native toxins as potential candidates as adjuvants towards co-administered antigens.

1.9.4 CyaA as an adjuvant

CyaA has been shown to behave as an adjuvant in mice by increasing total IgG levels to foreign antigens. Hormozi *et al.* (1999) showed increased total IgG anti-

ovalbumin antibody titres in mice immunised subcutaneously with 2 x 15 μg of recombinant CyaA plus ovalbumin. Ross *et al.* (2004) showed enhanced antigen-specific IL-5 and IL-10 production and anti-KLH IgG1 antibodies after subcutaneous immunisation with 2 x 1 μg of CyaA plus KLH. However, like CT, LT and PT, CyaA has toxic activity *in vitro* and its use in a vaccine may not be ideal. MacDonald-Fyall *et al.* (2004) compared the ability of a recombinant enzymically-inactive mutant, CyaA*, and wild-type recombinant CyaA to act as adjuvants towards FHA, PT and PRN. Mice that were immunised intraperitoneally with FHA, native PT and PRN + 25 μg of CyaA* showed greater total IgG antibody levels to FHA and PRN, and to a lesser extent, PT, compared with mice immunised intraperitoneally once with FHA, native PT and PRN + 25 μg CyaA or with FHA, native PT and PRN alone. Moreover, peritoneal macrophages from mice immunised with FHA, native PT and PRN + CyaA* produced more nitric oxide after stimulation with *B. pertussis* antigens. These data indicated that the enzymic activity of CyaA was not required for the adjuvant effects mediated by CyaA. In fact, the absence of AC activity appeared to enhance the adjuvant effects of CyaA perhaps due to the reduced toxicity of the CyaA* protein towards APCs.

The role of the AC domain and acylation of CyaA were investigated for their adjuvant contributions towards KLH (Boyd *et al.*, 2005). Mice immunised twice in the hind footpads with KLH plus 1 μg of acylated (native) rCyaA or 1 μg of non-acylated rCyaA, but not non-enzymic, acylated rCyaA, showed increased total IgG antibody responses to KLH. Moreover, acylated rCyaA and non-acylated rCyaA predominantly induced anti-KLH IgG1 antibodies. The cytokine profile of antigen-stimulated cells from the popliteal lymph nodes of immunised mice showed that rCyaA and non-acylated rCyaA in combination with KLH induced Th2- and IL-10-secreting type 1 regulatory T (Tr1) cells, as shown by increased IL-4, IL-5, IL-10 and IFNγ, a phenomenon also noted previously for CyaA (Ross *et al.*, 2004). As IL-10 suppresses IL-12, which is an inducer of cell-mediated responses, it is thought that Tr1 cells help up-regulate Th2 responses. The conclusion by Boyd *et al.* (2005) was that acylation was not required for the adjuvant effects of CyaA.

The differences in results between MacDonald-Fyall *et al.* (2004) and Boyd *et al.* (2005) could be due to differences in the amount of non-enzymic CyaA used for immunisation in the two studies. Nevertheless, both these studies show the potential for detoxified forms of CyaA to be used as adjuvants towards co-administered antigens.

1.9.4.1 CyaA as a delivery molecule for foreign antigenic epitopes

CyaA is tolerant to insertional sequences within its catalytic domain. Therefore abolishing AC activity does not affect translocation of the AC moiety into target cells

(Ladant *et al.*, 1992; Osicka *et al.*, 2000; Gmira *et al.*, 2001). Non-enzymic rCyaA forms containing $CD8^+$ T cell viral epitopes inserted in the AC domain have been investigated for their ability to induce protective antiviral and therapeutic antitumour $CD8^+$ CTLs in mice (Sebo *et al.*, 1995; Saron *et al.*, 1997; Fayolle *et al.*, 1999; Guermonprez *et al.*, 1999; Dadaglio *et al.*, 2003; Mascarell *et al.*, 2005). Foreign $CD4^+$ T cell epitopes have also been introduced into CyaA and investigated (Loucka *et al.*, 2002). It is thought that CyaA can deliver the AC domain carrying the $CD8^+$ or $CD4^+$ T cell epitopes into the cytosol of MHC class I or MHC class II APCs, which then are able to stimulate $CD8^+$ or $CD4^+$ T cells, respectively (Schlecht *et al.*, 2004; Wilkinson *et al.*, 2005). More impressively, Dadaglio *et al.* (2000) and Fayolle *et al.* (2001) both showed that CyaA could accommodate multiple epitopes, including MHC class I and class II epitopes from the nucleoprotein of the lymphocytic choriomeningitis virus, HIV peptides and chicken ovalbumin. In both studies, epitope-specific cytotoxic responses were induced *in vivo*. These findings represent a very versatile system where any desired synthetic peptide could be easily integrated into CyaA enabling the design of novel types of vaccines.

1.10 Microarrays

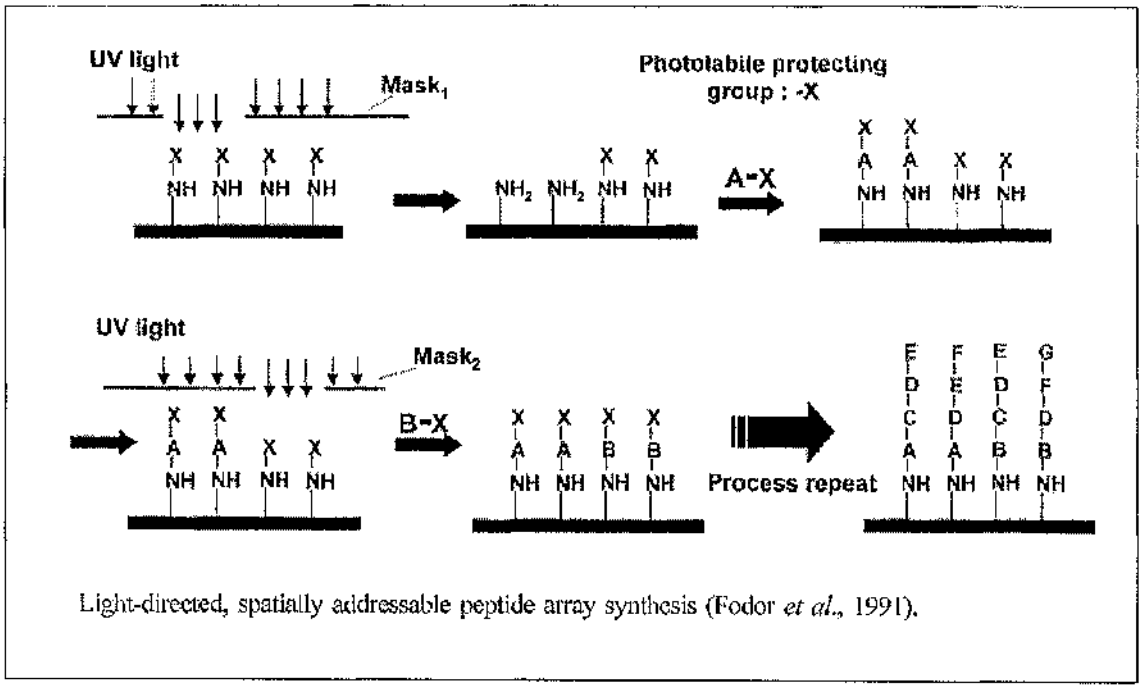
DNA microarray is an important technology for studying gene expression. The level of expression of thousands of genes, or even an entire genome, can be estimated from a sample of cells. Microarrays also allow the investigator to follow changes in the expression of many genes simultaneously. Microarray analysis functions on the basis of highly specific molecular recognition between cDNA strands or between cDNA and cRNA. Thus, a solution containing a complex mix of cRNA or cDNA (target) molecules can bind with high affinity to immobilised cDNA molecules (probes) on a solid surface.

1.10.1 Types of microarrays

The generation of microarrays is generally done in one of two ways. cDNA microarrays consist of cDNA gene probes that are robotically printed on glass by covalent attachment or by physisorption (Campbell and Ghazal, 2004). Covalent attachment involves the formation of a covalent bond between molecules on DNA with a surface and it results in a more stable attachment of DNA. Physisorption pairs areas of complimentary charge on DNA with a charged surface, such as poly-lysine or poly-amine (Schena *et al.*, 1995). Affymetrix GeneChips® have oligonucleotide probes lithographically synthesised *in situ* (Fodor *et al.*, 1991; Fig. 5). Firstly, amino groups (NH) on a glass slide are capped by photolabile protecting groups (X). The photolabile N-protecting group on the surface is site-specifically removed by the selective irradiation using a UV illuminator with a photomask. Thereafter, a nucleotide monomer (A) bearing the photolabile protecting group

Figure 5 Generation of oligonucleotide microarrays on solid surfaces by photolithography

All the oligonucleotides are synthesised in parallel on the glass chip. NH(X) is the amino group (containing a photolabile protecting group). A, is the first nucleotide. B, is the second nucleotide etc. The creation of oligonucleotides on the surface is described in Section 1.10.1.



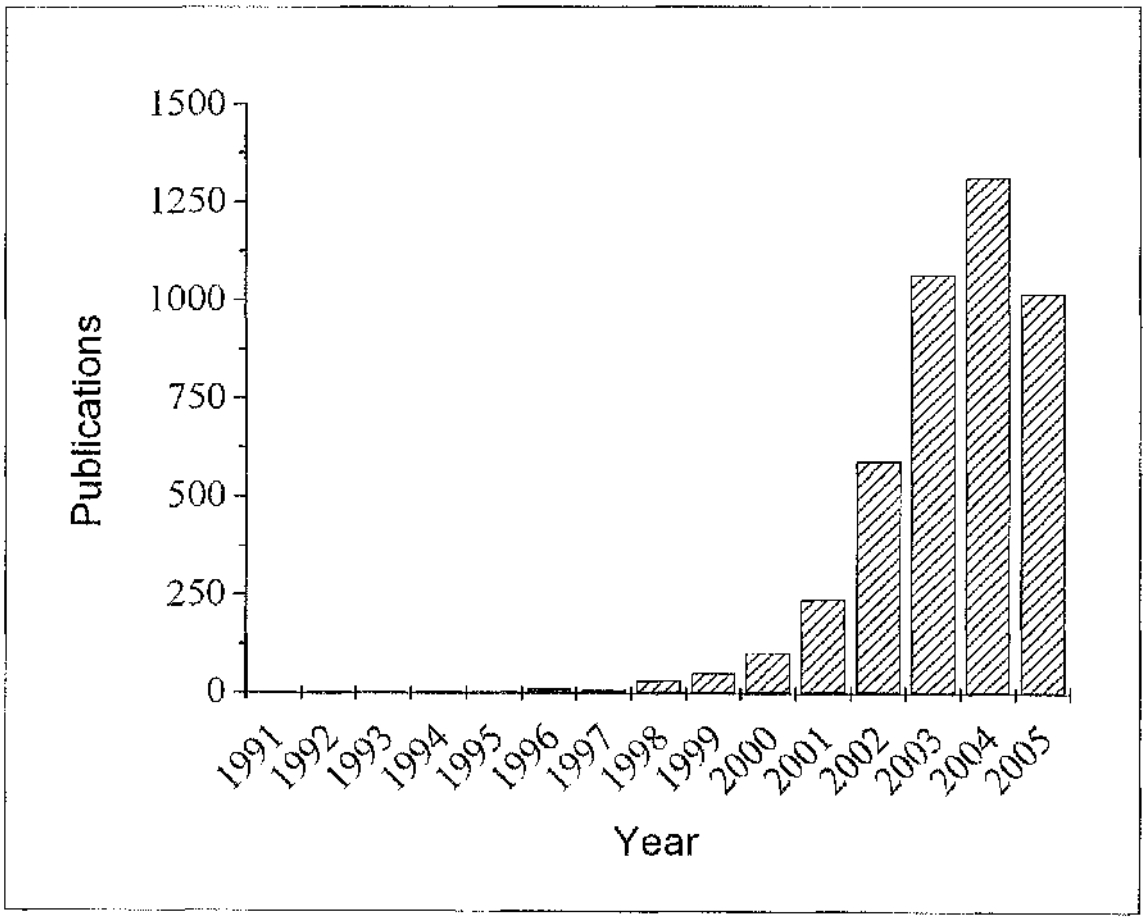
Light-directed, spatially addressable peptide array synthesis (Fodor *et al.*, 1991).

Taken from Shin *et al.* (2005).

is coupled to the exposed amino group (Fig. 5). Repetitive cycles of photodeprotection and coupling steps generate the desired microarray allowing up to 40,000 compounds to be synthesised in 1 cm^2 (Shin *et al.*, 2005). The frequency of sequence errors increase with the length of oligonucleotide probes which is why GeneChips® have settled with the production of 25-mers. Affymetrix probes are designed in pairs: one sequence is the exact complement of the target sequence (Perfect Match; PM), and the other differs from the exact complement by one mutation in the middle of the probe (MisMatch; MM). The MM probe acts as an internal control for the PM probe as it should not hybridise well to the target transcript but should hybridise non-specifically to many transcripts as well as the PM oligonucleotides. For any gene, up to 20 paired probes can be printed on the array and heterogeneous transcripts that would all bind to the same cDNA probe will bind differentially to each oligonucleotide probe. Using the difference signal between these two probes approximately cancels non-specific binding and background contributions providing a better estimate of the intensity due to hybridisation to the true target transcript. Creating 25-mers on solid bases using photolithography is costly, as 100 photomasks are needed per chip. Singh-Gasson *et al.* (1999) documented an alternative way of synthesising oligonucleotides on microarrays, *in situ*, using digital micromirror technology which replace the need for photomasks. The system is composed of an optical and microfluidic system. The optical system consists of an illuminator and a micromirror array which is controlled electronically. By controlling the deflection of illuminated UV light on the micromirror arrays, the photolabile protecting group on the chip surface can be cleaved at the specified site. The microfluidic system bathes the chip surface with a solution of monomers, allowing oligonucleotides to be synthesised after many repetitions.

Since the early 1990s, there has been an explosion in the number of scientific groups using DNA microarray technology. Figure 6 shows the number of published manuscripts using Affymetrix GeneChips® from 1991 to 2005 and it is evident that most of these publications have been within the last 3 - 4 years. These publications vary widely in their fields of research; from investigating gene expression in different cell types and tissues to identifying transcription profiles characteristic of physiological and pathological states, e.g. diseased cells or cells infected by a pathogen or infectious agent. A few examples of microarray technology being used to investigate host responses to bacterial pathogens include *in vitro* analysis of *B. pertussis* interaction with human bronchial epithelial cells (Belcher *et al.*, 2000), invasive wild-type *Salmonella* strain, *Salmonella dublin* with human colon epithelial cells (Eckmann *et al.*, 2000) and *P. aeruginosa* with human epithelial cells (Ichikawa *et al.*, 2000). In all cases, the transcription of previously undescribed genes were altered. In the case of *B. pertussis*-treated epithelial cells,

Figure 6 Cited publications using Affymetrix GeneChip® microarrays



Taken from http://www.affymetrix.com/community/publications/full_list.affx

microarray analysis showed that infected cells were in a pro-inflammatory state and evidence of NF-κB cell-signalling. DNA microarray technology enables new insights into disease mechanisms caused by infection.

Some vendors give customers the option of creating their own DNA chips. This is particularly useful for studying gene transcription in pathogens in response to different stimuli or after interaction with target cells. For example, Hot *et al.* (2003) used microarrays to investigate the types of genes expressed by *B. pertussis* in Bvg^- and Bvg^+ conditions by creating molecular probes based on the data generated from the publication of the complete *B. pertussis* genome (Parkhill *et al.*, 2003). Fifty genes, out of 184 genes used for microarray analysis, were found to be modulated by $MgSO_4$ or nicotinic acid (both of which cause *B. pertussis* to become avirulent; Bvg^- phase). In addition to confirming many Bvg-regulated *vags*, such as *cyaA* and *prn*, new *vags*, such as genes encoding potential autotransporters (*auto A*, *B*, *D* and *E*), genes encoding potential regulatory proteins (*sen5* and *reg11*) and genes encoding members of a type III secretion system (*bscJ*, *bcrD*) were discovered.

By screening for gene changes in bacteria in response to anti-microbials, it may be possible to identify new ways to combat anti-microbial resistance. This is particularly important for pathogens such as *H. pylori* because there are no efficacious vaccines against it (Aebischer *et al.*, 2005). On the other hand, microarrays can be used to generate expression profiles of different strains of pathogenic bacteria e.g. *E. coli* O157:H7 (Dowd and Ishizaki, 2006) or even identify unknown/uncatergorised genes which are expressed in antibiotic-resistant strains of bacteria. For example, the expression profile of a vancomycin (a glycopeptide class of antibiotics)-resistant *Staphylococcus aureus* strain (Cui *et al.*, 2005), showed increased expression of *graF* and *msrA2*. The importance of these genes were confirmed in a separate series of experiments: *graF* and *msrA2* were overexpressed in glycopeptide-susceptible *S. aureus* strains. This caused an increase in glycopeptide resistance as well as an increase in cell wall thickness compared with glycopeptide-susceptible *S. aureus* strains that did not express these genes. Moreover, Waddell *et al.* (2004) investigated the expression profiles of *Mycobacterium tuberculosis* to six anti-microbial compounds. In those studies, innate mechanisms which contributed to anti-microbial resistance were identified. Up-regulated genes included those that were involved in the mycobacterial stress response and efflux proteins/transporters. Microarray studies give a preliminary insight into the possible ways to tackle antibiotic-resistant strains of bacteria.

1.10.2 Labelling methods

A typical experiment using a cDNA microarray involves the preparation of two samples for hybridisation to the array: a control sample and an experimental sample. mRNA is extracted from cells and reverse transcribed into cDNA. During the reverse transcription step, a green-fluorescing dye called Cy3 or a red-fluorescing dye called Cy5 is incorporated into the newly formed cDNA so that samples can be differentially labelled. The samples can then be combined and hybridised to the microarray together. The two samples will competitively bind to the probes on the array and the sample containing more cDNA for a particular probe will hybridise to a greater extent. That is, if there is more of a cDNA transcript in the control sample than in the experimental sample (i.e. the gene is down-regulated in the experiment) then more Cy3-labelled control cDNA will bind to the probe on the array and the spot will fluoresce green. If there is more experimental transcript, the reverse will happen and the spot will fluoresce red. When the two samples have the same amount of transcript, the dyes will cancel each other out and the spot will fluoresce yellow. A scanner (laser scanning confocal microscope) or a charge-coupled device (CCD) camera is used to quantify the intensity of fluorescence at each pixel location on the microarray.

For Affymetrix GeneChip® arrays, the preparation of the sample is slightly different and the process is described in detail in Section 2.13. Briefly, mRNA is isolated then cDNA is synthesised. The cDNA is then used as a template for T7 RNA polymerase to create cRNA molecules labelled with biotin. The biotinylated cRNA molecules are then fragmented into smaller molecules which are between 80 – 100 nucleotides long to help improve their specificity for probes on the microarray. In addition, the structures of the fragmented targets are less complex, unlike the larger transcripts which often contain secondary structure and can interfere with hybridisation and increase the opportunity for non-specific cross-hybridisation to the probes on the GeneChip®. The biotin-labelled cRNA fragments are then hybridised to the GeneChip®. After hybridisation, the bound cRNA fragments are treated with streptavidin-phycoerythrin and with a biotinylated anti-streptavidin antibody to amplify the fluorescence signals. The processing of images to generate data is described in Section 2.14.

Using a wide choice of software programme packages, it is now becoming easier to manipulate the large amounts of data generated from a single microarray experiment. An extensive list of these software programmes can be found on http://www.ifom-ieo-campus.it/MICROARRAY/data_analysis.htm with GeneSpring® widely regarded as the gold standard for expression data analysis. GeneSpring® provides visualisation tools including Venn diagrams, bar charts, scatter plots, chromosome display and dendrograms.

Genes can also be clustered and, for some genomes, the data can be displayed according to the functional class or subcellular location of the gene product.

In summary, the use of microarray technology is applicable for almost every avenue of research. Not only can the gene responses of target cells be investigated using pre-designed arrays containing whole genomes, but the gene transcription in the pathogen can be investigated through the production of custom arrays containing probes for the whole genome of the pathogen. Microarray technology has given and will continue to give us new insights into the pathogenic mechanisms of many pathogens so that improved counter-measures can be made to fight against disease.

1.11 Aims and Objectives

The overall aim of this study was to compare the properties and immunomodulatory behaviour of four different recombinant forms of CyaA. These were: fully functional enzymically-active, acylated and invasive CyaA; an acylated and invasive CyaA form lacking adenylate cyclase enzymic activity (CyaA*); and the non-acylated, poorly-invasive forms of these toxins, proCyaA and proCyaA*, respectively. Specific objectives were:

1. To prepare large amounts of the purified CyaA forms which were low in LPS, so as to minimise the known immunomodulatory effects of this molecule.
2. To assess the relative contributions of the invasive and the enzymic activity of CyaA to cytotoxic activity against a mouse macrophage cell line using several *in vitro* assays which measured haemolysis, cell killing, apoptosis or inhibition of the zymosan-stimulated oxidative burst.
3. To use biophysical methods, such as circular dichroism and analytical ultracentrifugation, to investigate the molecular state of the different CyaA forms in solution. This was done to provide evidence that, at low concentrations, CyaA can invade cells in a monomeric form but, at higher concentrations, oligomers form in order to create pores in the target cell membrane.
4. To conduct an *in vivo* study in mice to investigate the protective effects of the different CyaA forms alone, and their ability to enhance the protective efficacy of a conventional acellular pertussis vaccine. The humoral and cell-mediated responses to these immunisations would be determined.
5. To examine the gene transcriptional responses induced in mouse bone marrow-derived macrophages (BMMs) by exposure to a concentration of proCyaA*, CyaA or CyaA* which induced only low-level cell killing. These forms were chosen to assess the importance of the invasive and AC enzymic activities for host cell responses. This was done by applying microarray analysis using Affymetrix™ mouse arrays to study global gene responses of the BMMs.

Chapter 2

Materials and Methods

2.1 Bacterial strains and growth conditions

E. coli BL21/DE3 (F⁻ *ompT* rB⁻ mB⁻) and *E. coli* BL21/DE3 *lpxM* (F⁻ *ompT* rB⁻ mB⁻) were used as expression hosts for the production of recombinant CyaA. The rB⁻ mB⁻ genotype allows cloning of DNA without cleavage by endogenous restriction endonucleases. BL21 strains are deficient in the OmpT and Lon proteases, which promotes recombinant protein stability. DE3 denotes the λDE3 lysogen that carries the gene for T7 RNA polymerase, which is located chromosomally, under the control of the *lacUV5* promoter (inducible by isopropyl-1-thio-β-D-galactoside (IPTG)). The polymerase targets plasmids containing a T7 promoter, driving expression of genes found downstream of the T7 promoter sequence. *E. coli* BL21/DE3 *lpxM* has a defective lipid A component of LPS due to a lack of myristoylation via the product of the *lpxM* gene (Cognet *et al.*, 2003). This strain was created by insertion of a chloramphenicol resistance gene within the *lpxM* gene and was kindly supplied by Dr. Jean-François Gauchat (Centre d'Immunologie Pierre-Fabre, Montreal).

2.1.1 *E. coli*

E. coli BL21/DE3 and *E. coli* BL21/DE3 *lpxM* were grown, from 50% (v/v) glycerol stocks, on Luria agar (LA - Appendix A.1.1) by incubating overnight (o/n) at 37 °C. Subsequent growth of *E. coli* was performed by inoculating single colonies onto LA or into Luria-Bertani broth (LB - Appendix A.1) or Terrific broth (TB) (Appendix A.2). Agar plates were incubated o/n at 37 °C. Broths were incubated at 37 °C with shaking after inoculation.

2.1.2 Extraction of LPS from *E. coli*

For isolation of LPS from *E. coli* BL21/DE3 (Promega, UK) and *E. coli* BL21/DE3 *lpxM*, 5 ml of o/n culture was added to a 2 L baffled flask containing 500 ml LB and incubated at 37 °C with shaking at 200 rpm until an $OD_{600\ nm}$ of 0.4 - 0.6 was reached. The cells were then allowed to grow for a further 3 h. For each bacterial strain, a total of 6 flasks of cultures were inoculated. For each strain, the cells were harvested and pooled together by centrifugation at 13,700 × *g* for 30 min (RC-5B, Sorvall), and then stored o/n at –20 °C. The cells were resuspended in 1.5 ml phosphate-buffered saline (PBS - Appendix A.4) and transferred to a 150 mm diameter spherical glass vial. Prior to freeze-drying, the cells were first frozen as a thin layer inside the glass container by briefly

immersing the flask, with rotation, in liquid nitrogen. The vial was then freeze-dried (Alpha 1-4, Christ technologies) until complete dehydration was achieved (~16 h). For each gram of dry weight, 10 ml of PBS [containing 5 mM EDTA] was added. After incubation for 2 h at 37 °C, for each gram of dry weight, 0.02 g of lysozyme (Sigma) was added and the mixture was incubated at 4 °C for 16 h, with constant stirring. The solution was further incubated at 37 °C for 20 min and then an equal volume of 20 mM magnesium chloride was added. RNase (Sigma) and DNase (Sigma) were added to the mixture, both at final concentrations of 1 μg/ml, and incubated at 37 °C for 10 min then at 60 °C for a further 10 min. An equal volume of pre-heated (70 °C) phenol solution, equilibrated with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 7.5-7.8) (Sigma), was added to the LPS solution and allowed to incubate for 20 min at 70 °C with constant stirring. The LPS/phenol mixture was placed on ice for 20 min with stirring, then o/n at 4 °C with no stirring. The upper aqueous phase (containing LPS) was removed and dialysed (M_w cut off was 12 – 14 kDa) against 4 L of distilled water for 3 days with 3 changes of distilled water per day. The dialysed LPS material was centrifuged at 4,220 × *g* (RC-5B, Sorvall) for 15 min at 4 °C. The supernatant was collected and freeze-dried, as described above. The fine LPS powder was stored at 4 °C. Once reconstituted with endotoxin-free water (Sigma), the LPS was stored at -20 °C.

2.1.3 *B. pertussis* 18.323 and *B. pertussis* 338

B. pertussis strain 18.323 (NCTC 10739) and *B. pertussis* strain 338, harbouring plasmid pGB5P1 (Weingart *et al.*, 1999), kindly supplied by Prof. Alison Weiss (University of Cincinnati), were grown on charcoal agar (CA) (Oxoid) containing 10% (v/v) defibrinated horse blood (Oxoid) from glycerol stocks. CA blood plates were incubated at 37 °C for 3 to 4 days in a humidified atmosphere. Plasmid pGB5P1 constitutively expresses green fluorescent protein (GFP) in *B. pertussis* 338 (Weingart *et al.*, 1999).

2.2 Plasmid DNA

The two genes required for the production of active CyaA in *E. coli* BL21/DE3 were expressed from separate compatible plasmids (Westrop *et al.*, 1996). Plasmid pGW44 expressed the gene encoding the enzymically-active CyaA protoxin, *cyaA*, and plasmid pGW54 expressed the gene, *cyaC*, which encodes the acyltransferase that post-translationally acylates the CyaA protoxin. In addition, plasmid pGW44/188 was used where a 1.1 kb *NdeI*/*BstB*I fragment at the 5' end of *cyaA* in pGW44 was replaced with another 1.1 kb *NdeI*/*BstB*I fragment from pACM188 (Ladant *et al.*, 1992) to create an enzymically-inactive CyaA protoxin mutant (MacDonald-Fyall *et al.*, 2004). As shown in

Table 2, the bacterial strain and combination of plasmid(s) determined which antibiotics were used. Co-expression of plasmids pGW44 or pGW44/188 with pGW54, in *E. coli* BL21/DE3, generated fully-active acylated CyaA or an enzymically-inactive, acylated CyaA (CyaA*) carrying a Leu-Gln di-peptide insertion between codons 188 and 189, respectively (Table 3). The same recombinant forms of CyaA were achieved in *E. coli* BL21/DE3 *lpxM* with plasmids pGW44 or pGW44/188 with pGCK54 (Section 2.2.1). Expression of plasmids pGW44 or pGW44/188 alone, in both *E. coli* BL21/DE3 and *E. coli* BL21/DE3 *lpxM*, produced non-acylated CyaA protoxins with enzymic activity (proCyaA) or without enzymic activity (proCyaA*), respectively (Table 3).

Table 2 Summary of plasmids and *E. coli* hosts used for expression

Plasmid	Antibiotic resistance (final concentration)	Use in *E. coli* BL21/DE3	Use in *E. coli* BL21/DE3 *lpxM*†
pGW44	Ampicillin (50 μg/ml)	Yes	Yes
pGW44/188	Ampicillin (50 μg/ml)	Yes	Yes
pGW54	Chloramphenicol (12.5 μg/ml)	Yes	No
pGCK54	Kanamycin (50 μg/ml)	No	Yes

† Created by insertion of a chloramphenicol resistance cassette.

Table 3 Characteristics of expressed CyaA preparations

CyaA form	Invasive/pore-forming	Adenylate cyclase activity
proCyaA	No	Yes
proCyaA*	No	No
CyaA	Yes	Yes
CyaA*	Yes	No

2.2.1 Construction of pGCK54

E. coli BL21/DE3 *lpxM* carried a chloramphenicol-resistance cassette inserted into the *lpxM* gene. Thus, in order to express fully functional CyaA in *E. coli* BL21/DE3 *lpxM*, a kanamycin resistance cassette was introduced into plasmid pGW54, which encodes chloramphenicol resistance, so that selection with kanamycin could be made for this plasmid in the *lpxM* background. For this, a 1.3 kb *Nco*I restriction fragment containing a kanamycin resistance cassette was removed from plasmid pGEM-T-Kan (pGEM-T from Promega, pGEM-T-Kan created by P. Blackburn (2000)) and ligated into *Nco*I-digested pGW54 to create pGCK54. Ligated DNA was electroporated (Section 2.4.2.1) into TOP10 competent *E. coli* cells (Invitrogen). Plasmid DNA was extracted (Section 2.2.2) from

single colonies that were grown o/n in LB and digested with *Nco*I (Promega). Digested plasmid DNA was subjected to electrophoresis (Section 2.3).

2.2.2 Plasmid purification

Plasmids were extracted using QIAprep® miniprep kits (Qiagen) according to manufacturer's instructions with the following modification: plasmid DNA, extracted from 5 ml of o/n bacterial culture in LB with appropriate antibiotics, was eluted from the miniprep columns with 50 µl of distilled water and stored at –20 °C.

2.2.3 DNA sequencing

Plasmid pGW44/188 was subjected to DNA sequencing to confirm the presence of a 6 bp insertion sequence which abolishes the AC enzymic activity in CyaA. Primers CYAGWF2 (ATGCAGCAATCGCATCAGG - 5' to 3', nucleotides 981 - 999) and CYAGWR2 (CACCGTTCCAGTACATCC - 5' to 3', nucleotides 2129 – 2111) (Invitrogen) were used to sequence the first 600 bases of the mutated *cyaA* gene in the plasmid. Plasmid pGW44, containing the *cyaA* gene, was also sequenced as a control. Sequencing was performed by Miss Julie Galbraith at the Sir Henry Wellcome Functional Genomics Facility (University of Glasgow). Samples were run on a MegaBACE 1000 (96 capillary) sequencer (GE Healthcare) using DYEnamic™ ET-Terminator chemistry according to the manufacturer's (GE Healthcare) instructions. The sequences generated were analysed using Chromas 2.3 software (http://www.technelysium.com.au/) which creates coloured electropherograms. DNA homology against other related sequences was performed using 'blastx' and comparisons between two sequences were performed using 'bl2seq' at http://www.ncbi.nlm.nih.gov/BLAST/.

2.3 Agarose gel electrophoresis

2.3.1 Sample preparation

DNA was mixed with 6× DNA loading buffer (Appendix B.1) prior to loading into the wells of an agarose gel (Section 2.3.2). A 1 kb molecular weight ladder (Invitrogen) was prepared and used according to manufacturer's instructions.

2.3.2 Gel preparation

Agarose type II-A (Sigma) was dissolved in 1× Tris-Borate EDTA (TBE) buffer (Appendix B.2) at 0.7% (w/v) or at 1% (w/v) for visualisation of large and small DNAs, respectively. The solutions were heated until the agarose had completely dissolved. Ethidium bromide (BioRad) was added to the solution, to a final concentration of 1 µg/ml, once the agarose had cooled. The agarose was poured into a gel tray with comb and allowed to set. The set gel was placed into an electrophoresis tank, filled with 1× TBE,

approximately 1 cm above the gel (Mini subcell GT™ electrophoresis system, BioRad). The comb was removed and samples were applied into each well. The gel was run at 90 mA until the dye front migrated to a distance from the wells such that single DNA bands could be visualised clearly under a high performance UV transilluminator (Ultra Violet Products) coupled to an image acquisition and analysis software package (Labworks).

2.4 Expression and purification of recombinant CyaA

2.4.1 Preparation of competent cells for heat shock

A 1 ml o/n culture of the recipient strain, grown in LB, was inoculated into 100 ml of LB in a 500 ml baffled flask and shaken vigorously at 37 °C to an $OD_{600\ nm}$ of 0.4 - 0.5. From this point, all incubations and re-suspensions were performed on ice. Cells were chilled for 2 h before being harvested by centrifugation at 6,900 × *g* (RC-5B, Sorvall) for 15 min at 4 °C. Cells were re-suspended with 45 ml of chilled competence solution (Appendix C.1). After 45 min, cells were recovered after centrifugation at 9,500 × *g* (RC-5B, Sorvall) for 10 min, 4 °C. The cells were gently re-suspended with 2.5 ml of chilled competence solution containing 900 µl of 80% (v/v) chilled sterile glycerol solution. Aliquots of 100 µl cell suspensions were 'snap-frozen' in a dry ice/ethanol mixture and stored at –70 °C until required. These cells remained viable for 6 months.

2.4.2 Heat shock transformation

A frozen aliquot of competent cells was thawed on ice and 2 µl of each required plasmid DNA(s) were added and left to incubate on iced water for 30 min. Cells were then transferred to a 42 °C water bath for 45 sec. After incubation, 1 ml of LB was immediately added to the cells and the transformed cell population was incubated at 37 °C for 1 h with shaking. The cells were then plated out onto LA with the appropriate antibiotics (Table 2, Appendix A.3) and incubated o/n at 37 °C.

2.4.2.1 Electroporation

An aliquot of competent TOP10 *E. coli* cells was removed from –80 °C and allowed to thaw on ice before 2 – 5 µl of plasmid DNA was added to the cell suspension. Electroporation was performed using a gene pulser with pulse controller (model 1652098, BioRad) under the following conditions: 2.5 kilovolts, 25 microfaradays, 200 Ohms in 0.2 cm gap cuvettes (BioRad). Immediately after electroporation, 1 ml of LB was added to the cells and the mixture was incubated for 1 h at 37 °C with shaking before being plated onto LA with the appropriate antibiotics (Table 2, Appendix A.3). Plates were incubated o/n at 37 °C.

2.4.3 Expression of recombinant proteins

2.4.3.1 Expression of protein from *E. coli* BL21/DE3 transformed with one plasmid

One colony of transformed *E. coli* BL21/DE3 was inoculated into 5 ml LB, containing the appropriate antibiotic (Table 2, Appendix A.3), and allowed to grow o/n at 37 °C with shaking. The o/n culture was added directly into a 2 L baffled flask containing 500 ml of LB with the same antibiotic. Cells were incubated at 37 °C with shaking at 200 rpm, until an $OD_{600\ nm}$ of approximately 0.4 was reached. IPTG (Melford) was added to a final concentration of 1 mM and the culture was allowed to grow at 37 °C for a further 3 – 4 h, until an $OD_{600\ nm}$ of 1.8 – 2 was reached. Cells were harvested by centrifugation at 13,700 × *g* (RC-5B, Sorvall) for 15 min and 4 °C and then stored at –20 °C until purification of CyaA (Section 2.4.4) could be performed.

2.4.3.2 Expression of protein from *E. coli* BL21/DE3 transformed with two plasmids

The same protocol was followed as described in section 2.4.3.1 with the exception that a 20 ml o/n *E. coli* culture transformed with two plasmids was inoculated into a 2 L baffled flask containing 500 ml of LB and the appropriate antibiotics (Table 2, Appendix A.3). A greater volume of *E. coli* transformed with two plasmids was inoculated into LB because such a strain grew slower compared with *E. coli* transformed with one plasmid (Section 3.2.1).

2.4.3.3 Expression of CyaA from *E. coli* BL21/DE3 *lpxM*

E. coli BL21/DE3 *lpxM*, transformed with plasmid(s), required a richer medium for growth (Section 3.2.2). Therefore, TB was used. The same methods were used to express the different CyaA forms as described in Sections 2.4.3.1 and 2.4.3.2 with the exception that expression of acylated CyaA and CyaA* required plasmid pGCK54 (Section 2.2.1) instead of plasmid pGW54.

2.4.4 Small-scale purification of CyaA

2.4.4.1 Urea extraction of inclusion bodies containing CyaA

After the expression of the recombinant proteins, the pelleted cells from 500 ml culture were resuspended in 10 ml of Solution A (Appendix C.2.1) and sonicated at 10% amplitude for 2 min, on iced water using a ½" diameter probe (Jencons). The sonicate was centrifuged at 48,000 × *g* (RC-5B, Sorvall) for 10 min, 4 °C, and washed twice with 25 ml of Solution B containing CHAPS [3[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate] (Appendix C.2.2), with centrifugation at 12,000 × *g* (RC-5B, Sorvall)

for 10 min at 4°C, between washes. Washing with several detergents, other than CHAPS was also investigated (Section 2.4.4.1.1). The inclusion bodies were then washed three times with 25 ml of Solution C (Appendix C.2.3), with the first two centrifugation steps at 17,200 × *g* (RC-5B, Sorvall), followed by a third at 27,000 × *g* (RC-5B, Sorvall), all for 10 min at 4 °C. The inclusion bodies were finally washed once with 25 ml of distilled water, followed by centrifugation at 48,000 × *g* (RC-5B, Sorvall) at 4 °C for 10 min, before being solubilised in 10 ml of Solution D (Appendix C.2.4) o/n at 4 °C (crude CyaA preparation). CyaA was collected after 30 min centrifugation at 48,000 × *g* (RC-5B, Sorvall) at 4 °C. The CyaA urea extract was stored at –20 °C prior to DEAE-Sepharose chromatography.

2.4.4.1.1 Endotoxin removal

Four different detergents; CHAPS, deoxycholic acid, n-octyl pyranoglucoside and Triton X100 (all purchased from Sigma), were compared for their ability to remove soluble proteins and endotoxin from inclusion bodies.

2.4.4.2 DEAE-Sepharose chromatography

A 1.5 cm × 25 cm glass column, with tap (BioRad), containing 8 ml of DEAE-Sepharose (Sigma) was washed first with 1 column volume (CV) of distilled water to remove excess alcohol and then equilibrated with Solution 1 (Appendix C.3.1). The solubilised crude CyaA preparation (10 ml), was adjusted to approximately 50 mM NaCl, by the addition of 500 µl of 1 M NaCl (pH 8.0), and the preparation was applied to the glass column containing equilibrated DEAE-Sepharose. Several 5 ml portions of Solution 1 were passed through the matrix. CyaA was then eluted using an increasing 50 mM NaCl step-gradient, up to 250 mM NaCl, of 5 ml portions (Appendix C.3.4) with caution to ensure that the matrix was not disturbed. Each fraction was analysed for protein and for LPS levels by Coomassie Blue and silver staining on 7.5% and 15% SDS-PAGE gels, respectively (Section 2.5).

2.4.4.3 Phenyl-Sepharose chromatography

Fractions containing CyaA, as determined by SDS-PAGE analysis, were pooled and adjusted to 2 M urea, 50 mM Tris-HCl, 800 mM NaCl (pH 8.0) by adding 3 parts of Buffer A (Appendix C.4.1) to 1 part of pooled CyaA. CyaA was then added to a 1.5 cm × 25 cm column, with tap (BioRad), containing 8 ml phenyl-Sepharose (Sigma) that was first washed with 1 CV of distilled water and then equilibrated with Buffer B (Appendix C.4.2). Ten × 25 ml portions of Buffer B were passed through the matrix and then CyaA was eluted by the addition of 10 × 1.5 ml portions of Solution D (Appendix C.2.4). Each

fraction was subjected to SDS-PAGE for determination of the presence of CyaA as described in Section 2.5.

2.4.5 Large-scale purification of CyaA

2.4.5.1 Urea extraction of inclusion bodies containing CyaA

For large-scale purification of CyaA preparations, cells from 24 × 500 ml of induced *E. coli* cultures (Section 2.4.3) were pooled and resuspended in 240 ml of Solution A (Appendix C.2.1) and 10 ml portions were sonicated in turn (Section 2.4.4.1) and pooled once again. Pooled 10 ml volumes, containing the inclusion bodies, were subjected to the wash procedure described in Section 2.4.4.1 and the CyaA preparations were solubilised in 240 ml of Solution D (Appendix C.2.4) and stored at –20 °C until DEAE-Sepharose purification.

2.4.5.2 DEAE-Sepharose chromatography

A 2.5 cm × 50 cm column with tap (BioRad), containing 80 ml of DEAE-Sepharose (Sigma), was first washed with 1 CV of distilled water and then equilibrated with Solution 1 (Appendix C.3.1). The column was stored o/n at 4°C, in the dark, so that the purification of CyaA could be performed the following day. The column and fractions containing CyaA were brought to RT. The crude solubilised CyaA material was adjusted to 50 mM NaCl with 12 ml of 1 M NaCl (pH 8.0) and then the total volume (~250 ml) was applied to the equilibrated column. Several 50 ml volumes of Solution 1 (Appendix C.3.1) were passed through the matrix. CyaA was eluted using 50 ml volumes of an increasing 50 mM NaCl step-gradient (Appendix C.3.4). Each fraction was analysed for protein and for LPS levels by Coomassie Blue and silver staining on 7.5% and 15% SDS-PAGE gels, respectively (Section 2.5).

2.4.5.3 Phenyl-Sepharose chromatography

A 2.5 cm × 50 cm column with tap (BioRad), containing 80 ml of phenyl-Sepharose (Sigma), was first washed with 1 CV of distilled water and then equilibrated with Buffer B (Appendix C.4.2). The column was stored o/n at 4 °C, in the dark, so that the last stage of CyaA purification could be performed the following day. The column and fractions containing CyaA were brought to RT. These fractions were pooled and adjusted to 2 M urea, 50 mM Tris-HCl, 800 mM NaCl (pH 8.0) by adding 3 parts of Buffer A (Appendix C.4.1) to 1 part of pooled CyaA and the mixture was added to the equilibrated phenyl-Sepharose column. Ten 250 ml volumes of Buffer B were passed through the matrix and then CyaA was eluted with 10 × 15 ml volumes of Solution D (Appendix C.2.4). Each fraction was subjected to SDS-PAGE for determining the presence of CyaA

(Section 2.5). Such fractions were pooled together and formed the final preparation for subsequent *in vitro* and *in vivo* analysis. CyaA preparations were stored at –20 °C.

2.5 SDS-PAGE

Samples were first mixed with 5× loading buffer (Appendix D.1) and boiled for 5 min. SDS-PAGE was performed according to the method of Laemmli (1970) in a vertical gel electrophoresis tank using the Mini Protean 3™ electrophoresis system (BioRad). Resolving gels (Appendix D.2) were poured between the assembled plates with space (1.5 cm) left on top for the later addition of the stacking gel. Immediately, 100% ethanol was added to eliminate bubble formation and form a horizontal interface for the addition of a stacking gel. Once set, the ethanol was removed and then a stacking gel (Appendix D.3) was poured onto the polymerised resolving gel and a comb (with 10 or 15 wells) was placed into the gel solution and was allowed to set. The wells were rinsed out with distilled water and any residual polymerised gel on the faces of the assembled glass plates was removed. The electrophoresis tank and gels were assembled according to the manufacturer's instructions. The middle chamber was filled with 1× electrode running buffer (Appendix D.4). Samples were loaded into each well and a protein ladder (Invitrogen) was included. The outside chamber was filled with 1× running buffer. The gels were run at 100 volts for approximately 40 min or until the dye front ran off the gel.

2.5.1 Coomassie Blue staining for the detection of protein

Protein samples, run on 7.5% SDS-PAGE gels, were stained with Coomassie Blue (Appendix D.5) for a minimum of 30 min on a rotating platform. After incubation, the Coomassie Blue stain was decanted and the gel was destained (Appendix D.5) several times, for a minimum of 30 min in between changes, on a rotating platform until protein bands could be visualised. At this stage, the gel was immersed in water and subsequently photographed.

2.5.1.1 Protein quantification

The Bradford's reagent (BioRad) was employed to provide a quantitative measurement of protein content in samples after purification using a microtitre plate protocol. When Coomassie dye binds protein in an acid medium, an immediate absorbance shift occurs from 465 nm to 595 nm with a simultaneous colour change of the reagent from green/blue to blue. The Bradford's reagent was diluted 1 in 5 with distilled water and filtered (Millipore) before use. Buffer A (Appendix D.6), 250 µg/ml of bovine serum albumin (BSA) (Sigma) prepared in Buffer A, and CyaA stored in 8 M urea, diluted ¼ in distilled water to give the same concentration of urea as in Buffer A, were diluted 2-fold

down a microtitre plate with distilled water. Buffer A alone served as a negative control and blank. To a new flat-bottomed 96-well microtitre plate, 25 µl of each dilution was mixed with 200 µl of diluted Bradford's reagent. After incubation for 10 min at RT, the absorbancies were read at $OD_{620\,nm}$ in a plate reader (Dynex technologies). Once the blank OD values had been subtracted from all OD values, the protein concentrations of the samples were determined from the BSA standard curve.

2.5.2 Detection of LPS

Silver staining was used for visual identification of LPS contaminated fractions. The *Limulus* amoebocyte lysate (LAL) assay was used to quantify the levels of LPS in the purified CyaA samples. This was done by two methods: the Chromogenic method (BioWhittaker) and by the gel clot method (Cape Cod).

2.5.2.1 Silver staining for LPS

A 20 µl sample from each column fraction was incubated with 1 µl of Proteinase K (50 units/ml, Sigma) for 1 h at 37 °C. The digested protein samples were subjected to SDS-PAGE on 15% gels (Section 2.5). The procedure for silver staining for LPS was performed essentially as described by Fomsgaard *et al.* (1990). Briefly, gels were bathed in oxidising solution (Appendix E.1) for 20 min at RT and then put into staining solution (Appendix E.2) for 10 min on an orbital shaker with three washes with distilled water in between solution incubations. The gels were bathed in developing solution (Appendix E.3) until LPS bands were visualised. The reaction was stopped (Appendix E.4) and the gels were transferred into distilled water.

2.5.2.2 LAL - Gel clot method

The LAL gel clot assay was performed according to the standard operations protocol at NIBSC. Briefly, 10 µl of Pyrotell® LAL reagent (0.03 endotoxin units (EU)/ml sensitivity, Cape Cod Inc.) was incubated with 10 µl of sample at different dilutions at 37 °C for 1 h (no CO_2). CO_2 interferes with clot formation. All other materials used were endotoxin-free. A drop of 0.2% (w/v) methylene blue (George T. Gurr Ltd.) in 70% (v/v) ethanol was added to each mixture. Clot formation indicated the presence of LPS. An average between the last dilution of sample which caused gel formation and the first dilution which showed no gel formation were used to provide the level of LPS in that sample. The NIBSC 2nd international endotoxin standard (94/580) (Poole *et al.*, 1997) was used as a positive control and for calculation of LPS levels. 10 endotoxin units (EU) were taken as equivalent to 1 ng of endotoxin (Poole *et al.*, 1997). The standard was vortexed for at least 10 min prior to making dilutions in endotoxin-free water (Baxter). The test was only valid if certain dilutions of the endotoxin standards (0.06 - 0.015 EU/ml) clotted.

2.5.2.3 LAL - Chromogenic method

Matched lysates (0.005 EU/ml – 50.0 EU/ml sensitivity, Kinetic-QCL™) and *E. coli* 055:B5 endotoxin standard were reconstituted according to the supplied certificates of analysis (LAL reagent, BioWhittaker, UK) using endotoxin-free water (BioWhittaker). All materials used were endotoxin-free. To each well of a 96-well plate (Costar), 100 µl of diluted sample and dilutions of the standard were added. A negative control consisted of endotoxin-free water (Sigma). For samples requiring a spike, 10 µl of 50 EU/ml or 5 EU/ml of standard were added if endotoxin values were expected to be greater or lower than 1 EU/ml, respectively. The inclusion of spikes was important to assess whether the samples inhibited the activity of the assay. 100 µl of reconstituted lysate was added to each well and the plate was read in a microplate reader (EL_X 808, Fisher Scientific) at $OD_{405\ nm}$ every 2.5 min up to 1 h 30 min at 37 °C. The programme produced endotoxin level data as EU/ml.

2.5.3 Western blotting

After running CyaA samples (150 µg per well) on 7.5% SDS-PAGE gels, proteins were transferred onto nitrocellulose membrane (Hybond C, Amersham Pharmacia Biotech) using the Mini Protean 3™ electrophoresis tank containing the Mini Transblot™ apparatus according to manufacturer's conditions (BioRad). The stacking gel was removed and the gel was placed in an electroblotting apparatus. This was placed into an electrophoresis tank, containing 1× transfer buffer (Appendix F.1) and an ice pack. After transfer for 1 h at 100 volts, the nitrocellulose membrane was soaked in Ponceau S solution (Sigma) for several minutes and then rinsed with distilled water to confirm the transfer of proteins onto the membrane. The membranes were then bathed in blocking buffer (Appendix F.3) for 30 min, followed by three washes with PBS-Tween (PBST) (Appendix F.2). The membranes were incubated for 2 h at RT with mouse anti-CyaA immune serum (Section 2.8) and then with goat anti-mouse horse-radish peroxidase conjugate secondary antibody (Sigma), both at 1:1000 dilutions in PBST diluent (Appendix F.4) with three washes with PBST between each step. The membranes were washed twice with PBS (Appendix A.4) before the addition of substrate solution (Appendix F.5). The reaction was stopped with excess water after the appearance of bands (5 - 10 min).

2.6 Characterisation of CyaA

2.6.1 Conductimetric assay of AC enzymic activity

2.6.1.1 Preparation and purification of calmodulin

100 g of frozen pigs testicle tissue was thawed in 300 ml of Buffer A (Appendix G.1.1). The testicle tissue, diced into 1 cm^3 blocks, was homogenised for 2 min with an

electric hand blender (HR 2810, Phillips) and was then heated for 5 min at 95 °C, mixing once every min. The sample was left to cool and was adjusted to pH 8.0 and to a final concentration of ~20 mM calcium chloride (Sigma). After most of the bulk precipitate settled, the supernatant was filtered through a 150 mm diameter GF/A glass fibre filter (Whatman) using a Buchner funnel. Calmodulin was purified from the filtrate using phenyl-Sepharose (Sigma). A 100 ml volume of resuspended phenyl-Sepharose was added to a 4.5 cm × 60 cm column with tap (Chemistry Dept., University of Glasgow) and was washed with 2 CVs of distilled water followed by 2 CVs of Buffer B (Appendix G.1.2). The filtered supernatant was added to the column and then several 20 ml volumes of Buffer B were added to remove contaminating proteins. Several 5 ml volumes of distilled water were added into the column and fractions were collected. Protein concentrations in each of the fractions were calculated from the UV absorbance at $OD_{280\ nm}$ using a spectrophotometer (V550, Jasco). Fractions containing protein were pooled and added to a 3.5 cm × 20 cm glass column with tap (Chemistry dept., University of Glasgow) containing 50 ml of resuspended phenyl-Sepharose (Sigma) which had been previously washed with 2 CVs of distilled water followed by 2 CVs of Buffer B. The calcium chloride concentration of the pooled sample was increased to 1 mM before being added to the equilibrated matrix. Distilled water was added slowly and 2 ml fractions were collected. Protein concentrations in each of the fractions were calculated from the UV absorbance at $OD_{280\ nm}$. Fractions containing protein were pooled and stored at –20 °C for the conductimetric assay (Section 2.6.1.2).

2.6.1.2 Conductimetric assay

The conductimetric assay is a non-radioactive means of determining the specific activity of the adenylate cyclase toxin (Lawrence *et al.*, 1998) based on the following reactions:

1. $$(ATP\ Mg)^{2-} + Bicine^{-} \xrightarrow{[CyaA]} cAMP^{-} + (BicineH) + (PP_iMg)^{2-}$$

2. $$(PP_iMg)^{2-} \xrightarrow{[Pyrophosphatase]} 2P_i^{2-} + Mg^{2+}$$

The presence of CyaA (AC enzymic activity) will convert ATP into cAMP with the release of an inorganic pyrophosphate-magnesium ion complex $[(PP_iMg)^{2-}]$. The addition of inorganic pyrophosphatase will cleave $(PP_iMg)^{2-}$ into $P_i^{2-} + Mg^{2+}$ ions. The system predicts little change in conductance unless the pyrophosphate complex is cleaved to release the Mg^{2+} from chelation. Briefly, the apparatus consists of 8 conductivity cells, each

containing platinum electrodes which have been fused into the walls of a glass tube. The cells are all linked to computer software that processes conductimetric readings and enables tangents to curves to be made in order to determine slopes and reactions rates and to measure total displacement between the initial and final baselines. One unit of AC enzymic activity produces 1 μmol of cAMP in 1 min at 37 °C and pH 8.0. The conductimeter was switched on prior to performing the experiment so that it reached 37 °C. Each cell was filled with 2 ml of degassed bicine buffer (appendix G.1.3) containing 0.5 mM ATP (Sigma) using a Finn pipette, followed by the addition of 1 μl of 0.5 units/μl pyrophosphatase (Sigma) and 1 μl calmodulin (1.8 mg/ml, section 2.6.1.1). CyaA was diluted to 60 μg protein/ml with distilled water and the enzymometer computer programme was set to record the conductance change after the addition of 2 μl of diluted CyaA. The total conductance changes and tangents to curves were measured by an on-line screen drawing. Thus for a substrate concentration of 1 mM giving a total change of 'x' units and an initial slope of 'y' units per min, the initial rate is;

$$\text{Initial rate} = (y/x)\ \mu\text{mol/ml/min}$$

2.6.2 Haemolysis assay

The haemolysis assay was performed essentially as described by Westrop *et al.* (1997) with minor modifications. For each microtitre plate, 10 ml of defibrinated sheep erythrocytes (E and O labs, UK) were centrifuged at 2,000 × *g* for 5 min (Heraeus Multifuge 3-SR). The supernate was discarded and erythrocytes were washed twice with 20 ml Hanks HEPES (HH) buffer (Appendix G.2) and finally re-suspended to a final concentration of 0.7% (v/v) in HH buffer. CyaA, diluted in HH buffer, was mixed with an equal volume of erythrocyte suspension to a final volume of 100 μl in each well of the U-bottomed 96-well microtitre plate (Costar). Cells incubated with HH buffer in the absence of presence of 1% (v/v) Triton-X100 (Sigma) served as controls for basal (blank/negative) and 100% haemolytic activity, respectively. The plate was incubated at 37 °C in an atmosphere of 5% CO_2 for 24 h unless otherwise stated. The supernate of each well was transferred to a new flat-bottomed plate (Costar) and a reading at $OD_{540\ nm}$ was made (Dynex technologies). Percentage haemolysis was calculated using the following equation: ((sample OD – blank OD)/(positive OD – blank OD)) × 100.

2.6.3 Tissue culture

The murine J774.2 macrophage cell line (ECACC number 91051511) was grown in either RPMI (Gibco) or DMEM (Gibco) tissue culture media (Appendix G.3.1). The monomac-6 (MM6) human monocyte/macrophage cell line (Nakagawa *et al.*, 2002) (a kind gift from Prof. L. Ziegler-Heitbrock, University of Leicester) was maintained in

RPMI (Gibco) containing a more complex mix of nutrients (Appendix G.3.2). An aliquot of 1 ml of frozen cells (maintained in liquid nitrogen) were immediately incubated at 37 °C (in a water bath) and gently washed with 10 ml of pre-warmed media. This was repeated a further two times. All cells were grown in 25 cm^2 tissue culture flasks at 37 °C in 5% CO_2 until about 85% confluent on the bottom of the flask. For J774.2 cells, this took approximately 1 - 2 days. For MM6 cells, this took up to 7 - 10 days. Cells were discarded after 10 passages.

2.6.4 Cytotoxicity assays

Cytotoxicity of CyaA against mouse macrophage-derived J774.2 cells was measured in three ways: using the CellTiter-Glo™ kit (Promega), CellTiter 96™ kit (Promega) and the CytoTox 96™ kit (Promega).

2.6.4.1 CellTiter-Glo™ luminescent cell viability assay

The CellTiter-Glo™ luminescent cell viability assay (Promega) measures cell viability by determining the amount of ATP present in the cells. Any ATP present will catalyse the conversion of luciferin into a luminescent signal by luciferase. The luminescent signal is proportional to the amount of ATP present. J774.2 cells were adjusted to 5×10^5 cells/ml in RPMI (Gibco) or DMEM (Gibco) non-phenol red culture media. To each well of a 96-well black tissue culture plate with clear bottom (Labtech), 50 µl of cells were added and allowed to adhere at 37 °C, in an atmosphere of 5% CO_2 for a minimum of 30 min. Equal volumes (50 µl) of CyaA dilutions, made in the same media, were added to the cells and the plate was incubated at 37°C, in an atmosphere of 5% CO_2 for 2 h, followed by a further 30 min at RT (in the absence of 5% CO_2). Then, 100 µl of reconstituted CellTiter-Glo™ substrate was added to each well and the plate was incubated for 2 min at RT (in the dark) with shaking. Luminescence was measured in a luminometer (LUCY-1, Anthos) with an integration time of 1 sec per well over three cycles with an interval of 125 sec between each cycle. Cells incubated in medium in the absence or presence of 1% (v/v) Triton-X100 (Sigma) served as controls for basal (negative/background) and 100% cytotoxic (positive) activity, respectively. Cell killing was calculated using the following formula: ((sample OD – positive OD)/(negative OD – positive OD)) × 100.

2.6.4.2 CellTiter 96™ (MTT) cell proliferation assay

The CellTiter 96™ assay (Promega) is based on the ability of active mitochondria in living cells to reduce a yellow (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT) dye into an insoluble MTT formazan purple product. Equal volumes of

J774.2 cells, at 5×10^5 cells/ml, were incubated with dilutions of CyaA in RPMI (Gibco) or DMEM (Gibco) non-phenol red culture media containing various concentrations of calcium chloride (Sigma) and 0.2 M urea for 2 h and 24 h, or as otherwise stated, at 37 °C, in 5% CO_2. Cells incubated with media in the absence or presence of 1% (v/v) Triton-X100 (Sigma) served as controls for basal (negative) and 100% cytotoxic (positive/background) activity, respectively. For this assay, 0.2 M urea was included in the assays to minimise any aggregation of the toxin. Cell viability was not affected by the presence of 0.2 M urea (negative controls). After the incubation period, 15 µl of MTT dye was added to each well and the plates were incubated at 37 °C in 5% CO_2 for 3 h. To each well, 100 µl of stop/solublisation solution was added and plates were incubated o/n at 37°C in 5% CO_2 and a reading at $OD_{570\ nm}$ was made (Dynex technologies). Cell killing was calculated using the following formula: 100 - (((sample OD – background OD)/(negative OD – background OD)) × 100). To investigate the rate at which CyaA and CyaA* killed cells, CyaA and CyaA* were diluted to 2.5 µg/ml and then 25 µl was added to the cells for different periods of time up to 120 min. Then 25 µl of a 1/100 dilution of anti-CyaA* reference serum (Section 2.8.3.4) was added to the wells to stop any additional cytotoxicity. The plates were developed as described above.

2.6.4.3 CytoTox 96™ (LDH release) assay

The CytoTox 96™ assay (Promega) is based on the conversion of lactate to pyruvate by lactate dehydrogenase released from cells as a result of membrane damage. Equal volumes of J774.2 cells, at 2.5×10^5 cells/ml, were incubated with dilutions of CyaA in RPMI or DMEM non-phenol red culture media supplemented with 5% (v/v) FBS, antibiotics/antimycotics, 2 mM L-glutamine and 0.2 M urea and varying concentrations of calcium chloride (Sigma) for 2 h and 24 h, or as otherwise stated, at 37 °C, 5% CO_2. Cell viability was not affected in the presence of 0.2 M urea (negative controls) which was included in the assays to minimise any aggregation of the toxin. To each well of a round-bottomed 96 well plate (Costar), 50 µl cell suspension was added and allowed to adhere at 37 °C, 5% CO_2 for 30 min. After incubation, 50 µl of toxin dilutions, made in the same media, were added to the cells and incubated for 2 h or 24 h, or as otherwise stated. After the incubation, the plate was centrifuged at $250 \times g$ (rotor 6455, Heracus Multifuge 3-SR) for 5 min, then 50 µl of the supernates were transferred to a new 96 well, flat-bottomed plate (Costar) and 50 µl of the LDH substrate, prepared according to manufacturers' instructions, was added to the supernates and the plate was incubated at RT for 30 min in the dark. After incubation, 50 µl of stop solution was added to each well and the absorbance was read at $OD_{490\ nm}$ (Dynex technologies). Cells incubated with media in the

absence or presence of 1% (v/v) Triton-X100 (Sigma) served as controls for basal/background and 100% cytotoxic activity, respectively. Cell killing was calculated using the following formula: ((sample OD – background OD)/(positive OD – background OD)) × 100. The rate at which CyaA and CyaA* killed cells in the LDH release assay was performed as described in Section 2.6.4.2.

2.6.5 Measure of the oxidative burst by chemiluminescence

Phagocytes, such as neutrophil PMNLs and macrophages, kill microorganisms by the combined activity of anti-microbial proteins and reactive oxygen species (ROS). The enzyme NADPH oxidase generates ROS, such as superoxide anions (O_2^-) from molecular oxygen and these can be detected by the use of lucigenin. Superoxide reacts with lucigenin causing its excitation. The emission of light when excited lucigenin returns to the ground state can be measured and is known as chemiluminescence. Therefore, lucigenin-enhanced chemiluminescence was used to study the effect of the different CyaA preparations on the zymosan-induced oxidative burst response in J774.2 cells as described recently by Prior *et al.* (2005). Briefly, an equal volume of non-phenol red DMEM (with 0.2 M urea) was mixed with 100 µl of 2×10^6 cells/ml in a series of luminometer tubes (DOP solutions Ltd). To these, 100 µl of dilutions of CyaA, made in the same medium, were added to the tubes and incubated at 37 °C and 5% CO_2 for 1 h. After incubation, 100 µl of zymosan (1.2 mg/ml) (Fluka, Buchs, Switzerland) was added and incubated for a further 30 min. Chemiluminescence was measured at RT immediately after the addition of 100 µl of lucigenin (0.2 mg/ml) (N,N'-dimethyl-9,9'-biacridinium dinitrate – Fluka), using a TD 20/20 luminometer (Turner Designs, CA, USA). Cells incubated with media in the absence or presence of zymosan, served as controls for basal and 100% cell oxidative burst activity, respectively. Cell viability was not affected in the presence of 0.2 M urea (negative controls) which was included in the assays to minimise any aggregation of the toxin. Results were expressed as percentage of chemiluminescence relative to the positive zymosan control after subtraction of basal activity.

2.6.6 Caspase 3/7 detection

Apoptosis of J774.2 macrophage cells was measured using the Apo-ONE™ homogeneous caspase 3/7 assay (Promega). Briefly, 50 µl of cells, at 5×10^5 cells/ml, were transferred to wells of a clear bottomed, 96-well, flat-bottomed black tissue culture plate (Labtech) and incubated for a minimum of 30 min before 50 µl of CyaA dilutions were added to the cell suspension. Toxin incubation was for 2 h or 24 h at 37 °C in 5% CO_2, unless stated otherwise. After incubation, 100 µl of reconstituted caspase 3/7 substrate was added to each well and allowed to incubate for 18 h at RT before fluorescence

measurements were made using a spectrofluorometer (LS-55, Perkin Elmer) configured to detect caspase 3/7 activity at an excitation wavelength range of 485 ± 20 nm and an emission wavelength range of 530 ± 25 nm. A positive control (supplied with kit) was used for maximal fluorescence. Values produced were in arbitrary relative fluorescent units (RLFU). CyaA-free media in the absence or presence of cells, served as controls for background and basal caspase activity, respectively. Incubations and dilutions were done in non-phenol red RPMI or DMEM media with different concentrations of $CaNO_3$ or $CaCl_2$, respectively, and without 0.2 M urea, unless otherwise stated. Urea interfered with the Apo-ONE™ homogeneous caspase 3/7 assay. Another apoptosis kit (Caspase Glo 3/7™, Promega) was used but the AC enzymic activity interfered with the assay (Section 3.4.5.1).

2.6.7 IL-6 release assay

2.6.7.1 Treatment of MM6 cells

Prior to the day of experiment, MM6 cells were adjusted to a concentration of 4×10^5 cells/ml in RPMI-assay medium (Appendix G.3.2) and then 50 ml have transferred into a 150 cm^2 tissue culture flask and allowed to grow o/n at 37 °C in 5% CO_2. The following day, samples to be tested and the NIBSC 2nd international endotoxin standard (94/580) (0.25 – 4 EU/ml) were diluted in PBS and added in 50 µl volumes to wells of 96-well U-bottomed tissue culture plate (Costar). Then 100 µl of RPMI-assay medium was added to all wells, followed by the addition of 100 µl of cells (at 2.5×10^6 cells/ml). After an incubation period of 24 h at 37 °C in 5% CO_2, the supernates were collected and stored at – 20 °C until ready for the detection of IL-6 (Section 2.6.7.2). All materials used were endotoxin-free.

2.6.7.2 IL-6 detection

Detection of IL-6 from supernates was assessed using multiplex fluorescent bead technology (BioRad) using the human IL-6 antibody bead kit (BioSource) as described in Section 2.8.10.

2.6.8 Detection of phosphorylated ERK 1/2 in J774.2 cells

J774.2 cells were exposed to CyaA to elucidate if the extracellular response kinase (ERK) signalling pathway was activated in mammalian cells as a result of CyaA binding to CR3 receptors. J774.2 cells were adjusted to 5×10^5 cells/ml in non-phenol red RPMI medium and 2 ml was distributed into each well of a 6 well plate and incubated at 37 °C, 5% CO_2 for a minimum of 30 min. The medium was replaced with 3 ml of fresh medium each day until there was 80 - 90% confluency. On the day of the treatment, CyaA diluted

in RPMI medium was added to the each well to a final concentration of 20 ng/ml at 2 min intervals up to 10 min. *E. coli* BL21/DE3 LPS, at 1 EU/ml, (Section 2.1.2) and 8 M urea buffer used for CyaA, both diluted in the same medium were used as positive and negative controls, respectively. After the last incubation, all media were aspirated from all the wells and each well was washed twice with 2 ml of sterile PBS before the addition of 150 µl of lysis buffer (Appendix G.4). Cells in each well were scraped off and the lysates were collected, using a 1 ml Gilson pipette, and stored on ice. The samples were then subjected to electrophoresis (Section 2.5) on 12% SDS-PAGE gels and then the bands were transferred onto a nitrocellulose membrane (section 2.5.3). Otherwise, the samples were stored at –20 °C. Phosphorylated ERK 1/2 was detected by Western blotting (Section 2.5.3) using mouse anti-phospho-ERK 1 and ERK 2 antibody (Cell-signalling) followed by sheep anti-mouse horseradish peroxidase conjugate (Amersham), both used at 1/1000 dilution. The bands were detected by X-ray development using Western blotting detection reagents (ECL, Amersham). This was done with the help of Dr. George Baillie (Division of Biochemistry and Molecular Biology, University of Glasgow).

2.7 Biophysical analysis of CyaA

2.7.1 Dialysis of CyaA

CyaA preparations, in 8 M urea, 50 mM Tris-HCl (pH 8.0), were dialysed using dialysis tubing with a M_w cut off of 12 – 14 kDa (Medicell International Ltd.). The tubing was boiled in 0.5 mM EDTA (pH 8.0) for 30 min and stored in 70% (v/v) ethanol at 4 °C until ready to be used. The tubing was rinsed thoroughly with distilled water before use. For each millilitre of CyaA, 500 ml of 10 mM Tris-HCl (pH 8.0) was used as the dialysis buffer. CyaA was dialysed twice for 1 h at 4 °C with constant stirring. All dialysed CyaA preparations were kept at 4 or –20 °C.

2.7.2 Urease assay

Urea, in the CyaA samples, was detected using an ammonia meter (HI 93715, Hanna Instruments). The CyaA samples were diluted with distilled water to a total volume of 9.5 ml and to this, 0.5 ml of 0.1 mg/ml urease (Fluka), in 0.1 M potassium phosphate buffer (pH 7.0), was added and incubated for 10 min at RT. Samples were diluted so that they contained a urea concentration which was within the detection limit of the ammonia meter (< 9.99 mg/l). The ammonia, produced by urease, is then converted into NH_3-N by addition of reagents containing sodium hydroxide and mercury (II) iodide (Hanna Instruments). NH_3-N production was measured at $OD_{480\ nm}$ according to manufacturer's instructions. A urea standard curve was used to calculate the theoretical concentration of urea in the samples.

A refractometer (ABBE 60/70, Bellingham and Stanley Ltd.) was also employed to measure the concentration of urea in the dialysed CyaA samples. As light passes through from one medium to another, it changes speed and bends (refraction). The measure of a material's refractivity is known as its refractive index. A solution containing urea will have a higher refractive index than an aqueous solution containing no urea. The concentration of urea can be calculated from the table of values given by Nozaki and Tanford (1963).

2.7.3 Circular dichroism

Circular dichroism (CD) is based on the differential absorbance of the left and right circularly polarised components of polarised light by chiral chromophores such as the amide bonds in the regular secondary structural elements of proteins (α-helices, β-sheets etc.). Protein concentrations were calculated from the UV absorbance at 280 nm using a spectrophotometer (V550, Jasco). For assessing the effects of calcium on CyaA structure, dialysed samples were diluted to 0.5 mg/ml in 10 mM Tris-HCl (pH 8.0) with or without different concentrations of $CaCl_2$ and incubated at 4 °C for 24 h. The blanks were 10 mM Tris-HCl (pH 8.0) plus the corresponding concentration of $CaCl_2$. For assessing the effect of a reduced concentration on CyaA structure, dialysed CyaA, diluted to 0.05 mg/ml with 10 mM Tris-HCl (pH 8.0), was incubated with or without 0.3 mM $CaCl_2$. The blank for these studies was 10 mM Tris-HCl ± 0.3 mM $CaCl_2$ (pH 8.0). For urea titration experiments, non-dialysed CyaA was diluted to 0.5 mg protein/ml containing concentrations of urea between 8 and 1 M urea in the presence and absence of 1 mM $CaCl_2$ and incubated at 4 °C for 24 h. The data for 0 M urea were obtained using samples of CyaA dialysed against buffer. Spectra were acquired using a spectropolarimeter (J810, Jasco) with 0.02 or 0.2 cm pathlength cells (Hellma) for CyaA at 0.5 or 0.05 mg/ml, respectively; each spectrum represented the average of 8 scans at a scan rate of 10 nm/min over the range 260 to 180 nm, with a time constant of 2 s. Structural analysis was performed using VARSELC, SELCON, CONTIN or CDSSTR in Dichroweb (http://www.cryst.bbk.ac.uk/cdweb/html/menu.html) (Lobley *et al.*, 2002; Whitmore and Wallace, 2004). VARSELC, SELCON, CONTIN and CDSSTR are widely used algorithms which provide an estimation of secondary structure composition of proteins from far UV CD data. For studies of structural stability, CyaA samples were stored at 4 °C or –20 °C, as indicated, before spectra and further *in vitro* characterisation were recorded. For near UV CD measurements, dialysed samples were diluted to 1.5 mg protein/ml in 10 mM Tris-HCl (pH 8.0) in the presence and absence of 1 mM $CaCl_2$ before being subjected to analysis in a 0.5 cm pathlength cell (Hellma) at a rate of 10 nm/min, at a 2 sec interval over the range of 320 to 260 nm. For fluorescence studies, dialysed samples were diluted to 0.1 mg/ml in 10 mM Tris-HCl (pH 8.0) in the presence and absence of 1 mM $CaCl_2$. Samples were

measured in a rectangular quartz cell of internal dimensions 1 cm × 0.4 cm (Hellma) over the range of 400 to 300 nm with excitation at 295 nm. Negative/blanks consisted of 10 mM Tris-HCl in the absence or presence of $CaCl_2$ or urea as indicated. The CD spectra were measured in units of molar ellipticity, [θ] (deg. cm^2 $dmol^{-1}$). Dr. Sharon Kelly and Mr. Thomas Jess (University of Glasgow) performed all measurements and analyses.

2.7.4 Crystallography

Dialysed CyaA (Section 2.7.1) was used in crystallisation trials. Trials were undertaken using the sitting drop vapour diffusion method at RT (20 °C) (Stura *et al.*, 1989). Crystallisation experiments were carried out using a number of commercially available crystal screens (Crystal screen 1 and Crystal Screen II (Hampton Research), Cryo I and Wizard II (Emerald Biosystems)). Trials were also carried using the 'foot print' screen (Stura *et al.*, 1992) in which precipitents of various concentrations were used at the same pH to discover the conditions at which the protein could precipitate. The vapour diffusion technique allows the slow loss of water (and other components) from the well solution to the reservoir solution, which effectively causes an increase in the concentration of the protein in solution (through water loss). Using a number of trials, it was hoped that conditions would be found that resulted in crystal growth (showers of micro crystals or small/large single crystals). Crystal trials were carried out using Cryschem 24-well sitting drop plates (Hampton Research). Each reservoir contained one of the conditions from the screens, whilst the well (sitting drop) contained equal volumes of protein and precipitent (2 µl : 2 µl). Trays were sealed using crystal clear tape (Hampton Research). Crystallisation trials were also undertaken using CyaA in the presence of 1 mM $CaCl_2$. For this, each reservoir contained one of the conditions from the screens, whilst the well (sitting drop) contained 1 µl protein and 3 µl precipitent. This work was done with the help of Dr. Alan Riboldi-Tunnicliffe (Department of Chemistry, University of Glasgow).

2.7.5 Analytical ultracentrifugation

During ultracentrifugation, proteins in solution are spun at high speeds (typically 3,000 – 60,000 rpm), resulting in movement of the particles through the solvent in the direction of the centrifugal force and sedimenting as a function of time. The sedimentation process is dependent on mass, shape and charge of a particle and causes depletion of the solute from the meniscus and the formation of distinct solute/solvent boundaries. In analytical ultracentrifugation (AUC) the movement of the boundaries can be observed by monitoring the solute concentration using absorbance or interference (Schachman *et al.*, 1962; Giebeler, 1992). This technique does not require labelling, chemical modification or interaction with a surface or supporting matrix. CyaA was dialysed as described in Section

2.7.1 with the exception that, for each ml of CyaA dialysed, a volume of 1000 ml of 10 mM Tris-HCl (pH 8.0) buffer was used and three buffer changes were made.

2.7.5.1 Calculation of buffer densities and viscosities

Calculations of buffer viscosity (η), buffer density (ρ) and partial specific volume ($\bar{v}$) were done using the programme, SEDNTERP (Version 1.08) (Laue *et al.*, 1992). SEDNTERP was downloaded from http://www.jphilo.mailway.com/download.htm.

2.7.5.2 Sedimentation equilibrium analytical ultracentrifugation

In sedimentation equilibrium (SE) AUC the opposing forces of sedimentation and diffusion lead to the establishment of a thermodynamic equilibrium with no net movement of molecules within the concentration gradient. SE experiments were performed at 4 °C in a Beckman Coulter Optima XL-1 analytical ultracentrifuge (Palo Alto, USA) using an An-50 Ti rotor and rotor speeds of 7,000 rpm, 11,000 rpm and 15,000 rpm. Concentrations of dialysed CyaA between 0.18 – 3 mg/ml in 10 mM Tris-HCl ± 1mM $CaCl_2$ (pH 8.0) were used. The samples (80 µl) were loaded into 12 mm pathlength charcoal-filled epon double sector centrepieces. Attainment of equilibrium was ascertained with WinMATCH (http://www.biotech.uconn.edu/auf/) until no net movement of protein was observed in scans recorded 3 h apart. SE data were analysed with the programme UltraScan (http://www.ultrascan.uthscsa.edu/). All analyses were performed by Dr. Olwyn Byron (Division of Infection and Immunity, University of Glasgow).

2.7.5.3 Sedimentation velocity analytical ultracentrifugation

In sedimentation velocity (SV) experiments, proteins are separated as a result of their different rates of migration through the centrifugal field. Generally, greater rotor speeds are used for SV experiments compared with SE experiments. The sedimentation is monitored over time by a set of absorbance/interference profiles. At the start of the experiment, the solute is distributed homogeneously throughout the radial range recorded. At later times, sedimentation profiles show movement of the solute boundary towards the bottom of the cell. The sedimentation coefficient (s) of a macromolecule is obtained from its sedimentation profile and defined by the Svedberg equation ($s = DM(1-\bar{v}\rho)/RT$, where M is the molecular mass (g mol^{-1}), D is the diffusion coefficient (cm^2 s^{-1}), R is the gas constant (8.314 erg mol^{-1} K^{-1}) and T the temperature (K)). SV experiments were performed at 4 °C in a Beckman Coulter Optima XL-1 analytical ultracentrifuge (Palo Alto, USA) using an An-50 Ti rotor and rotor speeds of 40,000 rpm to 48,000 rpm. Concentrations of dialysed CyaA between 0.18 – 3 mg/ml in 10 mM Tris-HCl ± 1mM $CaCl_2$ (pH 8.0) were used. Samples (360 µl) were loaded into 12 mm pathlength charcoal-filled epon double

sector centrepieces. Approximately 220 scans were taken at an interval of 1 minute using interference optics. Data were analysed with the programme SEDFIT (version 9.1, National Institute of Health, USA), with the help of Dr. Olwyn Byron (Division of Infection and Immunity, University of Glasgow).

2.8 *In vivo* experiments

2.8.1 Animals

Female NIH mice, aged 3 – 4 weeks were obtained from Harlan, UK and maintained in pathogen-free conditions.

2.8.2 Vaccines

A commercially-available Diphtheria Tetanus acellular Pertussis vaccine (DTaP/ACV, hereafter referred to as ACV) (Infanrix, Belgium) was used. Within 1 single human dose (SHD), there are 25 μg of detoxified pertussis toxin (dPT), 25 μg of FHA, 8 μg of PRN, 30 International Units (IU) of diphtheria toxoid, 40 IU of tetanus toxoid, 0.5 mg $Al(OH)_3$, 2-phenoxyethanol (preservative), sodium chloride and water. PT in the vaccine had been toxoided with glutaraldehyde solution and then later with formalin. FHA and PRN were treated with formalin only.

2.8.3 Immunisation

2.8.3.1 Immunisation with different CyaA preparations alone

Groups of five mice were injected intraperitoneally with 0.5 ml of Dulbecco's phosphate-buffered saline without calcium and magnesium (D-PBS, Gibco) containing different CyaA preparations (25 μg protein/dose) with or without 10% (v/v) alum [2% (w/v) $Al(OH)_3$, 1.3% (w/v) Al_2O_3] (JMF, Denmark) or with 0.5 ml of D-PBS with or without 10% (v/v) alum which served as a negative control. Mice were immunised on days 0 and 28 and sampled or challenged intranasally on day 42. For the aerosol challenge, mice were immunised on days 0 and 38 and challenged on day 56.

2.8.3.2 Immunisation with CyaA* with different doses of ACV

Groups of five mice were injected intraperitoneally with 0.5 ml of D-PBS containing a high (¼ SHD) or low (⅛ SHD) dose of ACV + 25 μg CyaA* without alum or with 0.5 ml high (¼ SHD) or low (⅛ SHD) dose of ACV alone or with 0.5 ml of D-PBS in 10% (v/v) alum which served as a negative control. Mice were immunised on days 0 and 28 and sampled or challenged intranasally on day 42. CyaA* was chosen because preliminary work showed that it provided better adjuvant activity than CyaA towards *B. pertussis* antigens (MacDonald-Fyall *et al.*, 2004).

2.8.3.3 Immunisation with different CyaA preparations plus ACV

Groups of five mice were injected intraperitoneally with 0.5 ml of D-PBS containing ⅛ SHD of ACV + 25 μg protein of different CyaA preparations without alum. Control groups consisted of mice immunised with 0.5 ml of D-PBS in 10% (v/v) alum or with 0.5 ml of D-PBS containing ⅛ SHD of ACV. Mice were immunised on days 0 and 28 and challenged intranasally on day 42.

2.8.3.4 Production of mouse anti-CyaA* reference serum

One hundred female NIH mice were injected intraperitoneally with 0.5 ml of D-PBS containing 25 μg protein of CyaA* adsorbed onto 10% (v/v) alum by incubation o/n at 4 °C. Mice were boosted twice with the same formulation on days 28 and 56 and sampled for blood on day 70. Sera, obtained from heart bleeds, were pooled and stored at – 20 °C. An arbitrary value of 30,000 ELISA units/ml was attributed to this serum for the calculation of geometric means of serum anti-CyaA antibody levels.

2.8.4 Preparation of *B. pertussis* suspension for aerosol and intranasal challenge

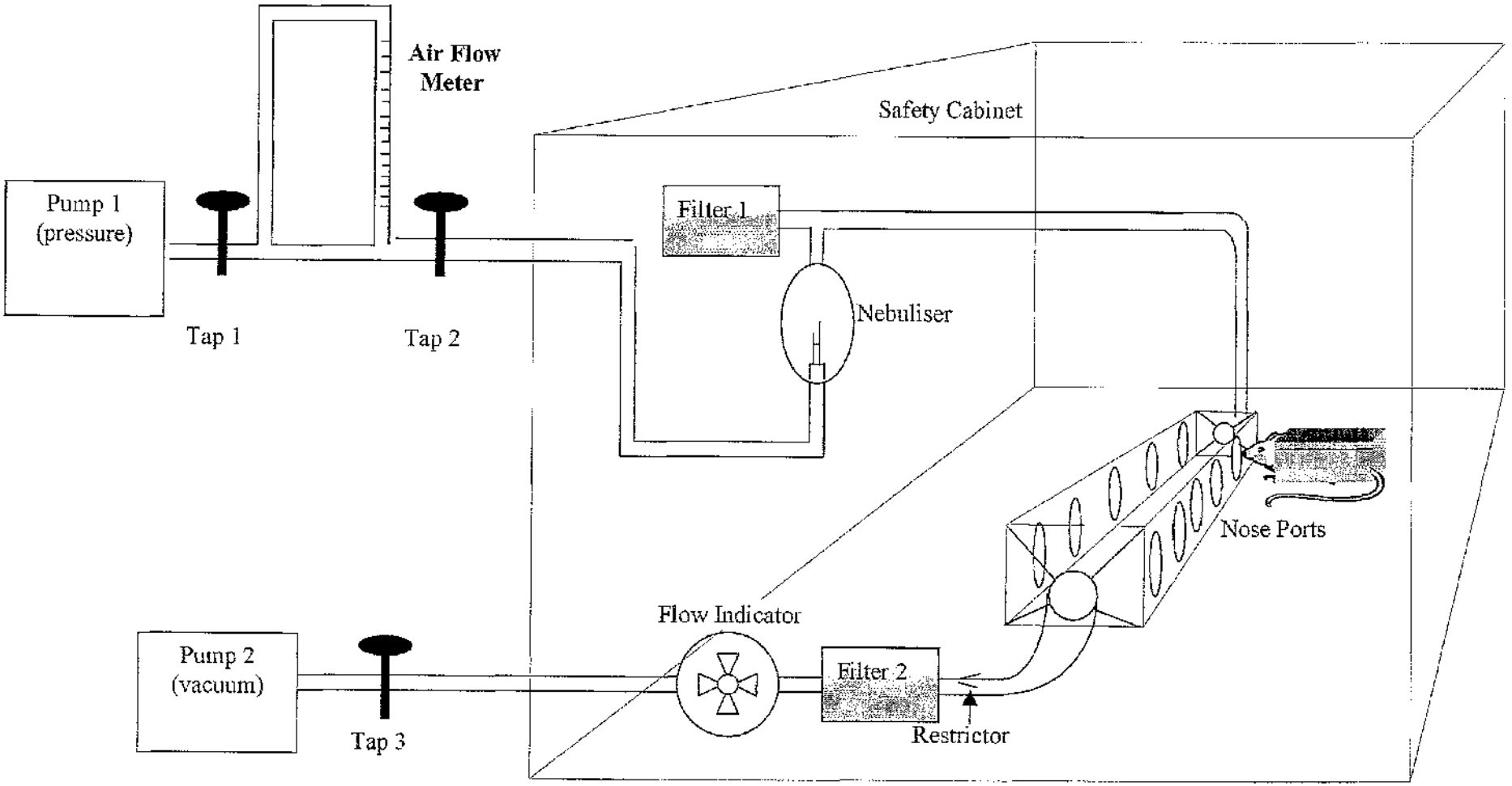
For aerosol challenge, *B. pertussis* 18.323 was grown on blood CA plates for 2 days and sub-cultured for 16 – 18 h twice at 37 °C. The bacterial cells were re-suspended in casamino acids solution (Appendix H.1) and adjusted to an $OD_{625\ nm}$ of 0.26 using a spectrophotometer (MSE, Fisons) to give 4.5×10^8 colony forming units (CFU)/ml. For intranasal challenge, *B. pertussis* 18.323 was grown on blood CA plates for 18 h followed by two further sub-cultures for 16 – 18 h. The bacterial cells were re-suspended in casamino acids solution and adjusted to an $OD_{625\ nm}$ of 0.2 using a spectrophotometer (MSE, Fisons) to give 4×10^8 CFU/ml. The bacterial suspensions were kept on ice until ready for challenge.

2.8.5 Aerosol infection of mice with *B. pertussis*

Aerosol challenge was performed as described previously by Xing *et al.* (1999). The aerosol apparatus was set up as shown in Figure 7. Briefly, two groups of five mice were placed in metal restraint boxes. Their noses were positioned through membranes of a metal nose port section of the apparatus and were exposed to aerosols produced by a nebuliser containing a *B. pertussis* suspension for 5 min. Lungs and trachea were removed from one PBS control group at 2 h post-challenge and from all groups at 7 days post-challenge. The lungs and trachea from each mouse of each group were homogenised in 1 ml of casamino acids solution. Viable counts were made by plating serial dilutions of the homogenate on blood CA plates and the numbers expressed as CFU/lung.

Figure 7 Schematic diagram of aerosol challenge apparatus

The bacterial suspension is added into a sterile glass nebuliser. Air is then pumped (pump 1) through to the nebuliser creating an aerosol of bacteria. A valve is released which allows the aerosol suspension of bacteria to flow through to the metal rack containing nose ports into which the noses of mice are positioned. The aerosol suspension continues to flow beyond the rack and into a filter and finally into pump 2.


Air Flow Meter
Safety Cabinet
Pump 1 (pressure)
Filter 1
Nebuliser
Tap 1
Tap 2
Nose Ports
Flow Indicator
Pump 2 (vacuum)
Filter 2
Tap 3
Restrictor

2.8.6 Intranasal infection of mice with *B. pertussis*

Anaesthetised mice were inoculated with 10 µl/nostril of *B. pertussis* strain 18.323 suspension. The lungs and tracheas of five mice from the PBS control group were sampled 2 h post-challenge and from all other immunised groups, and another group of five PBS control mice, 7 days post-challenge as described in Section 2.8.5. Viable counts were performed as described in Section 2.8.5.

2.8.7 Sampling for blood, spleen cells and peritoneal macrophages

Sera were obtained from heart bleeds for each mouse and stored at –20 °C. Extraction of spleen cells and peritoneal macrophages were performed at the same time and described below.

2.8.7.1 Culture of mouse peritoneal macrophages and spleen cells

Each peritoneal cavity was lavaged with 5 ml of sterile PBS wash buffer (Appendix G.3.3) and the lavage fluids were pooled from one group of five mice and cells recovered by centrifugation at 800 rpm for 10 min (rotor 6455, Heraeus Multifuge 3-SR). The cell pellets were resuspended in RPMI (Appendix G.3.1) to a concentration of 2×10^6 cells/ml. To each well of a 24-well tissue culture plate (Costar), 1 ml of cell suspension was added and incubated at 37 °C in 5% CO_2 for 2 h. The cells were washed three times with the PBS wash buffer to remove non-adherent cells. Single-cell suspensions from spleens were prepared from pooled spleens by passing the tissue through a cell strainer (Falcon) with sterile PBS wash buffer. Erythrocytes were depleted by suspension in sterile distilled water followed by centrifugation at 1000 rpm for 20 min, at 4 °C (rotor 6455, Heraeus Multifuge 3-SR, Fisher Scientific). The cells were then washed twice with PBS wash buffer and resuspended in RPMI medium to a concentration of 2×10^6 cells/ml. To each well of a 24-well tissue culture plate, 2 ml of cell suspension was added and cultured as above.

2.8.8 Treatment of macrophages and spleen cells

Macrophages and spleen cells were cultured with and without 5×10^7 heat-killed *B. pertussis* cells/well as described by Xing *et al.* (1998) or with combinations of CyaA*, formalin treated-PT, -FHA and -PRN (supplied by GSK, Belgium) used at 1, 2, 2 and 5 µg protein/ml, respectively. Formalin treated-PT, -FHA and -PRN were the same individual components which were used in the ACV for vaccination studies (Section 2.8.3.3). Cultures were incubated at 37 °C, in an atmosphere of 5% CO_2 for 24 h and for 48 h for macrophages and spleen cells, respectively. Cell viability was confirmed by trypan blue staining.

2.8.9 Determination of nitrite production

Nitrite determinations were made in triplicate on 50 µl volumes of pooled macrophage cell culture supernates following stimulation with antigens *in vitro* from immunised mice. The sample was mixed with 50 µl of Greiss reagent (Appendix H.2) and the absorbance at $OD_{540\ nm}$ was measured after incubation for 5 min at RT using an Anthos ELISA plate reader (Life Science International). The concentration of nitric oxide (NO) in the macrophage supernates was calculated by using a standard curve for sodium nitrite (Sigma).

2.8.10 Determination of cytokine production

Detection of cytokines in supernates of stimulated macrophage and spleen cells was done by Multiplex fluorescence bead technology (BioRad). The mouse cytokine 10-plex (BioSource) kit (which detects IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, TNFα, GM-CSF and IFNγ), with the addition of murine IL-8, was used according to the manufacturer's recommended procedure. Briefly, this technology is similar to a sandwich ELISA. Each cytokine is captured by antibodies which are coupled to beads with specific fluorescence markers to allow identification. Cytokine-specific biotinylated detector antibodies are added which bind to the appropriate immobilised cytokines. Streptavidin conjugated to a fluorescent protein, R-phycoerythrin (Streptavidin-RPE), was then added and binds to the biotinylated detector antibodies associated with the immune complexes on the beads, forming a four-member solid-phase sandwich. The beads were analysed with a Luminex 100™ (BioRad) instrument. By monitoring the spectral properties of the beads and the amount of associated RPE fluorescence, the concentrations of the cytokines were determined by comparison with supplied standards which served as positive controls. Cell culture medium alone served as a negative control. The sensitivities for each cytokine were as follows: IL-1β (10 pg/ml); IL-2 (20 pg/ml); IL-4 (5 pg/ml); IL-5 (10 pg/ml), IL-6 (10 pg/ml); IL-10 (15 pg/ml); IL-12 (15 pg/ml); TNF-α (5 pg/ml); GM-CSF (10 pg/ml) and IFN-γ (1 pg/ml). This work was performed with the help of Dr. Alastair Gracie (Division of Immunology, University of Glasgow).

2.8.11 Enzyme-linked immunosorbent assay (ELISA)

2.8.11.1 ELISA for IgG

ELISA plates (Maxisorp, Nunc) were coated o/n at RT with 100 µl of carbonate buffer (Appendix H.3.1) containing purified recombinant CyaA, PT, FHA or PRN, all at 2 µg protein/ml. PT, FHA and PRN were purified native proteins (GSK, Belgium). Plates were washed with PBS-Tween (PBST - Appendix H.3.2.1) and blocked by incubation with 200 µl of PBST blocking buffer (Appendix H.3.2.2) for 1 h at RT. After washing the plates

with PBST, 100 µl of serial dilutions in PBST of test sera, mouse anti-CyaA reference serum (Section 2.8.3.4), or First International Reference anti-*Bordetella pertussis* mouse serum (97/642, NIBSC) containing known levels of IgG antibodies to PT, FHA or PRN, were dispensed into the ELISA plate wells and incubated for 2 h at RT. For total IgG measurements, the plates were washed and then incubated with HRP-conjugated sheep anti-mouse IgG (Sigma) diluted 1 in 1000 in PBST (100 µl/well). Following incubation at RT for 2 h, 100 µl of peroxidase substrate solution was added (Appendix H.3.4) and incubated in the dark for 10 - 15 min. The reaction was stopped by addition of 50 µl per well of 1 M H_2SO_4 and the absorbance values at $OD_{450\ nm}$ were measured in an Anthos ELISA plate reader (Life Science International, UK). Total IgG was calculated as the geometric mean of the antibody titres (see below).

2.8.11.2 ELISA for IgG1 and IgG2a subclasses

For determination of IgG1 and IgG2a antibody levels to CyaA, PT, FHA and PRN, the same reagents and reference sera were used as described in Section 2.8.11.1 except that, for detection, 100 µl of biotin-conjugated rat anti-mouse IgG1 or IgG2a (BD Pharmingen) followed by streptavidin-HRP conjugate (BD Pharmingen), all at 1 in 1000 dilution, were added to each well and incubated for 90 min, in turn, in the dark with washing in PBST in between each step at RT. 100 µl of SigmaFast™ substrate [tablets of o-phenylenediamine di-hydrochloride and urea/hydrogen peroxide dissolved in 20 ml of sterile distilled water (Sigma)] was added per well and incubated for 10 - 15 min, in the dark. The reaction was stopped with 50 µl of 3 M HCl to all wells and the absorbance values were read at $OD_{493\ nm}$. For determination of total IgG, IgG1 and IgG2a antibody levels, potencies or antibody titres relative to a reference serum (which had been assigned an arbitrary value in ELISA units (EU)/ml), were obtained by parallel-line analysis of $\log_{10}$ sample OD against $\log_{10}$ dilution.

2.8.11.3 Neutralisation assays

Samples of the pooled serum from each group of mice were heat-treated for 30 min at 56°C to remove complement activity in the serum. Equal volumes of sera dilutions were incubated with concentrations of CyaA for 30 min at RT as follows: 2.5 µg protein/ml for cytotoxicity, 20 µg protein/ml for haemolysis, 60 µg protein/ml for enzymic activity. All assays were performed as described in Section 2.6.

2.9 Phagocytosis

It was of interest to find out whether anti-CyaA sera increased the ability of phagocytes to phagocytose *B. pertussis*. Blood, from two female NIH, 5-6 week old mice,

was collected into heparin-coated (250 units/ml – Sigma) syringes. Distilled water was added to the blood sample to lyse erythrocytes (Section 2.8.7.1) leaving a suspension of white blood cells (WBCs). The WBCs were washed twice with Hanks HEPES balanced salt solution (HBSS - Invitrogen) and then resuspended in HBSA (HBSS containing 0.25% (w/v) BSA) to a concentration of 7.4×10^4 cells/ml. *B. pertussis* 338 expressing (Section 2.1.3) were resuspended in HBSA to a concentration of 4×10^7 cells/ml and kept on ice. Different volumes (30, 60 or 90 µl) of bacterial suspension were incubated with 30 µl of heat-inactivated anti-PBS, different anti-CyaA sera (at 1/30 dilution) or anti-CyaA* reference serum (at 1/100 dilution) (Sections 2.8.3.4 and 3.6.3.1) in a total volume of 150 µl in HBSA for 30 min at 37 °C in 5% CO_2. Different volumes of bacteria gave different bacteria: leukocyte ratios or different multiplicities of infection (MOI). As a control, 30 µl of unopsonised bacteria were incubated with 120 µl of HBSA. Each assay was supplemented with 1 ml of WBC suspension and then the bacteria/WBC mixture was added to a well of a 24-well plate and incubated for 1 h at 37 °C in 5% CO_2 with shaking. After incubation, the cells were washed twice with HBSA and blocked with 15 µl of anti-CD16/32 monoclonal antibody for 15 min at RT followed by incubation with 10 µl anti-CD11b allophycocyanin (APC)- and 10 µl anti-CD45 R-phycoerythrin (RPE)-labelled monoclonal antibodies or with 10 µl of isotype-matched antibodies which served as negative controls (Caltag-MedSystems) for 25 min in the dark. Cells were washed twice with PBS-flow (Appendix H.3.5.1) and were then fixed in FACS-Fix (Appendix H.3.5.2) and transferred to a Fluorescence-Activated Cell Sorter (FACS) tube. Acquisition of 10,000 events was carried out in a FACS Calibur cytometer (Becton Dickinson) and analysis was performed using WinMDI v2.7 software. Control cells were incubated in the same medium in the absence of *B. pertussis* 338. The fluorescence associated with *B. pertussis*-treated cells was compared with the fluorescence associated with control cells. The extent of *B. pertussis* associated with cells expressing CD11b and CD45 was measured by the increased fluorescence caused by the GFP expressed by the bacteria. The results are expressed as percentage of median fluorescence intensity (MFI) associated with the cells treated with *B. pertussis* relative to the maximal MFI associated to the controls cells in the absence of *B. pertussis* (100% MFI).

2.10 Statistical analyses

Several groups were compared with one control group using one-way analysis of variance (ANOVA) – Dunnet's test. For multiple comparisons between groups (where no defined control group was used), Tukey's test (ANOVA) was used. For comparison of two groups, Student's *t* test was used. Statistical significance level was defined as $P < 0.05$.

2.11 Preparation of bone marrow-derived macrophages for microarray analysis

2.11.1 Animals

Male, 10 – 12 week old, Balb/c mice were purchased from Charles River Labs and maintained in pathogen-free conditions.

2.11.1.1 Extraction and maintenance of mouse bone marrow macrophages

Dr. Garwin Sing (Scottish Centre for Genomic Technology and Informatics, University of Edinburgh) performed extractions of bone marrow-derived macrophages (BMMs) from mouse femurs. Briefly, a 21G needle fitted with a 3 ml syringe was filled with Dulbecco's PBS (Sigma). The needle was then inserted into the cortex at one end of the dissected femur. The bone marrow was then flushed out into a 50 ml tube containing 5 ml PBS and was repeated 3 times on the other side of the dissected femur. The bone marrow cell progenitors were resuspended in complete DMEM/F12 (Gibco) growth medium containing GlutaMAX™ (Appendix I.1) and 10% (v/v) L929 cell conditioned medium (Section 2.11.1.2). BMMs were seeded at a density 1.6×10^6 cells/well in a 6-well tissue culture dish (Costar) in 4 ml of complete medium and incubated at 37 °C in 5% CO_2. After 3 days, non-adherent cells were washed off and the wells were replenished with 2 ml of fresh complete medium with additional medium changes every 48 h. Depending on confluency, the macrophages were used on day 6 or 7. Cells were either pooled to be used for the MTT assay (Section 2.11.2) or used for RNA extraction (Section 2.11.3).

2.11.1.2 Preparation of L929 conditioned medium

Conditioned medium from murine L929 connective tissue cells (ECACC #: 87031904) as a source of macrophage colony-stimulating factor (M-CSF) (Martinat *et al.*, 2002) was prepared as follows: A seed stock of L929 cells was grown to confluency in a 165 cm^2 tissue culture flask (Corning) in DMEM/F-12 growth medium (Gibco) supplemented with 10% (v/v) heat-inactivated FBS and 100 U/ml of penicillin and of streptomycin (Gibco). The monolayer was trypsinised and the cell pellet resuspended in 30 ml growth medium. One ml cell aliquots were then used to seed a number of 165 cm^2 flasks, each containing 49 ml of growth medium. Cultures were incubated at 37 °C in 5% CO_2 for 2 weeks, after which the conditioned medium was decanted, filtered and stored at –20 °C until use.

2.11.2 MTT assay

In order to determine a concentration of CyaA, CyaA* and proCyaA* suitable for BMM treatment without significant killing, a MTT assay was performed. Briefly, 50 µl of

BMMs in DMEM/F12 growth medium (Section 2.11.1.1), at 4×10^5 cells/ml, were dispensed into wells of a 96-well tissue culture plate (Costar) and incubated at 37 °C in 5% CO_2 until 70% confluency. The cells were treated with toxin for 2 h or 24 h and the assay was continued as described previously in Section 2.6.4.2.

2.11.3 Treatment and harvesting of cells for RNA extraction

The medium in each well of the 6-well plate, was replaced with 1 ml of DMEM/F12 serum-free medium with no other additives except for antibiotics and L-glutamine plus either 20 ng protein/ml of CyaA, CyaA* or proCyaA* and incubated for 2 h or 24 h at 37 °C in 5% CO_2. Urea was used at the same concentration as in the CyaA treatments and served as a negative control. Three or nine incubations of each treatment were performed. After incubation, the supernates were collected and stored at –20 °C for detection of cytokines (2.8.10).

2.12 RNA extraction

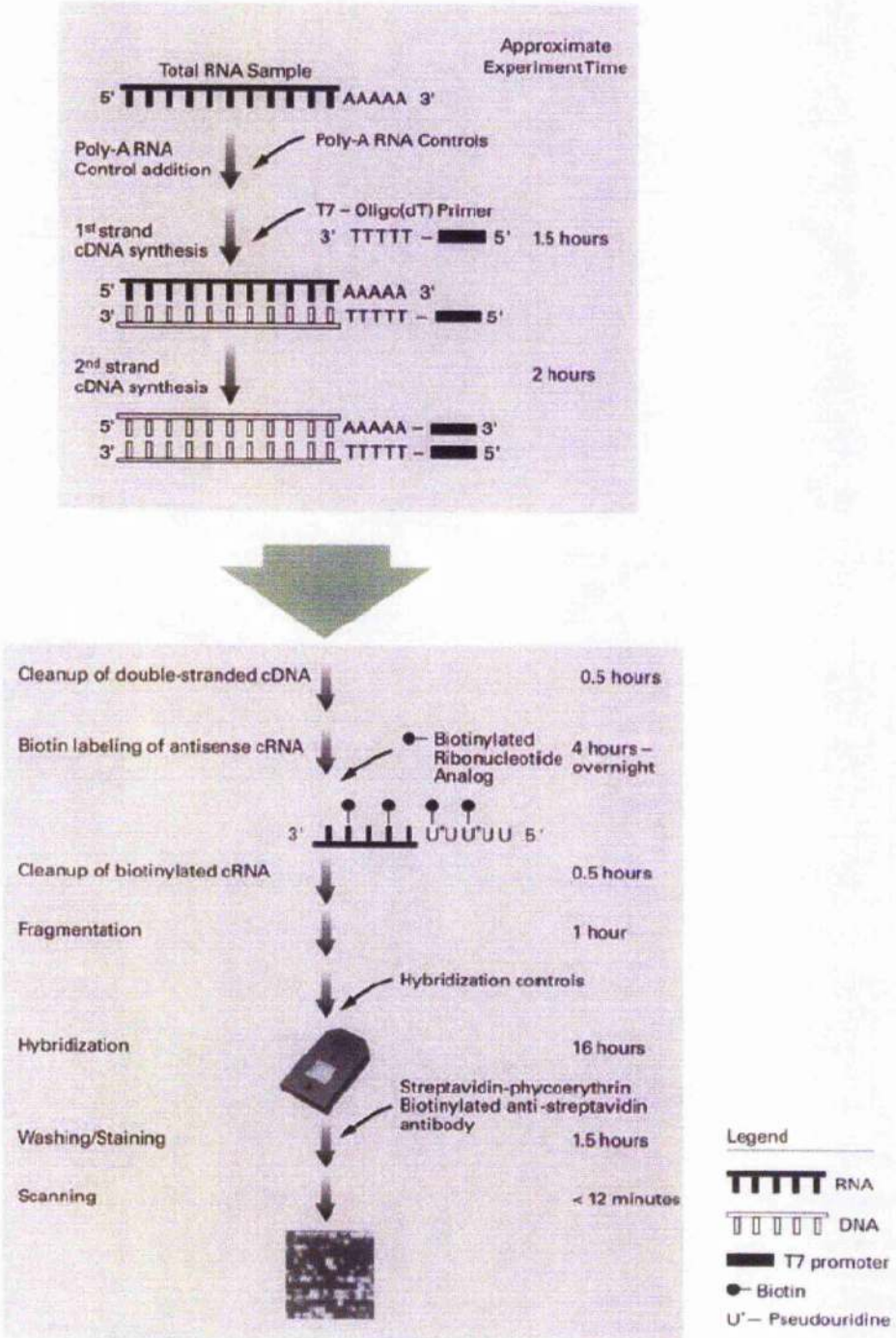
0.33 ml of Trizol (Invitrogen) was added into all wells and left for 5 min at RT. The cells in each well were manipulated with a cell scraper in order to ensure complete lysis. Trizol lysates were pooled from three wells of the same treatment generating three pooled total RNA samples for each treatment. The total RNA was then transferred into eppendorf tubes. To each tube, 200 µl of chloroform was added and shaken vigorously for 15 sec. The RNA mixture was incubated at RT for 2 – 3 min and then centrifuged at 12,000 × *g* for 15 min at 4 °C (model 5417c, Eppendorf). Using a pipette, the aqueous phase was removed and transferred into a new eppendorf tube. To each tube, 0.5 ml of isopropyl alcohol was added and tubes were inverted several times and left at RT for 10 min to precipitate the RNA. The tubes were centrifuged at 12,000 × *g* for 10 min at 4 °C (5417c, Eppendorf). The isopropyl alcohol was decanted and the RNA pellet was washed with 1 ml of 75% (v/v) ethanol. The tubes were vortexed briefly and then centrifuged at 7,500 × *g* for 5 min at 4 °C. The ethanol was decanted and the tubes were inverted to air dry for approximately 10 min. The RNA was dissolved in 10 µl of water and stored at –80 °C. The RNA was subjected to spectrophotometric determination of concentration by measurements at $OD_{260\ nm}$. The quality of RNA was also checked by electrophoresis on the Agilent Bioanalyser system (Agilent technologies) according to manufacturer's instructions by Dr. Paul Dickinson (Scottish Centre for Genomic Technology and Informatics, University of Edinburgh). All materials were RNAse free.

2.13 Synthesis of cRNA and hybridisation with Affymetrix chips

Synthesis of cRNA and hybridisation was performed by Ms Marie Craigon (Scottish Centre for Genomic Technology and Informatics, University of Edinburgh) and the procedure is shown diagrammatically in Figure 8. For the hybridisation of RNA onto Affymetrix chips for microarray analysis, double stranded cDNA was first synthesised from total RNA. T7-$(dT)_{24}$ primers and Superscript II reverse transcriptase were used for this process to generate single stranded cDNA, followed by incubation with DNA polymerase I to generate double stranded cDNA. The cDNA was subjected to a phenol/chloroform extraction and ethanol precipitation procedures. Biotinylated cRNA was generated from double stranded cDNA by using T7 RNA polymerase. Affymetrix use labelled cRNA because the process produces a linear amplification of the target material. The cRNA was purified using RNeasy (Qiagen) and checked for quantity by measurements at $OD_{260\ nm}$ and quality by running the samples in RNA 6000 Nano LabChips (Agilent Technologies) analysed in an Agilent 2100 Bioanalyser (Agilent Technologies). Agilent LabChips use microfluidics technology for the analysis of biological samples, such as RNA. The Agilent 2100 Bioanalyser produces gel-like images, electropherograms (peaks) and integrities of the RNA samples. Heating the sample to 94 °C for 35 min in the presence of Mg^{2+} ions was used to fragment the labelled cRNA. The fragmented cRNA was incubated with various controls and heated to 99 °C for 5 min. For example, a synthetic control, oligo (B2), was added to the mix to provide grid alignment signals used by the analysis software. *bioB*, *bioC* and *bioD*, from *E. coli*, and *cre*, from P1 bacteriophage, are antisense biotinylated cRNA used as hybridisation controls. *lys*, *phe*, *thr* and *dap*, from *Bacillus subtilis*, are poly-A-tailed sense RNA which are spiked into the isolated RNA samples as controls for the labelling and hybridisation process. The spikes were also used to estimate assay sensitivity. The sample was centrifuged to remove any particulate material and was then hybridised with a Test-3 array to check the integrity of the source RNA before proceeding onto the actual hybridisation; degraded RNA will not label evenly. Probes for the 5', middle and 3' end of genes for β-actin and GAPDH was assessed on the Test-3 array as a measure of RNA integrity. The cRNA was hybridised with MG-U74Av2 or with MOE430_2 GeneChips® for 16 h. After the incubation, the chips were subjected to an incubation/wash procedure to amplify the fluorescence signal according to manufacturer's instructions (Affymetrix). This involved the addition of streptavidin-phycoerythrin (SAPE) which binds to biotinylated cRNA target molecules hybridised to the array. Goat anti-SAPE IgG is then added followed by biotinylated anti-goat IgG antibody.

Figure 8 Eukaryotic target labelling for expression analysis

The preparation of cRNA from mRNA is described in Section 2.13.



Adapted from:

http://www.affymetrix.com/Auth/support/downloads/manuals/expression_s2_manual.pdf

Finally, more SAPE is added which will bind to the biotinylated antibody complex thereby amplifying the original fluorescence signal. Hybridised and washed microarrays were scanned using the Affymetrix GeneChip Scanner 2500 or 3000 to visualise phycoerythrin fluorescence by laser excitation at 532 nm and signal detection at 570nm. Fluorescence images were analysed using Microarray Suite 5.0 (MAS5) or Gene Chip Operating System 1.2 software (GCOS1.2, Affymetrix, USA) as detailed below.

2.14 Microarray analysis

MAS5 and GCOS1.2 were used to control analysis of Affymetrix GeneChip® microarrays from experimental set up to data output. GCOS1.2 provides control of GeneChip® Fluidics Stations and Scanners. In addition, GCOS1.2 acquires data, manages sample and experimental information, performs gene expression data analysis and supports other packages for resequencing, mapping data analysis and report generation. A number of data types are captured during analysis which can be distinguished by their file extensions (.EXP, .DAT, .CEL, .CHP. and .RPT). These file types correspond to different stages along the analysis path such that .EXP files contain experimental details, .DAT files contain raw image data, .CEL files are processed image data, .RPT files contain a report of particular image parameters and .CHP files are normalised image data to the urea control. The .CHP files are normalised to the global signal from the entire array for each individual chip i.e. the average brightness of each image is adjusted to the same brightness allowing comparisons of differential expression between chips to be made. Additionally to using this software, much of the analysis of Affymetrix arrays was performed using the Bioconductor1.4 package (http://www.bioconductor.org/) running under the R1.9 environment (http://www.r-project.org/). Bioconductor allows normalisation with the MAS5 algorithm as well as other normalisations such as rma, gcrma, d-chip etc. Empirical based statistical testing using Benjamini/Hochberg false discovery rate correction was used to determine significances between replicates and treatments ($P < 0.05$). GeneSpring 7.2 (Silicon genetics) was used to analyse the microarray data by enabling visual and analytical comparisons between different data sets.

One website which is particularly useful is the Database for Annotation, Visualisation and Integrated Discovery (DAVID) 2.1 (http://david.abcc.ncifcrf.gov/). DAVID 2.1 provides a comprehensive set of tools for investigators to visually summarise annotation from a large list of genes. One example is the Functional Classification Tool (FTC). FTC provides summaries for each gene entered into the database, enabling the user to discover the function of the proteins encoded by the genes in question.

Chapter 3

Results

3.1 DNA

A kanamycin-resistance cassette was introduced into plasmid pGW54 so that it could be used for the expression of CyaA in *E. coli* BL21/DE3 *lpxM* as both *E. coli* BL21/DE3 *lpxM* and pGW54 possessed genes for chloramphenicol resistance. Plasmid pGW44/188 was sequenced to confirm the presence of a 6 base pair sequence in the AC region of the *cyaA* gene.

3.1.1 Construction of a kanamycin resistant form of plasmid pGW54

The 1.3 kb kanamycin cassette was removed from plasmid pGEM-T-Kan by digestion with *Nco*I (Fig. 9, Lane 7) and was ligated into *Nco*I-cut plasmid pGW54 (Fig. 9, lane 3) to produce pGCK54 (Fig. 9, lane 4 and 5). Plasmid pGCK54 was transformed into *E. coli* BL21/DE3 *lpxM*, with plasmid pGW44, for the expression of CyaA (Section 2.4.3.3).

3.1.2 Sequencing of plasmids pGW44 and pGW44/188

DNA sequencing confirmed the presence of a 6 base pair sequence [CTGCAG] in the *cyaA* gene in pGW44/188 which was absent in the *cyaA* gene in pGW44. This sequence coded for Leu and Gln in between codons Asp 188 and Ile 189 (Appendix J). However, there were also two cytosine to thymine transitions in the *cyaA* gene sequence in pGW44/188 compared with that of pGW44 as confirmed in the chromatogram files. The changes, from CGC (Arginine) to TGC (Cysteine), corresponded to codons 206 and 246 in the amino acid sequence of CyaA (Appendix J).

3.2 Expression of recombinant CyaA from *E. coli*

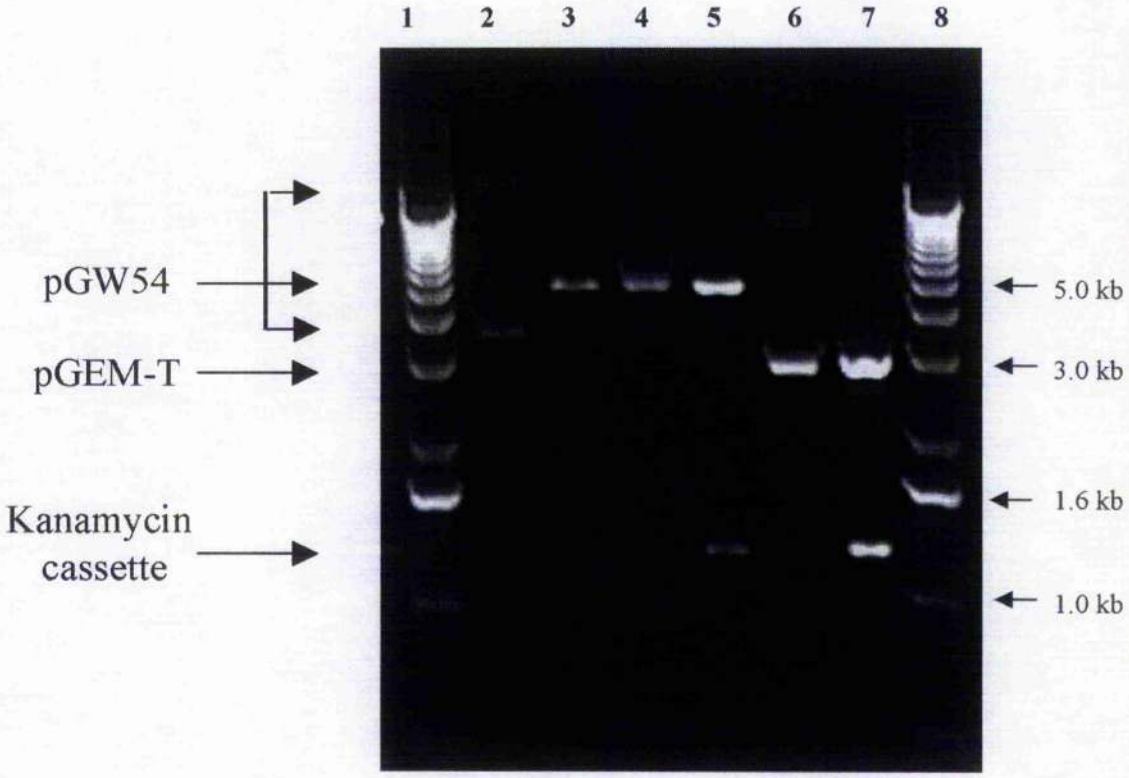
In addition to *E. coli* BL21/DE3, *E. coli* BL21/DE3 *lpxM* was also used to express CyaA. LPS from *E. coli* BL21/DE3 *lpxM* has a defect in the lipid A moiety (see section 2.1) and has reduced inflammatory properties compared with LPS from the wild-type strain (Cognet *et al.*, 2003). Any contaminating LPS in the final CyaA preparations expressed from *E. coli* BL21/DE3 *lpxM* should therefore have minimal immunomodulatory effects on the *in vitro* and bioassays.

3.2.1 Growth of transformed *E. coli* BL21/DE3 or *E. coli* BL21/DE3 *lpxM*

E. coli BL21/DE3 transformed with either one or two plasmids grew at different rates in LB with a starting inoculum of 5 ml (Fig. 10). Therefore, to reach an $OD_{600\,nm}$ of

Figure 9 1% agarose gel of plasmid DNAs digested with restriction enzyme *Nco*I

Volumes of 1 – 5 µl of uncut and plasmid DNAs digested with the restriction enzyme were separated on a 1% agarose gel and stained with ethidium bromide. Arrows indicate the position of the DNA standards.

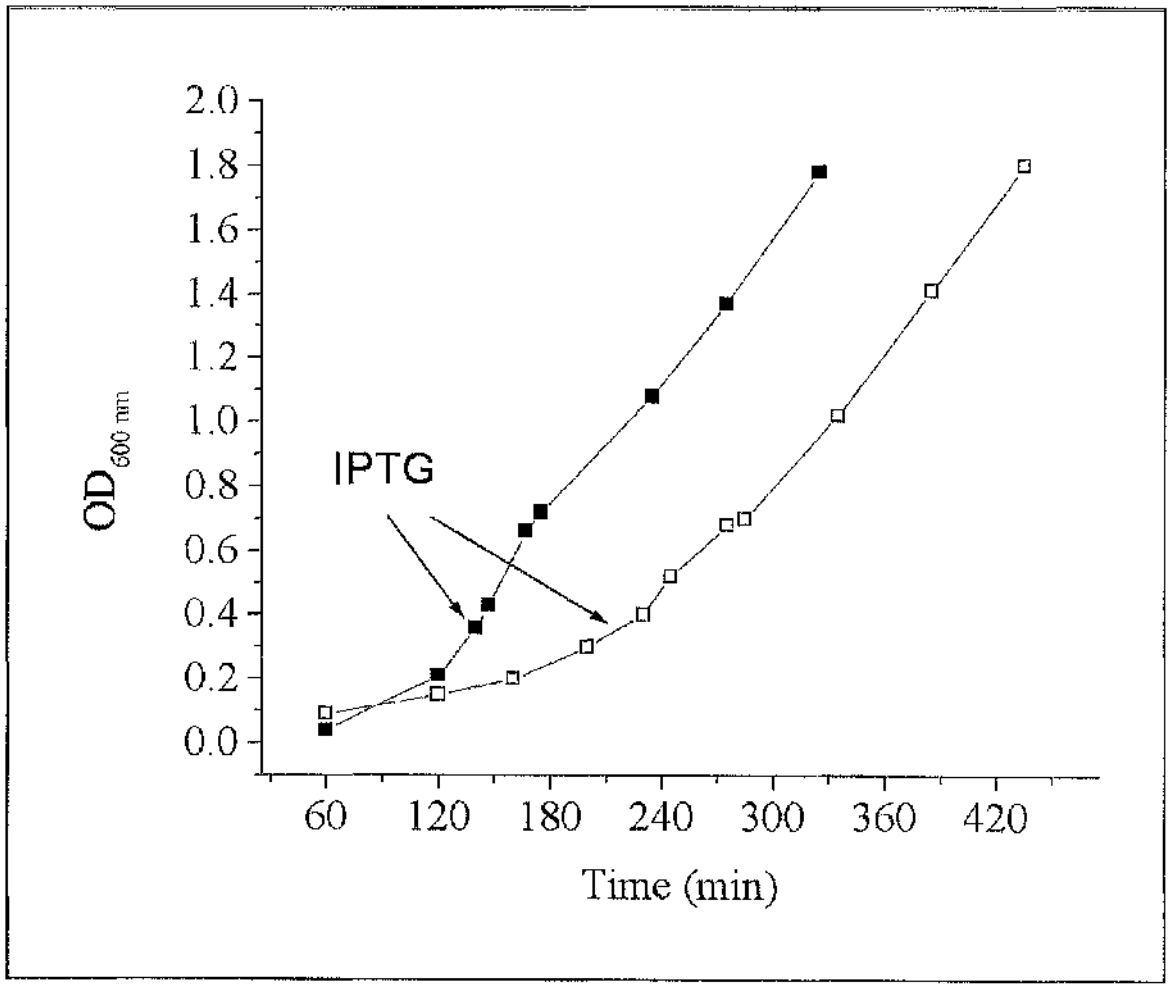


Lane	Sample	Restriction digest with:
1	1 kb ladder (Invitrogen)	-
2	pGW54	-
3	pGW54	*Nco*I
4	pGCK54	-
5	pGCK54	*Nco*I
6	PGEM-T-Kan	-
7	PGEM-T-Kan	*Nco*I
8	1 kb ladder	-

-, no restriction enzyme added.

Figure 10 Growth rates of *E. coli* BL21/DE3 transformed with one or two plasmids

A 5 ml o/n culture of *E. coli* BL21/DE3, transformed with plasmid pGW44 (solid squares) or with plasmids pGW44 and pGW54 (open squares), was inoculated into 500 ml of LB, with appropriate antibiotics, and then grown at 37 °C with shaking. Growth was monitored at $OD_{600\ nm}$. Arrows indicate the point at which IPTG (at 1 mM) was added.



~2.0, *E. coli* BL21/DE3 cells transformed with one and with two plasmid(s) had to be harvested at approximately 150 and 225 min, respectively, after IPTG induction at $OD_{600\ nm}$ of 0.3 - 0.4.

3.2.2 Expression of recombinant CyaA from *E. coli* BL21/DE3 *lpxM*

E. coli BL21/DE3 *lpxM* transformed with plasmids pGW44 and pGCK54 failed to grow after the addition of 1 mM IPTG when cultured in LB as determined by optical density (data not shown). However, when the strains were grown in Terrific broth (TB), the transformed *E. coli* BL21/DE3 *lpxM* grew at a rate comparable to transformed *E. coli* BL21/DE3 even after IPTG induction (data not shown). Therefore, LB was substituted with TB for growth of *E. coli* BL21/DE3 *lpxM*

3.3 Purification of recombinant CyaA

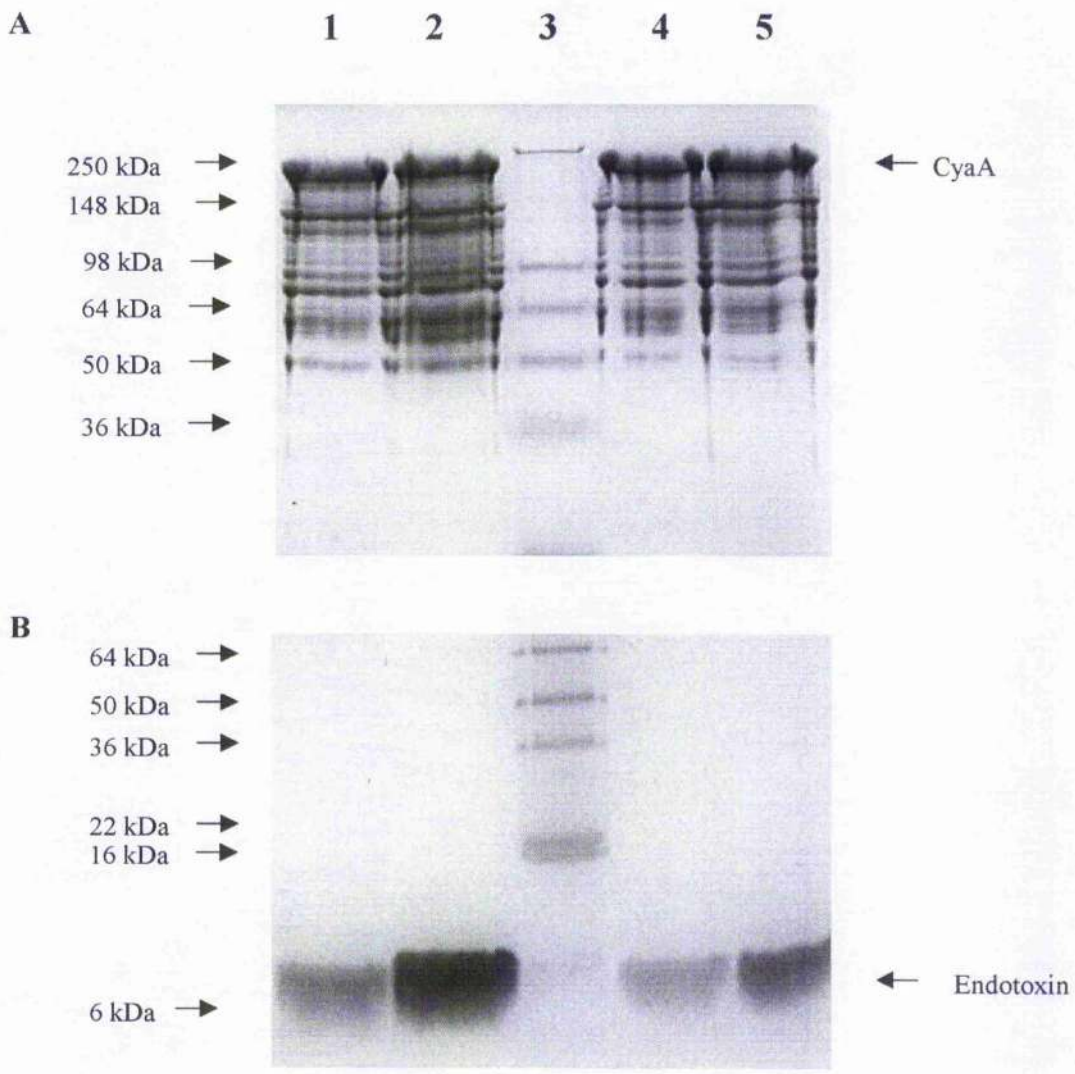
CyaA, expressed from *E. coli* BL21/DE3, was purified on a small- and large-scale using a modified purification procedure designed to reduce protein and LPS contamination. Different detergents were tested for their ability to remove contaminating proteins and LPS from the inclusion bodies. Two types of *Limulus* amoebocyte assays were used to compare LPS levels in the final CyaA preparations. In addition, the IL-6 release assay was used to investigate the inflammatory properties of CyaA expressed from *E. coli* BL21/DE3 and *E. coli* BL21/DE3 *lpxM*.

3.3.1 Removal of endotoxin from inclusion bodies

Inclusion bodies were washed once with either CHAPS, deoxycholic acid, n-octylpyranoglucoside or Triton-X100. All were washed with 2 M urea (solution C; Appendix C.2.3) then distilled water before being finally solubilised in 8 M urea, 50 mM Tris-HCl (pH 8.0). To each well of a 7.5 or 15% SDS-PAGE gel, 10 µl of 150 µg protein /ml of each crude CyaA sample was added for the detection of protein and LPS, respectively (Fig. 11). There were different levels of LPS in each sample. The order of the ability at which the detergents removed LPS, from high to low, was as follows: n-octylpyranoglucoside > CHAPS > Triton X-100 > deoxycholic acid. Although n-octylpyranoglucoside was the best at removing LPS during the washing procedure, it was also the most expensive. Therefore, CHAPS was used as the second best and cheaper alternative. Two washes with CHAPS removed substantially more LPS from inclusion bodies compared with one wash with CHAPS as indicated by the lack of precipitate on LPS silver stain gels after DEAE- and phenyl-Sepharose purification (data not shown). Thus, the potential for a third wash with CHAPS was not considered necessary and two washes were therefore incorporated into the routine purification procedure. In addition, a

Figure 11 SDS-PAGE analysis of CyaA solubilised from inclusion bodies after washing with different detergents

Cells from four flasks of 500 ml IPTG-induced *E. coli* BL21/DE3 were harvested, sonicated and pooled. The inclusion bodies, released from the cells, were separated into 4 equal volumes and each volume was washed with a different detergent. A 10 µl sample of each CyaA preparation, solubilised from the inclusion bodies, was subjected to SDS-PAGE electrophoresis on 7.5% and 15% gels for the visualisation of (A) protein and (B) LPS, respectively. For silver staining, samples were first digested with 1 µl of 50 units/ml of proteinase K prior to SDS-PAGE. Arrows indicate the position of the molecular weight standards, CyaA or LPS.



Lane	CyaA sample washed with:
1	CHAPS
2	deoxycholic acid
3	SeeBlue2 plus ladder (Invitrogen)
4	n-octyl pyranoglucoside
5	Triton X100

further 2 washes with solution C (Appendix C.2.3) were included into the purification procedure as LPS and soluble proteins were still present in large quantities after 1 wash with solution C (data not shown).

3.3.2 Small-scale purification of recombinant CyaA forms from *E. coli* BL21/DE3

The induction of recombinant CyaA expression from *E. coli* BL21/DE3 is shown in Figure 12. The solubilised CyaA material from inclusion bodies (as described in section 3.3.1) was then passed through a DEAE-Sepharose column (Fig. 13). All CyaA forms were eluted consistently by 100 – 200 mM NaCl as a 200 kDa band on a 7.5% SDS-PAGE gel (Fig. 13). Fractions containing CyaA were pooled and then loaded onto a phenyl-Sepharose column. Again, all CyaA forms migrated as a 200 kDa band on SDS-PAGE (Fig. 14). However, low molecular weight bands were always present (Fig. 14, lanes 15 - 16). These were confirmed as breakdown products of CyaA by Western blotting using mouse anti-CyaA serum (Fig. 15).

3.3.3 Large-scale purification of recombinant CyaA from *E. coli* BL21/DE3

CyaA was purified successfully (Fig. 16A and Fig. 17A) under the same conditions as with small-scale purification (Fig. 13 and 14). However, it was evident that a small amount of LPS co-eluted with CyaA as shown by silver stain during the large-scale purification procedures (Fig. 16B and 17B). Comparative yields of CyaA forms from small and large-scale purification procedures are shown in Table 4. Protein concentrations purified from a large-scale were more concentrated compared with small-scale purification. Concentrations ranged from 2 – 5 mg/ml. Moreover, large-scale purification produced yields from 30× - 130× more than small-scale purification (Table 4).

3.3.3.1 Endotoxin quantification by the LAL assay

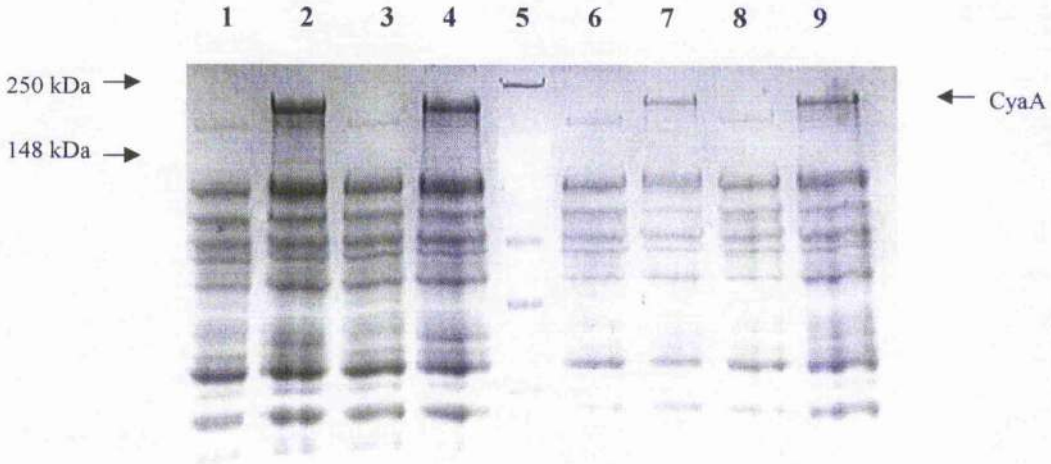
Endotoxin was quantified using two different types of LAL assay and the results were compared. Both assays showed the endotoxin concentration in the final preparations from small- and large-scale to be similar. The chromogenic assay values were in a narrow range (0.039 – 0.099 EU/μg protein) whereas the values from the gel clot assay ranged from 0.4 – 2.27 EU/μg protein such that there was a > 10-fold difference in LPS levels determined by the two assays (Table 5).

3.3.4 Purification of recombinant CyaA from *E. coli* BL21/DE3 *lpxM*

When *E. coli* BL21/DE3 *lpxM*, transformed with plasmids pGW44 and pGCK54, was cultured in TB, CyaA was successfully expressed and purified on a small-scale. For comparative analysis, CyaA was also expressed from *E. coli* BL21/DE3 transformed with

Figure 12 SDS-PAGE analysis of crude CyaA preparations from IPTG-induced and non-induced *E. coli* BL21/DE3

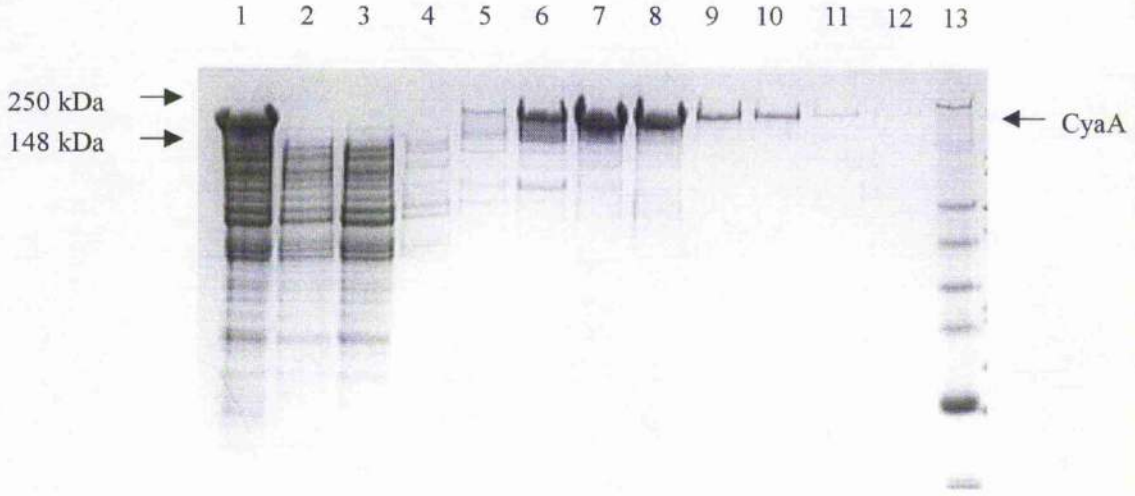
A 1 ml aliquot of cells was sampled from a growing culture of *E. coli* BL21/DE3, transformed with plasmid(s), before the addition of IPTG and then another aliquot was sampled 4 hours later, after the addition of IPTG. The cells were harvested and lysed in SDS-PAGE buffer and subjected to SDS-PAGE electrophoresis on a 7.5% gel and stained with 1% (w/v) Coomassie Blue. Arrows indicate the positions of the molecular weight standards or CyaA.



Lane	Sample
1	proCyaA before IPTG induction
2	proCyaA after IPTG induction
3	proCyaA* before IPTG induction
4	proCyaA* after IPTG induction
5	SeeBlue2 plus ladder (Invitrogen)
6	CyaA before IPTG induction
7	CyaA after IPTG induction
8	CyaA* before IPTG induction
9	CyaA* after IPTG induction

Figure 13 SDS-PAGE analysis of CyaA fractions from DEAE-Sepharose chromatography of CyaA from crude preparations (small-scale method)

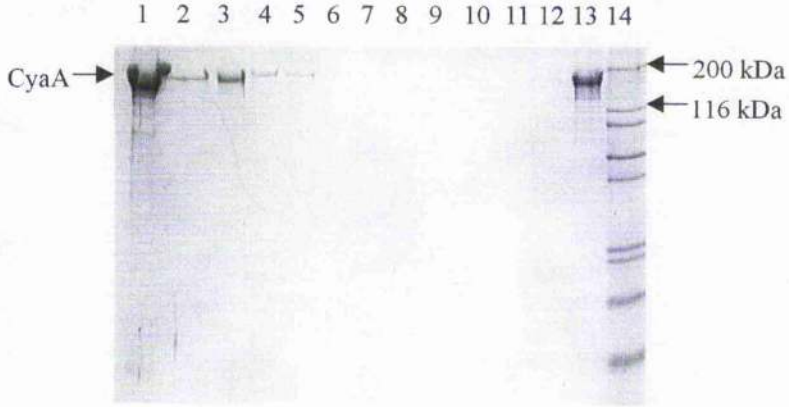
A volume of 10 µl from each fraction of DEAE-Sepharose purification was subjected to SDS-PAGE analysis on a 7.5% gel and stained with 1% (w/v) Coomassie Blue to visualise proteins. The figure is a representative example, showing CyaA being separated in the NaCl gradient from other proteins in the crude CyaA preparation. Arrows indicate the position of the molecular weight standards or CyaA.



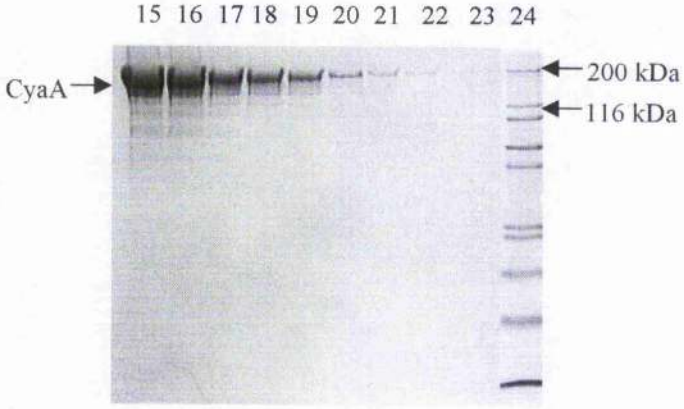
Lane	Sample
1	Crude CyaA fraction
2	Flow through
3	Wash 1
4	Wash 2
5	100 mM NaCl fraction
6	100 mM NaCl fraction
7	150 mM NaCl fraction
8	150 mM NaCl fraction
9	200 mM NaCl fraction
10	200 mM NaCl fraction
11	250 mM NaCl fraction
12	250 mM NaCl fraction
13	SeeBlue2 plus ladder (Invitrogen)

Figure 14 SDS-PAGE analysis of CyaA fractions after phenyl-Sepharose purification (small-scale method)

A volume of 10 µl from each fraction from phenyl-Sepharose purification was subjected to SDS-PAGE analysis on a 7.5% gel and stained with 1% (w/v) Coomassie Blue to visualise proteins. The figure is a representative example, showing CyaA eluting in the presence of 8 M urea, 50 mM Tris-HCl (pH 8.0). Arrows indicate the position of the molecular weight standards or CyaA. F, fraction. 8 M, 8 M urea. †Mark12 ladder (Invitrogen).



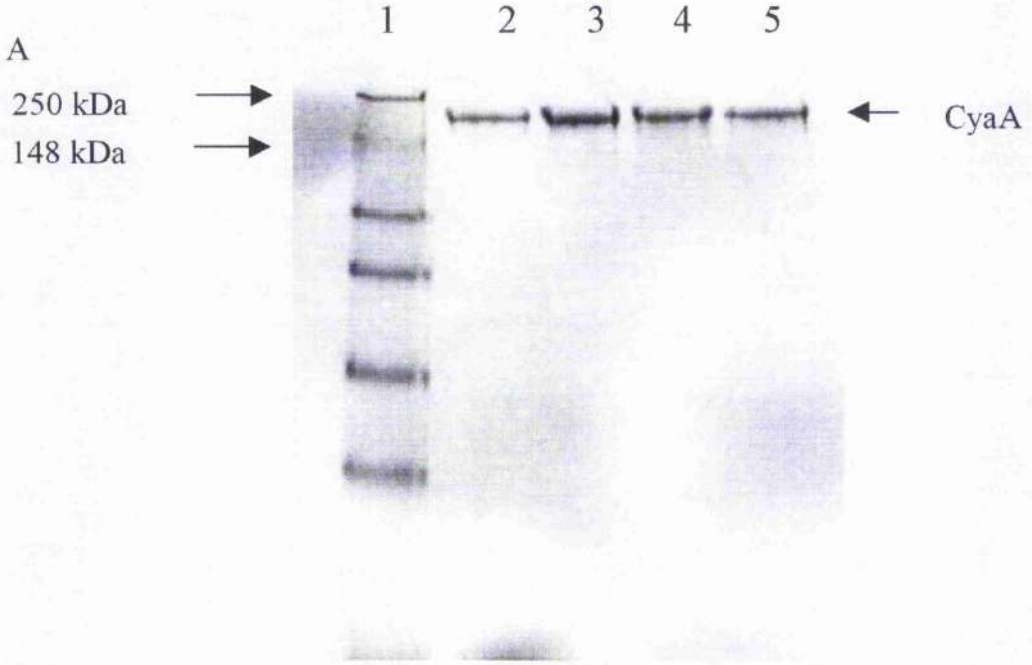
Lane	Sample
1	DEAE pooled
2	Flow through
3	Wash – F1
4	Wash – F2
5	Wash – F3
6	Wash – F4
7	Wash – F5
8	Wash – F6
9	Wash – F7
10	Wash – F8
11	Wash – F9
12	Wash – F10
13	8 M – F1
14	ladder†

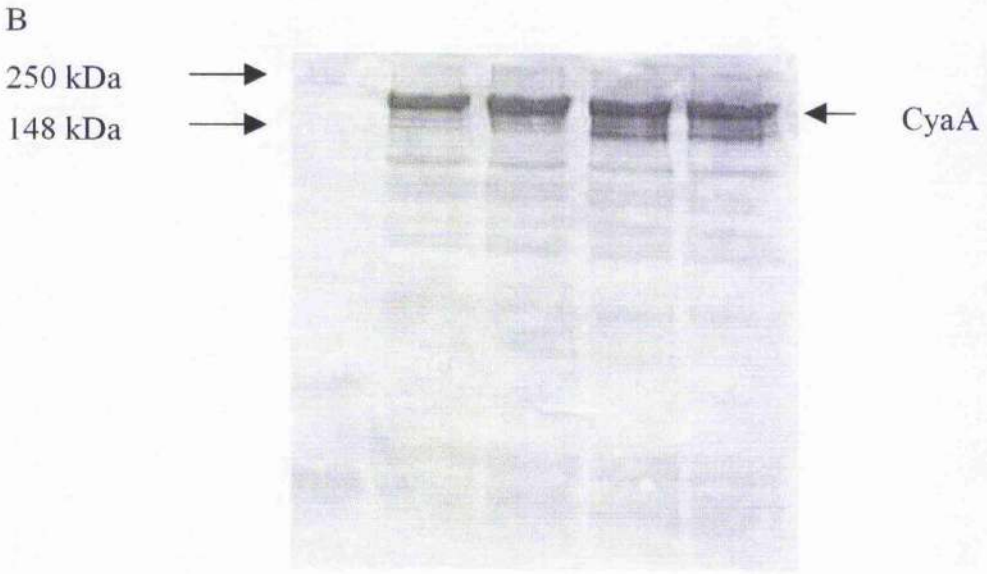


Lane	Sample
15	8 M – F2
16	8 M – F3
17	8 M – F4
18	8 M – F5
19	8 M – F6
20	8 M – F7
21	8 M – F8
22	8 M – F9
23	8 M – F10
24	ladder†

Figure 15 SDS-PAGE and Western blot analysis of purified CyaA preparations (small-scale method)

Different forms of CyaA purified by the small-scale method from phenyl-Sepharose chromatography were subjected to electrophoresis on a 7.5% polyacrylamide gel (A). Bands were then transferred to a Hybond-C nitrocellulose membrane (Section 2.5.3) which was then probed with mouse anti-CyaA* reference serum (Section 2.8.3.4) (B). Arrows indicate the position of the molecular weight standards or CyaA.

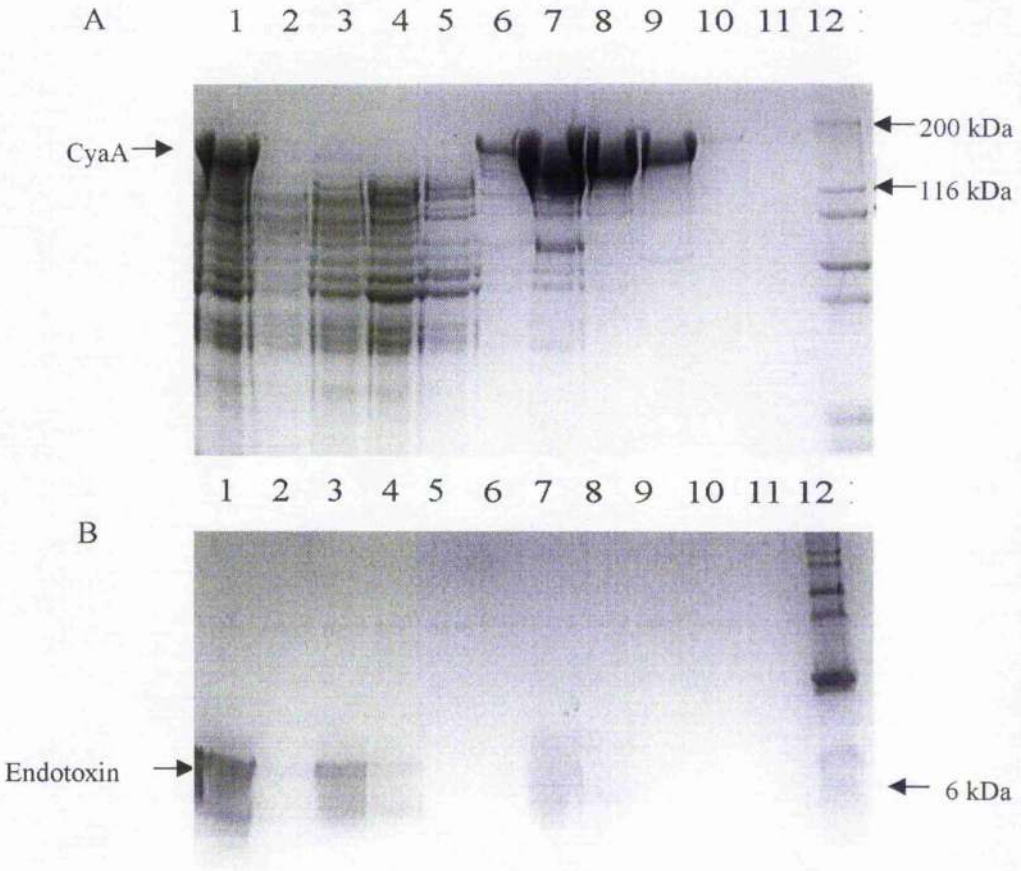




Lane	Sample
1	Mark12 ladder (Invitrogen)
2	proCyaA
3	proCyaA*
4	CyaA
5	CyaA*

Figure 16 SDS-PAGE analysis of fractions from large-scale DEAE-Sepharose purification of CyaA (large-scale method)

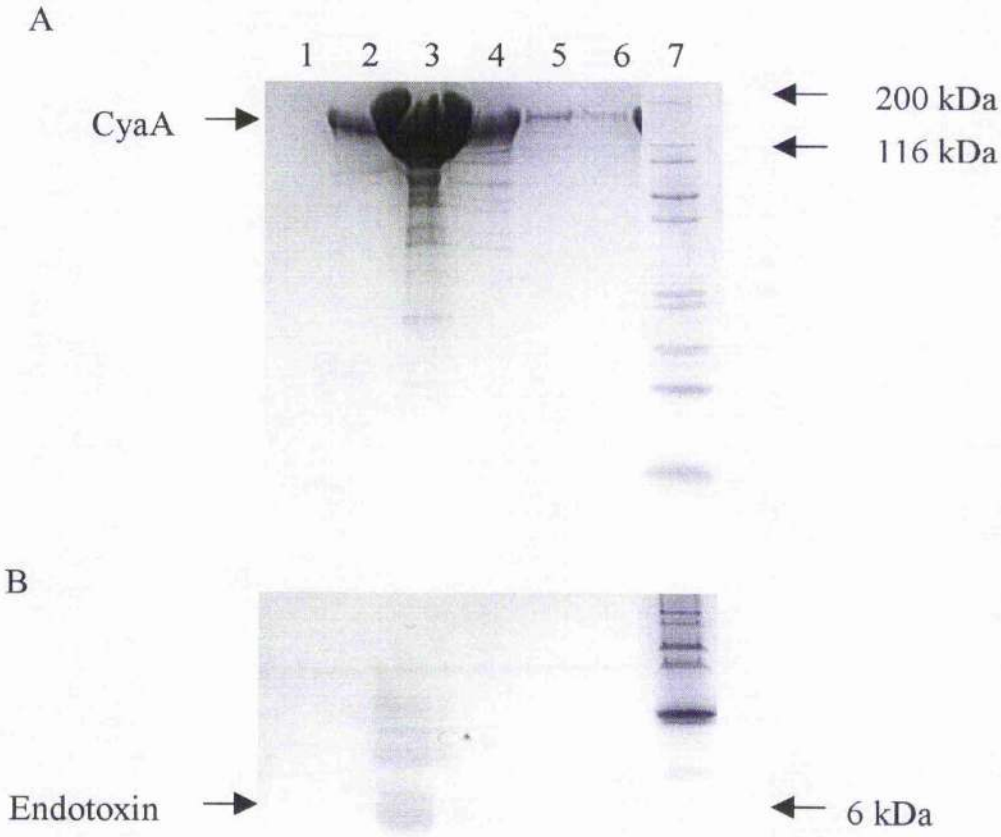
A volume of 10 µl from each fraction of DEAE-Sepharose purification was subjected to SDS-PAGE analysis on 7.5% and 15% gels for visualisation of proteins (A) and LPS (B), respectively, as described in Figure 11. Arrows indicate the position of the molecular weight standards, CyaA or LPS.



Lane	Sample
1	Crude CyaA fraction
2	Flow through
3	Flow through
4	Wash 1
5	Wash 2
6	100 mM NaCl fraction
7	100 mM NaCl fraction
8	150 mM NaCl fraction
9	150 mM NaCl fraction
10	200 mM NaCl fraction
11	200 mM NaCl fraction
12	Mark12 ladder (Invitrogen)

Figure 17 SDS-PAGE analysis of fractions from phenyl-Sepharose chromatography of CyaA (large-scale method)

A volume of 10 μl from each fraction of phenyl-Sepharose purification was subjected to SDS-PAGE analysis on 7.5% and 15% gels for visualisation of proteins (A) and LPS (B), respectively, as described in Figure 11. Arrows indicate the position of the molecular weight standards, CyaA or LPS. 8 M, 8 M urea.



Lane	Sample
1	8M – fraction 1
2	8M – fraction 2
3	8M – fraction 3
4	8M – fraction 4
5	8M – fraction 5
6	8M – fraction 6
7	Mark12 ladder (Invitrogen)

Table 4 Summary of yields of representative CyaA preparations purified on two scales

Protein concentrations were determined using the Bradford assay.

	Protein concentration (μg/ml)		Total yield (mg)	
Sample	small scale	large scale	small scale	large scale
proCyaA	258	5232	1.5	197
proCyaA*	541	2020	3.2	102
CyaA	407	4000	2.4	152
CyaA*	395	2551	2.3	96

Table 5 Comparison of LPS values in CyaA preparations by two types of LAL assays

	LPS content (Endotoxin units/μg protein)			
	Gel clot[1]		Chromogenic[2]	
Sample	small scale	large scale	small scale	large scale
proCyaA	1.74 ± 0.8	0.86 ± 0.4	0.047	0.059
proCyaA*	1.65 ± 0.8	0.44 ± 0.2	0.039	0.099
CyaA	1.10 ± 0.5	0.68 ± 0.1	0.064	0.074
CyaA*	2.27 ± 0.5	1.76 ± 0.8	0.054	0.069

[1]Gel clot assay was performed in triplicate with SEM.

[2]Chromogenic assay was performed once with three dilutions of the sample. Values represent the averages of three dilutions.

the plasmids pGW44 and pGW54 and grown in TB. *E. coli* BL21/DE3 grown in TB produced CyaA yields 5-fold greater than *E. coli* BL21/DE3 grown in LB (Tables 4, 6). Slightly lower yields of CyaA expressed from *E. coli* BL21/DE3 *lpxM* (~12 mg) were obtained compared with CyaA expressed from *E. coli* BL21/DE3 (> 15 mg). The enzymic and haemolytic activities of CyaA expressed from both host strains in TB were similar, although CyaA expressed from *E. coli* BL21/DE3 *lpxM* was ~20% less haemolytic than CyaA expressed from *E. coli* BL21/DE3 (Table 6). The cytotoxic activities of CyaA were not greatly affected by the choice of media or by the host for protein expression (Table 6). CyaA expressed from both *E. coli* strains contained low levels of LPS as measured by the gel clot LAL assay (Table 6). However, there appeared to be a 2-fold greater amount of LPS in the CyaA sample expressed from *E. coli* BL21/DE3 *lpxM* compared with CyaA expressed from *E. coli* BL21/DE3 (Table 6).

3.3.4.1 IL-6 release from MM6 cells

CyaA purified from the *E. coli* BL21/DE3 parent strain and from the *lpxM* strain were tested for their ability to induce IL-6 release from MM6 cells. This assay is another method used to measure the immunomodulatory activity of LPS. The EU content of the samples was standardised by the LAL gel clot assay (Table 6). Purified LPS from *E. coli* BL21/DE3 induced > 80× more IL-6 release ($P < 0.05$), calculated as pg of IL-6/EU, from MM6 cells than LPS purified from *E. coli* BL21/DE3 *lpxM* (Fig. 18). Similarly, CyaA expressed from BL21/DE3 stimulated > 6× more IL-6 release ($P < 0.05$) from MM6 cells than CyaA expressed from the *lpxM* strain when both samples contained the same LPS levels as determined by the LAL gel clot assay (Fig. 18). It was noteworthy that IL-6 production was reduced by 5.9-fold ($P < 0.05$) in MM6 cells after incubation with CyaA expressed from *E. coli* BL21/DE3 compared with LPS from *E. coli* BL21/DE3 containing the same amount of LPS as determined by the LAL gel clot assay.

3.4 *In vitro* characterisation of different CyaA forms

Several types of *in vitro* assays were employed to characterise the different forms of CyaA from the two scales of purification. These included *in vitro* and cell-based assays which measured adenylate cyclase, haemolytic, cytotoxic and apoptotic activities and inhibition of the oxidative burst. The effects of calcium on the cytotoxicity and structure of the different CyaA forms were also investigated using biophysical techniques such as circular dichroism and analytical ultracentrifugation. Co-transformation of *E. coli* BL21/DE3 with plasmids pGW44 or pGW44/188 with pGW54, generated fully-active and invasive CyaA or an enzymically-inactive, invasive CyaA (CyaA*), respectively. Expression of pGW44 or pGW44/188 alone in *E. coli* BL21/DE3 produced non-acylated

Table 6 Comparison of CyaA purified from different strains of *E. coli*

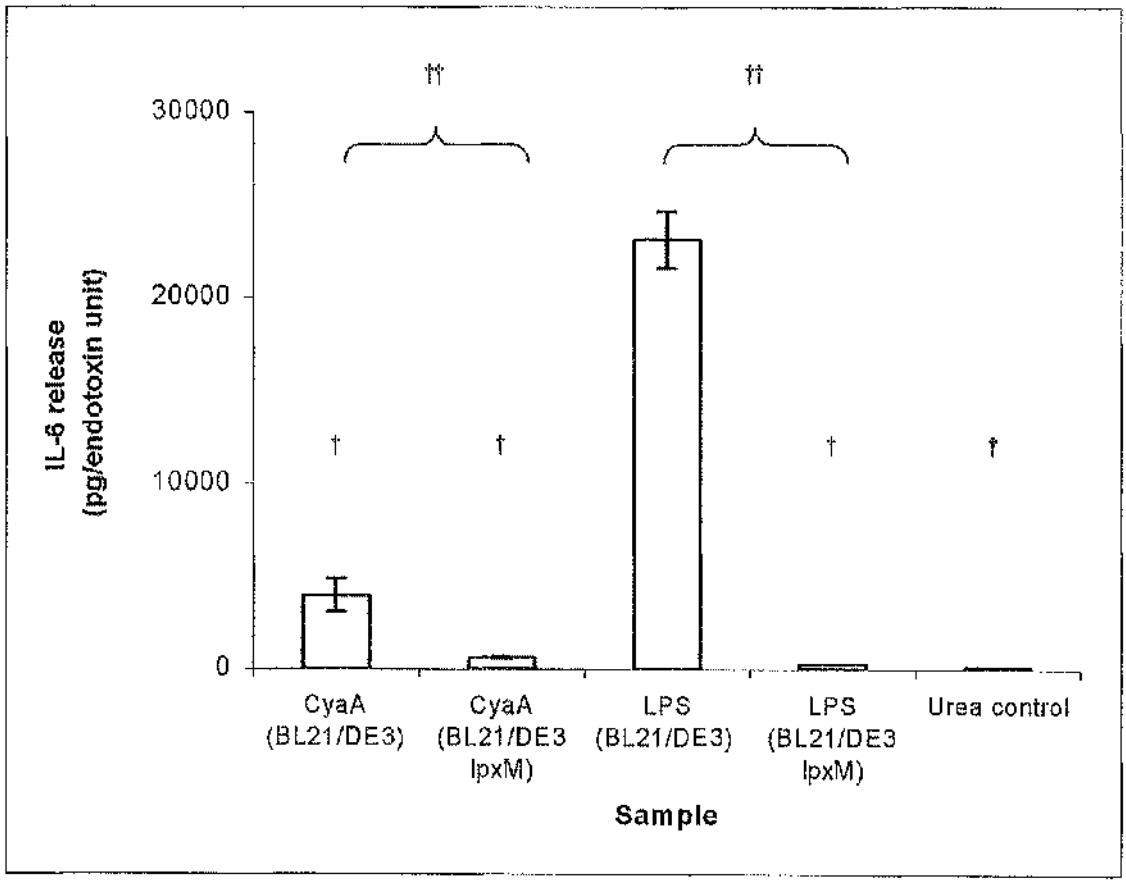
E. coli BL21/DE3 or *E. coli* BL21/DE3 *lpxM* containing plasmids pGW44 and pGW54 or pGW44 and pGCK54, respectively, were grown in TB and CyaA was purified after IPTG induction. Representative CyaA samples from each were assayed as described below.

E. coli expression strain	Protein concentration (μg/ml)	Total protein yield (mg)	LPS content[1] (Endotoxin units /μg protein)	Adenylate cyclase specific activity (μMoles cAMP /min/mg protein)[4]	Toxin concentration required for 50% killing in MTT[2,4] assay (μg/ml)	Toxin concentration required for 50% haemolysis[3,4] (μg/ml)
BL21/DE3	2.56	15.36	0.04	441 ± 30.22	0.0843 ± 0.004	4.55 ± 0.015
BL21/DE3 *lpxM*	1.99	11.93	0.09	462 ± 36.28	0.0824 ± 0.004	3.49 ± 0.16

[1]LPS contents were determined using the LAL gel clot assay. [2]Cytotoxicity towards J774.2 cells after incubation for 2 h with CyaA was assayed in DMEM. [3]Haemolysis of sheep erythrocytes by CyaA was assayed in HH buffer containing 1 mM $CaCl_2$. [4]Results represent the means of assays performed in triplicate with SEM

Figure 18 IL-6 release from MM6 cells after incubation for 24 h with CyaA expressed from *E. coli* BL21/DE3 or from *E. coli* BL21/DE3 *lpxM*, or with LPS purified from each *E. coli* strain

Purified LPS from both *E. coli* strains was diluted to the same concentration (~0.2 endotoxin units) as found in the CyaA samples. Results are the average of triplicate measurements with SEM (bars). Statistics: † (all groups vs *E. coli* BL21/DE3 purified LPS: ANOVA) and ††Student's *t*-test (linked brackets) were performed, $P < 0.05$.



CyaA protoxins with enzymic activity (proCyaA) or without enzymic activity (proCyaA*), respectively (Table 3).

3.4.1 Adenylate cyclase enzymic activity

Only proCyaA and CyaA showed enzymic activity (Table 7). There was approximately a 20 – 32% reduction in specific activity of CyaA and proCyaA purified on a large-scale compared with the same preparations purified on a small-scale.

3.4.2 Haemolytic activity of different CyaA forms towards sheep erythrocytes

At 1 mM $CaCl_2$, the activities of CyaA and CyaA* were similar and no haemolytic activity was detected in the proCyaA or proCyaA* preparations (Table 7). CyaA and CyaA* were assayed for their ability to induce haemolysis of sheep erythrocytes in increasing levels of $CaCl_2$ above 1 mM in HH buffer, after incubation for 24 h at 37 °C (Fig. 19). With increasing $CaCl_2$, the concentrations of CyaA and CyaA* required for 50% haemolytic activities diverged such that CyaA showed reduced haemolytic activity whereas CyaA* became more haemolytic (Fig. 19). When CyaA and CyaA* were assayed in the absence of $CaCl_2$, haemolytic activity was not detected (data not shown).

3.4.2.1 Kinetics of haemolytic activity over 24 h

The kinetics of haemolysis of sheep erythrocytes by CyaA and CyaA* were also monitored in HH buffer containing 1 or 3 mM $CaCl_2$. As shown in Figure 20, the haemolytic activities of CyaA and CyaA* (both at 7.5 μg/ml) became evident between 6 and 12 h and the toxins behaved in a similar manner, eventually lysing ~70% of sheep erythrocytes at 24 h when incubated in 1 mM $CaCl_2$. In contrast, CyaA lost approximately half of its haemolytic capacity when incubated in 3 mM $CaCl_2$ but CyaA* caused 100% haemolysis and at a rate which exceeded that of both CyaA and CyaA* in 1 mM $CaCl_2$.

3.4.3 Cytotoxicity of different CyaA forms on J774.2 macrophages

To determine the relative importance of the enzymic and invasive activities for the overall toxin function, the four different CyaA forms were assessed in three cytotoxicity assays that measure cell killing in different ways. Neither of the proCyaA forms had detectable activity in any of these assays. Cell killing by CyaA and CyaA* was compared after growth of J774.2 cells in either RPMI or DMEM because these media had different calcium contents (0.423 mM calcium nitrate ($CaNO_3$) and 1.27 mM calcium chloride ($CaCl_2$), respectively).

Table 7 Activities of different CyaA forms purified on two scales

Four forms of CyaA purified from *E. coli* BL21/DE3 were subjected to different *in vitro* assays. Representative samples of each CyaA form were assayed as indicated below.

	Adenylate cyclase specific activity (μMoles cAMP/min/mg protein)[1]		Toxin concentration causing 50% killing in MTT assay[1] (μg/ml)		Toxin concentration causing 50% LDH release[1] (μg/ml)		Toxin concentration causing 50% haemolysis[2] (μg/ml)		Toxin concentration giving 50% inhibition of oxidative burst[3] (μg/ml)	Toxin concentration giving peak caspase 3/7 activity[3] (μg/ml)
Sample	small-scale	large-scale	small-scale	large-scale	small-scale	large-scale	small-scale	large-scale	large-scale	large-scale
proCyaA	1120 ± 99	760 ± 110	n/a	n/a	n/a	n/a	n/a	n/a	1.4790 ± 0.065	n/a
proCyaA*	< 1	< 1	n/a	n/a	n/a	n/a	n/a	n/a	> 10	n/a
CyaA	1025 ± 134	854 ± 47	0.176 ± 0.006	0.145 ± 0.01	1.98 ± 0.12	2.11 ± 0.33	1.75 ± 0.018	2.17 ± 0.06	0.003 ± 0.0012	0.6 ± 0.2
CyaA*	< 1	< 1	> 10	> 10	> 10	> 10	1.29 ± 0.022	2.11 ± 0.15	1.7067 ± 0.111	n/a

[1]J774.2 cells were grown and assayed in RPMI medium with toxin for 2 h. [2]Sheep erythrocytes were washed and incubated with toxin in HH buffer containing 1 mM $CaCl_2$ for 24 h. [3]The different CyaA forms were incubated with J774.2 cells in DMEM for 90 min and 120 min for the measurement of zymosan-induced oxidative burst and caspase 3/7 activity, respectively. Results represent the means of assays performed in duplicate with SEM. n/a = no detectable activity.

Figure 19 Effect of different concentrations of $CaCl_2$ on the haemolytic activity of CyaA and CyaA* toward sheep erythrocytes

Haemolytic activity of CyaA (squares) and CyaA* (triangles) towards sheep erythrocytes was assessed after 24 h with different concentrations of calcium chloride in HH medium. Results represent the means of assays performed in duplicate with SEM (bars).

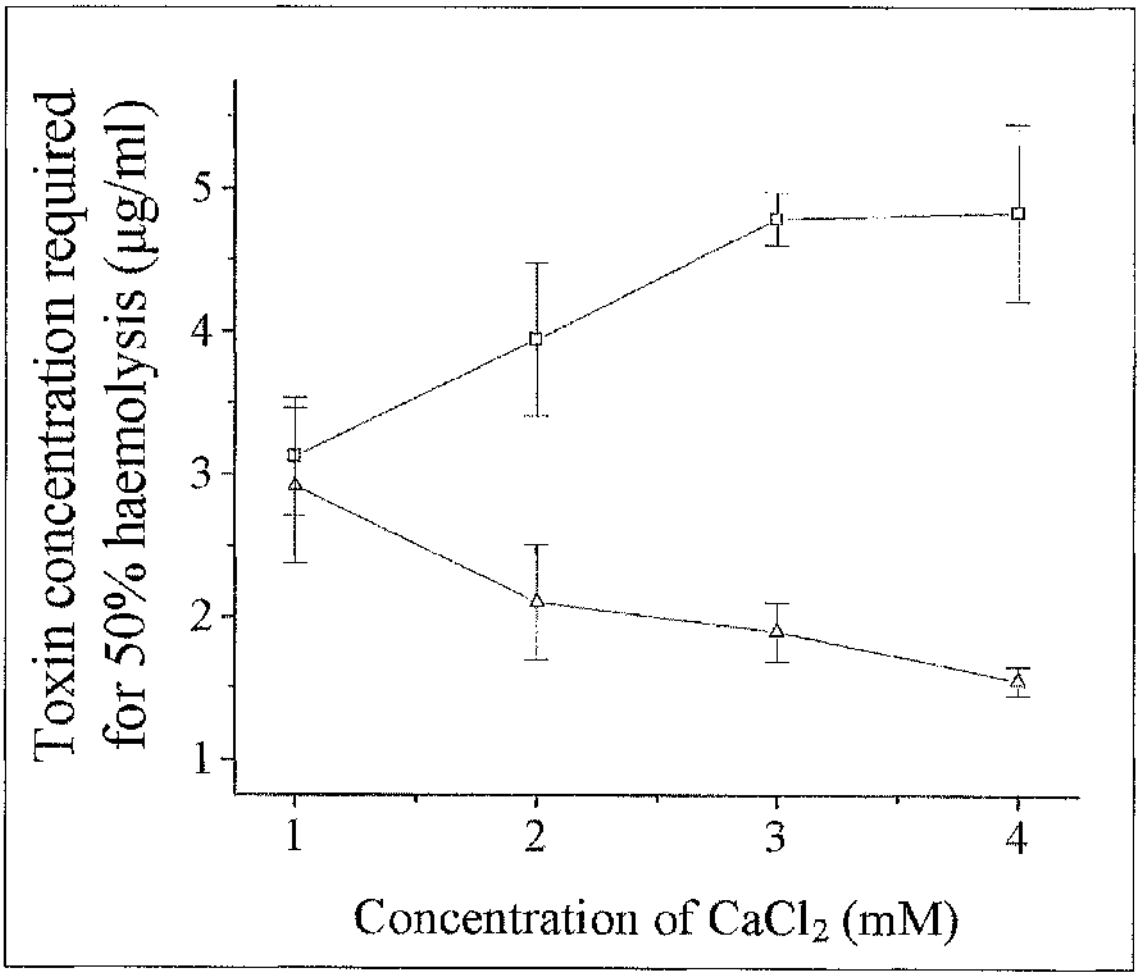
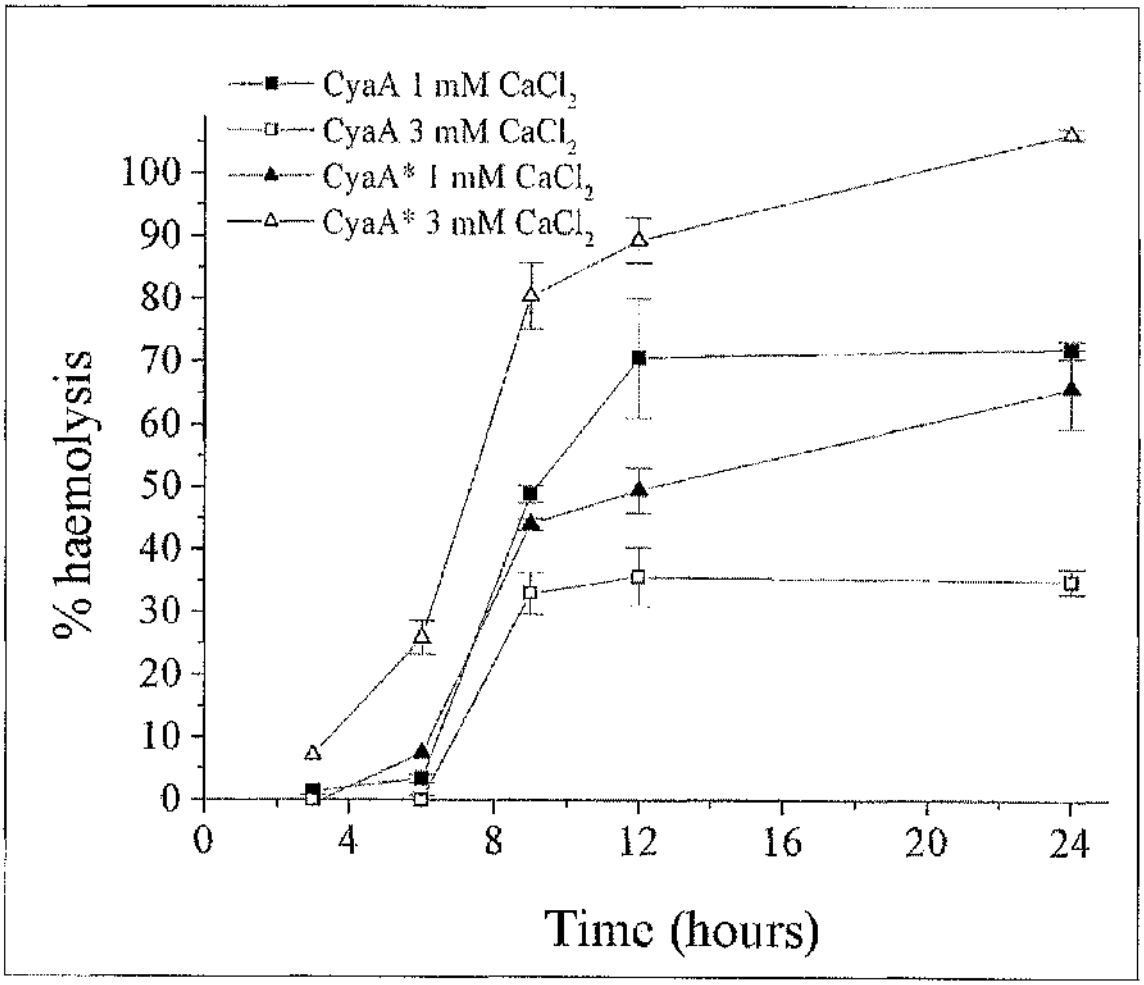


Figure 20 Kinetics of CyaA and CyaA* haemolysis of sheep erythrocytes in HH buffer with different concentrations of $CaCl_2$

CyaA and CyaA* were used at 7.5 μg/ml in the presence of 1 mM or 3 mM $CaCl_2$. Results represent the means of assays performed in duplicate with SEM (bars).



3.4.3.1 Comparison of assays for cytotoxicity

After incubation for 2 h of J774.2 cells with CyaA, the concentration required for 50% killing was 0.02 μg/ml in the ATP release assay (CellTiter-Glo®, data not shown). Compared with the MTT and LDH release assays, this concentration was very low (Table 7). This value was likely to be caused by a mixture of cell killing and the ability of CyaA to convert intracellular ATP into cAMP. This made the CellTiter-Glo® assay an unsuitable choice for measuring J774.2 viability. Therefore, cytotoxicity was measured using only the LDH and MTT assays as the AC activity of CyaA did not interfere with those assays. It can be seen in Table 6 that CyaA purified from small and large-scale purification showed similar levels of cytotoxicity. CyaA* was severely impaired in its ability to kill J774.2 cells when assayed in RPMI as shown by both the MTT and LDH assays. The concentration of CyaA required to kill 50% of J774.2 cells in 2 h as judged by the LDH assay was 10-fold greater compared with the results obtained for the MTT assay (Table 7).

3.4.3.2 Effect of calcium on the cytotoxicities of different CyaA forms in the MTT assay

When assayed in RPMI, CyaA was more cytotoxic than CyaA* (Table 7, Fig. 21A). CyaA* required a concentration of > 10 μg/ml to kill 50% of cells. There was a small increase in cell killing when the cytotoxicity of CyaA was assessed in DMEM compared with RPMI (Fig. 21A). However, when CyaA* was assayed in DMEM, there was a marked increase in cell killing compared with that seen in RPMI. This increase brought the dose of CyaA* required for 50% killing (0.37 μg/ml) very close to that of CyaA (0.12 μg/ml) (Fig. 21A). Therefore, to determine if calcium concentration was the factor responsible for the enhanced activities of CyaA*, cytotoxicity was monitored in RPMI and DMEM with 1 mM increments of $CaNO_3$ or $CaCl_2$, respectively. As shown in Figure 22, the addition of calcium increased the cytotoxicities exhibited by both CyaA and CyaA*. CyaA was most cytotoxic in RPMI supplemented with 2 mM $CaNO_3$ but appeared to be marginally less active in DMEM supplemented with $CaCl_2$ (Fig. 22). When CyaA* was assayed in RPMI with increasing concentrations of $CaNO_3$, there was about a > 20-fold increase in cytotoxicity over the range of calcium concentrations used (1 - 4 mM). ProCyaA and proCyaA* were not cytotoxic towards J774.2 cells in RPMI or DMEM in the MTT assay (data not shown).

3.4.3.3 Effect of calcium on the cytotoxicities of different CyaA forms in the LDH assay

Of the two acylated toxin forms, CyaA was the most active in RPMI (Table 7). Again, CyaA* required a concentration of > 10 μg/ml to kill 50% of cells (Table 7, Fig.

Figure 21 Cytotoxicity of CyaA and CyaA* on J774.2 cells in different media

Cytotoxicity was measured using (A) MTT or (B) LDH release assays. CyaA and CyaA* assayed in RPMI or in DMEM with cells for 2 h. Results represent the means of assays performed in duplicate with SEM (bars).

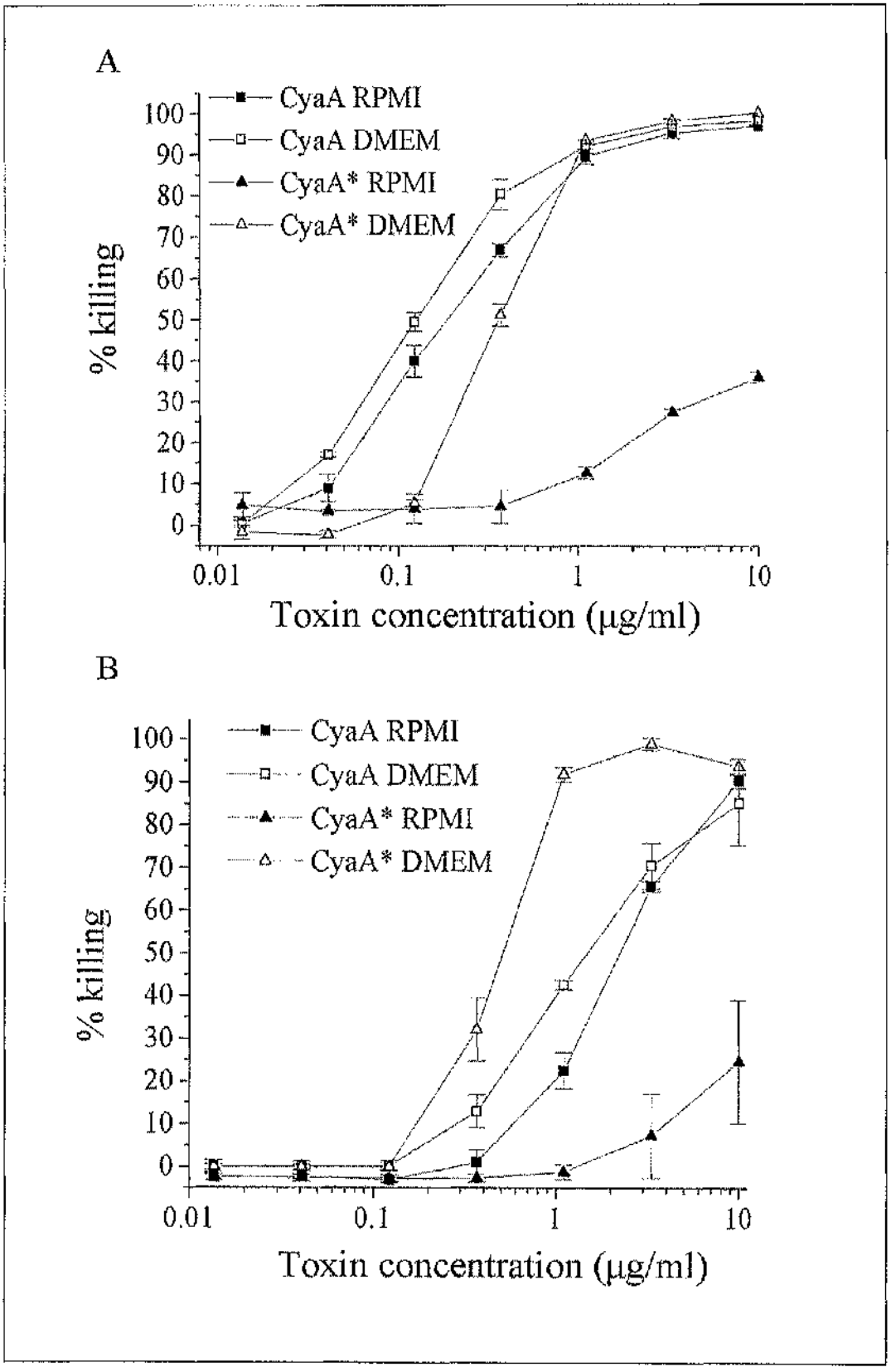
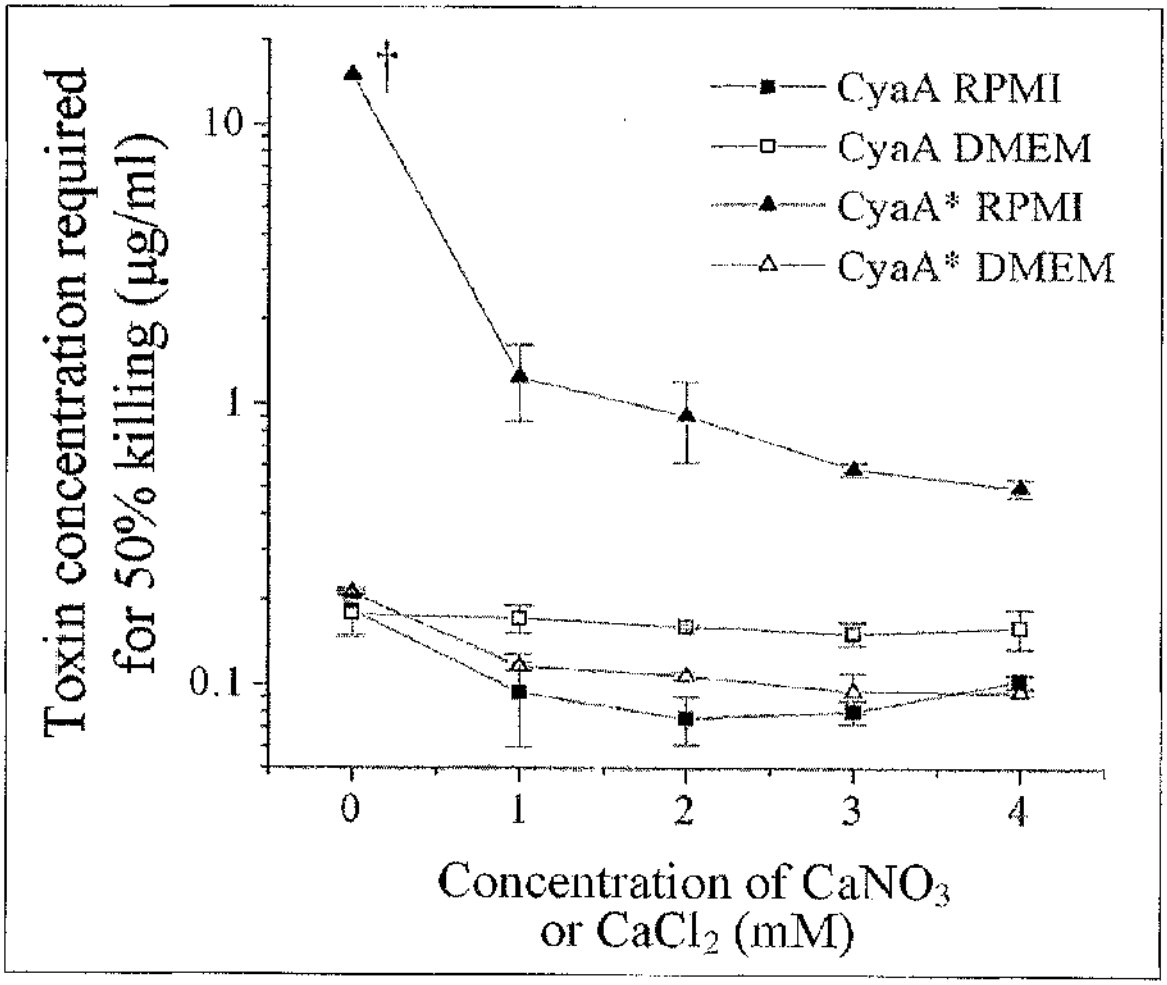

A
CyaA RPMI
CyaA DMEM
CyaA* RPMI
CyaA* DMEM
% killing
Toxin concentration (µg/ml)
B
CyaA RPMI
CyaA DMEM
CyaA* RPMI
CyaA* DMEM
% killing
Toxin concentration (µg/ml)

Figure 22 Effect of calcium on killing of J774.2 cells by CyaA and CyaA* as assessed in the MTT assay

The cytotoxicities of CyaA and CyaA* were assayed in RPMI or in DMEM supplemented with $CaNO_3$ or $CaCl_2$, respectively, after incubation for 2 h with J774.2 cells. Results represent the means of assays performed in duplicate with SEM (bars).



† indicates that the concentration required for 50% killing was greater than 10 μg/ml.

21B). It was interesting to note that the LDH release assay did not appear to be as sensitive as the MTT assay after incubation for 2 h with CyaA as > 10-fold higher concentration of toxin was required for 50% killing (Table 7). There was a small increase in cell killing when the cytotoxicity of CyaA was assessed in DMEM compared with RPMI (Fig. 21B). However, when CyaA* was assayed in DMEM, there was a marked increase in cell killing compared with that seen in RPMI. Moreover, CyaA* induced noticeably more release of LDH from J774.2 cells in DMEM than CyaA when used at 1 μg/ml (Fig. 21B). These data showed that RPMI did not provide the optimal conditions for CyaA* activity, and that this was probably due to the lower calcium content in this medium. The non-acylated proCyaA and proCyaA* forms were not cytotoxic (Table 6).

3.4.3.4 Kinetics of the cytotoxicities of CyaA and CyaA* towards J774.2 cells over 2 h

The kinetics of the cytotoxic activities of CyaA and CyaA* towards J774.2 cells were investigated over a 2 h period using 1.25 μg/ml of CyaA or CyaA*. The kinetics of CyaA cytotoxicity in both the MTT and LDH assays was similar in RPMI and DMEM media (Fig. 23A) with a slightly better activity achieved in DMEM, as noted previously (Fig. 21). There was a lag period of 1 h in the LDH release assay before CyaA showed an effect on the cells (Fig. 23B), compared with virtually no lag period in the MTT assay (Fig. 23A). This would explain the apparent greater sensitivity of the MTT assay after incubation with the toxin for 2 h (Table 7). In DMEM, CyaA* acted more rapidly than CyaA, reaching maximum activity within approximately 60 min of exposure of the cells to the toxin, in both cytotoxicity assays. Again, CyaA* had poor activity in RPMI medium in both assays.

3.4.3.5 The cytotoxicities of CyaA and CyaA* towards J774.2 cells after 24 h

The cytotoxicities of CyaA and CyaA* were assayed after incubation for 24 h with J774.2 cells. CyaA induced ~30% cell killing in DMEM at a concentration between 0.01 – 0.05 μg/ml in the MTT assay and this was somewhat reduced in RPMI (Fig. 24A). These killing trends were paralleled in the LDH release assay (Fig. 24B). This indicates that the toxic effect of CyaA, at low toxin concentrations, is accumulative over time and suggests that the intracellular concentration of CyaA is dependent on the extracellular concentration and does increase progressively over time. As expected, greater concentrations of CyaA* in RPMI were required to induce 50% killing in both the MTT and LDH release assays (Figs. 24A, B). However, in DMEM, the killing activity of CyaA* was as potent as CyaA at concentrations above 0.5 μg/ml but below 0.5 μg/ml, CyaA* was not cytotoxic (Fig. 24A, B). It was of interest to note how similar the dose-response activities of CyaA* were

Figure 23 Kinetics of cell killing of J774.2 cells by CyaA and CyaA* over 2 h

The cytotoxicities of CyaA and CyaA* (both at 1.25 μg/ml) were assayed in RPMI or in DMEM and measured using the (A) MTT and (B) LDH release assays after incubation with J774.2 cells over 2 h. Results represent the means of assays performed in duplicate with SEM (bars).

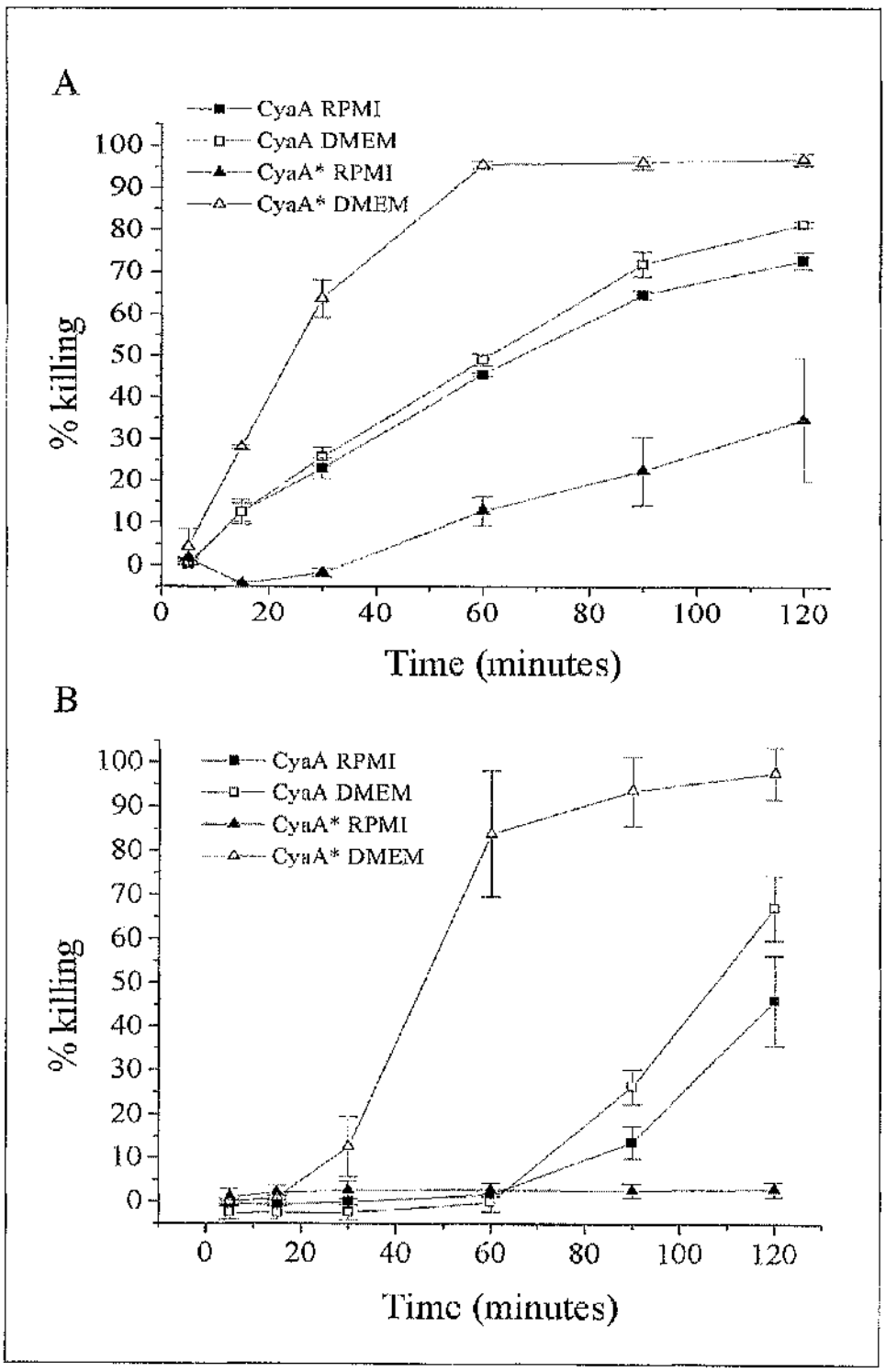
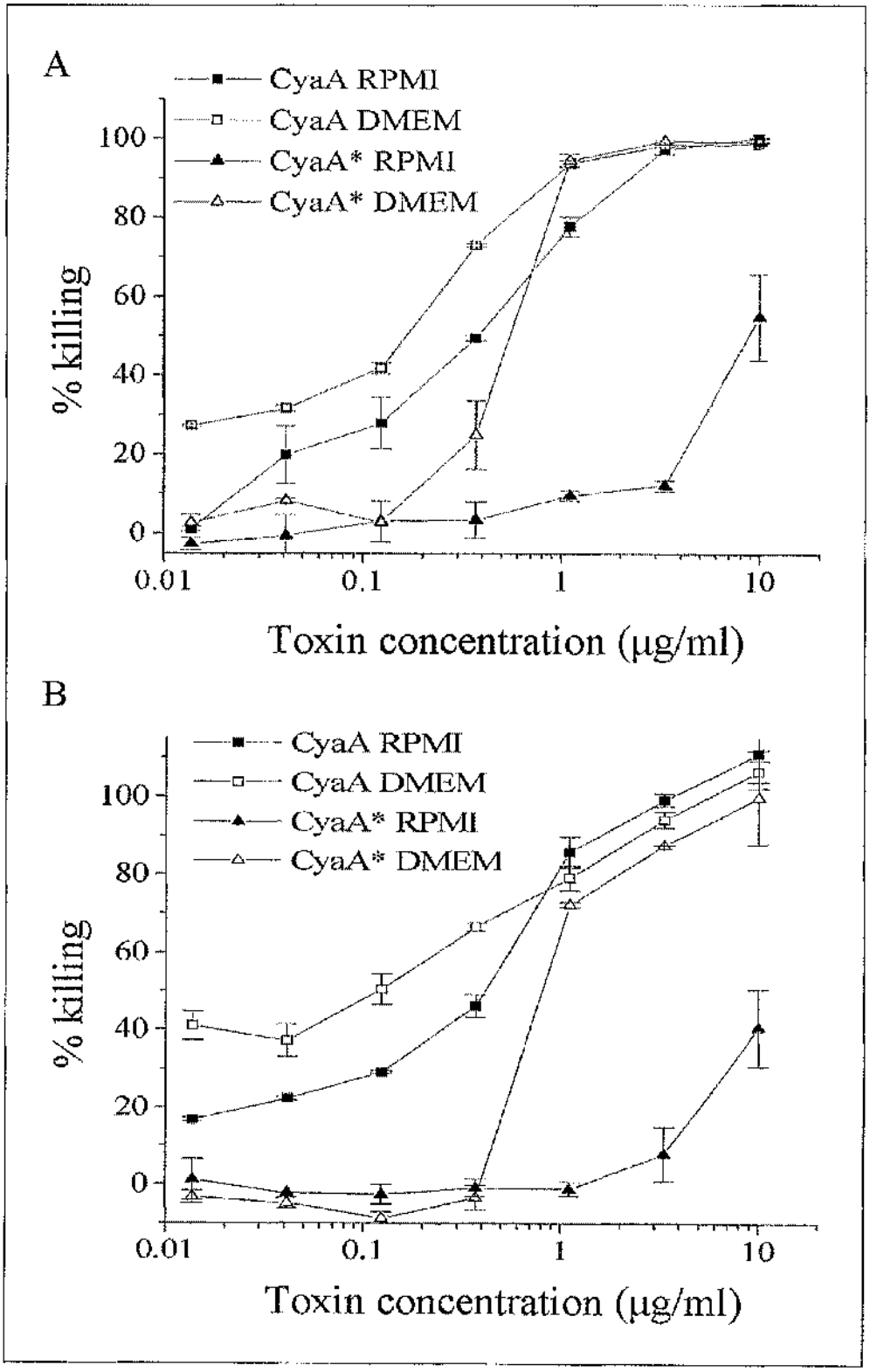


Figure 24 Cell killing by CyaA and CyaA* after incubation for 24 h with J774.2 cells in different media

Cytotoxicity was measured by (A) MTT and (B) LDH release. CyaA and CyaA* assayed in RPMI or in DMEM. Results represent the means of assays performed in duplicate with SEM (bars).



to those achieved after incubation for 2 h (Fig. 21).

3.4.3.6 CyaA and CyaA* cytotoxicity towards J774.2 cells in the presence of DTT

The presence of two cysteine residues in the proCyaA* and CyaA* forms that are not present in proCyaA or CyaA may allow a di-sulphide bridge to form either intra or intermolecularly which might, in turn, influence the behaviour of CyaA* in the various assays (Section 3.1.2). Therefore, to investigate this possibility a MTT experiment was performed with different concentrations of CyaA or CyaA* incubated with 0.5 mM dithiothreitol (DTT), a chemical which reduces di-sulphide bonds to sulphydryl groups within a protein. The cytotoxicities of CyaA and CyaA* towards J774.2 cells after treatment for 2 h in DMEM were similar in the presence and absence of 0.5 mM DTT, suggesting that the cysteine residues did not contribute to the enhanced CyaA* cytotoxicity in the presence of calcium (data not shown).

3.4.4 Inhibition of zymosan-stimulated oxidative burst in J774.2 cells by different CyaA forms

CyaA caused 50% inhibition of the zymosan-stimulated oxidative burst at a dose of 3 ng/ml (Table 7) whereas proCyaA and CyaA*, which are non-acylated and non enzymic, respectively, both required a ~500-fold greater concentration to have this effect. This assay was clearly very sensitive compared to the MTT and LDH release assays, which may have been the reason why some activity was detected with proCyaA. CyaA* was poorly active in this assay, even in DMEM, indicating that unlike the MTT and LDH release assays, inhibition of the oxidative burst required AC activity. ProCyaA* was inactive at the highest concentration used (10 μg/ml) in this assay.

3.4.5 Apoptosis

3.4.5.1 Measurement of apoptosis in J774.2 cells using Caspase Glo™ 3/7 luminescent assay

This assay relied on ATP to catalyse the production of luciferase as with the CellTiter-Glo® luminescent assay (Section 3.4.3.1). Therefore, this assay was not suitable to assay CyaA-induced apoptosis because of the AC activity which would deplete ATP. Interestingly, both CyaA and proCyaA at 10 μg/ml inhibited the assay (as shown by low luminescence compared with the control). The lack of luminescence after treatment suggests that, at this concentration of CyaA and proCyaA, the cells would be dead (Fig. 21). However, proCyaA does not kill cells at this concentration (Fig. 21) suggesting that proCyaA had the ability to penetrate cells and raise cAMP levels (data not shown). It was

only at a concentration of 0.1 μg/ml of proCyaA and below that levels of luminescence were similar to those of the urea control.

3.4.5.2 Measurement of apoptosis in J774.2 cells using Apo-ONE™ caspase 3/7 assay

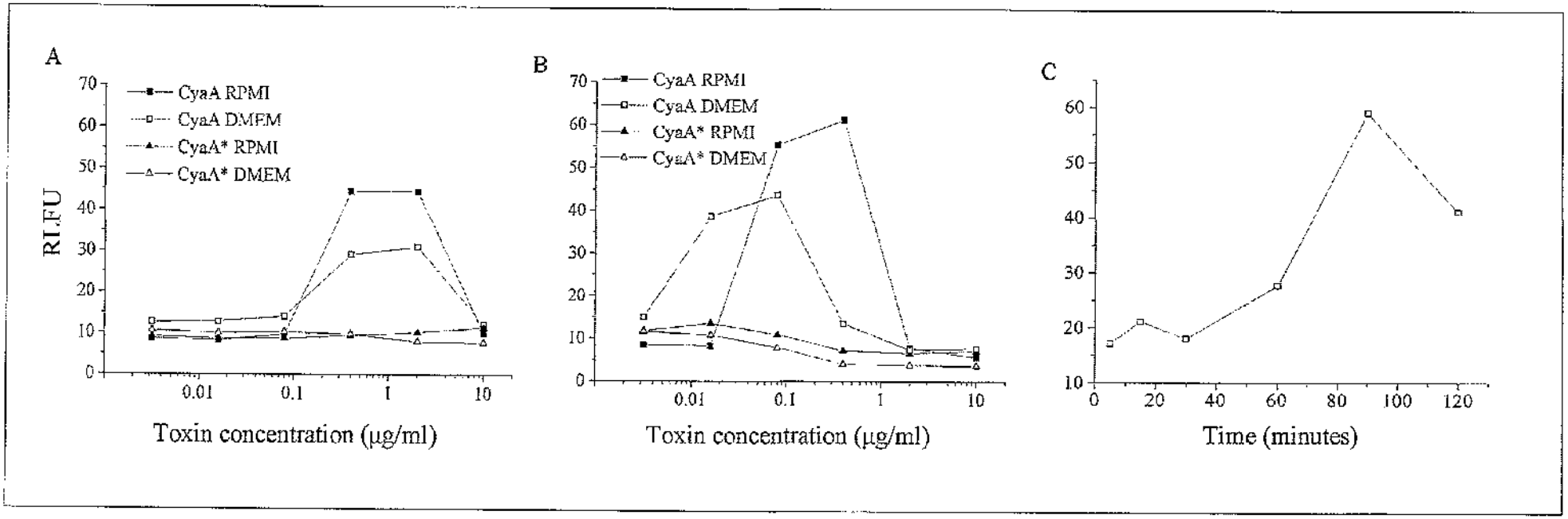
0.2 M urea was not used at any stage in this assay, unlike the MTT and LDH assays (Section 3.4.3), because there were signs of increased caspase 3/7 activation from control cells (data not shown). The small traces of urea (~0.02 M urea) in the CyaA samples diluted from the stock sample (in 8M urea, 50 mM Tris-HCl (pH 8.0) did not interfere with the assay. This was confirmed using a negative control with cells in the presence of 0.02 M urea. All four CyaA forms were tested for their ability to induce caspase 3/7 activity in J774.2 cells in RPMI and DMEM. CyaA was able to induce apoptosis of J774.2 cells after 2 h incubation, with a peak of caspase 3/7 activity at toxin concentrations between 0.3 – 1.1 μg/ml (Table 7, Fig. 25A). It was interesting to note that increasing the CyaA concentration above 1.1 μg protein/ml did not lead to increased caspase 3/7 activity in either medium. In fact, the induction of caspase 3/7 activity was adversely affected by increasing CyaA concentrations above 1.1 μg protein/ml. The data in Fig. 21A indicates that, at this level of toxin, > 50% of the cells are non-viable after incubation for 2 h in RPMI or DMEM. The progressive lack of induction of caspase 3/7 activity as toxin levels are increased is presumably due to increased cell death by means other than apoptosis. This interpretation is borne out by the kinetic data in Fig. 25C which shows that maximum induction is achieved after incubation for 90 min with 0.5 μg protein/ml CyaA, but thereafter declines on further incubation. As shown in Fig. 25B, the peak of caspase 3/7 activity induced by CyaA shifted from 0.3 – 1.1 μg protein/ml after incubation for 2 h to 0.01 - 0.1 μg protein/ml after incubation for 24 h with J774.2 cells. In addition, there was increased caspase 3/7 activity below 0.01 μg protein/ml after incubation for 24 h which was not observed after incubation for 2 h. CyaA* failed to induce any signs of caspase 3/7 activity (Fig. 25A, B) even after incubation for 24 h in DMEM, demonstrating that this form of CyaA was able to kill cells without causing apoptosis. Moreover, the results showed that apoptosis induction depended on AC activity and also on acylation of the toxin as proCyaA was inactive in this assay (Table 7) although it had slight activity in the Caspase Glo™ 3/7 luminescent assay (Section 3.4.5.1). ProCyaA* was inactive in both assays (Table 7 and Section 3.4.5.1).

3.4.6 Detection of phosphorylated ERK1/2

There was no phosphorylated ERK1/2 detected from J774.2 cells after incubation for 10 min with 20 ng/ml of CyaA or after incubation with a purified LPS preparation from

Figure 25 Induction of apoptosis by CyaA

Induction of caspase 3/7 activity in J774.2 cells by CyaA and CyaA* after (A) 2 h or (B) 24 h. (C) shows caspase 3/7 activity of CyaA (0.5 μg/ml) in DMEM over a period of 2 h. CyaA and CyaA* were incubated in RPMI or in DMEM.



E. coli BL21/DE3 at 1 EU/ml.

3.4.7 Urease assay

The urease assay was used to quantify the levels of urea in the CyaA samples after dialysis. Dialysed CyaA samples were only used for fluorescence (Section 3.4.8), circular dichroism (Section 3.4.9), AUC (3.4.10) and crystallography (3.4.11). It was important to assess if there was any residual levels of urea in the CyaA samples as the urea may have interfered with these biophysical studies. The concentration of urea in the dialysed samples was lower than the detection limits of the assay (9.99 mg/L or 0.00058 M), which indicated a minimal amount of urea in the samples (data not shown). In addition, the dialysed CyaA samples did not significantly refract light in the refractometer again confirming the lack of a significant amount of urea in the samples. Buffer, containing 8 M urea, was used as a positive control.

3.4.8 Fluorescence of CyaA

The results of fluorescence studies (Fig. 26) indicated that a conformational change had occurred in CyaA in the presence of 1 mM $CaCl_2$, with a blue shift of 2 nm and a 30% increase in emission intensity, consistent with a more pronounced burial of one or more tryptophan side chains in the interior of the protein. CyaA*, proCyaA and proCyaA* showed similar fluorescence spectra compared with CyaA in the presence of 1 mM $CaCl_2$ (data not shown). This suggests that CyaA*, proCyaA and proCyaA* adopted similar changes in structure upon exposure to calcium compared with CyaA

3.4.9 Circular dichroism

3.4.9.1 Near UV CD of CyaA

In the presence of 1 mM $CaCl_2$, there were increased spectral intensities at 293 nm and in the 265 to 285 nm region observed for CyaA compared to the spectrum obtained in the absence of 1 mM $CaCl_2$ (Fig. 27). Again, the proCyaA, proCyaA* and CyaA* forms produced similar shifts in spectra to those of CyaA in the presence of 1 mM $CaCl_2$ (data not shown) suggesting that all the CyaA forms showed similar changes in structure.

3.4.9.2 Far UV CD of different CyaA forms

The far UV CD spectra of dialysed CyaA and CyaA*, at 0.5 mg/ml, in different $CaCl_2$ concentrations are shown in Figures 28 A and B, respectively. Both CyaA and CyaA* show a marked change in the spectrum from 0 mM $CaCl_2$ to 0.5 mM $CaCl_2$ whereby a negative peak at 207 nm with a shoulder around 222 nm changes to one in which there is a negative peak at 220 nm with a shoulder around 208 nm (Fig. 28C). The spectra of CyaA showed slightly more variation in between 0.5 – 3 mM $CaCl_2$ (Fig. 28A)

Figure 26 Fluorescence spectra of CyaA

Fluorescence spectra of dialysed CyaA (0.1 mg/ml in 10 mM Tris-HCl (pH 8.0)) in the absence (thin solid line) and presence (thick solid line) of 1 mM $CaCl_2$. Samples were scanned over the range of 300 to 400 nm with excitation at 295 nm at 20 °C.

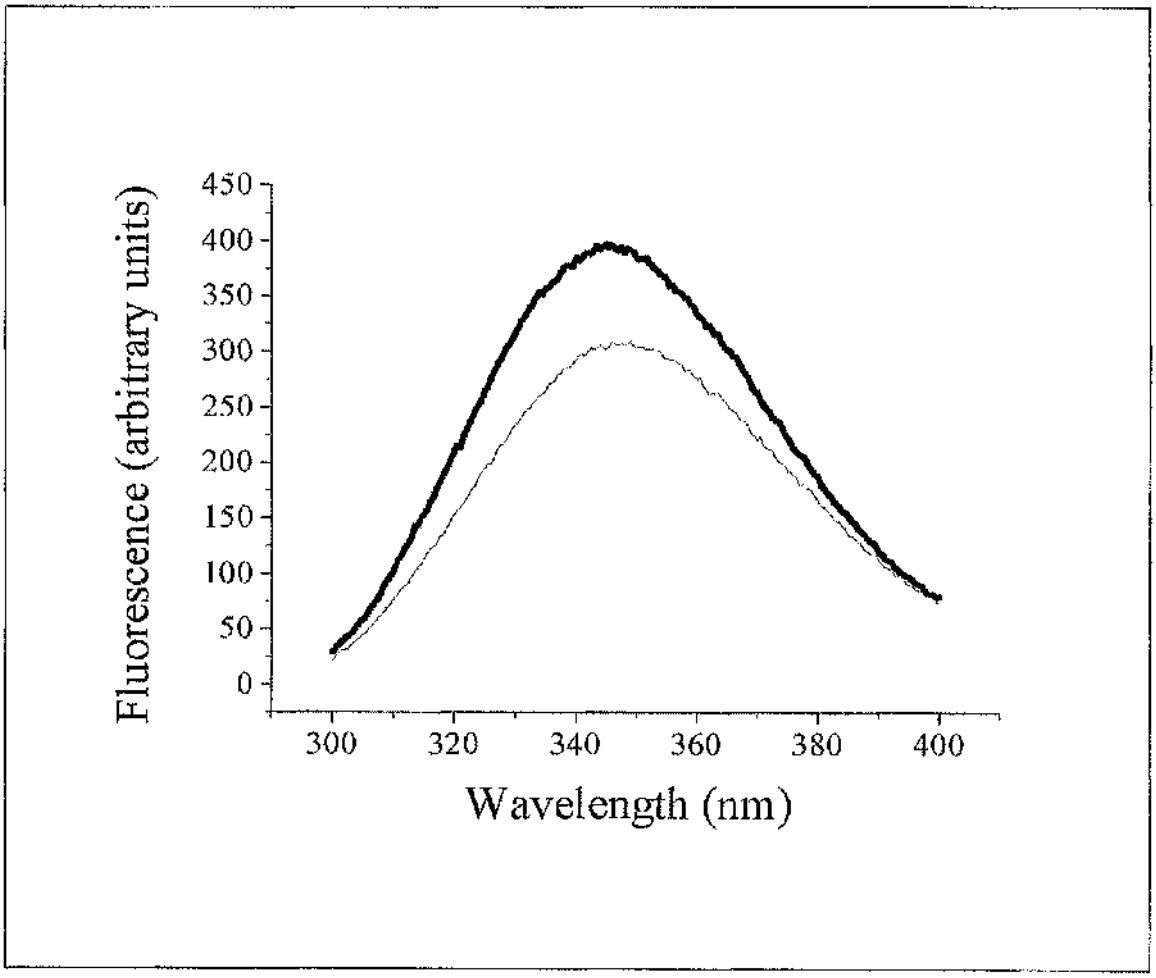


Figure 27 Near UV CD spectra of CyaA

Near UV CD spectra of dialysed CyaA (at 1.5 mg/ml in 10 mM Tris-HCl (pH 8.0)) in the absence (thin solid line) and presence (thick solid line) of 1 mM $CaCl_2$. Each scan represents the average of 8 scans, carried out at 20 °C between 320 and 260 nm, using 0.2-nm steps with a time constant of 2 s.

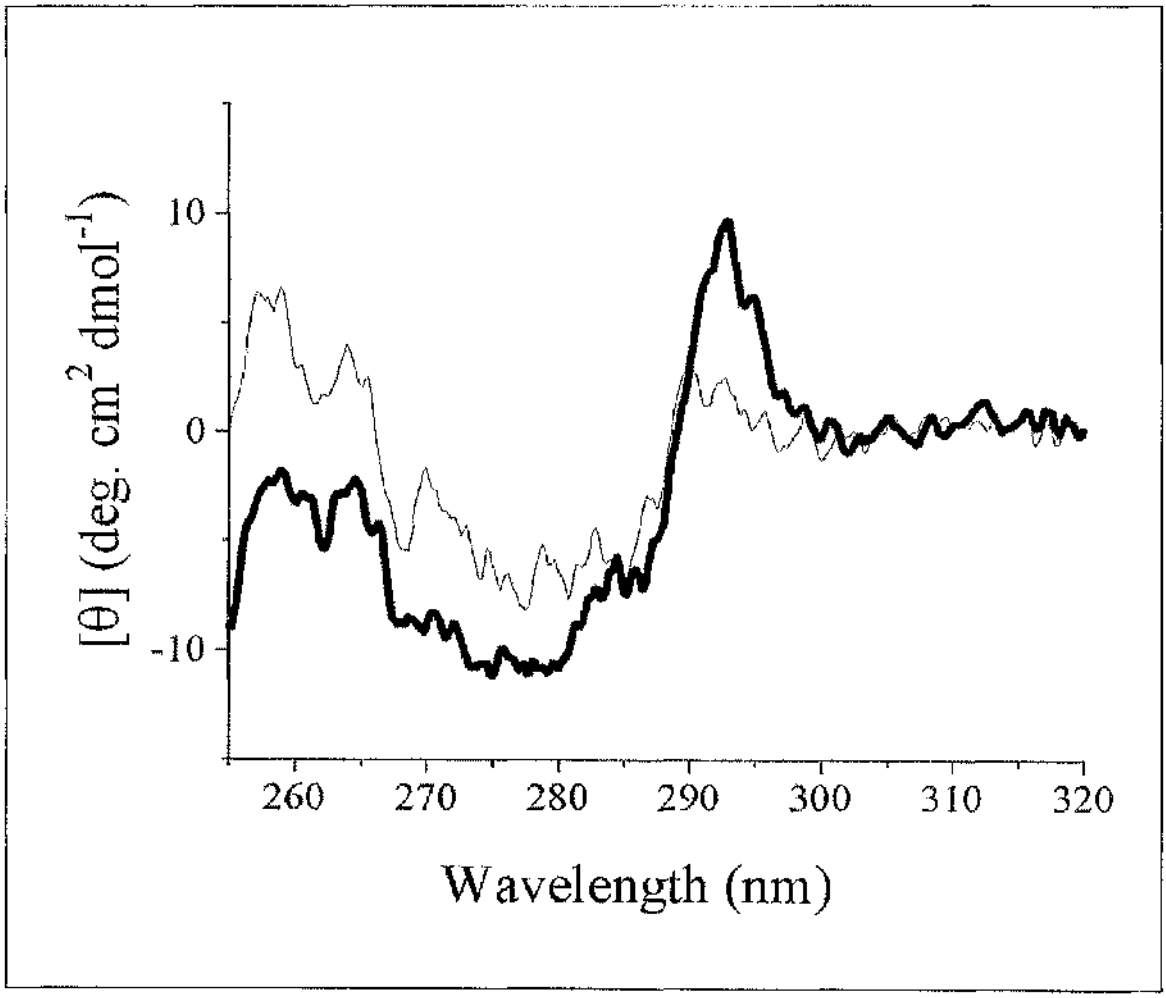
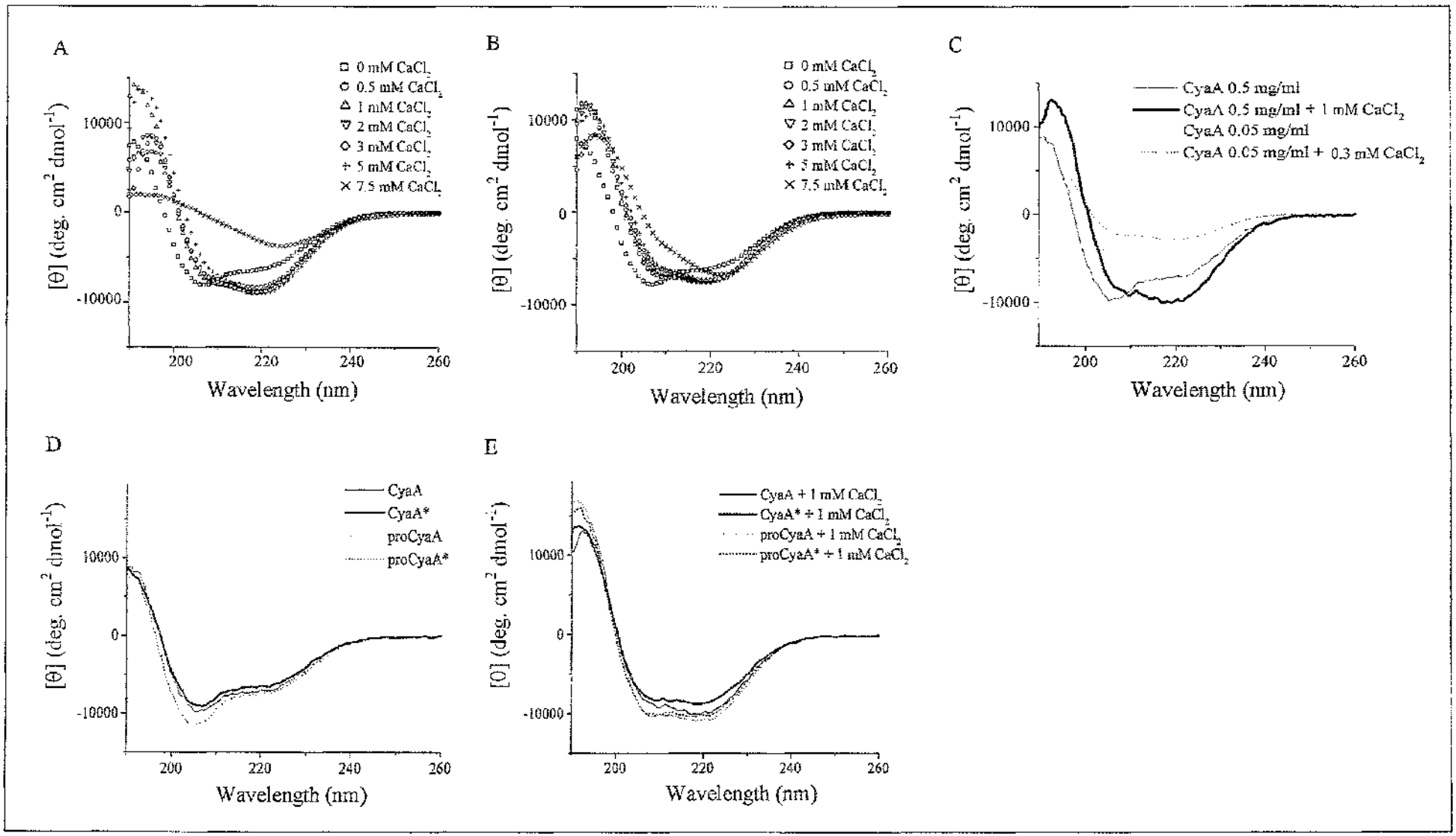


Figure 28 Far UV CD spectra of different CyaA forms

Far UV CD spectra of (A) CyaA and (B) CyaA* at 0.5 mg/ml in the absence (open squares) or presence of increasing concentrations of $CaCl_2$. (C) CyaA at 0.5 mg/ml in the absence (solid line) or in the presence (thick solid line) of 1 mM $CaCl_2$ or CyaA, at 0.05 mg/ml in the absence (dotted line) or in the presence (dashed line) of 0.3 mM $CaCl_2$. Far UV CD spectra of Results represent the means of titrations performed in duplicate with SEM (bars). Each spectrum represents the average of 8 scans, carried out at 20 °C between 190 and 260 nm, using 0.1-nm steps and a time constant of 2 s.


A
0 mM CaCl2
0.5 mM CaCl2
1 mM CaCl2
2 mM CaCl2
3 mM CaCl2
5 mM CaCl2
7.5 mM CaCl2
[θ] (deg. cm2 dmol-1)
10000
0
-10000
200
220
240
260
Wavelength (nm)
B
0 mM CaCl2
0.5 mM CaCl2
1 mM CaCl2
2 mM CaCl2
3 mM CaCl2
5 mM CaCl2
7.5 mM CaCl2
[θ] (deg. cm2 dmol-1)
10000
0
-10000
200
220
240
260
Wavelength (nm)
C
CyaA 0.5 mg/ml
CyaA 0.5 mg/ml + 1 mM CaCl2
CyaA 0.05 mg/ml
CyaA 0.05 mg/ml + 0.3 mM CaCl2
[θ] (deg. cm2 dmol-1)
10000
0
-10000
200
220
240
260
Wavelength (nm)
D
CyaA
CyaA*
proCyaA
proCyaA*
[θ] (deg. cm2 dmol-1)
10000
0
-10000
200
220
240
260
Wavelength (nm)
E
CyaA + 1 mM CaCl2
CyaA* + 1 mM CaCl2
proCyaA + 1 mM CaCl2
proCyaA* + 1 mM CaCl2
[θ] (deg. cm2 dmol-1)
10000
0
-10000
200
220
240
260
Wavelength (nm)

compared with CyaA* (Fig. 28B) although the CD spectra of CyaA and CyaA* at 1, 2 and 3 mM $CaCl_2$ were essentially superimposable (Figs. 28A, B) suggesting that the conformational changes in CyaA and CyaA* were already complete on incubation in 1 mM $CaCl_2$. It was noticeable, however, that with increasing concentrations of $CaCl_2$, the spectra of CyaA and CyaA* altered. This was particularly evident with CyaA at 7.5 mM $CaCl_2$ (Fig. 28A) and this spectrum is indicative of high β-sheet content. Interestingly, the spectral intensity of CyaA* (Fig. 28B) was ~10% less than that of CyaA (Fig. 28A). These small changes (up to 10% variation in the amplitudes and minor variations in shape) were observed consistently and did not appear to reflect errors in the determination of protein concentration. ProCyaA and proCyaA* showed similar spectral trends as observed for CyaA in the absence (Fig. 28D) and presence (Fig. 28E) of 1 mM $CaCl_2$. The far UV spectra of CyaA, at 0.5 mg/ml and 0.05 mg/ml, in the presence and absence of $CaCl_2$ are shown in Figure 28C. There was a marked dependence of the far UV CD spectrum of CyaA on protein concentration. As shown in Fig. 28C, the spectrum of CyaA at 0.05 mg/ml was of a similar (but not identical) shape to that observed at 0.5 mg/ml, but the molar ellipticity values were reduced by a factor of approximately 2. In addition, the response to addition of calcium was markedly different at the lower concentration (Fig. 28C). Similar effects of concentration on the far UV CD spectra were observed using proCyaA, proCyaA* and CyaA* (data not shown). This dependence of spectral changes on protein concentration implied an interaction between individual molecules that was promoted at higher protein concentrations. Because of the high level of noise below 195 nm in the spectra of CyaA at 0.05 mg/ml, it was not possible to undertake any reliable analysis of the spectra in terms of secondary structural content.

The CDSSTR program in Dichroweb was used to analyse the CD spectra of CyaA at 0.5 mg/ml over the wavelength range from 240 nm to 185 nm in terms of the secondary structure content. It was found that each of the other procedures such as VARSELC, SELCON and CONTIN in Dichroweb did not give satisfactory analyses for spectra in both the absence and presence of $CaCl_2$. For each sample, CDSSTR fitted both spectra satisfactorily as judged by 2 criteria:- (i) the Normalised Root Mean Square Deviation (NRMSD) values were low, in the range of 0.015–0.064 for all samples, and (ii) the reconstructed spectra using CDSSTR were essentially superimposable on the experimental data over the wavelength range 240 nm to 185 nm. The results of the CDSSTR analysis for the various forms of CyaA in the presence and absence of 1 mM $CaCl_2$ are shown in Table 8. The secondary structure contents of CyaA and CyaA* in the presence and absence of 1 mM $CaCl_2$ were similar with only relatively small (up to 4%) differences in the total content of α-helices, β-strands and turns (Table 8). Similarly, the secondary structure

Table 8 Secondary structure compositions of the different CyaA forms in the presence or absence of $CaCl_2$

	CyaA form							
	CyaA		CyaA*		proCyaA		proCyaA*	
$CaCl_2$ (1 mM)	-	+	-	+	-	+	-	+
Helix total (%)	22	22	20	24	32	32	25	28
Strand total (%)	24	23	26	25	17	20	23	22
Turns (%)	21	24	21	21	25	20	21	20
Unordered (%)	32	31	32	30	27	29	32	30
NRMSD	**0.03**	**0.028**	**0.029**	**0.026**	**0.033**	**0.026**	**0.064**	**0.015**

Deconvolution of spectra shown in Figs. 28 D and E was performed using CDSSTR, in Dichroweb (Section 2.7.3). Numbers represent percentage of predicted structural motifs within the protein in the absence or presence of 1 mM $CaCl_2$. [1]Normalised Root Mean Squared Deviation.

contents of proCyaA and proCyaA* were similar in the presence of 1 mM $CaCl_2$ with up to 4% differences in the content of α-helices, β-strands and turns, respectively. However, in the absence of calcium, the secondary structure contents of proCyaA appeared to be significantly different to the other forms (Table 8), with an increase in α-helices and a decrease in β-strands. However, given the overall similarity in spectral shape, it would appear unlikely that proCyaA has a markedly different secondary structure from CyaA or CyaA*. It is clear that the results of the analysis (especially in terms of the balance between α-helices and β-strands) are very sensitive to the amplitudes and shapes of the positive peak at 193 nm and of the negative peak and shoulder in the region 208 nm to 222 nm, with the positive peak being very sensitive to the level of noise due to residual traces of urea in the preparations. In general, not withstanding the slight differences detected in proCyaA in the absence of calcium, the data in Table 8 suggest that all the forms of CyaA have similar overall structures and show similar responses on the addition of $CaCl_2$.

Figure 29 shows the changes in ellipticity at 220 nm of CyaA as the urea concentration was varied between 0 M and 8 M urea (designated as 0% and 100%, respectively) in the presence and absence of 1 mM $CaCl_2$. In the absence of $CaCl_2$, the changes in structure occurred in a gradual fashion over the range of concentrations from 0 - 4 M urea. However, in the presence of $CaCl_2$, CyaA shows greater structural stability over the range of urea concentrations up to 4 M, with the changes occurring sharply over the range of 2 - 4 M urea. There was no immediate aggregation of dialysed CyaA samples upon addition of $CaCl_2$ even at toxin concentrations up to 3 mg/ml. However, white aggregates appeared after storage at 4 °C or –20 °C for periods longer than a month.

3.4.10 Analytical ultracentrifugation

3.4.10.1 Density and viscosity of buffers used

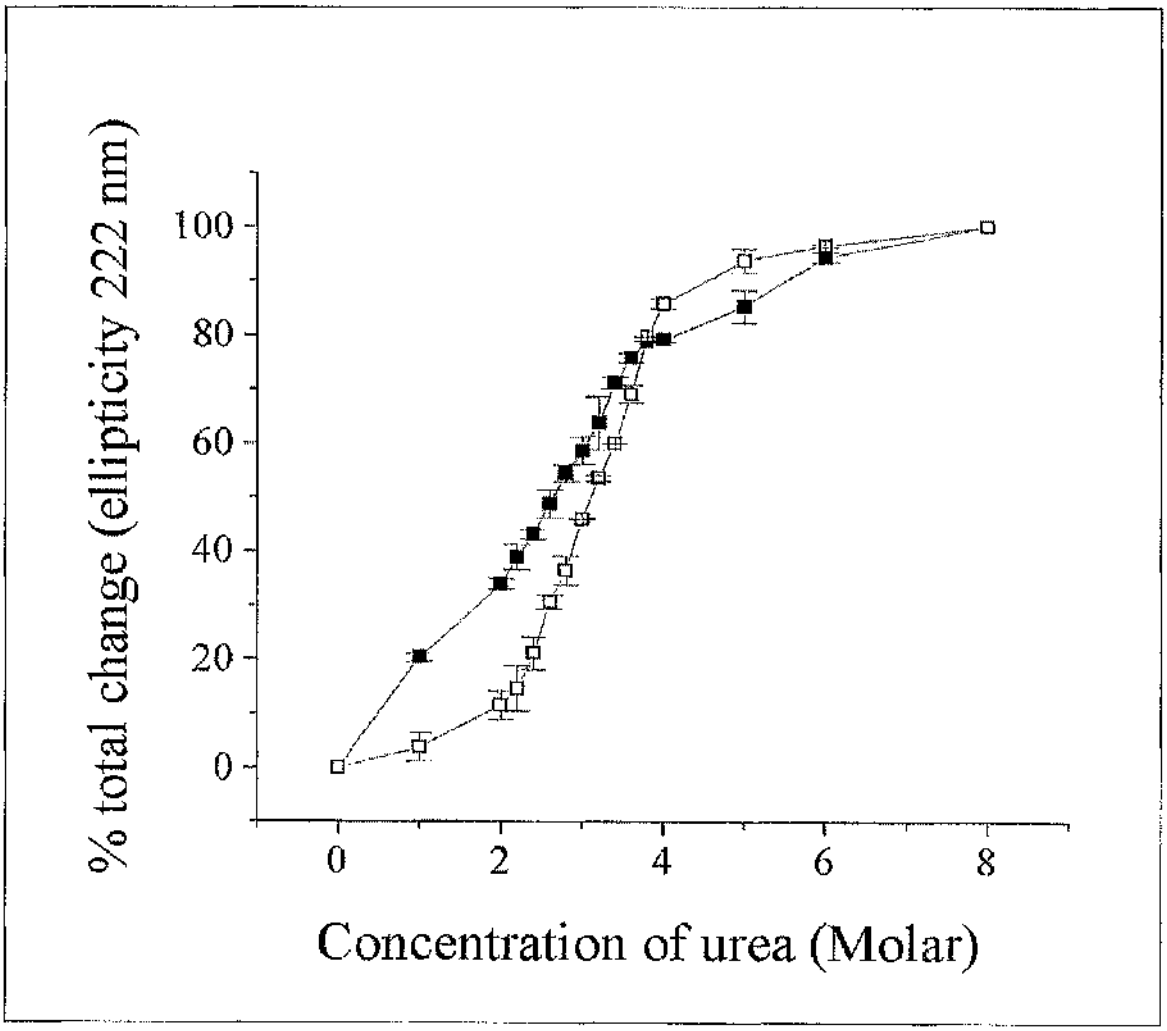
The computer programme SEDNTERP was used to calculate the densities and viscosities of the buffers used for analytical ultracentrifugation (Table 9).

Table 9 Density, viscosity and partial specific volume values of buffers used

Buffer	Temperature (°C)	Density (g/ml)	Viscosity (Poise)	Partial specific volume
10 mM Tris HCl, pH 8.0	4	1.00028	0.01504	0.7169
	20	0.99851	0.010042	0.7237
10 mM Tris HCl, 1 mM $CaCl_2$, pH 8.0	4	1.00035	0.01504	0.7169
	20	0.99858	0.010042	0.7237

Figure 29 Change in ellipticity at 222 nm from far UV CD spectra of CyaA in different urea concentrations

CyaA in different concentrations of urea in the absence (solid squares) or in the presence (open squares) of 1 mM $CaCl_2$. The data show the urea dependence of the mean residue ellipticity at 222 nm in the absence and presence of $CaCl_2$. Results represent the means of titrations performed in duplicate with SEM (bars). Each spectrum represents the average of 8 scans, carried out at 20 °C between 190 and 260 nm, using 0.1-nm steps and a time constant of 2 s.



3.4.10.2 Sedimentation equilibrium analytical ultracentrifugation

SE data showed that CyaA, in the absence of 1 mM $CaCl_2$, was a heterogeneous mixture of different molecular weight (M_w) species in solution. A histogram resulting from fitting the SE data with a molecular weight distribution model (using UltraScan) is shown in Fig. 30A. This model was used because the data were too complex to model with a self-association model or with a discrete multi-association model. The molecular weights in the distribution ranged from < 40 kDa to > 500 kDa. A species of 140 kDa was identified but there was no evidence of a 177.5 kDa (the M_w of a CyaA monomer) species. This may have been the result of a phenomenon known as non-ideality (Chapter 4) which depresses the observed molecular weight of highly charged and/or elongated particles. Accordingly, oligomers of 2 or 3 CyaA (apparent mass 140 kDa) monomers could be hypothesised to form in solution as indicated by the presence of 400 – 430 and 535 kDa M_w species, respectively (Fig. 30 Table).

In the presence of 1 mM $CaCl_2$, the SE data showed that CyaA, again, did not occur as a single homogeneous species in solution (Fig. 30B): low and high M_w species of CyaA were detected (Fig. 30B). Some of the species identified in the presence of $CaCl_2$ were not identified in the absence of $CaCl_2$. This included the observation of a 170 kDa species. Based on a M_w of 170 kDa, oligermeric states of 2 or 3 CyaA monomers could be modelled as indicated by the presence of 360 and 510 – 560 kDa species, respectively (Fig. 30 Table). Moreover, in both the absence and presence of calcium, there were many species that could not be assigned to a specific oligomeric state (Fig. 30 Table).

3.4.10.3 Sedimentation velocity analytical ultracentrifugation

In the absence of 1 mM $CaCl_2$, the SV data (Fig. 31A) showed that CyaA in solution, consisted of 4 main peaks (species) at concentrations of 3 and 2.25 mg/ml. At concentrations lower than 2.25 mg/ml, the fourth peak could not be observed (Fig. 31A; Fig. 31 Table). In addition, as the concentration of CyaA was decreased, the sedimentation coefficient for each peak increased and this was indirectly proportional to the concentration of each species ($c(s)$) (Fig. 31A; Fig. 31 Table). This feature is characteristic of non-ideality (Chapter 4). The sedimentation coefficients for peaks ranged from 2.5 S to ~5 S (Fig. 31 Table). Dialysed CyaA at 3 mg/ml, in the presence of 1 mM $CaCl_2$, produced 3 more peaks than dialysed CyaA in the absence of $CaCl_2$ in solution (Fig. 31B). These peaks were greater in their sedimentation coefficient values, ranging from 5 - 18 S, which is in contrast to the smaller sedimentation coefficients obtained by CyaA in the absence of calcium (Fig. 31A). In addition, there was little or no shift of sedimentation coefficients for each of the main peaks as the concentration of CyaA was reduced. Instead, the $c(s)$ of each peak was reduced (Fig. 31 Table). There was a small peak (between peaks 1 and 2) for

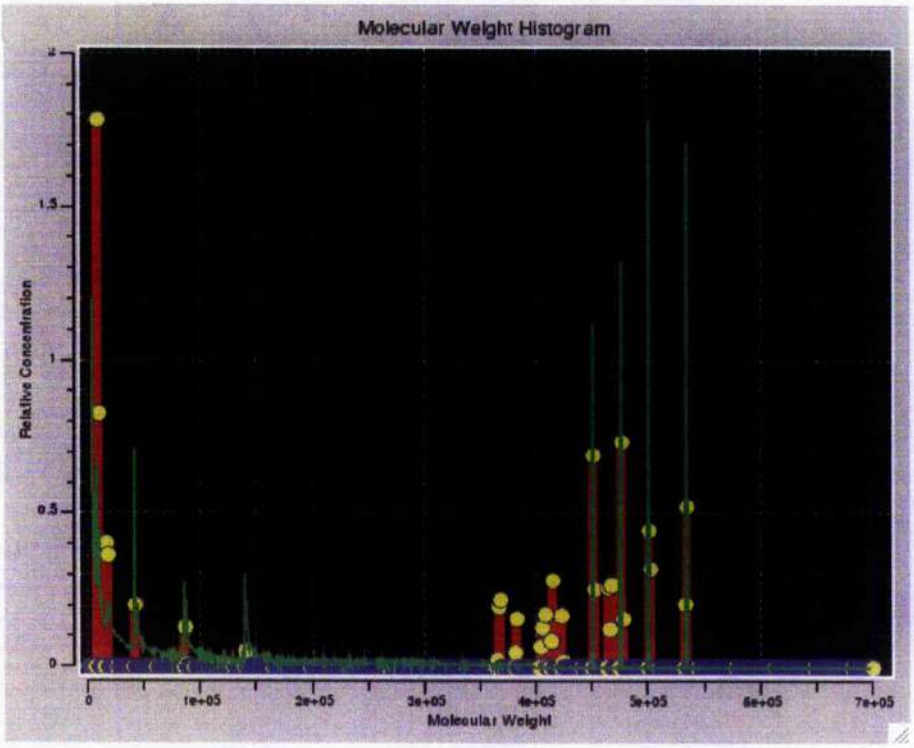
Figure 30 Sedimentation equilibrium analysis of purified CyaA in the absence and presence of $CaCl_2$

Data from each cell containing different concentrations of purified CyaA in the (A) absence or (B) presence of $CaCl_2$ were recorded at different rotor speeds (7, 11 and 15 k rpm) and analysed simultaneously using the programme, UltraScan, which allows the data to be fitted with a molecular weight distribution that assumes no model at all. UltraScan produced histograms showing the distribution of molecular species vs their abundances in solution. Red bars are histograms of the main species. The green line is a different treatment of the same data that lies outwith the scope of this analysis.

	(A) CyaA without $CaCl_2$		(B) CyaA with $CaCl_2$	
Species	**M_w (kDa)**	**Comment**	**M_w (kDa)**	**Comment**
1	0 - 30	Cleavage product?	20	Cleavage product?
2	40	Cleavage product – AC domain?	40	Cleavage product – AC domain?
3	80	Cleavage product?	60	Cleavage product?
4	140	Monomer (M_w of 177550 kDa depressed by non-ideality)	70	Cleavage product?
5	365 - 380	-	170	Monomer (M_w of 177550 kDa may no longer be depressed by non-ideality due to presence of $CaCl_2$)
6	400 - 430	3-mer of 140kDa?	210	-
7	450	-	225	-
8	460 - 475	-	280	-
9	500	-	360	2-mer of 177 kDa?
10	535	4-mer of 140kDa?	510	3-mer of 177 kDa?
11	n/d		550 - 560	3-mer of 177 kDa?
12	n/d		580	-
13	n/d		625	-

-, no comment. n/d, not determined.

A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Relative Concentration
Molecular Weight
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1e+05
2e+05
3e+05
4e+05
5e+05
6e+05
7e+05

B

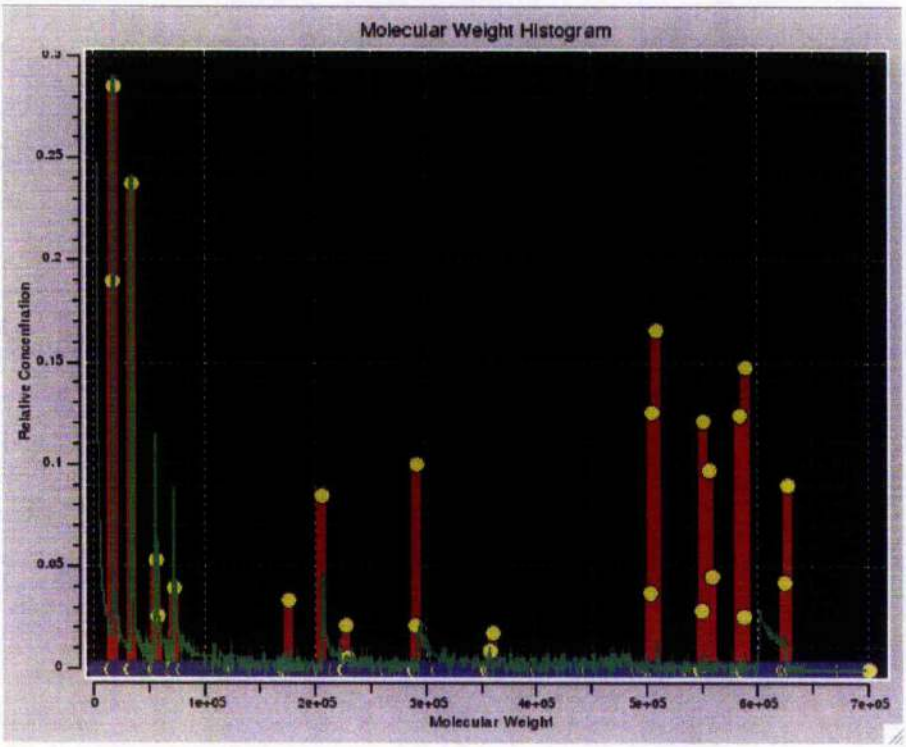
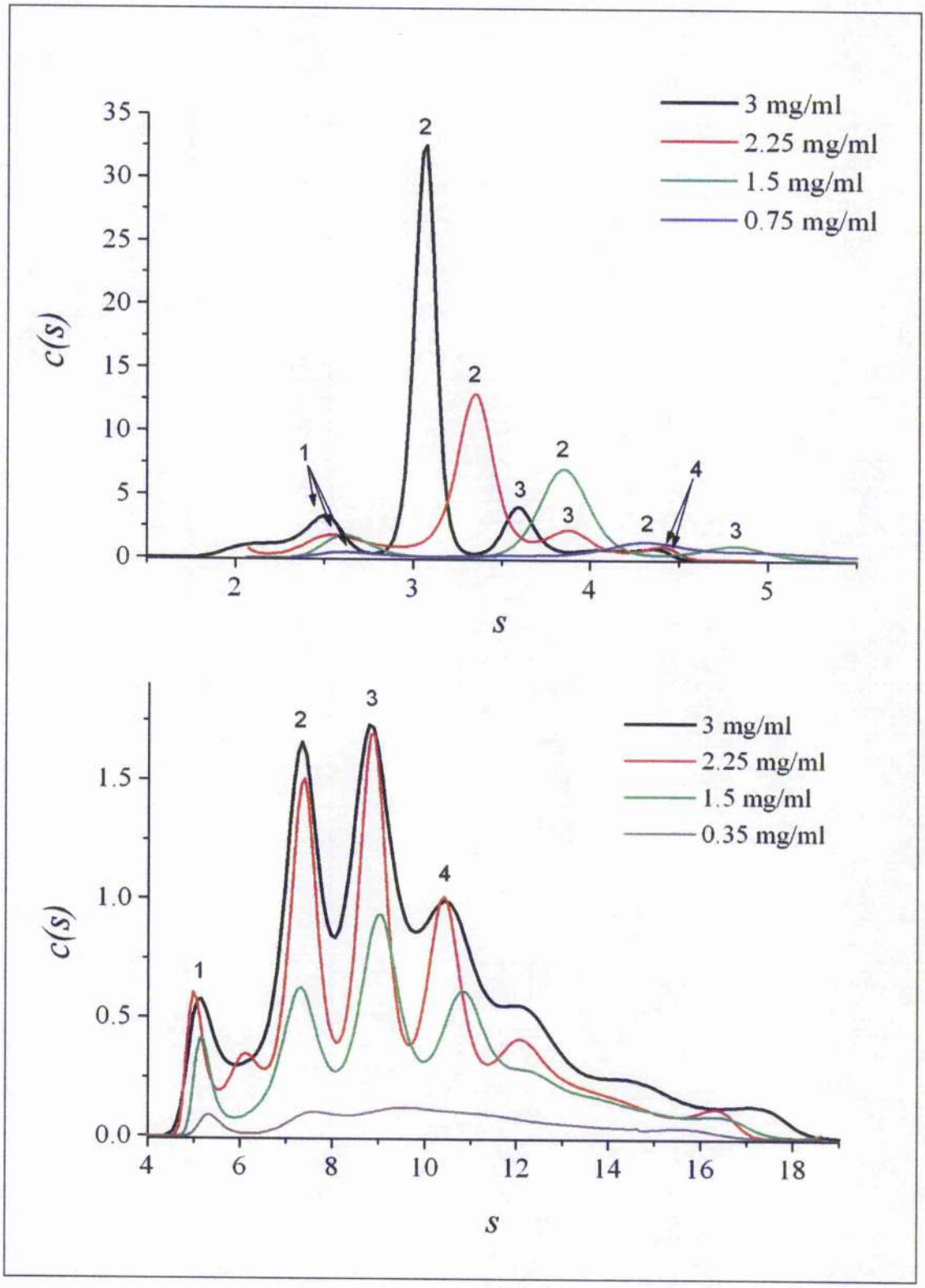

Molecular Weight Histogram
Relative Concentration
Molecular Weight
0
0.25
0.2
0.15
0.1
0.05
1e+05
2e+05
3e+05
4e+05
5e+05
6e+05
7e+05

Figure 31 Sedimentation velocity analysis of purified CyaA in the absence and presence of $CaCl_2$

c(s) distribution derived from SV interference data collected for various concentrations of CyaA in the (A) absence or (B) presence of 1 mM $CaCl_2$. The sediment coefficients (*s*) and concentrations (*c(s)*) for 4 main peaks (assigned 1 – 4 in the figure) in the absence and presence of 1 mM $CaCl_2$ have been documented in the table below. Due to leakage of sample from cells, data for 0.35 mg/ml CyaA (no $CaCl_2$) could not be obtained. This also occurred for 0.75 mg/ml CyaA (+ 1 mM $CaCl_2$).

			Peak			
Sample	Concentration		1	2	3	4
CyaA no $CaCl_2$	3 mg/ml	*s*	2.51	3.05	3.59	4.36
		c(s)	3.27	32.46	4.15	0.87
	2.25 mg/ml	*s*	2.54	3.34	3.89	4.41
		c(s)	1.79	12.94	2.31	1.044
	1.5 mg/ml	*s*	2.61	3.85	4.811	-
		c(s)	1.77	7.108	1.117	
	0.75 mg/ml	*s*	2.63	4.32	4.97	-
		c(s)	0.46	1.37	0.605	
CyaA + 1 mM $CaCl_2$	3 mg/ml	*s*	5.15	7.34	8.83	10.43
		c(s)	0.57	1.66	1.73	0.99
	2.25 mg/ml	*s*	5.01	6.12	7.37	8.901
		c(s)	0.6	0.347	1.49	1.68
	1.5 mg/ml	*s*	5.16	7.306	8.97	10.82
		c(s)	0.4	0.62	0.939	0.61
	0.35 mg/ml	*s*	5.31	7.57	9.57	11.06
		c(s)	0.09	0.105	0.12	0.102

-, no peak.


3 mg/ml
2.25 mg/ml
1.5 mg/ml
0.75 mg/ml
c(s)
s
1
2
3
4
3 mg/ml
2.25 mg/ml
1.5 mg/ml
0.35 mg/ml
c(s)
s

CyaA at 2.25 mg/ml (Fig. 31B). At the lowest toxin concentration used (0.289 mg/ml, data not shown), the spectrum was essentially superimposable on the spectrum produced by CyaA at 0.35 mg/ml in the presence of $CaCl_2$. These changes in peak patterns may be due to the phenomenon of non-ideality being overcome in the presence of $CaCl_2$ (Chapter 4).

In summary, the SV data correlate with the SE data in that CyaA, in solution, is not a single species. Furthermore, the addition of $CaCl_2$ to CyaA in solution causes a significant change in the pattern of peak intensities.

3.4.11 Crystallography

No protein crystals were grown during the period of these trials.

3.5 *In vivo* experiments: mouse protection against *B. pertussis* challenge

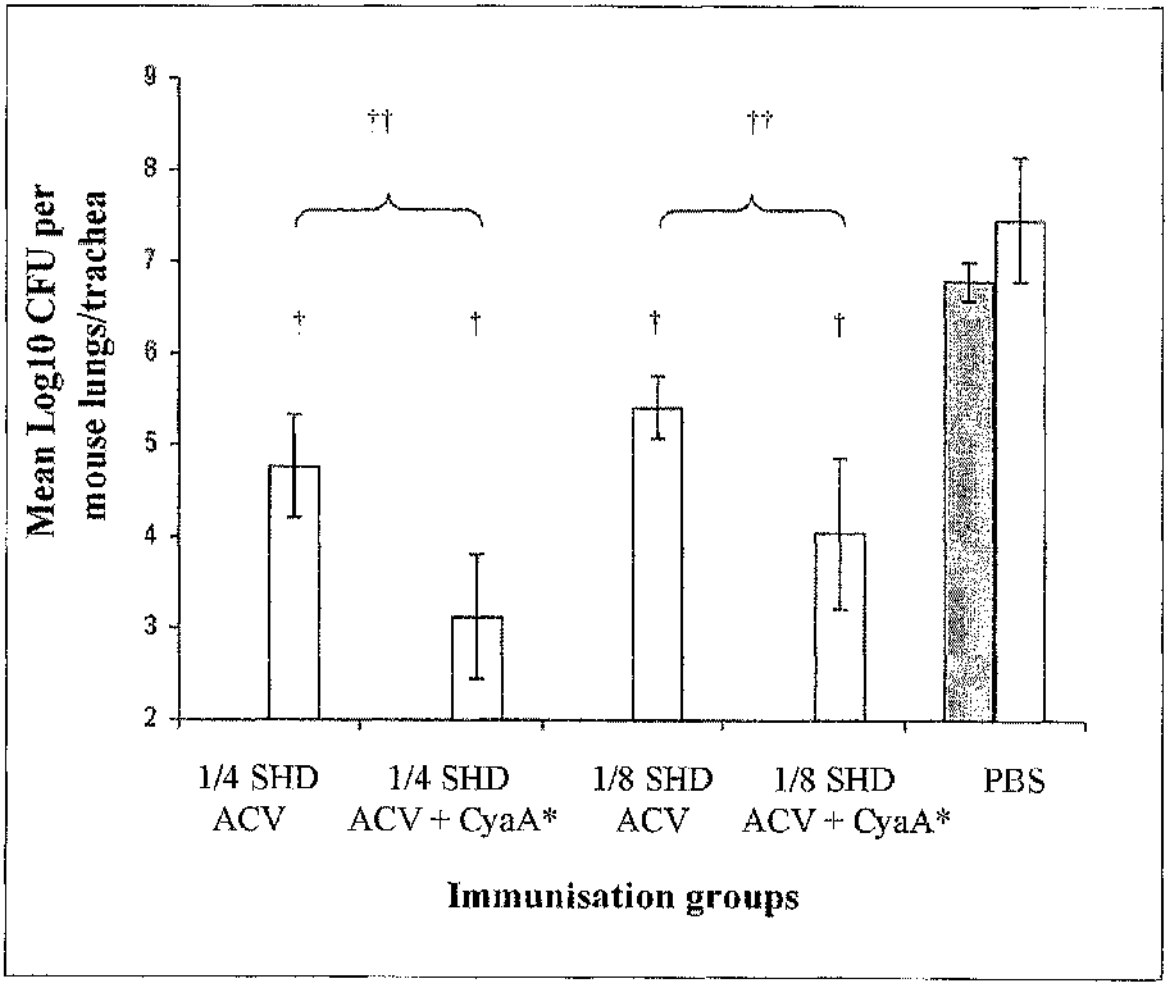
The ability of the different CyaA forms alone, and their ability to enhance the protective efficacy of a conventional acellular pertussis vaccine in mice against *B. pertussis* intranasal challenge was investigated. The ability of the different CyaA forms to protect mice against aerosol challenge was also tested.

3.5.1 Selection of vaccine dose for sub-protection of mice against intranasal challenge with *B. pertussis*

Groups of 10 mice were immunised intraperitoneally on days 0 and 28 with ¼ or ⅛ single human dose (SHD) of ACV with or without 25 μg CyaA*. Mice were challenged intranasally with *B. pertussis* 18.323 on day 42 (two weeks post-boosting). Lungs and tracheas were sampled on day 7 post-challenge and for the PBS group, an additional sampling was carried out at 2 h post-challenge to confirm the inoculation. A comparison of the bacterial counts from the lungs and tracheas of two PBS control mouse groups, sampled at 2 h and 7 days post-challenge, showed a 0.7 $\log_{10}$ increase in bacteria after 7 days, confirming the growth and persistence of the challenge strain (Fig. 32). There was significant ($P < 0.05$) protection in all ACV-immunised groups of mice at 7 days post-*B. pertussis* challenge compared with the PBS control group, with the ¼ SHD of ACV providing greater protection than the ⅛ SHD, as expected (Fig. 32). There was a further significant ($P < 0.05$) reduction of bacteria in mice that were immunised with either ¼ or ⅛ SHD of ACV + 25 μg of CyaA* compared with mice immunised with ¼ or ⅛ SHD of ACV alone, respectively (Fig. 32). These data indicated that ¼ and ⅛ doses provided partial protection against challenge and that this protection was enhanced by inclusion of CyaA*. A SHD of ⅛ of ACV was chosen to be used in further experiments because it gave

Figure 32 Protection of mice against intranasal challenge with *B. pertussis* after immunisation with ¼ or ⅛ of SHD of ACV with or without 25 µg of CyaA*

Groups of mice were immunised intraperitoneally on days 0 and 28 with ¼ or ⅛ of a SHD of ACV alone or these doses of ACV + 25 µg of CyaA* and challenged intranasally on day 42 with *B. pertussis* 18.323. Mice injected with PBS served as controls. Five mice from a PBS control group were sampled at 2 h post-challenge for enumeration of bacteria in lungs and tracheas (▨). All remaining mice were sampled at 7 days post-challenge (□). Results represent the means of five mice per group with SEM (bars). Symbol: †, $P < 0.05$ (groups vs PBS group at day 7: ANOVA) or ††, $P < 0.05$ (as linked by brackets).



less protection than ¼ SHD of ACV against *B. pertussis* intranasal challenge. Any additional protective effects due to inclusion of CyaA would therefore be apparent.

3.5.2 Protection of mice against intranasal challenge with *B. pertussis* after immunisation with ACV plus different forms of CyaA

Groups of 10 mice were immunised intraperitoneally on days 0 and 28 with ⅛ SHD of ACV with or without the four different CyaA forms (25 µg/dose). On day 42, five mice from each group were challenged intranasally with *B. pertussis* 18.323 and the remaining five mice were sampled for serum, spleens and peritoneal macrophages at the same time to measure humoral and cellular responses (Sections 3.6.1, 3.7 and 3.8). A comparison of the bacterial numbers from the lungs and tracheas of two PBS control mouse groups, sampled at 2 h and 7 days post-challenge, showed a 0.5 $\log_{10}$ increase after 7 days (Fig. 33). Mice immunised with the four CyaA forms alone were not protected against *B. pertussis* intranasal challenge (Section 3.5.4.1). Mice immunised with ACV alone showed significant ($P < 0.05$) protection, as indicated by a ~3 $\log_{10}$ reduction in bacterial numbers compared with mice from the PBS control group (Fig. 33). Although mice immunised with ACV + CyaA showed a further ~1 $\log_{10}$ reduction in bacterial numbers compared with ACV alone, there were no significant differences in bacterial numbers in the lungs of mice immunised with ACV+ CyaA, + proCyaA or + proCyaA* compared with ACV alone. However, mice immunised with ACV + CyaA* were significantly more protected ($P < 0.05$) than mice immunised with ACV alone as shown by a reduction of ~1.6 $\log_{10}$ in bacterial numbers compared with the ACV alone group (Fig. 33). Protection by CyaA alone is discussed in Section 3.5.4.

3.5.3 Protection of mice against intranasal challenge with *B. pertussis* after immunisation with ACV plus graded doses of CyaA*

Since CyaA* showed the best adjuvant activity, a further study was performed to examine if the enhanced protection of CyaA* towards ACV was dose-dependent. Mice were immunised with ⅛ SHD of ACV plus graded doses of CyaA* on days 0 and 28 and challenged intranasally at day 42 with *B. pertussis* 18.323. A comparison of the bacterial numbers from the lungs and tracheas of two PBS control mouse groups, sampled at 2 h and 7 days post-challenge, showed a 0.5 $\log_{10}$ increase after 7 days (Fig. 34). Mice immunised with ACV alone gave significant ($P < 0.05$) protection 7 days post-challenge, as indicated by a ~2 $\log_{10}$ reduction in lung counts compared with control mice (PBS group) (Fig. 34). However, mice that had received ACV + 25 µg or 12.5 µg of CyaA* showed significantly ($P < 0.05$) greater protection compared with the ACV alone group. This enhanced protection was dose-dependent and ACV + 25 µg CyaA gave a further 2.49 $\log_{10}$ reduction

Figure 33 Protection of mice against intranasal challenge with *B. pertussis* after immunisation with ACV plus different CyaA forms

Mice were immunised intraperitoneally on days 0 and 28 with PBS, ACV (⅛ SHD) or ACV plus CyaA forms at 25 μg. Mice were challenged intranasally with *B. pertussis* 18.323 on day 42. Five mice from the PBS control group were sampled at 2 h post-challenge for enumeration of bacteria in lungs and tracheas (▩). All remaining mice were sampled at 7 days post-challenge (□). Results represent means of five mice per group with SEM (bars). Symbol: †, $P < 0.05$ (groups vs ACV: ANOVA) or ††, $P < 0.05$ (groups vs PBS: ANOVA) or †††, $P < 0.05$ (as linked by brackets).

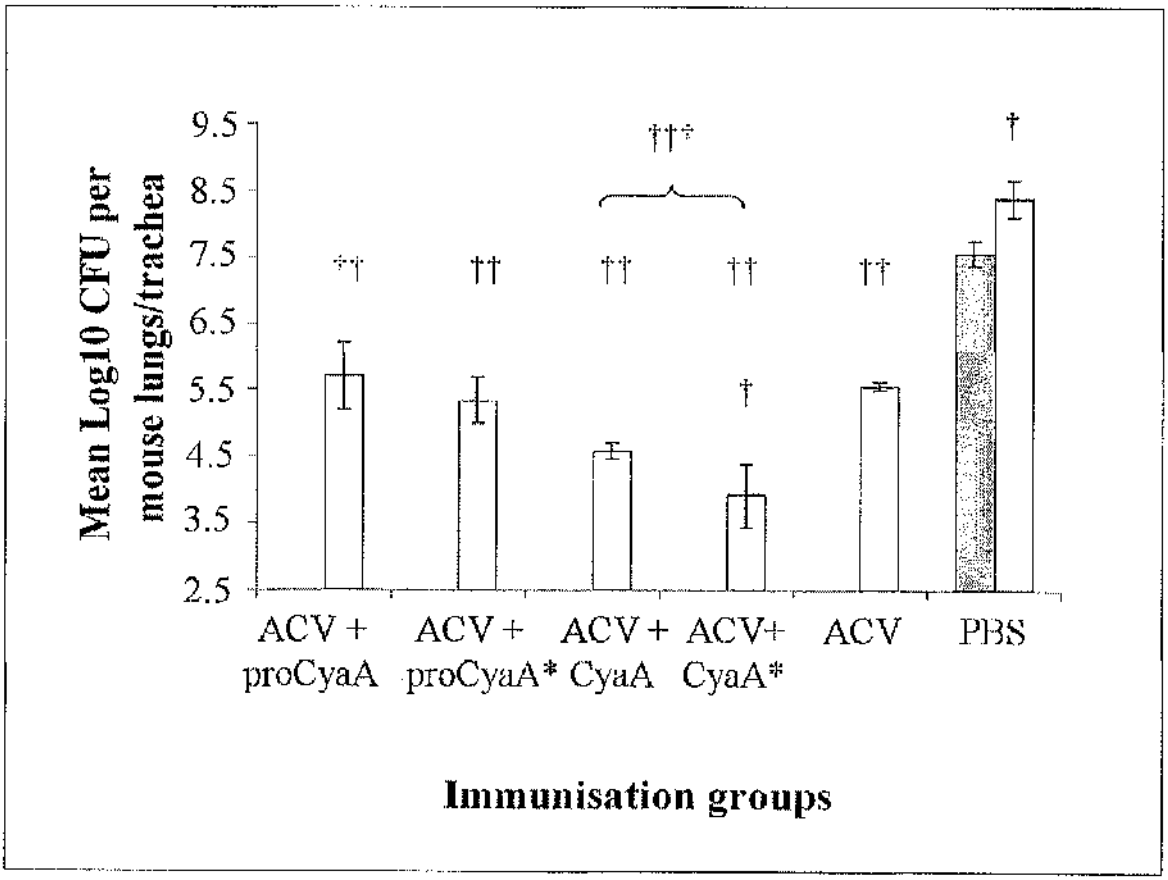
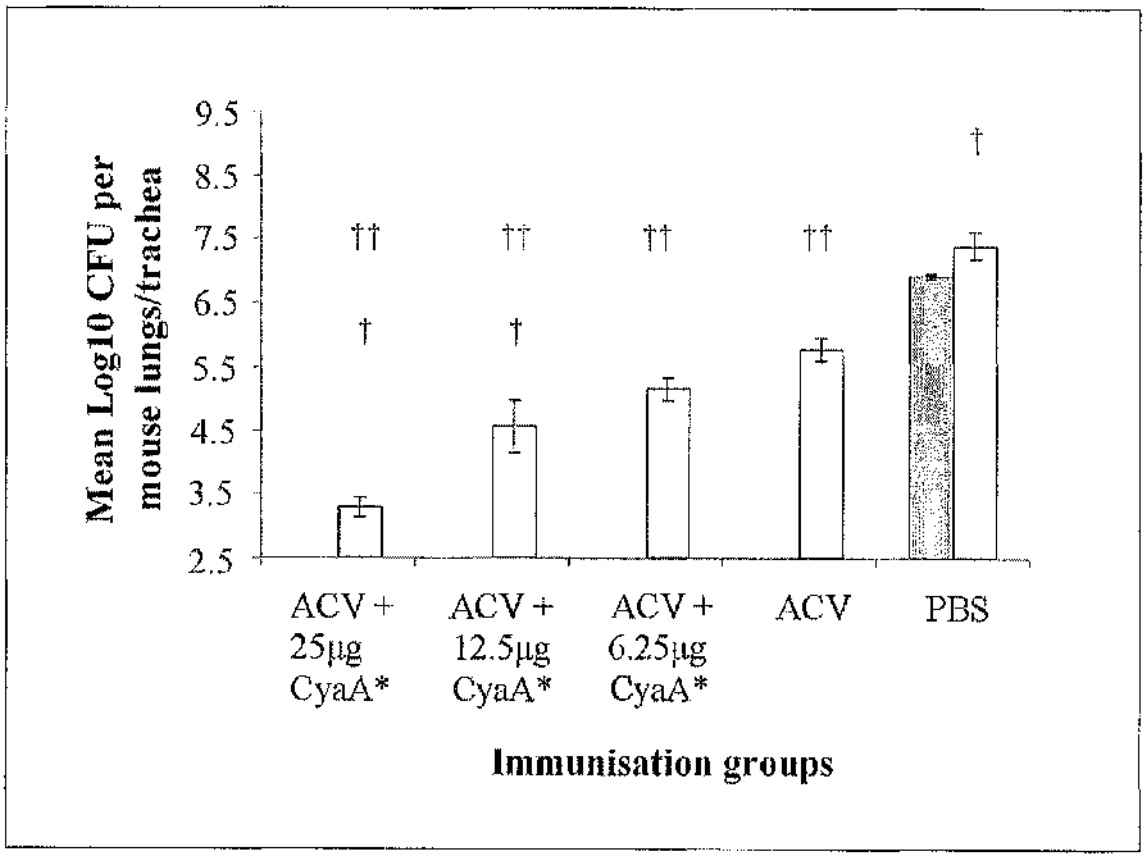


Figure 34 Protection of mice against intranasal challenge with *B. pertussis* after immunisation with ACV plus graded doses of CyaA*

Mice were immunised intraperitoneal with ⅙ SHD of ACV plus graded doses of CyaA* on days 0 and 28 and challenged intranasally at day 42 with *B. pertussis* 18.323. Mice immunised with ACV or PBS alone served as controls. Five mice from the PBS control group were sampled at 2 h post-challenge (▨). All remaining mice were sampled at 7 days post-challenge (□). Results represent means of five mice per group with the SEM (bars). Symbol: †, $P < 0.05$ (groups vs ACV: ANOVA) or ††, $P < 0.05$ (groups vs PBS: ANOVA).



in lung counts compared with the ACV alone (Fig. 34). There was no significant ($P > 0.05$) protection in mice immunised with any dose of CyaA* alone (data not shown).

3.5.4 Protection with CyaA alone

Groups of mice were immunised intraperitoneally twice with the four different CyaA forms alone (25 μg/dose) and then either challenged intranasally or by aerosol with *B. pertussis* 18.323.

3.5.4.1 Intranasal challenge

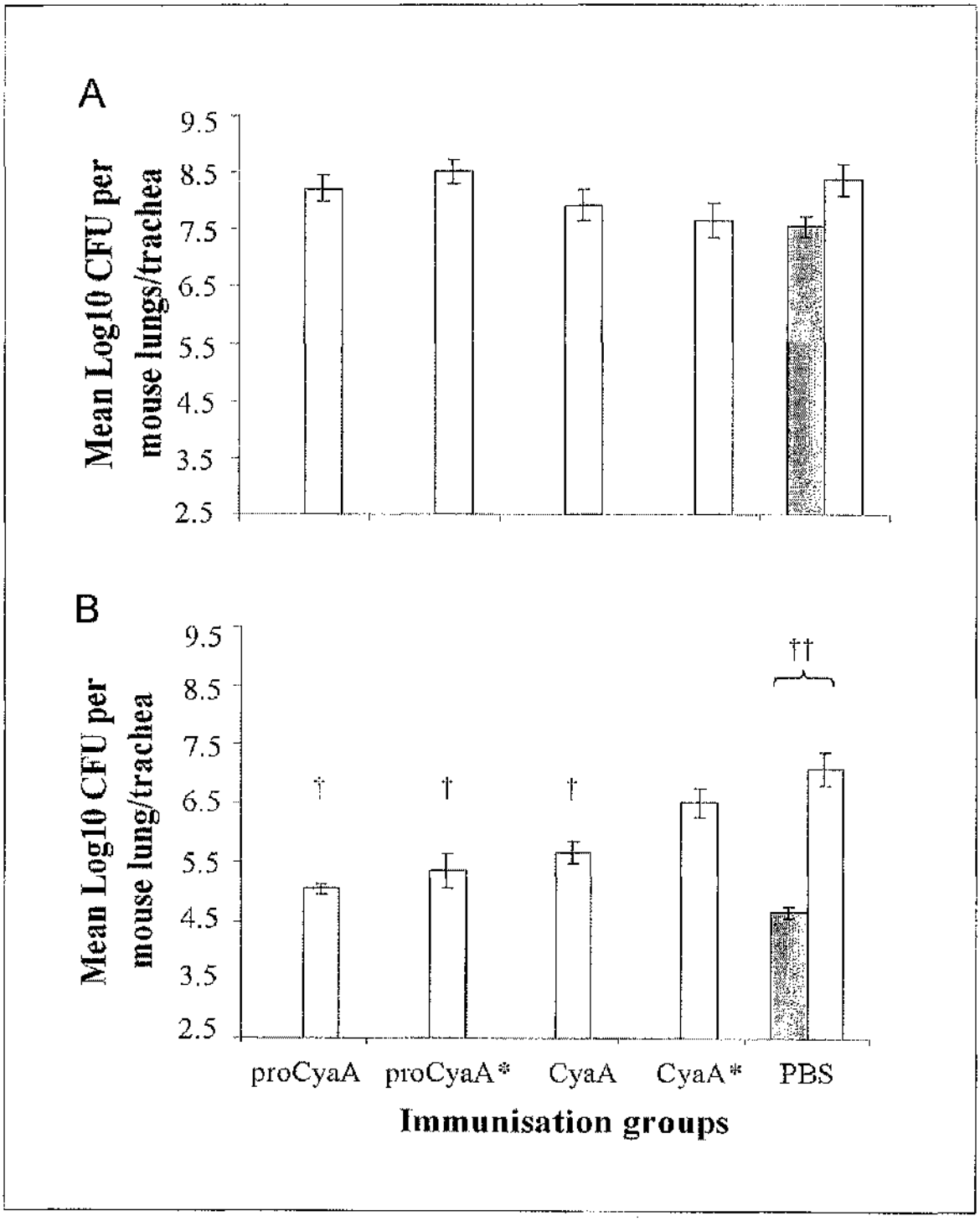
Groups of 10 mice were immunised intraperitoneally on days 0 and 28 with the four different CyaA forms in 10% (v/v) alum or injected with PBS in 10% (v/v) alum. On day 42, five mice from each group were challenged intranasally with *B. pertussis* 18.323 and the remaining five mice were sampled for serum, spleens and peritoneal macrophages to measure humoral and cell-mediated responses (Sections 3.6.3, 3.7 and 3.8). A comparison of the bacterial numbers from the lungs and tracheas of two PBS control groups, sampled at 2 h and 7 days post-challenge, showed that there was a 0.8 $\log_{10}$ increase ($P > 0.05$) in bacterial numbers after 7 days. This confirmed the growth and persistence of the challenge strain (Fig. 35A). Mice given two doses of the CyaA forms alone showed bacterial numbers at day 7 that were not significantly different ($P > 0.05$) from the PBS control mouse group (Fig. 35A) inferring that these mice were not protected to any significant extent against intranasal challenge with *B. pertussis*.

3.5.4.2 Aerosol challenge

To assess whether the high challenge dose in the intranasal model was the factor responsible for the lack of protection seen in the immunised mice, an aerosol challenge was performed. Here, the bacterial suspension for aerosol challenge was identical (4×10^8 CFU/ml) to that used in the intranasal challenge but the nature of the aerosol challenge procedure is such that fewer bacteria are introduced into the lungs (Xing *et al.*, 1999). Groups of 5 mice were immunised intraperitoneally on days 0 and 38 with the four different CyaA forms in 10% (v/v) alum or injected with PBS in 10% (v/v) alum. On day 56, each mouse from each group were challenged intranasally with *B. pertussis* 18.323 and sampled for lungs and tracheas at 7 days post-challenge. Another group of PBS control mice were also included in the study but were sampled at 2 h post-challenge. Indeed, there were 4.65 $\log_{10}$ CFU/ml in mice at 2 h post-aerosol challenge (Fig. 35B) compared with 7.54 $\log_{10}$ CFU/ml in mice at 2 h post- intranasal challenge (Fig. 35A). There was also a 2.44 $\log_{10}$ increase in bacterial numbers after 7 days, demonstrating bacterial virulence in the aerosol challenge model. With this alternative challenge procedure, intraperitoneal immunisation with all CyaA forms at 25 μg/dose reduced the bacterial numbers in lung

Figure 35 Protection of mice against intranasal or aerosol challenge with *B. pertussis* after immunisation with different CyaA forms alone

For intranasal challenge (A) mice were immunised on days 0 and 28 with different CyaA preparations (25 μg per mouse) or PBS and challenged at day 42 with *B. pertussis* 18.323. For aerosol challenge (B) mice were immunised on days 0 and 38 and then challenged at day 56. Five mice from the PBS control group were sampled at 2 h post-challenge for enumeration of bacteria in lungs and tracheas (▩). All remaining mice were sampled at 7 days post-challenge (□). Results represent means of five mice per group with SEM (bars). Symbol: †, $P < 0.05$ (groups vs PBS group: ANOVA) or ††, $P < 0.05$ (as linked by brackets).


A
Mean Log10 CFU per mouse lungs/trachea
9.5
8.5
7.5
6.5
5.5
4.5
3.5
2.5
B
Mean Log10 CFU per mouse lung/trachea
9.5
8.5
7.5
6.5
5.5
4.5
3.5
2.5
†
†
†
††
proCyaA
proCyaA*
CyaA
CyaA*
PBS
Immunisation groups

tissue compared with the PBS control group (Fig. 35B). However, only mice immunised with the CyaA, proCyaA and proCyaA* preparations were significantly ($P < 0.05$) protected compared with the PBS control group (Fig. 35B).

3.6 *In vivo* experiments: murine humoral responses to immunisation

To examine further the ability of CyaA and CyaA* to enhance the protective efficacy of an ACV, total IgG, IgG1 and IgG2a antibody levels to FHA, PRN, PT and CyaA were measured. The sera from the different immunisation groups were also tested for their ability to neutralise CyaA adenylate cyclase, haemolytic and cytotoxic activities.

3.6.1 Mice immunised with ACV plus different forms of CyaA

3.6.1.1 Total IgG responses to PT, FHA, PRN and CyaA

Groups of 10 mice were immunised intraperitoneally on days 0 and 28 with the four different CyaA forms (25 μg/dose) with ACV. On day 42, five mice from each group were sampled for serum, spleens and peritoneal macrophages and the remaining five mice were challenged intranasally with *B. pertussis* 18.323 (Section 3.5.2) at the same time. Intraperitoneal immunisation of mice with ACV plus the different CyaA forms did not greatly influence the total IgG antibody responses to PT, FHA or PRN compared with mice immunised with ACV alone (Table 10). In fact, the total IgG levels for each antigen were similar across all groups. In addition, there were no significant differences in the total IgG antibody responses to CyaA in any of the immunised mice that received a CyaA form (Table 10).

3.6.1.2 IgG1 and IgG2a antibody responses to PT, FHA, PRN and CyaA

The IgG1 antibody levels to FHA, PT and PRN in sera were similar to those in mice immunised intraperitoneally with ACV plus the different CyaA forms compared with the ACV control group (Fig. 36A - C). By contrast, mice immunised with ACV + CyaA* produced significantly ($P < 0.05$) more anti-CyaA IgG1 antibodies than the ACV + proCyaA* group but not with the ACV + CyaA or proCyaA groups (Fig. 36D). Although there appeared to be an increase in IgG2a antibody levels to FHA, PT and PRN in mice immunised with ACV + CyaA or ACV + CyaA* compared with the ACV-immunised group, only mice that were immunised with ACV + CyaA* produced significantly ($P < 0.05$) greater levels of IgG2a to PRN than mice immunised with ACV alone (Fig. 36C). There were no significant differences in the anti-CyaA IgG2a antibody levels between any of the immunised groups (Fig. 36).

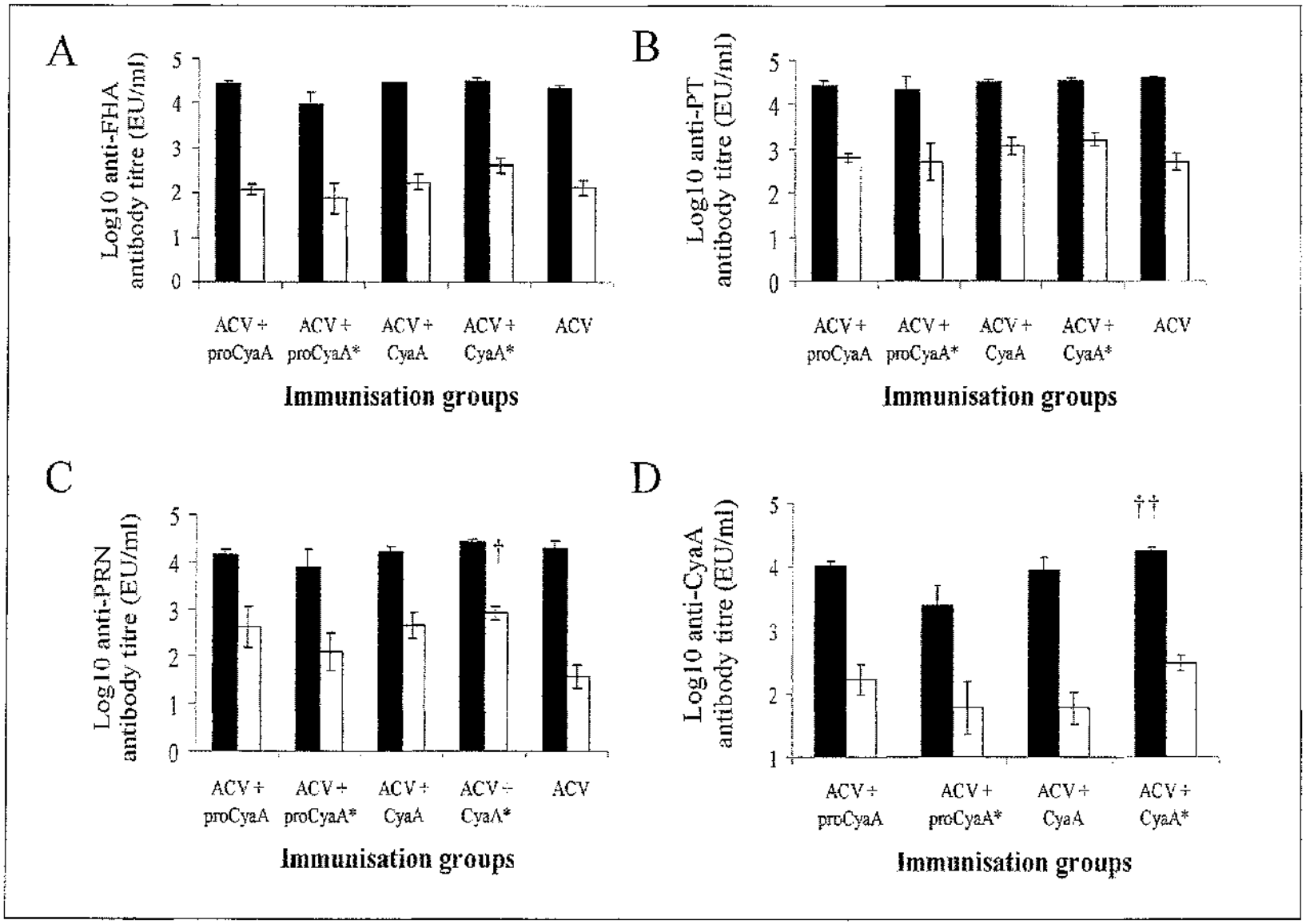
Table 10 Antibody responses to PT, FHA, PRN and CyaA in mice after immunisation with ACV with and without different CyaA forms

Sera were collected from mice at day 42, after intraperitoneal immunisation with ⅛ SHD of ACV with and without different CyaA forms (25 μg) on days 0 and 28, and then assessed for total IgG levels against PT, FHA, PRN and CyaA. Total IgG levels are the geometric means of five mice per group with upper and lower 95% confidence intervals (CI). n/d, not detected.

Immunisation groups	Total IgG levels (ELISA units/ml)			
	anti-PT	anti-FHA	anti-PRN	anti-CyaA
ACV alone	462 (284, 749)	3968 (2924, 5384)	653 (436, 977)	< 1
ACV + proCyaA	695 (249, 1383)	2439 (747, 7964)	543 (212, 1391)	1903 (1277, 2836)
ACV + proCyaA*	506 (367, 697)	3801 (3448, 4190)	541 (218, 932)	938 (307, 2858)
ACV + CyaA	658 (476, 911)	4425 (3537, 5536)	557 (142, 2181)	1860 (938, 3867)
ACV + CyaA*	649 (530, 795)	2906 (1930, 4375)	415 (218, 790)	2832 (2076, 3861)
PBS	n/d	n/d	n/d	n/d

Figure 36 IgG1 and IgG2a antibody responses to PT, FHA, PRN and CyaA in mice after immunisation with ACV with and without different CyaA forms

Sera were collected on day 42, after intraperitoneal immunisation with ⅛ SHD of ACV with and without different CyaA forms at 25 μg per dose on days 0 and 28. The sera were then assessed for IgG1 (■) and IgG2a (□) antibody levels, expressed as ELISA units (EU)/ml. Results represent means of from five mice per group with the SEM (bars). Symbol: †, $P < 0.05$ (IgG2a ACV + CyaA* vs IgG2a ACV; Student's *t* test). ††, $P < 0.05$ (IgG1 ACV + CyaA* vs IgG1 ACV + proCyaA*; Student's *t* test).


A
Log10 anti-FHA antibody titre (EU/ml)
0
1
2
3
4
5
ACV + proCyaA
ACV + proCyaA*
ACV + CyaA
ACV + CyaA*
ACV
Immunisation groups
B
Log10 anti-PT antibody titre (EU/ml)
0
1
2
3
4
5
ACV + proCyaA
ACV + proCyaA*
ACV + CyaA
ACV + CyaA*
ACV
Immunisation groups
C
Log10 anti-PRN antibody titre (EU/ml)
0
1
2
3
4
5
†
ACV + proCyaA
ACV + proCyaA*
ACV + CyaA
ACV + CyaA*
ACV
Immunisation groups
D
Log10 anti-CyaA antibody titre (EU/ml)
1
2
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4
5
††
ACV + proCyaA
ACV + proCyaA*
ACV + CyaA
ACV + CyaA*
Immunisation groups

3.6.1.2.1 Neutralisation of CyaA functions

The capacity of the above sera to neutralise CyaA functions was tested using various *in vitro* assays (Table 11). Only serum from ACV + CyaA*-immunised mice was capable of neutralising CyaA enzymic activity. However, sera from mice immunised with ACV plus the different CyaA forms neutralised the haemolytic and cytotoxic activities of CyaA. Again, serum from ACV + CyaA*-immunised mice was best, followed by serum from ACV + CyaA- and ACV + proCyaA-immunised mice. Serum from ACV + proCyaA*-immunised mice was poorest at neutralising CyaA functions. These results reflected the anti-CyaA total IgG antibody titres found in the sera of each immunised group (Table 11). Sera from mice immunised with ACV alone and the PBS control group failed to neutralise any of the CyaA activities tested (Table 11).

3.6.2 Mice immunised with ACV plus graded doses of CyaA*

3.6.2.1 Total IgG responses to PT, FHA, PRN and CyaA

Sera were collected from mice on day 42, after intraperitoneal immunisation on days 0 and 28 with ⅛ SHD of ACV with and without different doses of CyaA* at 25, 12.5 and 6.25 μg per dose. Sera were assessed for antibody levels to PT, FHA, PRN and CyaA. There were no significant differences in IgG antibody levels to PT, FHA or PRN in mice co-immunised with ACV plus graded doses of CyaA* compared with mice immunised with ACV alone (Table 12). However, as more CyaA* was used in the presence of ACV, there appeared to be an increase in anti-CyaA total IgG antibodies (Table 12) but this was not significant ($P > 0.05$) between the highest (25 μg) and lowest (6.25 μg) doses of CyaA* used.

3.6.2.2 IgG1 and IgG2a antibody responses to PT, FHA, PRN and CyaA

The levels of IgG1 antibodies to PT, FHA, PRN or CyaA were similar in all mice immunised with ACV with or without different doses of CyaA* (Fig. 37). Again, mice immunised with the highest dose (25 μg) of CyaA* in combination with ACV produced significantly ($P < 0.05$) greater levels of IgG2a towards PRN (Fig. 37) than mice immunised with ACV plus lower doses of CyaA* or with ACV alone. There was also significantly greater ($P < 0.05$) anti-CyaA IgG2a antibodies produced by mice immunised with ACV plus the highest dose (25 μg) of CyaA* compared with ACV plus the lowest dose (6.25 μg) of CyaA*. Although the absolute anti-PRN IgG2a antibodies in the dose-response experiment (Fig. 37) was lower compared with the first experiment (Fig. 36), both experiments showed a similar trend of greater anti-PRN IgG2a antibody production (Figs 36, 37). There were no significant differences in the anti-PT or anti-FHA IgG2a antibody levels between any of the immunised groups (Fig. 37).

Table 11 Neutralisation of enzymic, haemolytic and cytotoxic activities of CyaA by different immune sera

Sera were collected from mice on day 42, after intraperitoneal immunisation with ⅛ SHD of ACV with and without different CyaA forms at 25 μg per dose on days 0 and 28. The serum from mice injected intraperitoneally on days 0 and 28 with PBS served as a negative control. These sera were assessed for the capacity to neutralise the activities of CyaA used at [1]60 μg/ml for adenylate cyclase activity, [2]20 μg/ml for haemolysis, [3]2.5 μg/ml for cytotoxicity. CyaA was pre-incubated with dilutions of sera for 30 min prior to performing conductimetry, haemolysis and MTT cytotoxicity assays (Section 2.6). Values represent the mean of two independent assays which produced similar results for each assay. A column containing the geometric means of the anti-CyaA total IgG antibody titre for each immunisation group, expressed as ELISA units (EU/ml), are also included.

Immunisation groups	Geometric mean anti-CyaA IgG antibody titre (EU/ml)	Reciprocal of serum dilution giving 50% neutralisation		
		Adenylate cyclase activity[1]	Haemolysis[2]	Cytotoxicity[3]
ACV + proCyaA	1903	< 2	35	46
ACV + proCyaA*	938	< 2	8	8
ACV + CyaA	1860	< 2	33	24
ACV + CyaA*	2832	4	66	74
ACV	< 1	< 2	< 2	< 2
PBS	< 1	< 2	< 2	< 2

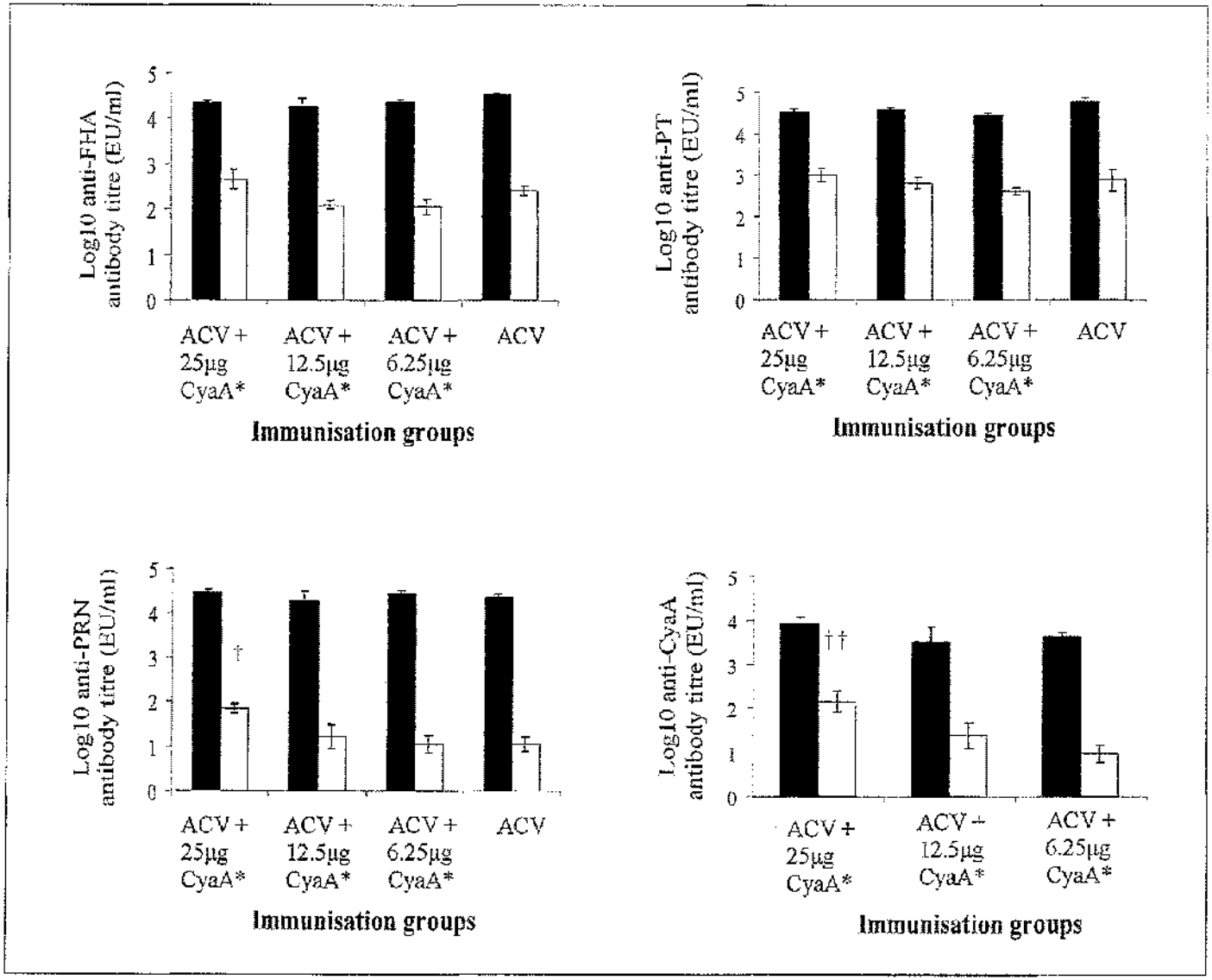
Table 12 Antibody responses to PT, FHA, PRN and CyaA from mice immunised with ACV plus graded doses of CyaA*

Sera were collected on day 42, after intraperitoneal immunisation on days 0 and 28 with ⅛ SHD of ACV with and without different doses of CyaA* at 25, 12.5 and 6.25 μg per dose, and then assessed for total IgG levels against PT, FHA, PRN and CyaA. Total IgG levels are the geometric means of five mice per group with upper and lower 95% CI. n/d, not determined.

Immunisation groups	Total IgG levels (ELISA units/ml)			
	anti-PT	anti-FHA	anti-PRN	anti-CyaA
ACV alone	867 (590, 1275)	3530 (2859, 4358)	589 (315, 1101)	n/d
ACV + 6.25ug CyaA*	604 (421, 866)	2853 (1878, 4335)	686 (368, 1280)	665 (308, 1435)
ACV + 12.5ug CyaA*	721 (476, 1093)	2272 (719, 7176)	445 (111, 1786)	536 (81, 3533)
ACV + 25ug CyaA*	667 (497, 895)	3488 (2706, 4392)	830 (447, 1541)	1104 (492, 2474)
PBS	n/d	n/d	n/d	n/d

Figure 37 IgG1 and IgG2a antibody responses to PT, FHA, PRN and CyaA from mice immunised with ACV plus graded doses of CyaA*

Sera were collected on day 42, after immunisation on days 0 and 28 with ⅙ SHD of ACV with and without different doses of CyaA* at 25, 12.5 and 6.25 μg per dose, and then assessed for IgG1 (■) and IgG2a (□) antibody levels. Results represent the means of five mice per group with the SEM (bars). Symbol: †, $P < 0.05$ (IgG2a ACV + 25 μg CyaA* vs IgG2a ACV; Student's *t* test). ††, $P < 0.05$ (IgG2a ACV + 25 μg CyaA* vs IgG2a ACV + 6.25 μg CyaA*; Student's *t* test).


Log10 anti-FHA antibody titre (EU/ml)
0
1
2
3
4
5
ACV + 25µg CyaA*
ACV + 12.5µg CyaA*
ACV + 6.25µg CyaA*
ACV
Immunisation groups
Log10 anti-PT antibody titre (EU/ml)
0
1
2
3
4
5
ACV + 25µg CyaA*
ACV + 12.5µg CyaA*
ACV + 6.25µg CyaA*
ACV
Immunisation groups
Log10 anti-PRN antibody titre (EU/ml)
0
1
2
3
4
5
†
ACV + 25µg CyaA*
ACV + 12.5µg CyaA*
ACV + 6.25µg CyaA*
ACV
Immunisation groups
Log10 anti-CyaA antibody titre (EU/ml)
0
1
2
3
4
5
††
ACV + 25µg CyaA*
ACV + 12.5µg CyaA*
ACV + 6.25µg CyaA*
Immunisation groups

3.6.3 Mice immunised with different forms of CyaA

3.6.3.1 Total IgG responses to CyaA

Sera were collected from mice on day 42, after intraperitoneal immunisation on days 0 and 28 with different forms of CyaA at 25 μg per dose in the presence of absence of 10% (v/v) alum. These sera were assessed for total IgG anti-CyaA antibody levels. Mice immunised with proCyaA, proCyaA* and CyaA* with alum, but not CyaA with alum, produced significantly ($P < 0.05$) greater total anti-CyaA IgG antibodies than mice immunised with the CyaA forms alone (without alum) (Table 13). However, there were no significant differences ($P > 0.05$) in the total IgG anti-CyaA antibody responses between the groups of mice that received the different CyaA forms with alum (Table 13).

It is noteworthy that mice immunised with ACV plus different CyaA forms produced significantly ($P < 0.05$) less anti-CyaA IgG antibodies compared with mice immunised with different CyaA forms alone plus alum ($P < 0.05$) (Tables 10, 13). There was no significant difference in antibody levels for mice immunised with CyaA with or without alum. In addition, there were no significant differences in the antibody levels between mice that were immunised with ACV plus different CyaA forms compared with the different CyaA forms alone (without alum) (Tables 10, 13), even though the ACV itself contained adjuvant. Mice immunised with proCyaA*, regardless of presence of alum adjuvant, produced the lowest levels of total anti-CyaA IgG antibodies compared with the other groups (Table 10).

3.6.3.2 IgG1 and IgG2a antibody responses to CyaA (plus alum)

Sera were collected at day 42 after intraperitoneal immunisation on days 0 and 28 with different CyaA preparations (25 μg/dose) with alum and the anti-CyaA IgG1 and IgG2a antibody levels were measured. Mice immunised with different forms of CyaA (plus alum) did not show significant differences in the anti-CyaA IgG1 or IgG2a antibody levels (Fig. 38) except that sera from mice immunised with CyaA* produced significantly ($P < 0.05$) greater anti-CyaA IgG1 and IgG2a antibodies than sera from mice immunised with proCyaA* alone (Fig. 38). The IgGa and IgG2a levels were not measured for sera from mice immunised intraperitoneally with the different CyaA forms without alum.

3.6.3.3 Neutralisation of CyaA functions by sera from mice immunised with different CyaA forms alone (plus alum)

Both sera from CyaA*- and CyaA-immunised mice neutralised CyaA AC enzymic activity, although serum from CyaA*-immunised mice was more effective, even taking into account the slightly higher antibody titre (Table 14). Although serum from the CyaA*-immunised group more efficiently neutralised AC enzymic activity compared with that

Table 13 Antibody responses to CyaA in mice after immunisation with different CyaA preparations with or without alum

Sera were collected at day 42 after intraperitoneal immunisation on days 0 and 28 with different CyaA preparations (25 μg/dose) with or without alum. Sera were assessed for total IgG anti-CyaA levels. Total IgG anti-CyaA levels are the geometric means of five mice with 95% CI in brackets. Symbol: †, $P < 0.05$ (proCyaA vs proCyaA + alum; Student's t test), ††, $P < 0.05$ (proCyaA* vs proCyaA* + alum; Student's t test), †††, $P < 0.05$ (CyaA* vs CyaA* + alum; Student's t test).

Immunisation groups	Total IgG levels (ELISA units/ml)	
	anti-CyaA (no alum)	anti-CyaA (plus alum)
proCyaA	1581 (1160, 4361)†	6893 (4087, 11627)
proCyaA*	872 (610, 2035) ††	3933 (2033, 5661)
CyaA	2500 (2074, 12177)	6965 (3756, 12915)
CyaA*	1233 (950, 4141) †††	10326 (4280, 22124)

Figure 38 IgG1 and IgG2a antibody levels to CyaA

Sera were collected on day 42, after intraperitoneal immunisation on days 0 and 28 with different CyaA forms at 25 μg/dose with or without alum, and then assessed for IgG1 (■) and IgG2a (□) antibodies against CyaA. IgG1 and IgG2a antibody levels represent the means of 5 mice per group with SEM (bars). A one-way ANOVA (Tukey's test) was performed to determine significance ($P < 0.05$) between groups as linked by brackets. Symbol: †, $P < 0.05$ (IgG1 proCyaA* vs IgG1 CyaA*; Student's t test); †, $P < 0.05$ (IgG2a proCyaA* vs IgG2a CyaA*; Student's t test).

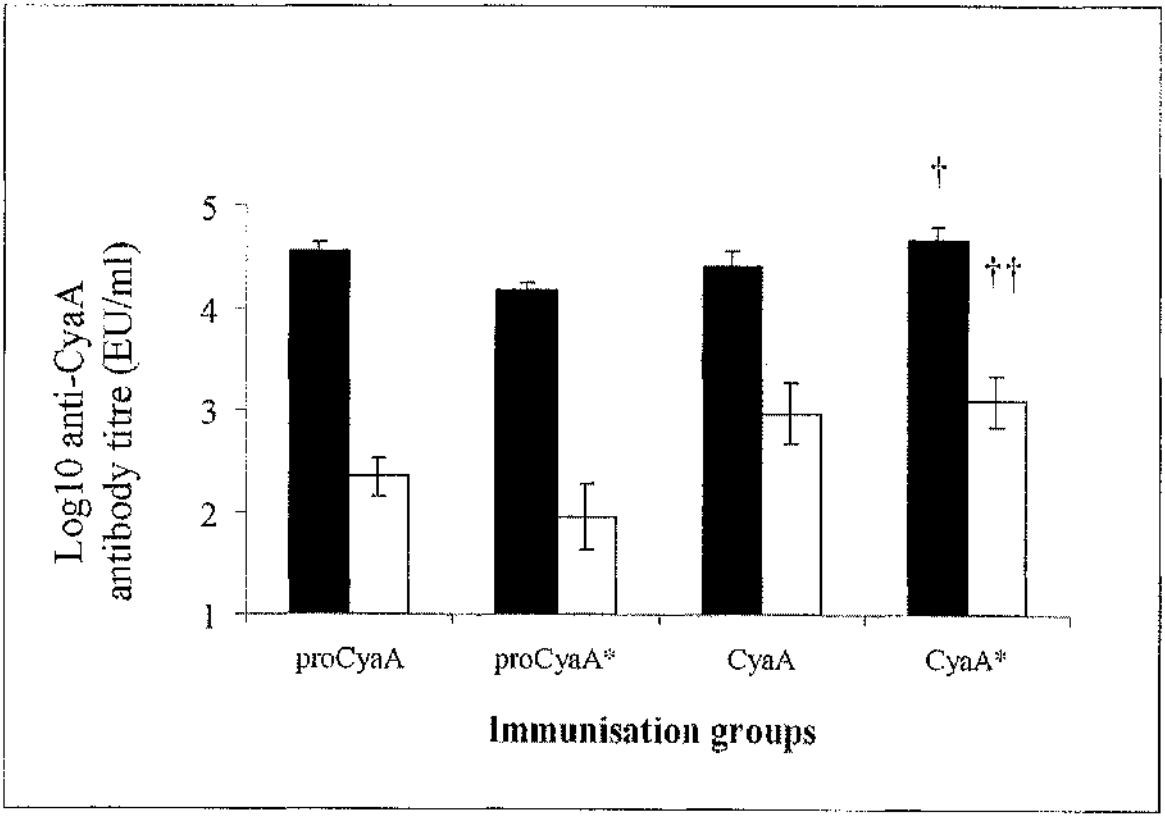


Table 14 Neutralisation of enzymic, haemolytic and cytotoxic activities of CyaA by different immune sera

Sera were collected on day 42, after intraperitoneal immunisation on days 0 and 28 with different CyaA forms at 25 μg/dose with alum, and then assessed for the capacity to neutralise the activities of CyaA used at [1]60 μg/ml for AC, [2]20 μg/ml for haemolysis, [3]2.5 μg/ml for cytotoxicity. CyaA was pre-incubated with dilutions of sera (Sections 2.8.3.4, 3.6.3.1) for 30 min prior to performing conductimetry, haemolysis and MTT cytotoxicity assays (Section 2.6). Values represent the means of two independent assays which produced similar results for each assay.

Immunisation groups	Geometric mean anti-CyaA IgG antibody titre (EU/ml)	Reciprocal of serum dilution required for 50% neutralisation		
		Adenylate cyclase activity[1]	Haemolysis[2]	Cytotoxicity[3]
ProCyaA	6893	< 2	174	169
ProCyaA*	3393	< 2	78	80
CyaA	6965	2	345	410
CyaA*	10326	32	118	196
PBS	< 1	< 2	< 2	< 2
Positive control serum[†]	30000	48	123	568

[†] The positive control serum used corresponds to the anti-CyaA* reference serum produced in Section 2.8.3.4.

from the CyaA group, the latter serum was more potent at neutralising CyaA haemolytic and cytotoxic activities, despite having a lower anti-CyaA antibody titre. Sera from the proCyaA- and proCyaA*-immunised groups were unable to neutralise AC enzymic activity, but effectively neutralised haemolytic and cytotoxic activities with serum from the proCyaA-immunised group showing the greater potency. As expected, due to its higher antibody titre, the positive control serum (the anti-CyaA* reference serum) was best at neutralising AC enzymic and cytotoxic activities, but was surprisingly poor at neutralising haemolysis. All the immune sera also neutralised haemolysis and cytotoxicity caused by CyaA* to a similar degree as recorded for CyaA in Table 14 (data not shown).

3.6.3.4 Phagocytosis assays

3.6.3.4.1 Identification of CD11b$^+$ cell populations from mouse blood

Leukocytes were labelled with anti-mouse CD11b antibodies conjugated with allophycocyanin (APC) to identify a CD11b-positive population of cells within in the leukocyte suspension isolated from normal mouse blood and subjected to FACS analysis. Treated cells were scanned for APC fluorescence and the amount of light diffracted from the surface of the cells (forward light scattering). Forward light scattering is a measurement of cell size. Thus, the greater the value scatter, the greater the size. Figure 39 shows increased APC fluorescence, from a group of cells with large forward scatter values, arising from a CD11b-specific antibody compared with the control antibody. This indicates the presence of CD11b$^+$ cells (e.g. macrophages) in the mouse leukocyte cell suspension.

3.6.3.4.2 Opsonisation of *B. pertussis* with different anti-CyaA mouse sera

Serum from CyaA-immunised mice (Section 3.6.3.1) and positive control serum (Section 2.8.3.4) were incubated with different multiplicities of infection (MOI) of *B. pertussis* cells expressing GFP prior to incubation with mouse leukocytes labelled with anti-mouse CD11b antibody conjugated with APC or with an APC-conjugated matched isotype control. MOI represents the bacteria:leukocyte ratio. In addition, *B. pertussis* cells treated with anti-PBS serum or no serum were used as negative controls.

Figure 40 gives an example of FACS analysis showing increased GFP fluorescence of CD11b$^+$ cells incubated with a high MOI (48) compared CD11b$^+$ cells incubated with a MOI of 0. Even in the absence of opsonising serum, the fluorescence increases as a result of greater numbers of GFP-expressing *B. pertussis* with CD11b$^+$ cells. Thus, an efficient opsonising serum should increase the levels of fluorescence. As shown in Table 15 there was a slight increase in association of CD11b$^+$ cells with *B. pertussis* expressing GFP,

Figure 39 Scatterplot showing the presence of CD11b$^+$ cell populations in mouse blood

FACS analysis showing allophycocyanin (APC) fluorescence against forward scatter of mouse leukocytes labelled with (A) a rat IgG2b-APC-conjugated monoclonal antibody (isotype control) or with (B) rat APC-conjugated anti-mouse CD11b monoclonal antibody. Each dot represents an event (or antibody-labelled cell). The horizontal and vertical lines in (A) indicate arbitrarily selected baselines used for (B).

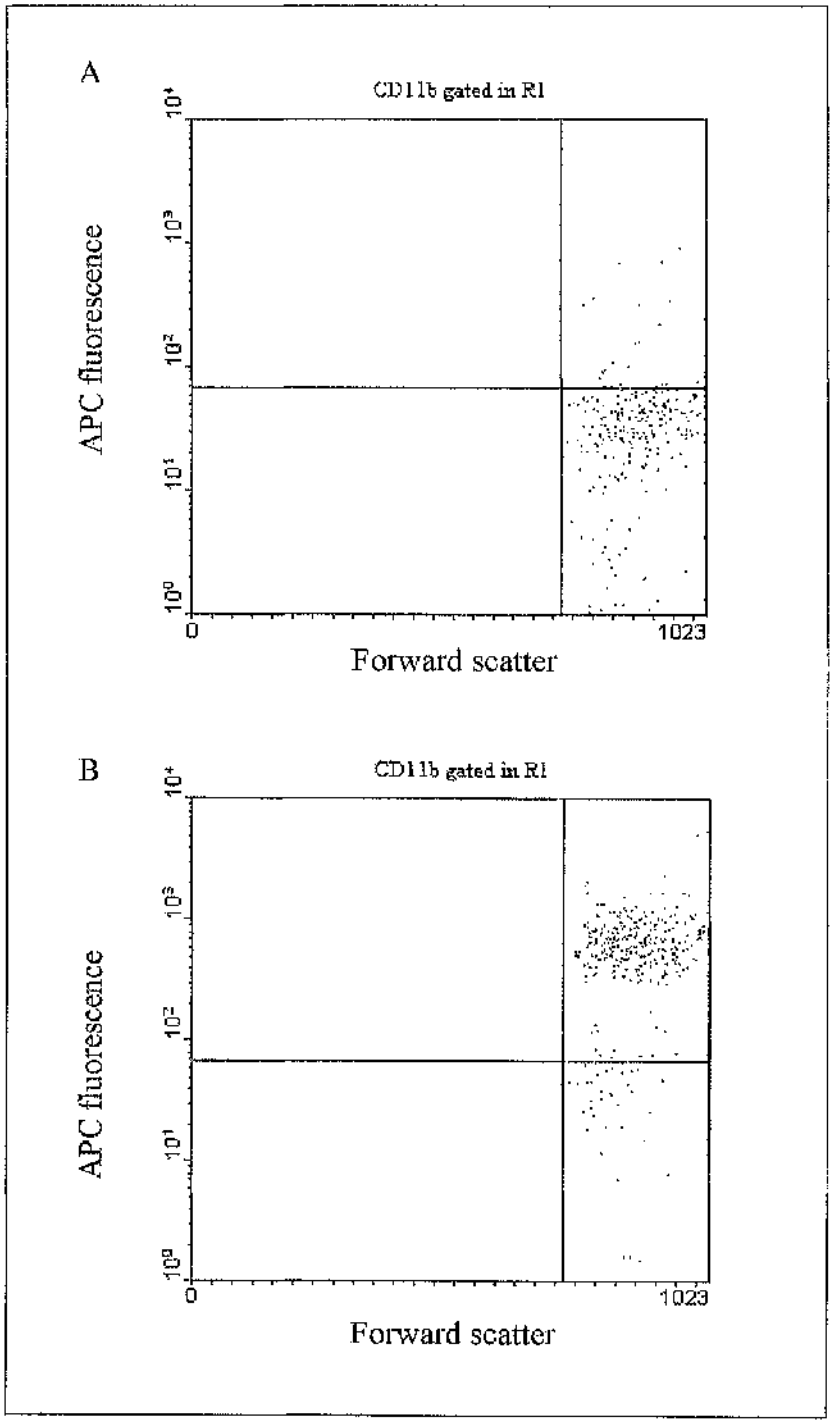


Figure 40 Example of increased association of GFP *B. pertussis* with CD11b$^+$ cell populations

Histogram obtained by FACS analysis displaying an increased of GFP fluorescence (of *B. pertussis*) against the number of events (CD11b$^+$ cells). CD11b$^+$ cells (gated from Fig. 39) incubated with a multiplicity of infection (MOI) of 0 (dark shaded area) compared with an MOI of 48 bacteria associated with one leukocyte (non-shaded area). Arrows indicate the median fluorescence intensity (MFI) for each cell population.

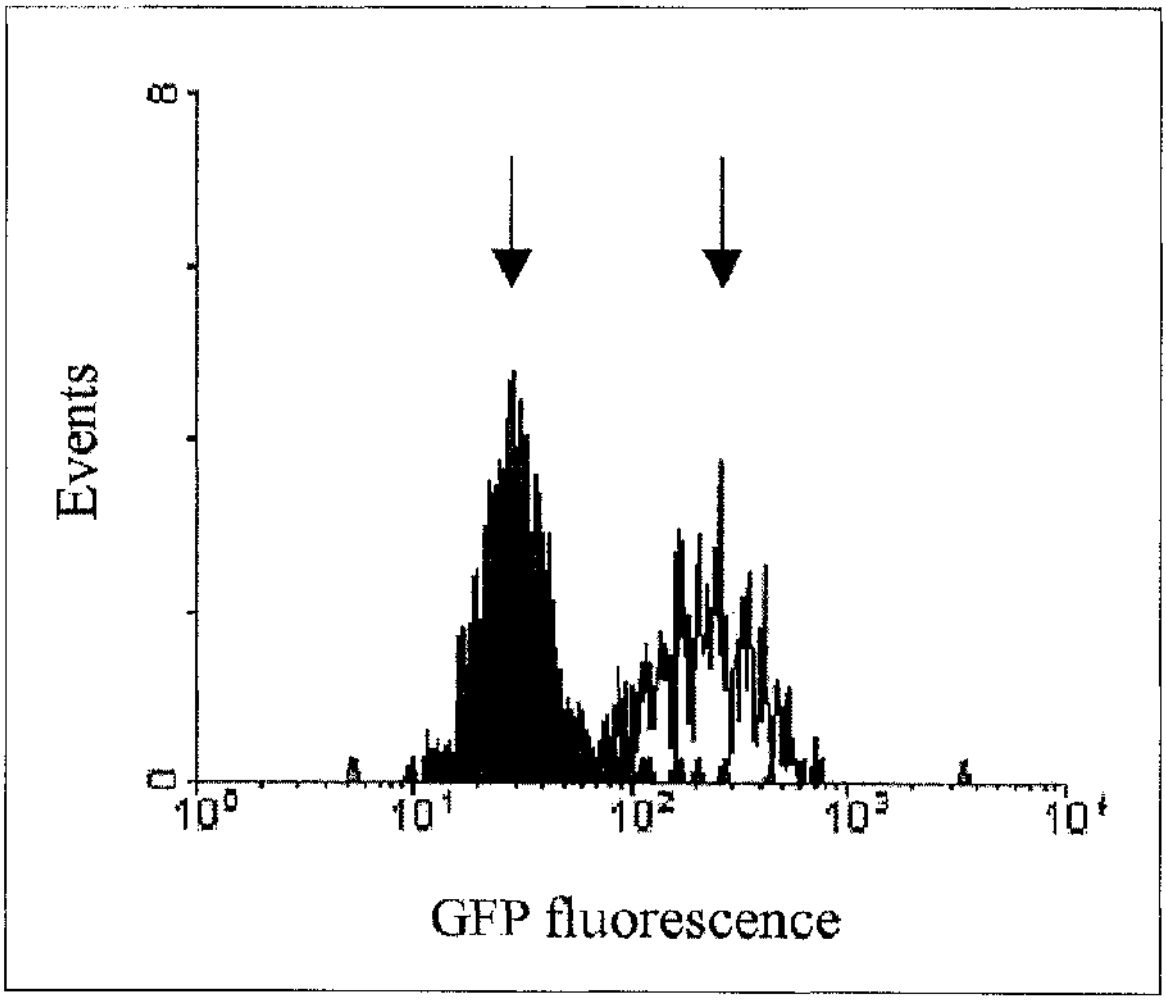


Table 15 Mean fluorescence intensity values of *B. pertussis* expressing GFP incubated with CD11b$^+$ cells after pre-incubation with different mouse anti-CyaA sera

Mouse leukocytes were incubated for 1 h at 37 °C with *B. pertussis* 338 expressing GFP that had been pre-incubated (30 min at 37 °C) with mouse serum from CyaA-immunised mice, positive control serum (anti-CyaA* reference serum) or serum from the PBS group (negative control) (Sections 2.8.3.4, 3.6.3). *B. pertussis* incubated in the absence of serum served as an additional negative control. In addition, different *B. pertussis* to leukocyte ratios (or multiplicity of infections -- MOI) were used. Leukocytes were then washed and stained with antibodies specific for CD11b and CD45 (a general leukocyte marker) or with isotype-matched controls (Section 2.9). The values represent the median fluorescent intensity (MFI), as determined by the WinMDI software, of the treated cells for GFP fluorescence. Values are representative, and data shown are from 1 experiment with the average of 2 replicates.

	Median fluorescence intensity			
MOI	No serum	Anti-PBS	Positive control serum	Anti-CyaA
0	19.17	14.89	-	-
6	35.57	37.26	50.14	38.48
12	52.13	54.29	64.87	53.39
48	67.33	69.17	85.63	73.74

-, not tested.

indicated by an increase in the MFI of GFP fluorescence, when the positive control serum was used at 1/100 dilution compared with the serum from the PBS control group (Table 15). This suggests that the positive control serum has some opsonising activity. However, there was little increase in the MFI of GFP fluorescence when mouse leukocytes were incubated with serum from CyaA-immunised mice compared with serum from the PBS control group, both used at 1/30 dilution (Table 15). Mouse leukocytes incubated in the absence of serum showed no differences in MFI values compared with serum from the PBS control group. As serum from CyaA-immunised mice did not provide any evidence for anti-phagocytic activity, other sera from other mice immunised with the other three CyaA forms were not tested.

3.7 *In vivo* experiments: cytokine production from spleen cells

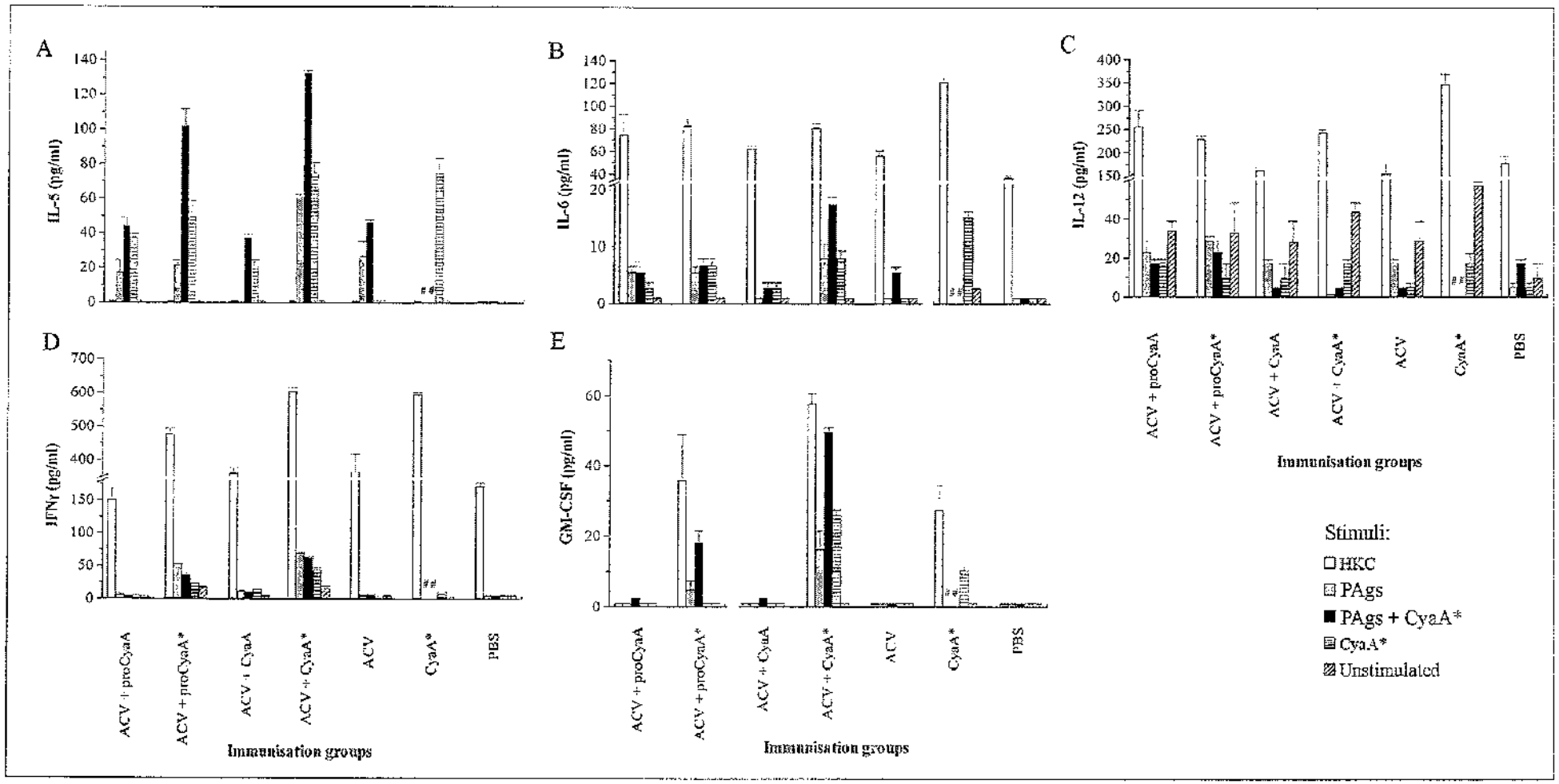
The cytokine responses from antigen-stimulated spleen cells and peritoneal macrophages from mice immunised with the different CyaA forms plus ACV were investigated to see if cytokine production could provide evidence of the type of immune response responsible for enahnced protection. Figure 41 shows the results for five cytokines released from spleen cells obtained from mice at day 42 after intraperitoneal immunisation on days 0 and 28 with ⅛ SHD of ACV or ⅛ SHD of CV plus different forms of CyaA (at 25 μg dose). Cells were stimulated *in vitro* with heat-killed *B. pertussis* 18.323 cells (HKC) or with a mixture of *B. pertussis* antigens (PAgs, consisting of detoxified-PT, FHA, PRN) with or without CyaA*, or with CyaA* alone for 48 h. Antigen-stimulated spleen cells from mice immunised with CyaA* alone or PBS served as controls. CyaA* was the form of CyaA chosen as the stimulant because it was less toxic than the native toxin and non-toxic for spleen cells at 1 μg/ml. Cytokine secretion was influenced by the nature of the antigens used for immunisation and by the antigens used for stimulation.

3.7.1 Stimulation by HKCs

No IL-5 was produced from any of the groups with HKCs as stimulant (Fig. 41A). HKCs stimulated high levels of IL-6 (40 - 120 pg/ml), IL-12 (180 - 250 pg/ml) and IFNγ (150 - 600 pg/ml) from spleen cells from all mice, including the PBS control group (Fig. 41B - D). However, for GM-CSF, only cells from mice immunised with ACV + proCyaA* (36 ± 13 pg/ml), ACV + CyaA* (58 ± 3 pg/ml) or CyaA* alone (27 ± 7 pg/ml) produced GM-CSF in response to HKC stimulation (Fig. 41E).

Figure 41 Cytokine production by spleen cells from immunised mice after stimulation *in vitro* with *B. pertussis* antigens

Groups of mice were immunised intraperitoneally on days 0 and 28 with ⅛ SHD of ACV alone, CyaA* alone or ⅛ SHD of ACV plus different CyaA forms (25 μg per mouse) or were given PBS only. Spleen cells were obtained on day 42. Production of (A) IL-5, (B) IL-6, (C) IL-12, (D) IFNγ and (E) GM-CSF was assessed from spleen cells of immunised mice stimulated *in vitro* with different *B. pertussis* antigens for 48 h. HKCs (□), PAgs (□), PAgs + CyaA* (■), CyaA* alone (⊟) were used as stimuli. Other cells were not stimulated, and served as controls (◪). PAgs consisted of a mixture of formalin-treated-PT, -FHA and -PRN used at a final concentration of 2, 2 and 5 μg/ml, respectively. CyaA* and HKCs were used at a final concentration of 1 μg/ml and $5x10^7$ cells/ml, respectively. Results represent the means of duplicate assays with SEM (bars). #, stimulation with PAgs or PAgs + CyaA* not tested.


A
IL-5 (pg/ml)
B
IL-6 (pg/ml)
C
IL-12 (pg/ml)
D
IFNγ (pg/ml)
E
GM-CSF (pg/ml)
ACV + proCyaA
ACV + proCyaA*
ACV + CyaA
ACV + CyaA*
ACV
CyaA*
PBS
Immunisation groups
Stimuli:
HKC
PAgs
PAgs + CyaA*
CyaA*
Unstimulated

3.7.2 Mice immunised with ACV

Greater levels of IL-5 and IL-6 were produced by spleen cells from this group compared with the PBS control group using PAgs + CyaA* as stimulant (Fig. 41A, B). As expected, the spleen cells from mice immunised with ACV alone did not responded to CyaA* as a stimulant. Spleen cells from the ACV alone group did not produce any more IFNγ or GM-CSF than cells from PBS control mice (Fig. 41D, E). IL-12 production from unstimulated cells from the ACV-immunised group exceeded that of cells stimulated with specific antigens (Fig. 41C). This effect was not observed in the PBS control group.

3.7.3 Mice immunised with ACV + CyaA or ACV + proCyaA

The cytokine profiles of spleen cells from these two groups were generally similar to the ACV control group upon antigen stimulation (Fig. 41A-E), although they responded well to CyaA* as a stimulant (Fig. 41A, B, D, E). Again, IL-12 production from unstimulated cells exceeded that of antigen-stimulated cells (Fig. 41C).

3.7.4 Mice immunised with ACV + CyaA*, ACV + proCyaA* or CyaA* alone

Spleen cells from mice immunised with ACV + CyaA* produced higher levels of IL-5 (~130 pg/ml), IL-6 (~20 pg/ml), IFNγ (~60 pg/ml) and GM-CSF (~50 pg/ml) than spleen cells from mice immunised with ACV (~45 pg/ml for IL-5, < 5 pg/ml for IL-6, IFNγ and GM-CSF) when stimulated with PAgs + CyaA*. Spleen cells from the ACV + proCyaA* group also responded better with regard to IL-5, IFNγ and GM-CSF production, than cells from the ACV alone group after stimulation by PAgs + CyaA* (Fig. 41A, D, E). Spleen cells from mice immunised with CyaA* alone produced lower levels of IFNγ (<4 pg/ml) and GM-CSF (~10 pg/ml) than cells from mice immunised with ACV + CyaA* when stimulated with CyaA*, although similar levels of IL-5 (~70 pg/ml) and IL-6 (~15 pg/ml) were recorded. It was noteworthy that only spleen cells from mice immunised with ACV + CyaA*, ACV + proCyaA* or CyaA* alone produced higher amounts of GM-CSF than cells from the PBS control group (Fig. 41E) in response to antigen stimulation. In addition, only spleen cells from mice immunised with ACV + CyaA* or ACV + proCyaA* produced high amounts of IFNγ in response to specific antigen stimulation.

As expected, the spleen cells from mice immunised with ACV + CyaA*, ACV + proCyaA* or CyaA* alone generally responded well to CyaA* as a stimulant (Fig. 41A, B, D, E). This was not true, however, for IL-12 where again production by spleen cells from these groups was lower after PAgs + CyaA* or CyaA* stimulation compared with unstimulated cells (Fig. 41C).

3.7.5 Other cytokines

None of the antigens stimulated spleen cells to produce detectable IL-1β, IL-2, IL-4 and IL-10 (data not shown). However, HKC-stimulated spleen cells from all the immunised groups, and the PBS control group, produced TNFα at high levels (180 - 270 pg/ml), but only low-level responses (10 - 20 pg/ml) were seen upon stimulation with the other antigens.

Overall, the cytokine responses of spleen cells from mice immunised with ACV + CyaA, ACV + proCyaA or ACV alone were similar in most cases, whereas spleen cells from mice immunised with ACV + CyaA*, ACV + proCyaA* or CyaA* alone, particularly the former, secreted higher levels of cytokines in response to antigen stimulation (Fig. 41A – E). For IFNγ and GM-CSF production, spleen cells from ACV + CyaA* immunised mice were more responsive to CyaA* stimulation than the CyaA* alone group.

3.8 *In vivo* experiments: cytokine production from peritoneal macrophages

Figure 42 shows the results for six cytokines released from peritoneal macrophages isolated on day 42 from mice immunised intraperitoneally on days 0 and 28 with ⅛ SHD of ACV or ⅛ SHD of ACV plus different forms of CyaA at 25 μg/dose. Macrophages were then stimulated *in vitro* with HKCs or PAgs with or without CyaA*, or with CyaA* alone. Antigen-stimulated peritoneal macrophages from mice immunised with CyaA* alone or PBS served as controls.

3.8.1 Stimulation by HKCs

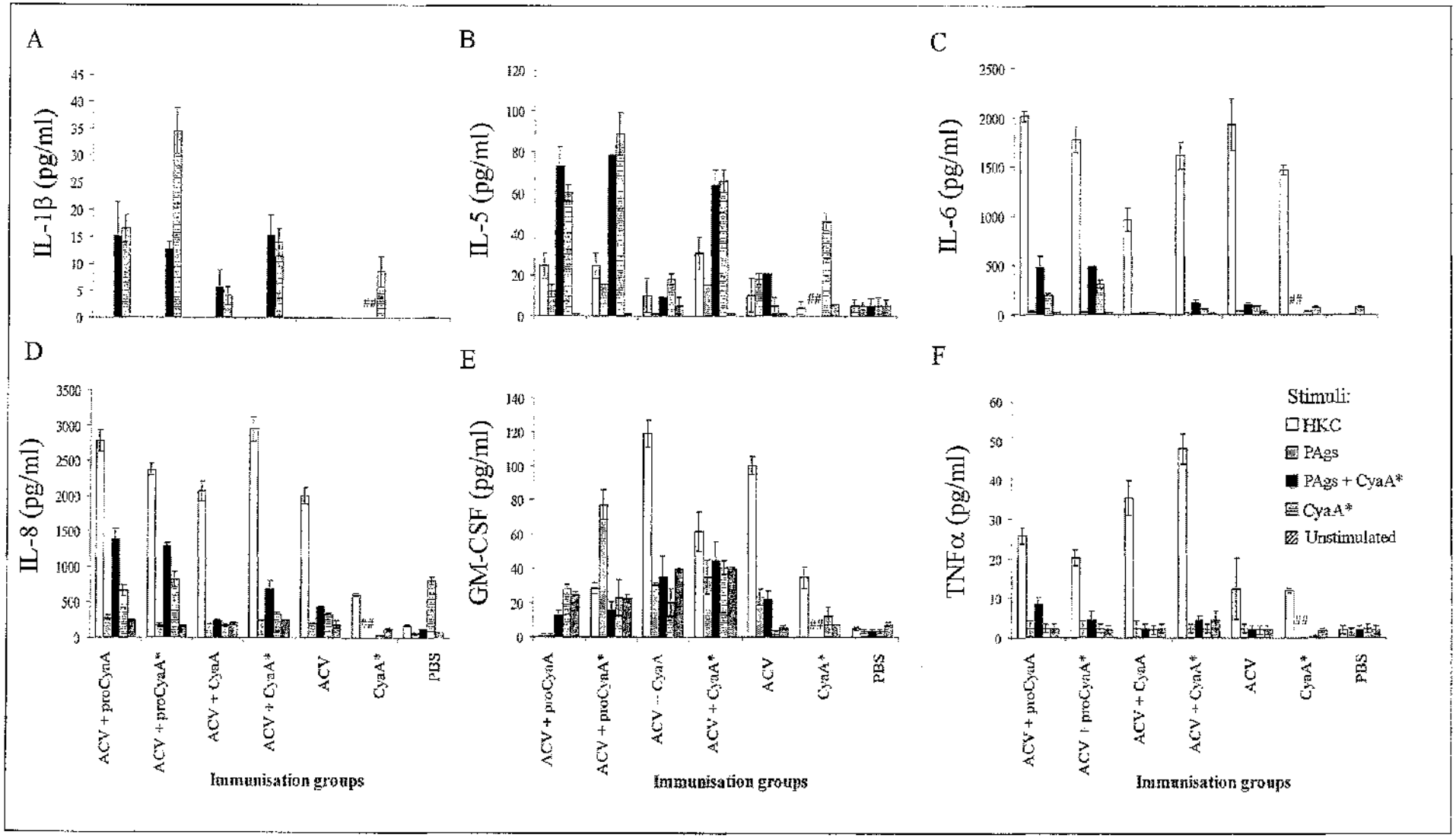
Peritoneal macrophages from all antigen-immunised groups secreted high levels of IL-6 and IL-8 compared with the PBS control group after stimulation with HKC (Fig. 42C, D), but HKCs were the best stimulant for GM-CSF production by macrophages from mice immunised with ACV, ACV + CyaA or ACV + CyaA*. For TNFα secretion, the levels were similar for all immunised groups after HKC stimulation, although they were marginally higher in macrophages from mice co-immunised with ACV + CyaA or ACV + CyaA* compared with the other immunised groups (Fig. 42F).

3.8.2 Stimulation by specific antigens

The levels of GM-CSF secreted by PAgs + CyaA*-stimulated macrophages from mice immunised with ACV plus a CyaA form were only marginally higher than those from the ACV alone group (Fig. 42E). The exception to this was stimulation with CyaA* where,

Figure 42 Cytokine production by peritoneal macrophages from immunised mice after stimulation *in vitro* with *B. pertussis* antigens

Groups of mice were immunised intraperitoneally on days 0 and 28 with CyaA*, ⅛ SHD of ACV alone or ⅛ SHD of ACV plus different CyaA preparations (25 μg/dose) or were given PBS only. Peritoneal macrophages were collected on day 42 and stimulated with HKCs (□), PAgs (▨), PAgs + CyaA* (■) and CyaA* (▤) for 24 h. Other cells were untreated and served as negative controls (▨). See Fig. 41 legend for details on the final concentration of antigens used. Results represent the means of duplicate assays with SEM (bars). #, stimulation with PAgs or PAgs + CyaA* not tested.


A
IL-1β (pg/ml)
B
IL-5 (pg/ml)
C
IL-6 (pg/ml)
D
IL-8 (pg/ml)
E
GM-CSF (pg/ml)
F
TNFα (pg/ml)
Stimuli:
HKC
PAgs
PAgs + CyaA*
CyaA*
Unstimulated
ACV + proCyaA
ACV + proCyaA*
ACV + CyaA
ACV + CyaA*
ACV
CyaA*
PBS
Immunisation groups

as expected, only the macrophages from mice immunised with ACV plus a CyaA form responded. Macrophages from mice immunised with CyaA* alone did not respond well to CyaA* stimulation. Surprisingly, unstimulated cells from the groups vaccinated with ACV plus all the 4 types of CyaA produced GM-CSF, but unstimulated cells from the ACV alone group only produced a marginal level similar to that of PBS control group (Fig. 42E). There was variable production of GM-CSF from stimulated macrophages of all immunised groups compared with the PBS control and no clear trend was observed. IL-1β was detected, albeit at a low level, from macrophages of mice immunised with ACV plus a CyaA form or CyaA* alone after PAgs + CyaA* or CyaA* stimulation, respectively, but was not detected from cells from ACV-immunised mice or from PBS control mice. Higher levels of IL-5 were secreted by antigen-stimulated macrophages from immunised mice compared with stimulated macrophages from the PBS control group. IL-5 is principally produced by T cells and the fact that it was detected suggests that there was some T cell contamination from the peritoneal lavage cell suspension (Fig. 42B). Thus, although most of the cell population consisted of peritoneal macrophages, there appeared to be residual numbers of T cells present. No IL-2, IL-4, IL-10, IL-12 or IFNγ were detected.

3.9 *In vivo* experiments: nitric oxide production from peritoneal macrophages

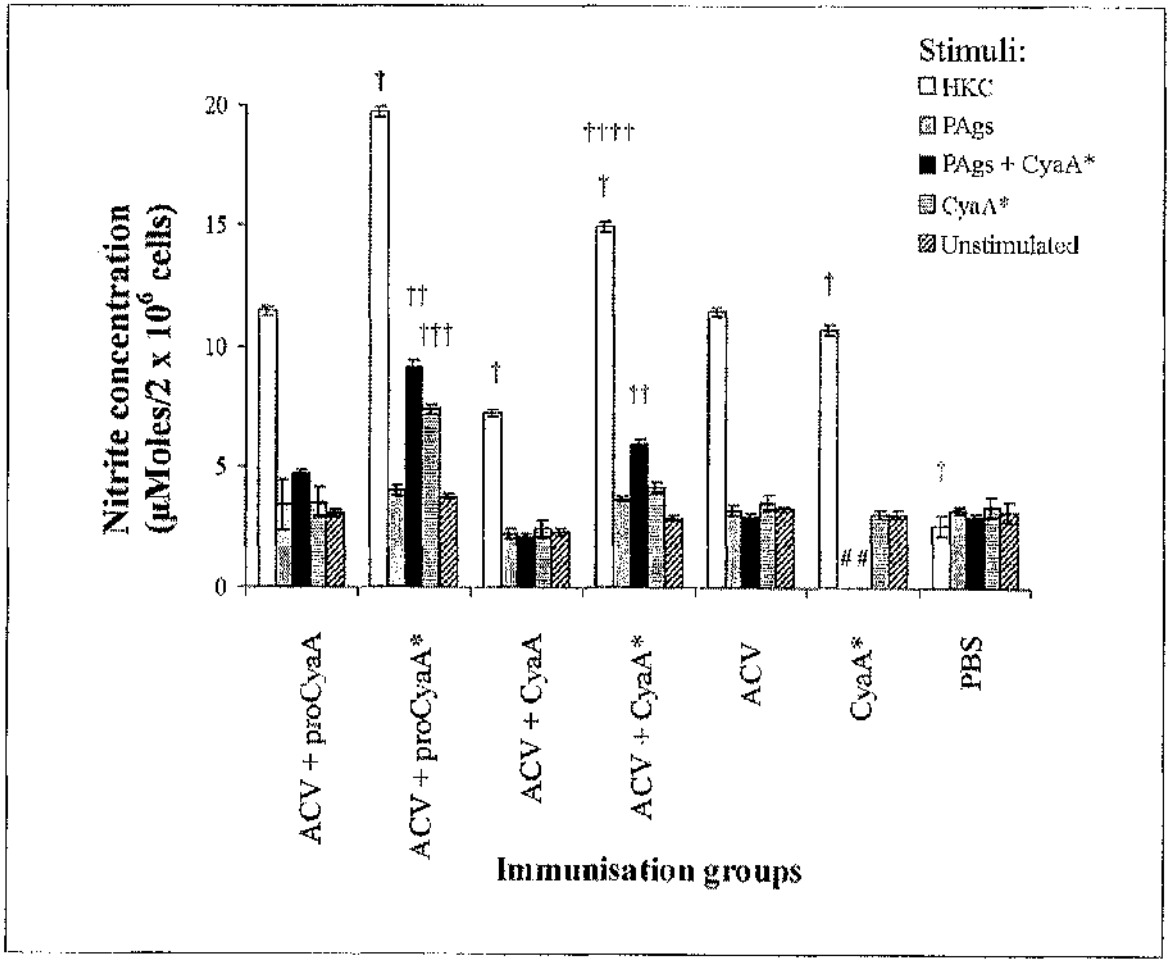
Nitric oxide production by peritoneal macrophages from immunised mice was measured as an indication of their activated state and the induction of cell-mediated immunity.

3.9.1 Nitric oxide production from peritoneal macrophages from mice immunised with ACV plus different CyaA forms after stimulation *in vitro* with *B. pertussis* antigens

Groups of mice were immunised intraperitoneally on days 0 and 28 with CyaA*, ⅛ SHD of ACV alone or ⅛ SHD of ACV plus different CyaA preparations (25 μg/dose) or were given PBS only. Peritoneal macrophages were isolated from mice on day 42. After antigen stimulation for 24 h, the supernates were collected and assessed for NO production by the macrophages. The levels of NO from antigen-stimulated peritoneal macrophages from the ACV control group were similar to the PBS control group (Fig. 43). Stimulation of peritoneal macrophages with PAgs + CyaA* induced significantly greater ($P < 0.05$) production of NO from mice immunised with ACV + proCyaA* or ACV + CyaA* compared with the ACV or PBS control groups (Fig. 43). In addition, only macrophages from mice immunised with ACV + proCyaA* or ACV + CyaA* produced significantly ($P < 0.05$) greater levels of NO compared with the CyaA* immunised group in response to

Figure 43 Nitric oxide production by peritoneal macrophages from immunised mice after stimulation *in vitro* with *B. pertussis* antigens

Groups of mice were immunised intraperitoneally on days 0 and 28 with ⅛ SHD of ACV alone or ⅛ SHD of ACV plus different CyaA forms (25 μg per mouse) or were given PBS only. Peritoneal macrophages were isolated from immunised mice on day 42 and then stimulated with antigen. HKCs (□), PAgs (▩), PAgs + CyaA* (■) and CyaA* (⊟) were used as stimuli. Other cells were untreated and served as negative controls (▨). Nitric oxide production was assessed from peritoneal macrophages of immunised mice stimulated with different *B. pertussis* antigens for 24 h. See Fig. 41 legend for details on the final concentration of antigens used. For NO release, results represent the means of triplicate assays with SEM (bars). Symbol: †, $P < 0.05$ (HKC stimulated groups vs HKC stimulated ACV group: ANOVA); ††, $P < 0.05$ (PAgs + CyaA* stimulated groups vs PAgs + CyaA* stimulated ACV group: ANOVA); †††, $P < 0.05$ (CyaA* stimulated groups vs CyaA* stimulated ACV group: ANOVA).



CyaA* stimulation (Fig. 43). Macrophages from the ACV + CyaA group were the least responsive to antigen stimulation. HKCs stimulated greater NO production from macrophages from all immunised mice than from macrophages from the PBS control group. Significantly higher levels ($P < 0.05$) of NO were produced in response to HKC by macrophages from mice immunised with ACV + proCyaA* (20 ± 0.2 μM/ 2×10^6 cells) or ACV + CyaA* (15 ± 0.2 μM) compared with the ACV- (12 ± 0.2 μM), ACV + proCyaA- (11 .4 ± 0.2 μM), ACV + CyaA- (7.2 ± 0.1 μM) or CyaA* alone- (10.6 ± 0.2 μM) immunised groups or the PBS control group (2.5 ± 0.4 μM). (Fig. 43).

3.9.2 Nitric oxide production by peritoneal macrophages from mice immunised with ACV plus graded doses of CyaA* after stimulation *in vitro* with *B. pertussis* antigens

Groups of mice were immunised intraperitoneally on days 0 and 28 with CyaA*, ⅛ SHD of ACV alone or ⅛ SHD of ACV plus different doses of CyaA* (25, 12.5 or 6.25 μg/dose) or were given PBS only. Peritoneal macrophages were isolated from mice on day 42. After antigen stimulation for 24 h, the supernates were collected and assessed for NO production by the macrophages. Peritoneal macrophages from mice immunised with ACV + 25 or 12.5 μg of CyaA* stimulated with PAgs + CyaA* produced significantly ($P < 0.05$) more NO compared with macrophages from mice immunised with ACV alone or the PBS control group (Fig. 44). Macrophages from all immunised groups did not respond to the PAgs stimulation any better than the PBS control group. In this experiment, HKC-stimulation of peritoneal macrophages did not induce significant NO production. This may have been due to the 100-fold lower concentration of HKCs used for stimulation compared with the other experiments where 5×10^7 cells/ml were used (Section 3.9.1; Fig. 43).

3.10 Global gene responses of murine bone-derived macrophages in response to CyaA

The gene transcriptional responses induced in mouse bone marrow-derived macrophages (BMMs) by exposure proCyaA*, CyaA or CyaA* were examined. A cytotoxicity assay (MTT) was used to determine a suitable toxin concentration hich was not cytotoxic to cells. Affymetrix technology was used to investigate the gene transcriptional responses.

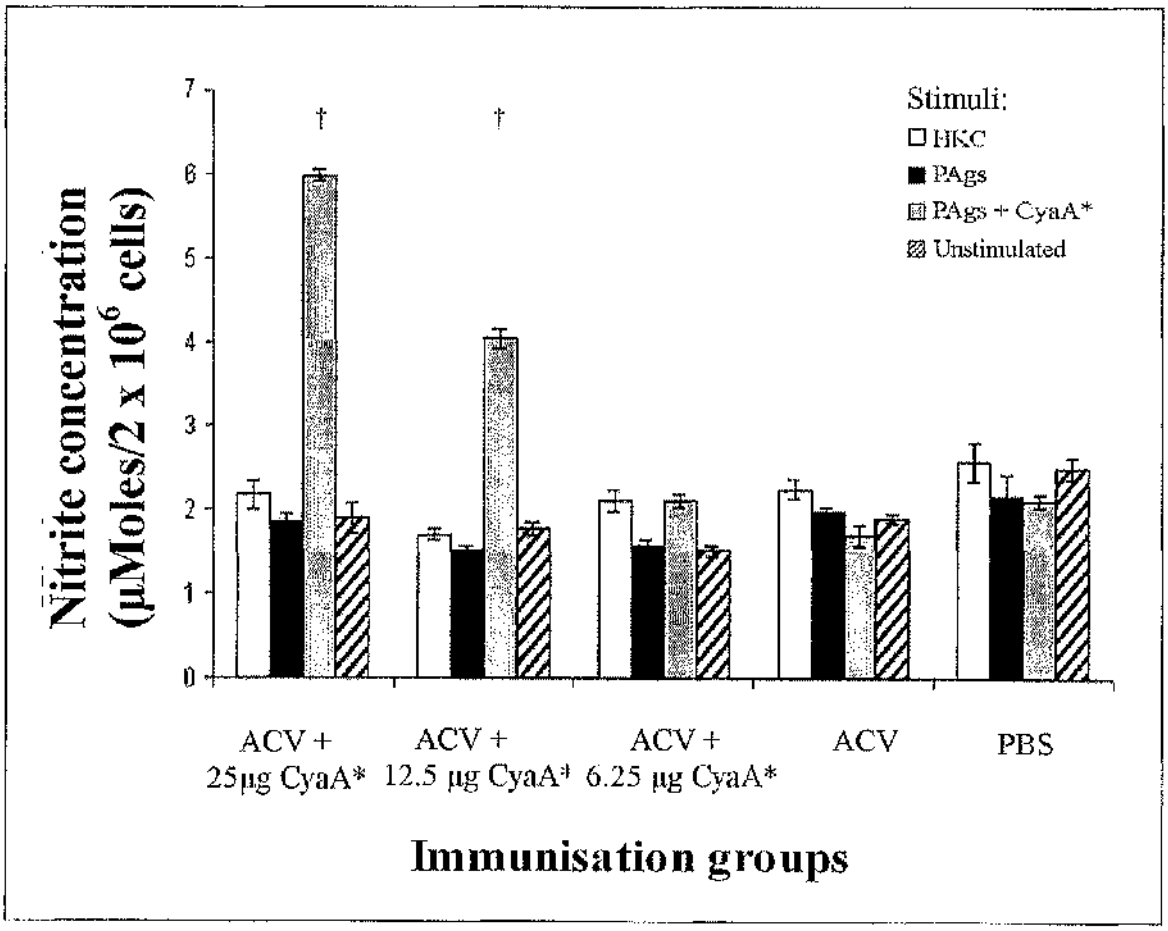
3.10.1 MTT assay

3.10.1.1 BMM viability after incubation for 2 or 24 h with CyaA

A MTT assay was first performed to determine a toxin concentration that was relatively non-cytotoxic for the mouse bone marrow derived macrophages (BMMs).

Figure 44 **Nitric oxide production by peritoneal macrophages from mice immunised with ACV plus graded doses of CyaA* after stimulation *in vitro* with *B. pertussis* antigens**

Peritoneal macrophages from mice immunised intraperitoneally with ⅛ SHD of ACV with or without graded doses of CyaA* (6.25, 12.5 or 25 μg) or given PBS only were stimulated *in vitro* for 24 h with HKCs (□), PAgs (■) or PAgs + CyaA* (▩) for 24 h. Other spleen cells were not stimulated, and served as controls (▨). PAgs consisted of a mixture of detoxified-PT, -FHA and -PRN used at a final concentration of 2, 2 and 5 μg/ml, respectively. HKCs were used at a final concentration of 5×10^5 cells/ml. Results represent the means of triplicate assays with SEM (bars). Symbol: †, $P < 0.05$ (groups vs ACV group: ANOVA).



As shown in Fig. 45, after incubation for 24 h with CyaA, more BMMs were killed by CyaA compared with incubation for 2 h. At a toxin concentration of 20 ng/ml, ~5% or ~20% of BMMs were killed after incubation with CyaA for 2 h or 24 h, respectively. This toxin concentration was chosen for incubation of BMMs with CyaA for 2 and 24 h.

3.10.1.2 BMM viability after incubation for 24 h with CyaA, CyaA* or proCyaA*

As shown in Fig. 46, although both CyaA and CyaA* were cytotoxic towards BMMs, CyaA* was not as cytotoxic as CyaA: only CyaA was cytotoxic at a concentration of 20 ng/ml. The observation that CyaA* was less cytotoxic towards BMMs may be that there is less calcium in DMEM/F12 ($CaCl_2.2H_2O$: 154.5 mg/l) compared with DMEM ($CaCl_2.2H_2O$: 264 mg/l). CyaA* requires more calcium for cell killing than CyaA (Section 3.4.3.5). ProCyaA* showed slight cytotoxicity at concentrations above 5 μg/ml. The negative control (which contained the same levels of urea as in the CyaA samples) did not affect cell viability compared with untreated cells. For comparative purposes, a final concentration of 20 ng/ml was used for proCyaA*, CyaA and CyaA* for incubation with BMMs for 24 h.

3.10.2 RNA quantification and quality assessment

Good quality RNA was obtained as shown by two major RNA bands (Fig. 47). These small and large bands corresponded to 18S and 28S ribosomal RNA respectively. High RNA integrity values, for each RNA preparation, were produced by the Agilent 2100 Bionalyser software (Appendices K1 and K2). The integrity values are essentially a measure of a comparison of the 18S and 28S rRNA peaks and indicate the state of rRNA degradation. A value of 10 indicates the least degradation. In addition, A_{260}/A_{280} ratios were roughly ~1.6 indicating low levels of protein contamination in the RNA preparations. The cRNA, made from RNA from BMMs incubated with CyaA for 2 h and 24 h, were hybridised with MG-U74Av2 GeneChips® for 16 h or cRNA, made from RNA from BMMs incubated with proCyaA*, CyaA or CyaA* for 24 h, were hybridised with MOE430_2 Affymetrix arrays for 16 h.

3.10.3 Preliminary experiment to assess gene responses in BMMs after incubation for 2 or 24 h with CyaA

BMMs were incubated with 20 ng/ml of CyaA (in 400 μM urea) or with urea (400 μM) for 2 and 24 h, and the RNA samples, each pooled from triplicate BMM treatments, were processed and applied to MG-U74Av2 GeneChips®, using 1 chip for each cRNA. This experiment had 2 purposes. 1) To investigate which time of CyaA treatment would yield the most informative data and 2) To check that 20 ng/ml CyaA would provide

Figure 45 Cytotoxicity of CyaA towards murine BMMs

BMMs were incubated with CyaA for 2 h (solid squares) or 24 h (open squares). Cytotoxicity was assessed by the MTT assay. Results represent the means of assays performed in triplicate with SEM (bars).

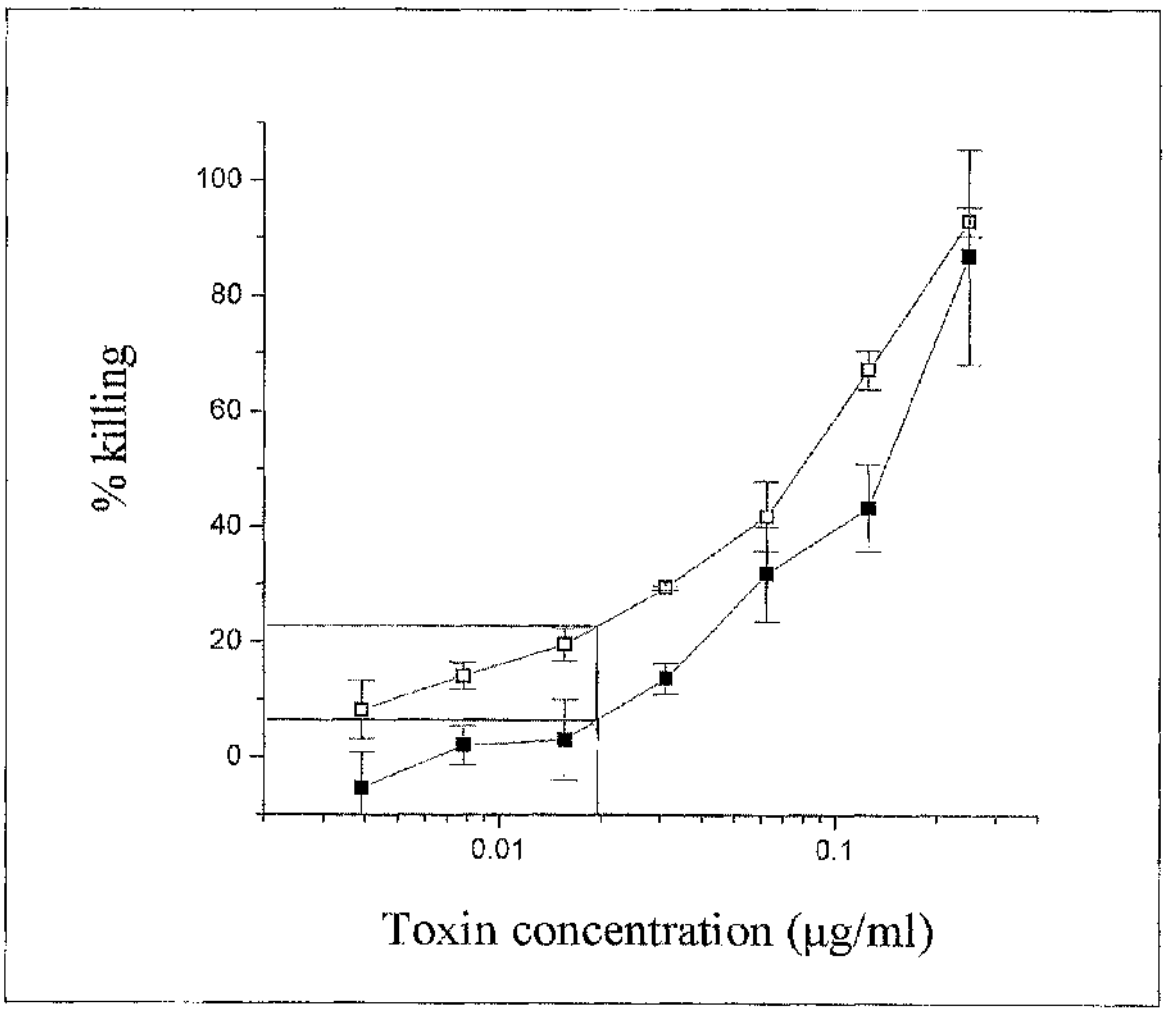


Figure 46 Cytotoxicity of CyaA forms for murine BMMs after incubation for 24 h

ProCyaA* (open squares), CyaA (open circles) or CyaA* (open triangles) were incubated for 24 h with BMMs. Cells treated with urea at the same concentration as in the CyaA samples served as a control (solid squares + dotted lines). Results represent the means of assays performed in triplicate with SEM (bars).

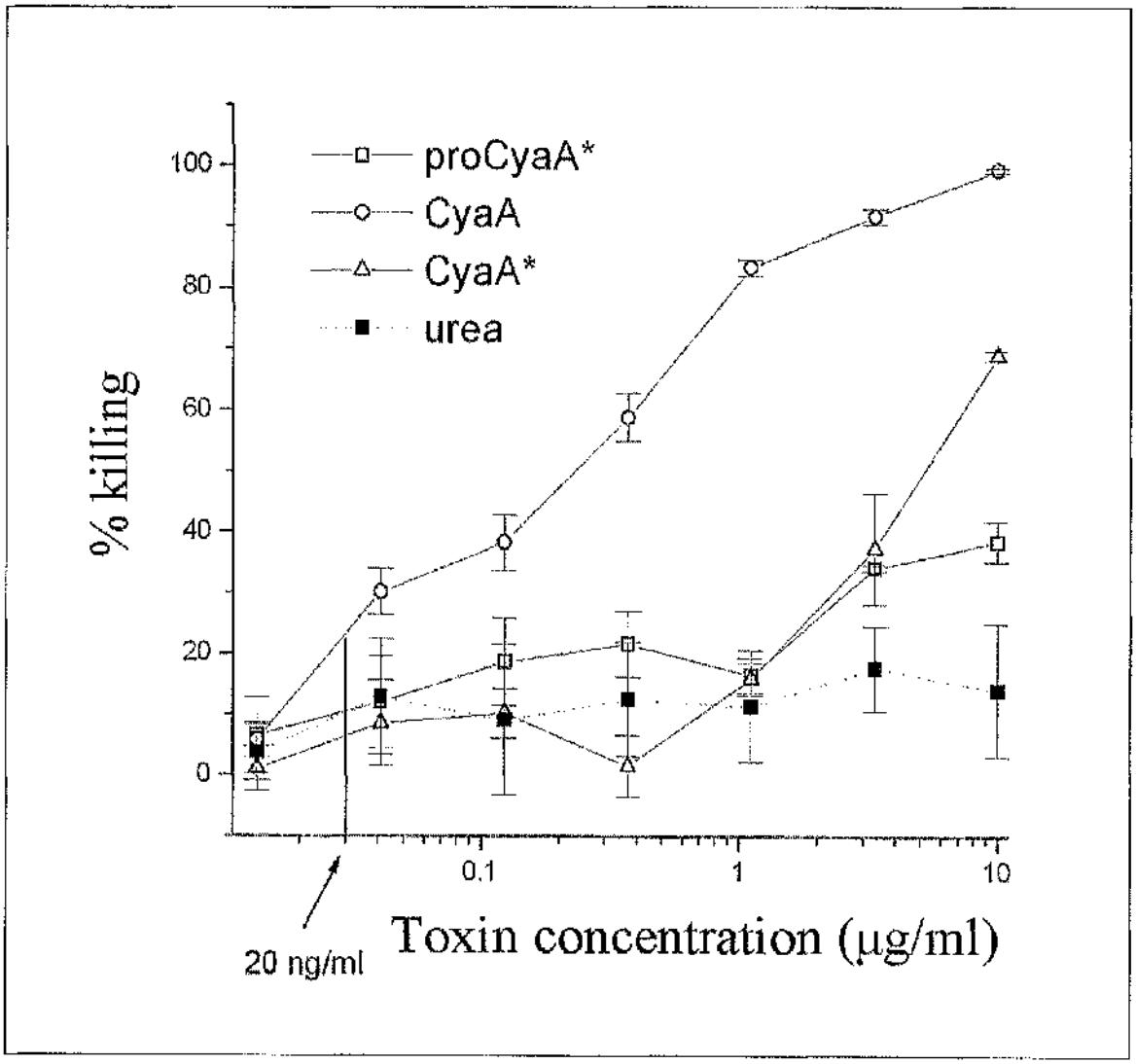
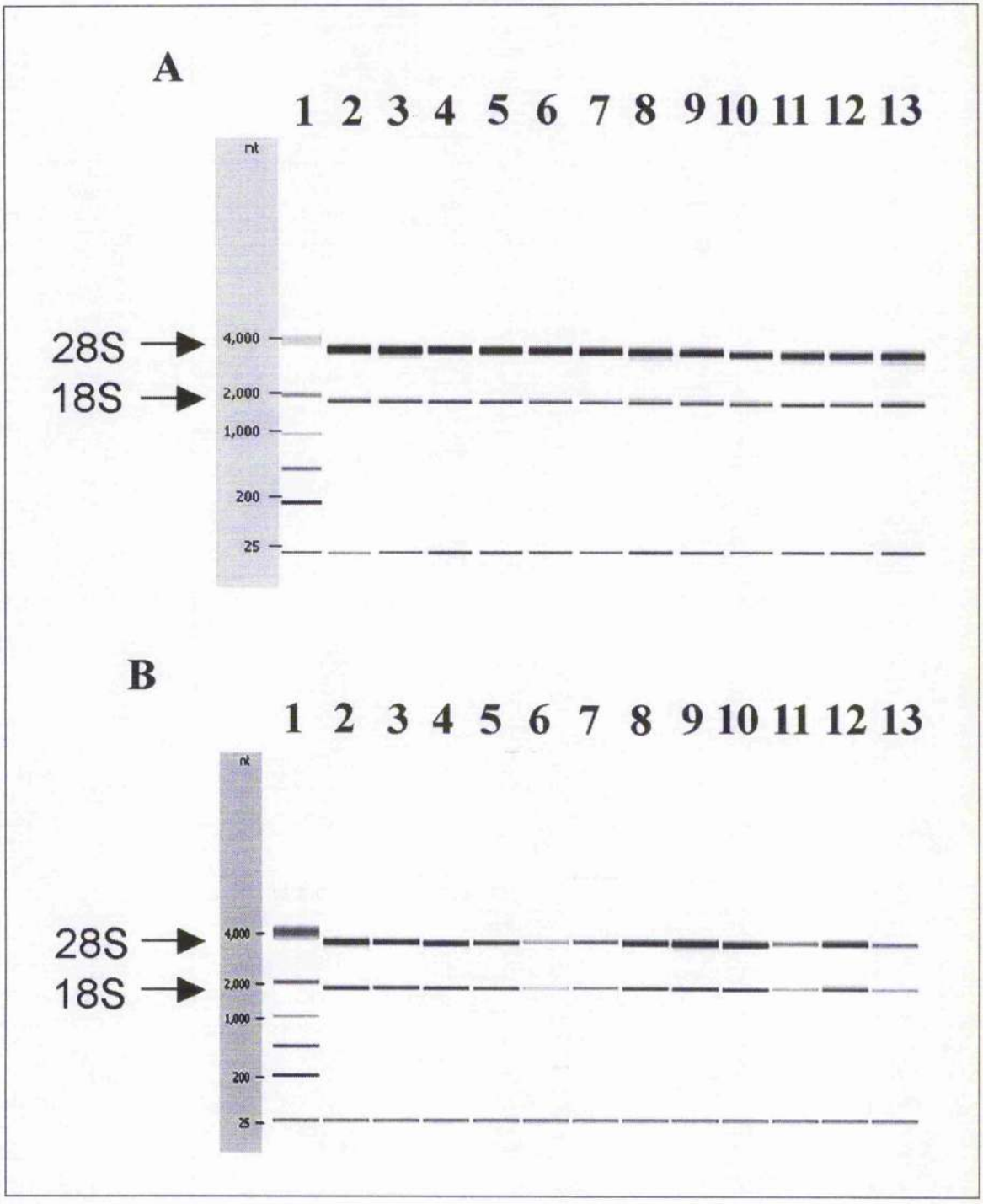


Figure 47 Quality of RNA extracted from BMMs after incubation with CyaA

Images of RNA from BMMs incubated with (A) CyaA for 2 h or 24 h or with (B) proCyaA*, CyaA or CyaA* for 24 h after electrophoresis on a Nano 6000 LabChip. The LabChips were then analysed on Agilent 2100 Bioanalyser which produced images as shown on the opposite page. Each treatment was performed in triplicate as indicated by # followed the number 1, 2 or 3.

Fig. 47A	
Lane	Sample
1	RNA ladder
2	2 h urea #1
3	2 h urea #2
4	2 h urea #3
5	2 h CyaA #1
6	2 h CyaA #2
7	2 h CyaA #3
8	24 h urea #1
9	24 h urea #2
10	24 h urea #3
11	24 h CyaA #1
12	24 h CyaA #2
13	24 h CyaA #3

Fig. 47B	
Lane	Sample
1	RNA ladder
2	proCyaA* #1
3	proCyaA* #2
4	proCyaA* #3
5	CyaA #1
6	CyaA #2
7	CyaA #3
8	CyaA* #1
9	CyaA* #2
10	CyaA* #3
11	Urea #1
12	Urea #2
13	Urea #3


A
1 2 3 4 5 6 7 8 9 10 11 12 13
nt
28S
18S
4,000
2,000
1,000
200
25
B
1 2 3 4 5 6 7 8 9 10 11 12 13
nt
28S
18S
4,000
2,000
1,000
200
25

sufficient gene activation compared with the urea-treated control. The experiment involved a total of four arrays, each with ~6,000 characterised murine genes and ~6,000 expressed sequence tags. Sequences used in the design of the array were selected from GenBank®, dbEST, and RefSeq. The expressed sequence tags were created from the UniGene database (Build 107, June 2002) and then refined by analysis and comparison with the publicly available draft assembly of the mouse genome from the Whitehead Institute Center for Genome Research (April 2002). Affymetrix GeneChips® have oligonucleotide probes (25-mers) lithographically synthesised *in situ* (Section 1.10.1). Affymetrix probes are designed in pairs: one sequence is the exact complement of the target sequence (Perfect Match; PM), and the other differs from the exact complement by one mutation in the middle of the probe (MisMatch; MM) (Section 1.10.1). The MM probes are designed to account for non-specific binding of cRNA (Section 1.10.1). For any gene, up to 20 paired probes can be printed on the array. An expressed sequence tag is a tiny portion of an entire gene that can be used to help identify unknown genes and to map their positions within a genome. They are generated by sequencing either one or both ends of an expressed gene. The data from each array were analysed using GeneSpring 7.2 software. A total of three pairwise comparisons was performed (2 h CyaA plus urea *versus* 2 h urea treatment, 24 h CyaA plus urea vs 24 h urea treatment and 24 h CyaA plus urea treatment vs 2 h CyaA plus urea treatment). To view a complete list of genes in the database, visit http://www.gti.ed.ac.uk/GPX/, accession number GPX-00031.1. A password, which can be requested from Dr. Paul Dickinson [Paul.Dickinson@ed.ac.uk], is required to access the database.

3.10.3.1 Gene responses in BMMs after incubation for 2 h with CyaA

There were 247 and 223 genes up- and down-regulated > 2-fold in BMMs, respectively, after treatment for 2 h with CyaA (plus urea) compared with treatment with urea alone. Genes which were up- and down-regulated > 3-fold are listed in Appendix L.1 and L.2, respectively. The more stringent criterion of a minimum 3-fold change was used to select genes most likely to be associated with CyaA treatment since no statistics could be performed as only 1 array had been used for each treatment in this preliminary experiment. Up-regulation of genes involved in immunity included the IL-1β gene, *Il1b* (> 16-fold) and the cytotoxic T lymphocyte-associated protein gene, *Ctla2a* (> 4-fold).

Some up-regulated genes which coded for cell-surface molecules included *Adora2b* (~4-fold) and *Gja1* (~5-fold). *Dusp6*, which encodes a protein that desphosphorylates signalling molecules was up-regulated by 3.26-fold. Genes involved in transcription included *Ahr* (~4-fold), *Nfkbie* (~4.3-fold), *fos* (~5.39-fold) and *Nr4a2* (~10-fold). Regarding cell differentiation and growth, *Vegfa* and *Btg2* were transcribed the greatest.

Other types of up-regulated genes were associated with apoptosis, cellular structure, metabolism and adhesion (Appendix L.1). There was also strong increased transcription of other genes such as *Thbs1* (Thrombospondin) (> 15-fold) and *Arg1* (Arginase) (> 25-fold).

CyaA also down-regulated a number of genes that spanned a wide range of cell functions (Appendix L.2). These included *Tnfa* (~8-fold), genes that coded for cell-surface receptors (*Abcf2*, > 8-fold), cell-signalling (*Map3k8*, > 15-fold), transcription (*Np95*, > 6-fold) and apoptosis (*Traf5*, > 4-fold). Overall, there was a wide spectrum of genes which were up- and down-regulated which have a role in all aspects of cell function in BMMs, after treatment with CyaA for 2 h.

3.10.3.2 Gene responses in BMMs after incubation for 24 h with CyaA

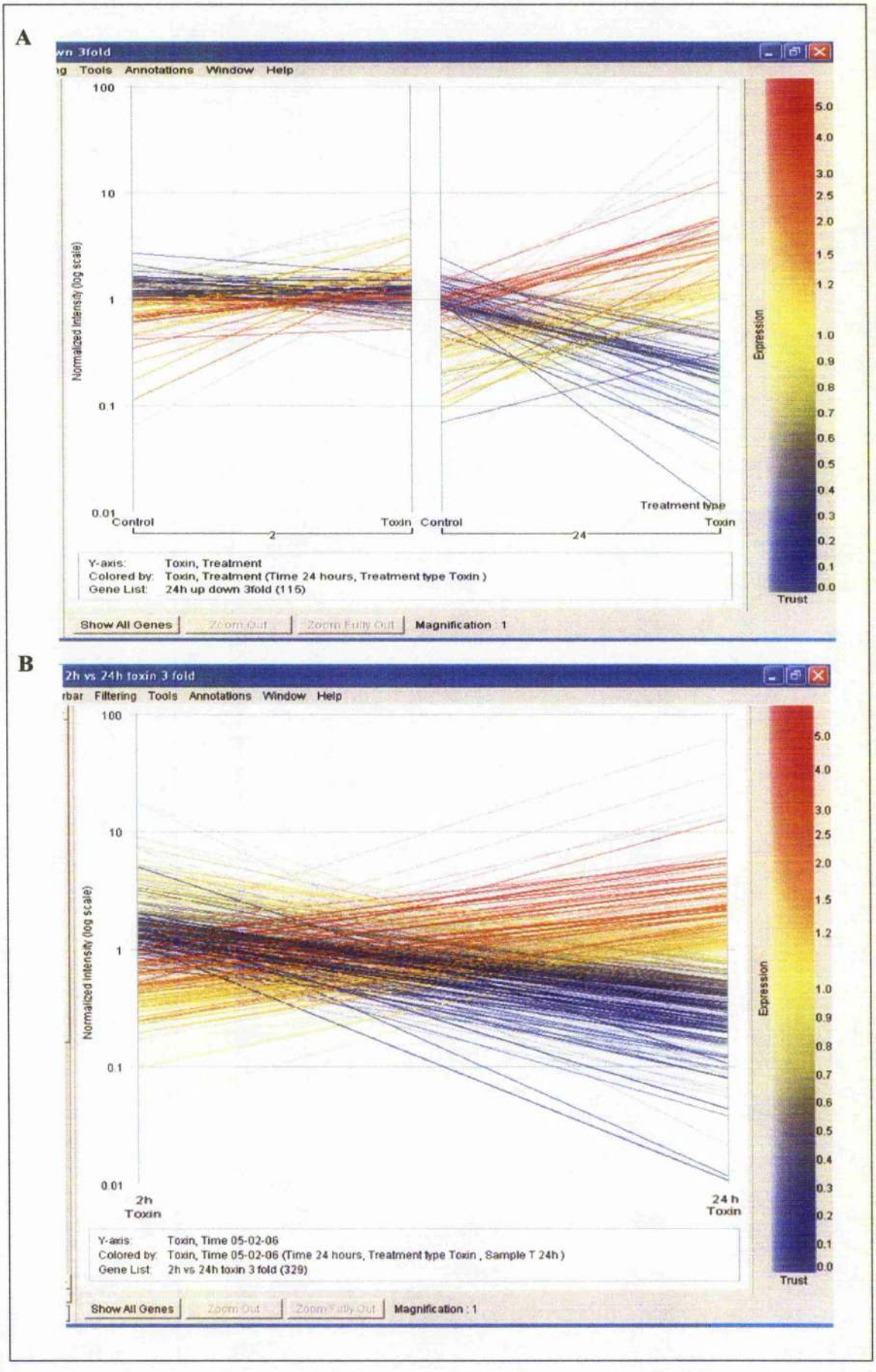
There were 226 and 204 genes up- and down-regulated > 2-fold in BMMs, respectively, after treatment with CyaA (plus urea) for 24 h compared with treatment with urea alone. Genes which were up- and down-regulated > 3-fold are listed in Appendices L.3 and L.4, respectively. Interestingly, only 115 genes were found up- or down-regulated > 2-fold in BMMs after treatment for both 2 h and 24 h with CyaA. However, 12% of the 115 genes were regulated greater than 3-fold by both treatments. The gene for IL-1β was again highly up-regulated, but most of the 3-fold up-regulated genes common to both time intervals were cell-surface receptors (*Edg2*, *Adora2b* and *Gja1*) or associated with adhesion (*Col9a1* and *Thbs1*). The only gene that was down-regulated > 3-fold, and common to both treatments, was the cell cycle regulator gene, *Np95*. Generally, there were greater changes in the fold of transcription of most of the genes after treatment for 24 h compared with 2 h (Fig. 48A, B). The best examples of this were *Gja1* (~5-fold to > 16-fold), *Il1b* (> 16-fold to > 21-fold) and *Arg1* (> 25-fold to > 164-fold), after 2 h or 24 h, respectively.

Some genes that were detected after treatment for 24 h which were not detected after 2 h included the up-regulation of *CD86* (> 3.5-fold), several chemokines (*Cxcl2*, > 9.5-fold; *Cxcl1*, > 10-fold), integrins (*Itga5*, > 14-fold) and receptors for chemokines (*Ccr1*, > 5-fold). Other types of up-regulated genes were associated in different cell processes, such as signalling (*Cav*), transcription (*Nr4a2*), cell growth (*Hus1*), inducing apoptosis (*Bnip3*) and adhesion (*Thbs1*) (Appendix L.3).

CD5l was the only gene down-regulated at 24 h but not at 2 h that was involved in the immune response (Appendix L.4). Some of the down-regulated signalling and transcription-associated genes included *Dusp2*, *Racgap1*, *Ptp4a3*, *Tk1*, *Np95* with *Np95* being down-regulated the greatest (~14-fold). Interestingly, there were 11 down-regulated genes involved in cell proliferation and DNA replication after treatment for 24 h with

Figure 48 Line diagram showing the differences in gene transcription in BMMs after incubation for 2 h or 24 h with CyaA compared with the negative control

Differences in gene transcription > 3-fold after incubation with CyaA for (A) 2 h (left) or 24 h (right) and (B) comparison of gene transcription (> 3-fold) between 2 and 24 h with CyaA treatment. Each line represents a single gene. Greatest up- or down-regulation of genes is represented by red or blue, respectively, as shown on the right hand panel.


A
wn 3fold
g Tools Annotations Window Help
100
10
1
0.1
0.01
Normalized intensity (log scale)
Control
Toxin
2
Control
Toxin
24
Treatment type
Y-axis: Toxin, Treatment
Colored by: Toxin, Treatment (Time 24 hours, Treatment type Toxin)
Gene List: 24h up down 3fold (115)
Expression
5.0
4.0
3.0
2.5
2.0
1.5
1.2
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Trust
Show All Genes
Zoom Out
Zoom Fully Out
Magnification : 1
B
2h vs 24h toxin 3 fold
rbar Filtering Tools Annotations Window Help
100
10
1
0.1
0.01
Normalized intensity (log scale)
2h
Toxin
24 h
Toxin
Y-axis: Toxin, Time 05-02-06
Colored by: Toxin, Time 05-02-06 (Time 24 hours, Treatment type Toxin , Sample T 24h)
Gene List: 2h vs 24h toxin 3 fold (329)
Expression
5.0
4.0
3.0
2.5
2.0
1.5
1.2
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Trust
Show All Genes
Zoom Out
Zoom Fully Out
Magnification : 1

CyaA. Other genes which were down-regulated strongly included *Psat1* (> 5.5-fold) and *Lpl* (> 80-fold) which are involved in metabolism and biosynthesis.

A further comparison was made to investigate the types of genes up- and down-regulated between 2 h and 24 h after treatment with CyaA, excluding any genes found up- and down-regulated after treatment with urea alone. This produced a gene list of 846 genes up- or down-regulated > 2-fold. About 39% of these genes were up- or down-regulated > 3-fold and these genes are listed in Appendices L.5 and L.6, respectively. There were more genes detected using this comparison than by comparison of 2 h CyaA *versus* 2 h urea or 24 h CyaA *versus* 24 h urea (Section 3.9.3.1 and 3.9.3.2). This situation would arise if a gene is up-regulated < 2-fold after treatment for 2 h with CyaA (and therefore not included in the gene list) but is then down-regulated > 2-fold after treatment for 24 h with CyaA, and *vice versa*.

There was increased expression of 7 genes associated with the immune response in BMMs between treatment of 2 h and 24 h with CyaA. In particular, expression of *Traf5* and *Cxcl5* was increased by > 8-fold and > 30-fold, respectively. Two pro-apoptotic genes were also detected. Moreover, there were genes involved in signalling, transcription and cell growth, such as *Map3k8* (> 28-fold), *Nmyc1* (> 10-fold) and *Ptgs1* (> 4-fold), respectively (Appendix L.5). Some genes associated with the immune response that were down-regulated from 2 to 24 h included *Mip1b* (> 4-fold), *Ly86* (> 7-fold), *Ifit2* (> 20-fold), *Ccrl2* (> 3.5-fold) and *H2-Ab1* (> 3.5-fold). Interestingly, there was decreased transcription of the anti-apoptotic genes *Bcl2* and *Birc5* and the down-regulation of 8 signalling genes e.g. *Rgs2* (> 7-fold), and 5 transcription genes, e.g. *Crem* (~12-fold). There were 9 genes down-regulated that were associated with cell division (*Gadd45b* and *Rrm2*) accompanied by 2 genes associated with mitochondrial function (*Clic4* and *Cox6a2*). An interesting observation was the up- and down-regulation of *slc* (solute carrier protein) genes involved in transportation of different substrates. The conclusions drawn from this preliminary study must be treated with caution as no statistics could be performed because only one chip was used for hybridisation with each test sample.

3.10.4 BMM gene responses to the different CyaA forms after incubation for 24 h

The conclusions drawn from the data in this section should be more reliable as three chips were hybridised with each test sample and demonstrate the reproducibility between the three hybridisations. Although fewer genes (430) were up- or down-regulated > 2-fold in BMMs after treatment for 24 h with CyaA compared with 470 genes up- or down-regulated > 2-fold in BMMs after 2 h with CyaA, there were greater levels of increased and decreased transcription after treatment for 24 h (Figs. 48A, B). In addition,

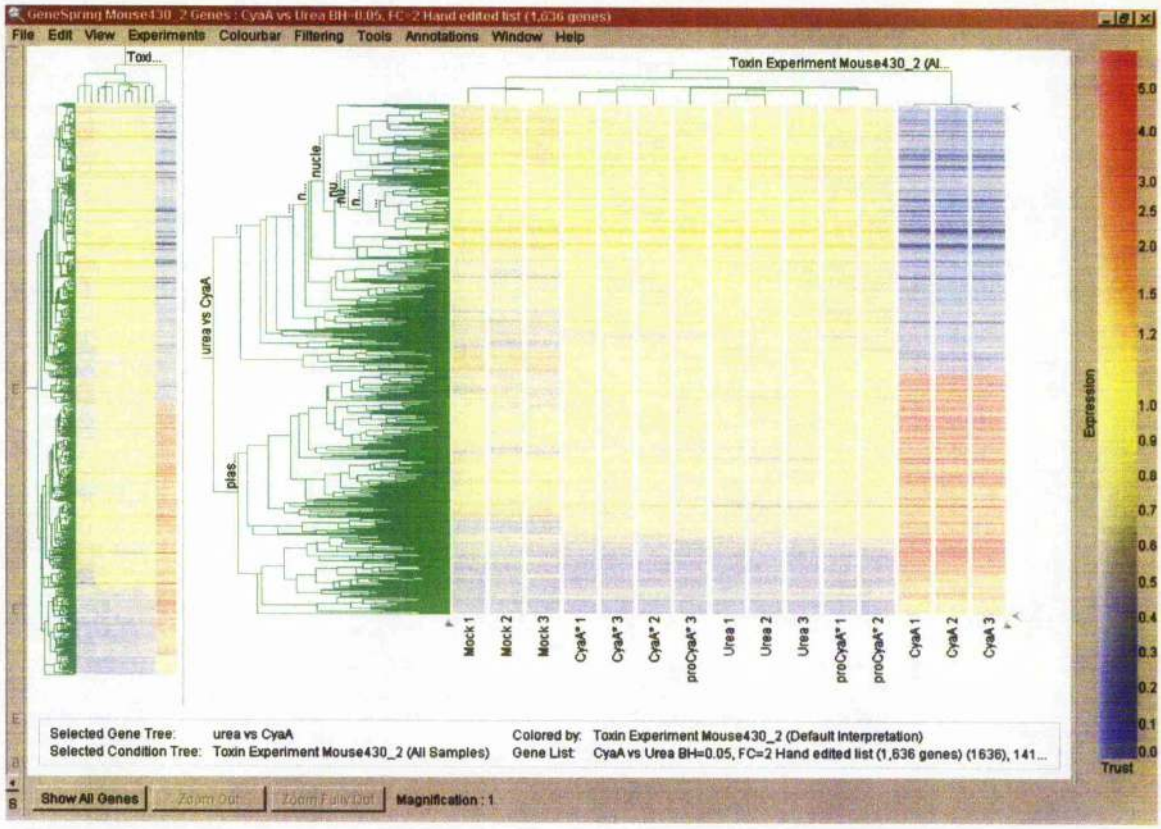
approximately 30% of genes altered in transcription at 2 h were still detected after prolonged exposure to CyaA. Therefore, a period of 24 h was chosen to treat the BMMs with 20 ng/ml CyaA, CyaA*, proCyaA* and urea alone. In addition, the number of replicates for each hybridisation was increased to three so that statistical comparisons could be made. A new gene chip (MOE430_2, Affymetrix) containing 45,000 probe sets representing over 39,000 transcripts and variants from over 34,000 well characterised mouse genes was used for the subsequent experiment. Newly isolated BMMs were treated with CyaA, CyaA* or proCyaA* (all at 20 ng/ml in 400 μM urea) or with 400 μM urea alone for 24 h. For each treatment, the RNA was pooled from three incubations, made into cRNA and hybridised to a MOE430_2 GeneChip (Affymetrix). This was done in triplicate for each treatment. Thus, the analysis was done in triplicate using pools of RNA from 3 incubations for each of the three hybridisations, including the control, generating a total of twelve arrays. The data were analysed using GeneSpring 7.2 software. A total of 3 comparisons was performed: CyaA treatment *versus* urea treatment; CyaA* treatment *versus* urea treatment; and proCyaA* treatment *versus* urea treatment. A change in gene expression was reported if the fold change was at least 2.0. Data from a mock experiment (BMMs incubated in DMEM/F12 for 24 h in the absence of urea, kindly provided by Mr Andrew Livingston, University of Edinburgh) was used as a second negative control and as a comparison for any gene changes caused by urea itself. As shown in Fig. 49, 400 μM urea caused changes in gene transcription in BMMs after treatment for 24 h as indicated by differences in colour patterns for each column of the urea treatment compared with the mock treatment. In addition, the condition tree grouped the three mock treatments together and separate from the urea control indicating that the gene transcription profile after urea treatment was different from the mock treatment.

3.10.4.1 Gene responses to CyaA treatment

Transcripts for 3.5% of the probe sets were detected, representing 1,636 transcripts which were up- or down-regulated greater than 2-fold ($P < 0.05$) in BMMs after treatment with CyaA for 24 h. To view a complete list of genes in the database, visit http://www.gti.ed.ac.uk/GPX/, accession number GPX-00050.1. Access to the database is password encrypted and the password can be requested from Dr. Paul Dickinson [Paul.Dickinson@cd.ac.uk]. 61.5% of these genes were up- and down-regulated > 3-fold with a more stringent P value (< 0.01) and, of these, approximately half (486 gene transcripts) were up-regulated. A further criterion was created to select up-regulated genes which were flagged as "present", as determined by the MAS5 programme (Section 2.14), in all three replicates. Only 1 gene (Affy ID, 1428851_at) was excluded from the 486 transcripts. Thus, it can be said with confidence that the genes listed in Appendix L.7 are

Figure 49 Overview of gene transcription for different treatments of BMMs

A condition tree takes the data for each condition and hierarchically clusters the most similar treatments or samples together (green lines above each column). Green lines on the left of the columns groups genes according to location and function within the cell. The columns, each representing a treatment, is made up of horizontal bars (genes) and colour coded depending on their degree of gene expression (e.g. red and blue indicating high and low gene expression, respectively) compared with the 24 h urea control. A Mock treatment (BMMs incubated in the absence of urea for 24 h) was also included in this condition tree (kindly provided by Mr Andrew Livingston, University of Edinburgh).



up-regulated in BMMs after treatment with CyaA. A majority (32.5%) of up-regulated genes were associated with inflammation. These included *Trem1* (> 50-fold) and *Cmkor1* (> 55-fold), CD antigen genes (*CD80*, *CD86* and *CD207*), chemokine genes (*Ccl7*, *Cxcl2*, *Cxcl5*, *Cxcl1* and *Cxcl7*), the IL-1β cytokine gene, the integrin α_M gene (*Itgam*), a colony stimulating factor receptor (*Csf2rb2*) and an IL-1, type II receptor. Interestingly, the cytokine regulator *Il1rn* was also up-regulated. Genes associated with the cell membrane included *Ltb4r1* (> 5-fold), *Ier3* (> 11-fold), *Gja1* (~20-fold) and *Fxyd2* (> 40-fold). Genes involved in cell-signalling, *Dusp6*, *Procr*, *Fpr1* and *Cav* as well as transcription, *Cebpb*, *Junb*, *Crem* and *Ahr* were all up-regulated and detected previously (Section 3.9.3). Up-regulated pro-apoptotic genes included *Bcl2l11*, *Tnfrsf21*, *Stk17b* and *Egln3*. No anti-apoptotic genes were detected. Other genes that were strongly increased in expression, were *Notch1* (> 7-fold), *Arg2* (> 27-fold), *Nt5e* (> 60-fold) and *Thbs1* (> 360-fold) (Appendix L.7). There was up-regulation of Ras homologues (*Rab20*, *Rasa2* and *Rhoe*) and transcription factors found downstream of important signalling pathways (*Elk3*, *c-fos*, *Junb* and *Fosl2*). There was a whole suite of solute carrier proteins found to be up-regulated, such as cationic transporters, as well as genes involved in the degradation of the extracellular matrix (*Mmp9* and *Mmp19*).

There was a slightly greater number of genes which demonstrated a > 3-fold reduction ($P < 0.01$) in transcription (519 gene transcripts) compared with the number of up-regulated genes using the same criteria. A selection of the down-regulated genes is listed in Appendix L.8. These included pro-inflammatory genes such as *Ccl3*, *Ccl4*, *Ifit2*, *H2-Aa*, *H2-Ab1* and *Scarb1*. Amongst the down-regulated genes involved with the immune response, were *CD109* (~5-fold), *Cscl10* (~4-fold), *Igh6* (~14-fold), *Trem2* (> 7-fold), *Ptger4* (> 9-fold) and *Itga6* (> 12-fold). Interestingly, only 10% of the down-regulated genes listed in Appendix L.8 were associated with the immune response compared with 32.5% of up-regulated genes (Appendix L.7). There was a strong decrease in transcription of the anti-apoptotic gene *Birc5* (> 16-fold). Approximately 40% of the down-regulated genes listed in Appendix L.8 were involved in cell growth, division and differentiation compared with only ~3% of up-regulated genes associated with the same functions (Appendix L.7). A large collection of genes involved in the cell cycle, e.g. minichromosome maintenance deficient genes (*Mcm3* and *Mcm5*), *Plk4*, cell division cycle genes (*Cdc25a* and *Cdc20*), cyclin genes (*Ccnd1* and *Ccnf*), kinesin genes (*Kif2c* and *Kif22*) and genes involved in DNA replication (*Top2a* and *Rrm2*) were found down-regulated in BMMs after CyaA treatment. Some down-regulated genes involved in cell-signalling included *Rgs2* (~8-fold), *Il6st* (~12-fold), *Racgap1* (~15-fold) and *Pbk* (~30-fold). *Tk1* and *Uhrf1*, genes associated with transcription, were down-regulated by ~22-

fold and ~34-fold, respectively. Two genes associated with the mitochondrion (*Clic4* and *Cybb*), 1 gene associated with cellular structure (*Ckap2*) and 2 genes associated with metabolism/biosynthesis (*Psat1* and *Lpl*) showed decreased transcription. There was a decrease in the transcription of several solute carrier transport genes. This was an interesting observation as there was also an increase in transcription of several different types of solute carrier transport genes (Appendix L.7). Other down-regulated genes included *Tyms* and *Asns* (both ~12-fold), *Trib3* (4.5-fold), *Ect2* (> 6.5-fold) and *Mthfd2* (~15-fold). Microarray analysis also identified 276 hypothetical transcripts or transcripts with unknown function (Visit http://www.gti.ed.ac.uk/GPX/, accession number GPX-00050.1) which were up- or down-regulated.

In summary, there were about 1000 genes that either were up- or down-regulated after treatment for 24 h with CyaA (> 3-fold, $P < 0.01$), but there were clear differences in the types of genes up- and down-regulated. Generally, there was an increase in expression of pro-inflammatory genes and genes that coded for receptors as well as increased transcription of genes associated with signalling pathways but there was decreased transcription of many genes involved in all aspects of cell function. Most strikingly, there was a substantial down-regulation of many genes involved in cell proliferation.

3.10.4.1.1 Morphology of BMMs in response to CyaA treatment

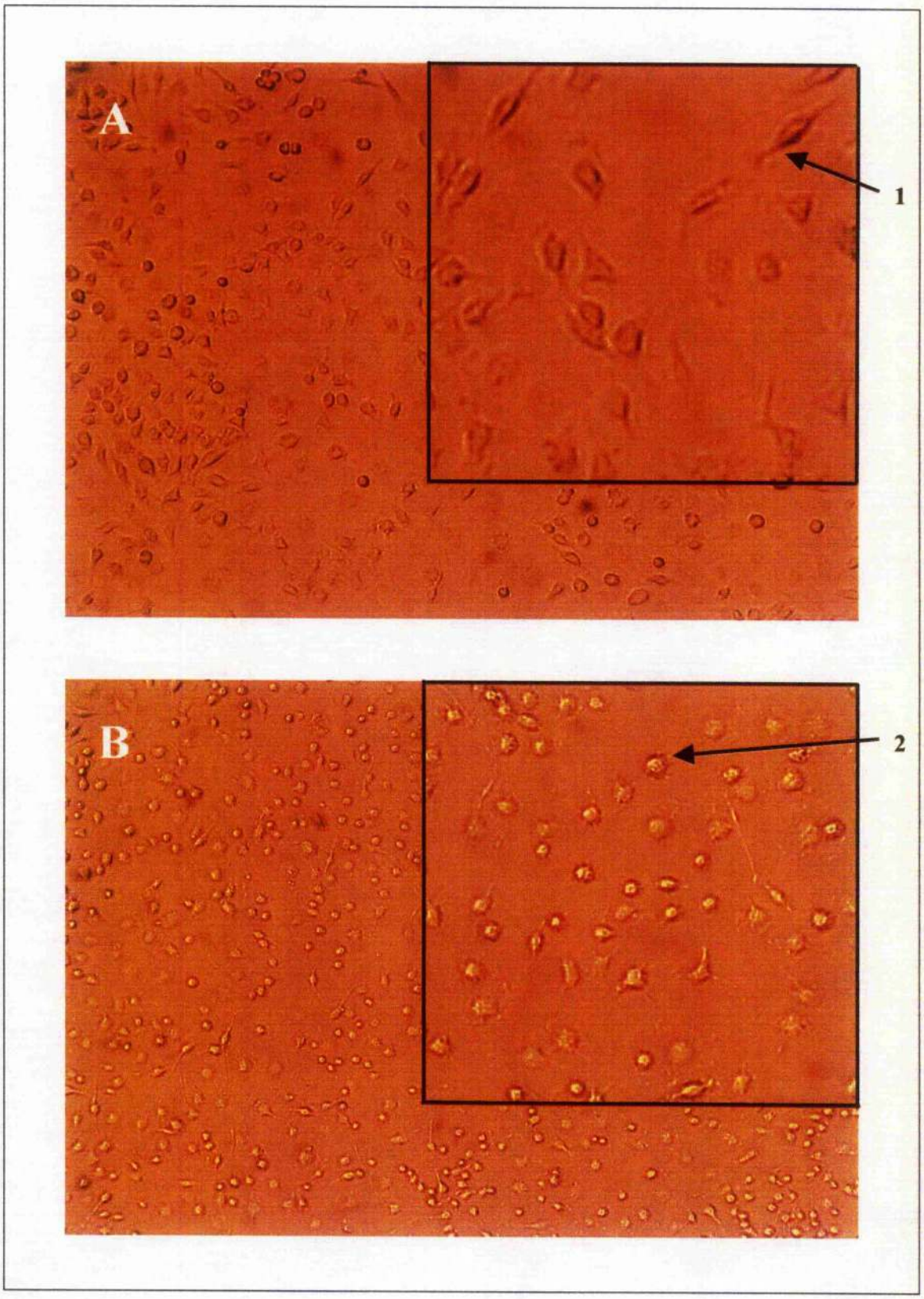
The morphology of BMMs after treatment with CyaA was different from that of control cells incubated with urea alone (Fig. 50). A majority of the BMMs treated with urea alone for 24 h appeared to be actively differentiating or proliferating as indicated by a swollen cell body and two (or three) short processes (Fig. 50A). In contrast, CyaA treated BMMs showed a spherical morphology with ruffled membranes. These cells also produced long and thin processes (Fig. 50B) which were distinctly different from the protruding arms of urea-treated BMMs (Fig. 50A).

3.10.4.2 Gene responses to CyaA* treatment

After treatment of BMMs with 20 ng/ml of CyaA* for 24 h, no changes in transcription were detected using the same criteria as for CyaA treatment (Section 3.9.4.1) i.e. a P value, < 0.01 and minimum 3-fold change in gene transcription. Therefore, a lower criterion was used (P value < 0.05 with a minimum of 2-fold change). With this criterion, only 2 genes passed. Immunoresponsive gene 1 (*Irg1*, [Affy ID, 1427381_at]) and acyloxyacyl hydrolase (*Aoah* [Affy ID, 1450764_at]) were up-regulated by 4.3-fold (P value, 0.002) and 2.17 (P value, 0.0006), respectively, after CyaA* treatment.

Figure 50 BMM morphology after treatment with urea buffer or CyaA (in urea buffer) for 24 h

Cell morphology of BMMs in DMEM/F12 medium after treatment for 24 h, at 37 °C and 5% CO_2 in a humidified atmosphere, with (A) urea buffer or (B) CyaA (20 ng/ml) in urea buffer. Arrow 1 indicates a typical appearance of a proliferating cell after urea buffer treatment - note short processes on opposing sides of the cell body. Arrow 2 shows a rounded cell with an irregular 'ruffled' membrane after CyaA treatment – note the production of long and thin processes from the cell bodies. The inserts in (A) and (B) are enlarged images of BMMs treated with urea and CyaA, respectively.


A
1
B
2

3.10.4.2.1 Morphology of BMMs in response to CyaA* treatment

After treatment of BMMs with 20 ng/ml of CyaA* for 24 h, BMMs treated with CyaA* showed similar cell morphologies to BMMs treated with urea buffer alone (data not shown).

3.10.4.3 Gene responses to proCyaA* treatment

After treatment of BMMs with 20 ng/ml of proCyaA* for 24 h, no genes were found up- or down regulated compared with the urea control even with the less stringent criteria of $P < 0.05$ and a minimum of 2-fold change.

3.10.4.3.1 Morphology of BMMs in response to proCyaA* treatment

After treatment of BMMs with 20 ng/ml of proCyaA* for 24 h, BMMs treated with proCyaA* showed similar cell morphologies to BMMs treated with urea buffer alone (data not shown).

Chapter 4

Discussion - CyaA structure and function

4.1 CyaA preparation

In order to reduce the levels of LPS further in the final purified CyaA preparations than described previously (MacDonald-Fyall *et al.*, 2004), a slightly different purification protocol was devised. This is important because LPS has immunomodulatory, pro-inflammatory and adjuvant properties. In addition, LPS synergises with CyaA and may therefore influence its activity *in vitro* and *in vivo* (Ross *et al.*, 2004). A majority of the LPS was removed from the inclusion bodies by increasing the number of wash steps. In addition, two washes with the detergent, CHAPS, was also a cheaper alternative to n-octylpyranoglucoside, as used previously by MacDonald-Fyall *et al.* (2004). CHAPS also successfully removed contaminating proteins as well as LPS from the inclusion bodies prior to DEAE-Sepharose and phenyl-Sepharose purification. The LPS levels within the purified CyaA preparations from *E. coli* BL21/DE3 were all extremely low, < 0.1 EU/μg protein as deduced by the chromogenic LAL assay. However, there was a slight discrepancy with the LPS levels between the chromogenic and gel clot assays, such that the gel clot LAL assay produced values slightly greater than the chromogenic LAL assay. This may have been due to the fact that the gel clot LAL is a semi-quantitative assay and the values given are the average of two values whereas the chromogenic assay is quantitative producing one value from a standard curve. Nevertheless, both assays confirmed that the levels of LPS in the CyaA preparations from small- and large-scale purification were significantly lower than those reported previously (MacDonald-Fyall, 2002) which ranged from 14 – 257 EU/μg protein. The problem of LPS contamination in the CyaA preparations was overcome by the use of *E. coli* BL21/DE3 *lpxM* strain. CyaA was expressed and purified successfully from this strain and the CyaA preparations were similar to that of CyaA expressed from the parent strain. Moreover, the IL-6 release assay showed that CyaA expressed from the *E. coli* BL21/DE3 *lpxM* induced significant less IL-6 release from MM6 cells compared with CyaA expressed from the parent strain despite containing similar levels of LPS. The use of CyaA expressed from *E. coli* BL21/DE3 *lpxM* would be ideal for future *in vivo* studies. In this study, large-scale purification of the different CyaA forms expressed from the *E. coli* BL21/DE3 parent strain allowed subsequent *in vitro* and *in vivo* studies to be done using the same batch for all experiments, for maximum consistency.

4.2 The roles of enzymic activity and acylation for CyaA function

The MTT and LDH release assays were compared to detect the cytotoxicity of CyaA towards J774.2 cells. Only CyaA and CyaA* were cytotoxic and haemolytic and no activity was detected in these assays with proCyaA or proCyaA* up to a concentration of 10 μg protein/ml. CyaA* required a greater concentration of calcium for cytotoxicity as shown by the severely reduced ability of CyaA* to kill J774.2 cells in RPMI medium compared with the marked increase in cytotoxicity when assayed in DMEM or RPMI supplemented with various amounts of calcium. CyaA* at concentrations below 1 μg/ml in DMEM was not as cytotoxic as CyaA in the MTT assay after incubation with J774.2 cells for 2 h but induced greater LDH release, and at a faster rate, than CyaA at the same concentrations (Fig. 21). After incubation for 24 h, only CyaA showed any cytotoxicity, at concentrations below 0.4 μg/ml in both the MTT and LDH release assays (Fig. 24). This killing at very low toxin concentrations may be related to the fact that caspase 3/7 activity was detected in J774.2 cells after incubation for 24 h with similar low concentrations of CyaA (Fig. 25B). Induction of apoptosis by CyaA has been reported previously (Khelef *et al.*, 1993b; Khelef and Guiso, 1995; Bachelet *et al.*, 2002; Hewlett *et al.*, 2006). In contrast, the enzymically-inactive form, CyaA*, did not induce caspase 3/7 activity and was less efficient at killing J774.2 cells at low toxin concentrations. CyaA* could only kill cells at a toxin concentration greater than 0.1 μg/ml suggesting that a 'quota' of toxin molecules had to be present in order to kill cells in a manner which would lyse cells and release LDH. The possible explanation for this is the ability of CyaA* monomers to self-associate to create oligomeric pores. Native CyaA produced dose-response curves in the MTT and LDH assays similar to CyaA* at toxin concentrations greater than 0.5 μg/ml after incubation for 24 h with J774.2 cells in DMEM, suggesting that native CyaA may also be oligomeric in these conditions.

In agreement with Hewlett *et al.* (2006), the present study showed that the type of cell killing was dependent on toxin concentration whereby, at low toxin concentrations (< 0.1 μg/ml), CyaA principally kills by apoptosis, but at higher toxin concentrations (> 0.1 μg/ml), CyaA efficiently lyses cells. It is likely that these two mechanisms overlap with each other at concentrations between 0.1 and 0.5 μg/ml. Time was an important factor for CyaA-mediated killing, particularly at low toxin concentrations (< 0.5 μg protein/ml) as cell killing was only observed after 24 h but not after 2 h incubation with CyaA. The major difference between CyaA and CyaA* was the greater cytotoxicity exhibited by CyaA* at higher calcium concentrations. This may be related to a greater capacity to self-associate to form oligomeric pores as shown by the faster kinetics of cell killing by CyaA* compared to CyaA (Fig. 23). CyaA* required only increased calcium levels to become at least as

cytotoxic as CyaA. In this regard, CyaA* may be considered functionally similar to HlyA, which lacks the enzymic AC domain. HlyA has a wide spectrum of calcium-dependent cytocidal activity against a variety of cells including human monocytes (Bhakdi *et al.*, 1990) and T-lymphocytes (Jonas *et al.*, 1993).

This study confirmed that AC activity was not required for haemolysis (Sakamoto *et al.*, 1992) as both CyaA and CyaA* were equally haemolytic towards sheep erythrocytes in 1 mM $CaCl_2$ (Table 7). However, maximal haemolytic activity by CyaA for sheep erythrocytes occurred at 1 mM $CaCl_2$ but declined with increasing calcium concentrations (Figs. 19, 20). This confirms previous similar observations by Bellalou *et al.* (1990b) and Ehrmann *et al.* (1991). It has been suggested that higher calcium concentrations may interfere with its activity by promoting aggregation rather than oligomerisation (Rose *et al.*, 1995). In contrast, CyaA* behaved differently. At concentrations of $CaCl_2$ greater than 1 mM, more extensive and faster haemolysis was observed (Figs. 19, 20). This greater haemolytic activity may have been due to the fact that CyaA* requires a greater number of calcium ions for maximum activity (Figs. 19, 22) and may therefore be less susceptible to calcium-mediated aggregation. Indeed, far UV CD analysis showed that CyaA completely changed structure upon the addition of 7.5 mM $CaCl_2$ (~37% β-strands) whereas CyaA* showed only a small decrease of CD spectral amplitude between 200 - 220 nm. This suggests that CyaA is more sensitive in sequestering calcium for changes in secondary structure compared with CyaA* to the extent that an entirely different structure is formed at $CaCl_2$ concentrations > 7.5 mM (Fig. 28A). Although the highest concentration of $CaCl_2$ used in HH buffer was 3 mM, the presence of several other salts may have helped facilitate aggregation of CyaA. It is noteworthy in this context that concentrations of $CaCl_2$ used for haemolysis range from 1 mM to 10 mM (Westrop *et al.*, 1997; Martin *et al.*, 2004; Vojtova *et al.*, 2006) making it difficult to compare the haemolytic activity of CyaA from different laboratories.

The present data and that of Prior *et al.* (2005) showed that the concentration of CyaA required for 50% LDH release from J774.2 cells after incubation for 2 h when assayed in DMEM was greater than the concentration required for 50% killing by CyaA in the MTT assay when assayed in DMEM (Fig. 21). This could in part be explained by the lag period required for LDH release whereas killing measured by the MTT assay occurred without a lag period (Fig. 23). It is known that upon exposure of cells to CyaA, cAMP accumulation is almost immediate and the accumulation of intracellular CyaA proceeds without any noticeable lag period (Farfel *et al.*, 1987; Gentile *et al.*, 1988; Gordon *et al.*, 1989). Killing, measured by the MTT assay, may therefore reflect accumulation of the toxin within the cell. It is noteworthy that AC enzymic activity is not required for the

killing measured by the MTT assay as CyaA* is as active as CyaA, at higher calcium concentrations, and in fact has faster kinetics (Fig. 23). The requirement for greater toxin concentrations to kill 50% of cells in the LDH assay also suggests that formation of pores large enough to release LDH involves more toxin molecules in an oligomeric form. The immediate onset of cell death in the MTT assay may well be due to the ability of CyaA to uncouple oxidative phosphorylation of mitochondria (Bachelet *et al.*, 2002) which is believed to be the basis for the MTT assay (Slater *et al.*, 1963). The present data indicate that CyaA* acts in a similar way, at an even faster rate (Fig. 23) showing that this cytotoxic effect is independent of AC enzymic activity.

Other reports have indicated that the non-enzymically active form of CyaA, equivalent to CyaA* used here, were non-cytotoxic (MacDonald-Fyall *et al.*, 2004; Prior *et al.*, 2005; Hewlett *et al.*, 2006) or required high protein concentrations (Boyd *et al.*, 2005; Basler *et al.*, 2006a) for lysis of J774.2 cells, yet were haemolytic towards erythrocytes (Hewlett *et al.*, 2006). In the present study, cytotoxicity of CyaA* alone was dependent on calcium for oligomerisation and pore-formation and it might be expected that this type of activity would be operative against both erythrocytes and macrophages. Hewlett *et al.* (2006) reported that their enzymically-inactive CyaA form was cytotoxic, but not apoptotic, towards J774.2 cells when assayed in DMEM, although this apparently occurred only after pre-incubation with a monoclonal antibody which blocked delivery of the N-terminal enzymic domain into target cells. It would therefore be of interest to investigate if the lack of cytotoxicity of enzymically-inactive CyaA forms by other groups can be restored in the presence of high calcium concentrations.

In the present study, proCyaA was not cytotoxic or apoptotic towards J774.2 cells (data not shown) which is in contrast to the findings of Boyd *et al.* (2005) and Hewlett *et al.* (2006). However, although proCyaA and proCyaA* were inactive up to 10 μg protein/ml in the cytotoxicity and haemolysis assays used here, proCyaA was able to inhibit the zymosan-stimulated oxidative burst by J774.2 cells with 50% inhibition at a concentration of 1.48 μg/ml. Inhibition of the zymosan-stimulated oxidative burst was the most sensitive assay used to assess cytotoxicity in the present study, with CyaA giving 50% inhibition at 0.003 μg/ml. Thus, this assay provided evidence that proCyaA was able to intoxicate cells, albeit at a level some 500-fold less than CyaA in the oxidative burst assay. This agrees with the work of Hewlett *et al.* (2006) who showed that at toxin concentrations greater than 1 μg/ml, non-acylated CyaA was able to increase cAMP levels in J774.2 cells. The fact that proCyaA* was ineffective and CyaA* was 500-fold less effective than CyaA, agrees with previous work that this inhibition is cAMP dependent (Pearson *et al.*, 1985). CyaA* may inhibit the zymosan-stimulated oxidative burst by

causing a loss of cell membrane integrity by the production of pores. In addition, this inhibition could be due to the dissociation of the NADPH oxidase complex or by interference with phosphorylation of the cytosolic protein components (El-Benna *et al.*, 2005). Thus, taken together, our data imply that the efficient inhibition of the zymosan-stimulated oxidative burst and the induction of apoptosis in J774.2 cells is dependent on both acylation and the AC enzymic activity of CyaA but that acylation is most important for general cytotoxicity, implying that this effect is dependent on pore-formation at toxin concentrations above 0.1 μg/ml.

To assess whether conformational changes related to the interaction with calcium in the different CyaA forms could account for the temporal and quantitative differences in cell killing by CyaA or CyaA* observed *in vitro*, the different CyaA forms were subjected to analysis by far UV CD, near UV CD and fluorescence. The spectra of CyaA observed in the presence and absence of 1 mM $CaCl_2$ were comparable to previous CD spectra with regard to spectral intensities (Rose *et al.*, 1995; Bauche *et al.*, 2006). However, our analyses suggested there were, in general, only small changes in the secondary structure contents in the presence of $CaCl_2$ (Table 8). CyaA*, as well as the two non-acylated forms, showed similar changes in spectra compared with CyaA, suggesting that they were all similar in structure and that the addition of calcium induced the same type of changes. In addition, a high resolution tertiary structure model for the N-terminal domain of CyaA* was constructed, with the help of Dr. Olwyn Byron (Division of Infection and Immunity, University of Glasgow), based on its sequence homology with the N-terminal domain of native CyaA (for which an atomic resolution structure is available - 1YRT.pdb (Guo *et al.*, 2005)). From this model, it was apparent that the di-peptide insertion in the N-terminal domain did not affect the overall protein folding or structure, as it was comparable to that of native CyaA (data not shown).

Although, there were little changes in the estimated secondary structure on addition of $CaCl_2$, the spectral changes from far UV CD observed on addition of $CaCl_2$ to the CyaA forms may have been due to a rearrangement or reorientation of the secondary structural elements with respect to each other (Fig. 28C). The near UV CD and fluorescence data pointed to a calcium-induced conformational change in CyaA which resulted in partial burial and immobilisation of aromatic side chains. It has been demonstrated that small backbone conformational distortions can lead to marked changes in CD signal (Manning *et al.*, 1988). Indeed the ratio of ellipticities at 222 nm and 208 nm moves from a value of 0.73 to 1.10 for all CyaA forms on addition of $CaCl_2$. Such a change would be consistent with isolated helices interacting to form structures of a coiled coil type (Lau *et al.*, 1984; Zhou *et al.*, 1992) or could indicate the rearrangement of β-structures into a helical-like

pattern (Perczel and Fasman, 1992) of the type proposed for a parallel β-roll motif within CyaA (Rose *et al.*, 1995). Studies on the alkaline protease from *P. aeruginosa* containing similar nonapeptide sequence repeats have shown that these repeats are involved in calcium binding and give rise to a parallel β-helix or parallel β-roll structure (Baumann *et al.*, 1993). Similarly, chemically-synthesised proteins containing 6 glycine-rich repeats have been shown to give rise to a marked increase in the ellipticity value obtained at 220 nm following calcium binding and analysis by circular dichroism (Lilie *et al.*, 2000). One of the weaknesses of circular dichroism as a structural technique is the fact that contributions from β-structures are generally masked in α/β proteins by more dominant n to π^* and π to π^* transitions contributed by the α-helices. For this reason, it is difficult to distinguish whether the structural rearrangements observed are due to those involving α-helices, β-structures or indeed both. Some model proteins containing all β-structures have been shown to give rise to CD spectra which resemble α/β proteins. In the case of the parallel β-roll structure, it would not be surprising if the β-helical nature of this motif gave rise to a spectrum resembling that of α-helices given the number of β-turns present in this conformation.

An interesting observation from the far UV CD spectra was that there was an approximately 2-fold decrease in spectral amplitude and a small change in spectral shape when CyaA was diluted from 0.5 mg/ml to 0.05 mg/ml (Fig. 28C). This type of spectral change has been previously observed in the case of dilution of a solution of a 43-residue model peptide derived from the N-terminal domain of tropomyosin (Greenfield and Hitchcock-DeGregori, 1993), and has been interpreted as reflecting dissociation of a coiled-coil structure to form isolated helices. In the case of CyaA, the far UV CD data may indicate that CyaA can assume both monomeric and oligomeric forms in solution, depending on the protein concentration. This would help support the concept that a monomeric form of CyaA induces cAMP accumulation and apoptosis in J774.2 cells at low toxin concentrations, whereas at greater concentrations, an oligomeric form of CyaA kills cells through pore formation as indicated by the *in vitro* experiments in this study. Lee *et al.* (2005) demonstrated the self-association of CyaA monomers, a process that they indicated was dependent on acylation and not calcium but the fact that the spectra of proCyaA and proCyaA* were identical to that of native CyaA in the presence and absence of calcium suggests that non-acylated CyaA is oligomeric in solution. Acylation may only facilitate interaction with target cells expressing the CR3 receptor (El-Azami-El-Idrissi *et al.*, 2003).

In an attempt to investigate the behaviour of CyaA in solution, analytical ultracentrifugation was used. Preliminary SE studies indicated that dialysed CyaA was not

a single homogenous species in solution as the data revealed that there were many different molecular weight species in the absence and presence of $CaCl_2$. Both SE and SV analyses were affected by thermodynamic non-ideality in the absence of $CaCl_2$ (Sections 3.4.10.2 and 3.4.10.3). Non-ideality can be caused by at least two factors: 1. repulsion between charged molecules (proteins) that are not shielded by solvent counter-ions; 2. size-exclusion effects arising from extended/elongated conformations. In both cases, the proteins do not compact efficiently when a centrifugal force is applied, thus interfering with the data analysis. In addition, the greater the centrifugal force, the greater the effect of non-ideality. The effects of non-ideality are decreased when less protein is present. This was particularly evident for CyaA in the absence of $CaCl_2$ whereby the sedimentation coefficient of the main peak increased as the protein concentration was decreased (Fig. 31A). In addition, a M_w of 140 kDa was observed, but not 170 kDa (the M_w of CyaA). The addition of NaCl, which would deliver monovalent Na^+ and Cl^- cations could help to reduce the effects of non-ideality by shielding the proteins so that the charges present on the surface of the proteins cannot repel each other as significantly. This would also indicate whether the perceived non-ideality is being caused by charges on the protein surface or by elongation of CyaA. It is noteworthy that interpreting the SE and SV data for CyaA in the presence of $CaCl_2$ is not a simple matter. In addition to possibly helping to reduce the effects of non-ideality, $CaCl_2$ also induces changes in CyaA structure (Hewlett *et al.*, 1991; Rose *et al.*, 1995; Section 3.4.9) that may also influence the data analysis. Therefore, the results from these studies must be interpreted with caution.

SE analyses of CyaA in the absence and presence of 1 mM $CaCl_2$, indicated the appearance of many M_w species ranging from 20 to > 600 kDa. Studies have shown that CyaA can undergo auto-proteolysis (Ladant *et al.*, 1986; Rogel *et al.*, 1988; Bellalou *et al.*, 1990a) to release a 40 kDa fragment which has AC enzymic activity (Gilboa-Ron *et al.*, 1989). The fact that a 40 kDa M_w species, as well as other species at 20 – 80 kDa, were detected by SE analysis suggests that CyaA had degraded. Reducing centrifugation times could reduce degradation. On the other hand, high M_w CyaA species were detected in both the absence and presence of calcium, although more species were identified in a solution of dialysed CyaA containing 1 mM $CaCl_2$ compared with no $CaCl_2$. Such high M_w species could be obtained by the formation of oligomers composed of two or three CyaA monomers. This is in agreement with other studies (Iwaki *et al.*, 1995; Lee *et al.*, 2005) that have suggested the requirement of 2 – 4 CyaA monomers for pore formation in target membranes. However, the presence of several other species, which do not fit with the 2 or 3-mer model (Fig. 30 Table), may be due to aggregation between monomers and degradation products (Rose *et al.*, 1995). Interestingly, amongst the different species, one

at 170 kDa was observed in the presence of $CaCl_2$ suggesting that CyaA may freely associate and dissociate between an oligomeric and monomeric form in solution in the presence of calcium.

These SV data correlated with the SE data suggesting that CyaA did not occur as a single species in solution as indicated by the numerous peaks at high toxin concentrations. SV analysis of CyaA, in the presence of $CaCl_2$, at lower toxin concentrations (0.35 mg/ml) still showed the presence of several peaks (Fig. 31B). To answer if CyaA could exist as a single M_w species at low concentrations, as suggested by CD studies, further studies could be performed with AUC using toxin concentrations lower than 0.35 mg/ml. Until the crystal structure of CyaA is solved, the mechanism of the association of the CyaA monomers remains elusive.

Chapter 5

Discussion - Protective and immunological responses in mice to ACV plus different CyaA forms

One of the objectives of this study was to assess the relative contributions of the AC enzymic activity and the pore-forming/invasive features of CyaA to the protective and adjuvant properties when co-administered with an ACV. This was done by using four different purified recombinant forms of CyaA. The properties of these different CyaA forms have been discussed in Chapter 4. Importantly, all the CyaA forms in the present study contained very low levels of LPS which, at the concentrations used, minimised any potential synergistic effect of CyaA with LPS (Ross *et al.*, 2004).

5.1 Protection experiments

In this present study, post-translational acylation of CyaA was essential for the enhanced protective effect towards the ACV against intranasal challenge with *B. pertussis*. Mice immunised intraperitoneally twice with ACV + CyaA or CyaA*, but not with the two non-acylated forms, proCyaA or proCyaA*, had reduced bacterial numbers in the lungs at 7 days post-challenge compared with the ACV alone group. Significantly more protection ($P < 0.05$) was afforded when CyaA* (CyaA which lacks AC enzymic activity) was co-administered with ACV compared with CyaA and the enhanced protective effect of CyaA* was found to be dose-dependent. However, the protection experiment with ACV plus different forms of CyaA was performed only once. Thus, whether CyaA* could reproducibly enhance the protection afforded by the ACV to a greater extent than CyaA was not tested. On the other hand, the ability of CyaA* to enhance the protective effect of ACV was tested three times. The extent of reduction in bacterial colonisation of the lungs and tracheas between mice that had received ACV alone or ACV + CyaA* (25 μg/dose) was variable ($\log_{10}$ reductions of 1.38 (Section 3.5.1), 2.49 (Section 3.5.2) and 1.65 (Section 3.5.3)) due to the nature of the *in vivo* bioassay but all three independent experiments showed a similar trend such that the bacterial counts in mice immunised with ACV + CyaA* was statistically lower compared with those of the ACV control group ($P < 0.05$).

Mice immunised with 25 μg of the CyaA forms were not significantly protected against intranasal challenge. This indicated that the enhanced protective effect of CyaA* for ACV was more than the sum of the protective effects of CyaA* and ACV alone. The lack of protection afforded by any of the CyaA forms alone contrasted with previous

reports where recombinant CyaA was shown to act as a protective antigen in mouse models of *B. pertussis* infection (Betsou *et al.*, 1993; Hormozi *et al.*, 1999). Although there was little protection provided by any of the CyaA forms in our intranasal challenge model, a clear protective effect was seen in mice by all of the CyaA forms in an aerosol challenge model with a lower challenge dose. However, only mice immunised with proCyaA, proCyaA* and CyaA were significantly more protected than the PBS control group. MacDonald-Fyall (2002) showed that in mice immunised intraperitoneally once with CyaA or CyaA* there was a 50% reduction in bacterial numbers in the lung by 2-fold compared with the PBS control group after aerosol challenge with *B. pertussis*. This agrees with previous results from intranasal challenge models where CyaA protected mice when a lower challenge dose was used (Guiso *et al.*, 1991; Khelef *et al.*, 1992; Betsou *et al.*, 1993; Hormozi *et al.*, 1999; MacDonald-Fyall, 2002). Non-acylated proCyaA and proCyaA* also protected mice against *B. pertussis* challenge in the aerosol model. This contrasts with previous work where proCyaA was reported to be non-protective, even with a low intranasal challenge dose (Khelef *et al.*, 1992; Betsou *et al.*, 1993; Hormozi *et al.*, 1999). The reasons for this discrepancy may be related to the longer immunisation schedule (56 days) adopted for our protection experiment, the route of immunisation, and differences in antigen dose, adjuvant and mouse strains compared with those used previously (Betsou *et al.*, 1993; Hormozi *et al.*, 1999). The protection experiment with the different CyaA forms alone and aerosol challenge was performed only once. Thus, whether the different forms of CyaA could reproducibly enhance the protection against *B. pertussis* aerosol challenge was not determined. A possible explanation as to why mice immunised with the different CyaA forms, in the presence of alum, were protected in the aerosol challenge could have been because of the high total IgG anti-CyaA antibody levels (Section 3.6.3.1, Table 13). The spleen cells and peritoneal macrophages from mice immunised with CyaA* alone were also responsive to antigen stimulation (Section 5.2) suggesting that both humoral and cell-mediated responses to CyaA could have a role in protection. However, this warrants further investigation.

5.2 Humoral and cell-mediated responses

The enhanced protection in mice immunised with ACV + CyaA* against *B. pertussis* intranasal challenge did not correlate with the total IgG antibody levels to FHA, PRN or PT because there were no statistically significant differences between groups that received ACV alone compared with the combination groups. This again contrasts with previous work (Hormozi *et al.*, 1999; MacDonald-Fyall *et al.*, 2004; Ross *et al.*, 2004) which showed that CyaA or CyaA* could act as an adjuvant by enhancing the levels of total IgG antibodies to co-administered antigens. This discrepancy may be due to the fact

that, in one report, the data were obtained after a single vaccination via the intraperitoneal route (MacDonald-Fyall *et al.*, 2004) or, in the other reports, where two vaccinations were used, a smaller dose of CyaA or CyaA* was administered subcutaneously (Hormozi *et al.*, 1999; Ross *et al.*, 2004). In addition, the ACV used here contained alum as adjuvant which may have increased the antibody levels to FHA, PRN and PT irrespective of the presence of the CyaA form. The levels of anti-CyaA total IgG antibodies were significantly ($P < 0.05$) lower in mice that were co-immunised with ACV plus CyaA forms compared with mice immunised with the CyaA forms alone in the presence of alum (Tables 10, 13). However, mice immunised with different CyaA forms in the absence of alum produced similar levels of anti-CyaA total IgG antibodies to mice immunised with ACV plus the different CyaA forms. This might suggest that the increased levels of anti-CyaA total IgG antibodies were due to the types and amounts of aluminium compounds in the immunising preparations. Alum (containing $Al(OH)_3$ and Al_2O_3) was used with CyaA forms alone, compared with the ACV which contained $Al(OH)_3$ only and where the CyaA forms added did not contain alum. Alternatively, the differences in anti-CyaA antibody titres may be due to antigenic suppression which was observed in other cases of combined vaccines (Vidor *et al.*, 1999). For example, when the *Haemophilus influenzae* capsular polysaccharide (conjugated to tetanus toxoid) vaccine is co-administered with DTaP, antibodies to the polysaccharide were significantly reduced in infants compared with those who received the vaccines separately (Vidor *et al.*, 1999). Further investigation is needed to confirm the reasons for the differences in anti-CyaA total IgG titre.

ACVs have been shown to induce high levels of anti-pertussis IgG1 antibodies in mice (Barnard *et al.*, 1996) and this was also observed in this study. However, the presence of the different CyaA forms did not alter the levels of anti-FHA, -PRN or -PT IgG1 antibodies in mice although mice immunised with ACV + CyaA* produced significantly greater anti-CyaA IgG1 antibodies than the ACV + proCyaA* group. Mice immunised with ACV + CyaA* also promoted significantly higher levels of anti-PRN IgG2a antibody production compared with mice immunised with ACV alone. IgG2a has been implicated in opsonisation and complement fixation of *B. pertussis* and associated with superior protection (Mahon *et al.*, 1997; Mills *et al.*, 1998). PRN has been reported to play a role in adhesion of *B. pertussis* to mammalian cells (Everest *et al.*, 1996). Therefore, increased levels of anti-PRN IgG2a antibodies could presumably decrease the ability of *B. pertussis* to adhere and could promote its clearance from the respiratory tract.

Humoral immunity alone may not be sufficient to confer long-term protection against *B. pertussis* infection and the importance of cell-mediated immunity in the clearance of *B. pertussis* has been demonstrated (Mills *et al.*, 1993; Mahon *et al.*, 1996;

Barbic *et al.*, 1997; Ryan *et al.*, 1997a; Leef *et al.*, 2000). ACVs typically induce a Th2-associated T-cell response in mice, characterised by high levels of anti-pertussis IgG1 antibodies as well as Th2-associated cytokines, including IL-4 and IL-5, with little IFNγ production (Redhead *et al.*, 1993; Barnard *et al.*, 1996; McGuirk and Mills, 2000b). In humans, ACVs produce a more mixed Th1/Th2 response, including IL-4, IL-5, IFNγ and IgG2a production (Ausiello *et al.*, 1997; Ryan *et al.*, 1998b). Cytokines secreted by immune effector cells, such as T-cells, play a key role in determining IgG isotype production and the outcome of immune responses to infectious agents. In the present study, the cytokine profiles of spleen cells and NO production from peritoneal macrophages from immunised mice after stimulation with various antigens were rather complex but some conclusions could be drawn from these data.

The spleen cells of mice immunised with ACV plus the non-enzymic CyaA forms (CyaA* or proCyaA*) responded well to antigen stimulation by secreting higher levels of IL-5, IL-6, IFNγ and GM-CSF than cells from mice immunised with ACV alone. CyaA*-stimulated spleen cells from mice immunised with CyaA* alone were less able to produce IFNγ and GM-CSF upon antigen stimulation than spleen cells from mice immunised with ACV + CyaA* although they produced comparable levels of IL-5 and IL-6. Thus, the proCyaA* and CyaA* forms of CyaA appeared to be promoting a mixed Th1/Th2 response to *B. pertussis* antigens, which was most pronounced with the ACV + CyaA* group. In particular, spleen cell production of IFNγ and GM-CSF was greatly enhanced compared to that obtained from mice immunised with ACV or CyaA* alone, indicating that proCyaA* and particularly CyaA* had an adjuvant effect for production of these cytokines when administered with ACV. Spleen cells from mice immunised with ACV + proCyaA or ACV + CyaA responded to antigen stimulation in a similar way to cells from ACV-immunised mice producing little or no IFNγ or GM-CSF.

GM-CSF, produced principally by fibroblasts, monocytes/macrophages, endothelial cells and T cells (Liles and Van Voorhis, 1995), has been shown to activate monocyte/macrophages and neutrophil PMNLs *in vitro* and to promote maturation of DCs *in vivo* (Wang *et al.*, 2000). Such effects include enhanced chemotaxis (Wang *et al.*, 1987), phagocytosis (Fleischmann *et al.*, 1986), respiratory burst and superoxide anion generation (Weisbart *et al.*, 1987). They also include adjuvant effects to boost maturation of dendritic cells for increased antigen presentation (Bowne *et al.*, 1999; Lu *et al.*, 2002, Wang *et al.*, 2000), to promote a protective Th1-orientated immune response in mice vaccinated with a BCG vaccine (Wang *et al.*, 2002) or *Chlamydia trachomatis* elementary bodies (Lu *et al.*, 2002) and to promote an antigen-specific inflammatory response to acrosolised ovalbumin characterised by the production of the Th2-associated cytokines IL-4 and IL-5 (Stampfli *et*

al., 1998). Thus, enhanced GM-CSF production due to the presence of CyaA* in ACV could enhance presentation of the antigens and promote both Th1 and Th2 immune responses.

Higher levels of IL-8, a pro-inflammatory cytokine with lymphocyte chemo-attractant properties (Baggiolini *et al.*, 1994), were detected from HKC-stimulated peritoneal macrophages from mice immunised with ACV plus the different CyaA forms compared with the CyaA* control group. Interestingly, IL-8 mRNA has also been detected from *B. pertussis*-infected bronchial epithelial cells (Belcher *et al.*, 2000). Both IL-8 (Detmers *et al.*, 1991) and GM-CSF (Williams *et al.*, 1995) have been reported to promote the expression of the CD11b/CD18 (CR3) receptor found on the surface of neutrophil PMNLs and monocytes. CR3 is a receptor for several *B. pertussis* virulence-associated factors, including FHA, PRN and CyaA (Everest *et al.*, 1996; Mahon *et al.*, 1996; Guermonprez *et al.*, 2001). Therefore, it is conceivable that increased CR3 expression, that might result from greater GM-CSF production, could enhance phagocytosis of *B. pertussis*. In addition, neutralising anti-CyaA antibodies induced by mice immunised with ACV + CyaA or ACV + CyaA* (MacDonald-Fyall *et al.*, 2004; Section 3.6.1.2.1) would block the inhibitory action of CyaA on neutrophil PMNLs (Mobberley-Schuman *et al.*, 2003, Weingart and Weiss. 2000; Weingart *et al.*, 2000) and also enhance clearance of the organism from the lungs.

Macrophages from mice immunised with ACV + CyaA* produced more NO after HKC and antigen stimulation than macrophages from mice immunised with CyaA* alone or ACV alone (Fig. 37), suggesting that these macrophages are highly activated (Torre *et al.*, 1996; Xing *et al.*, 1998; Canthaboo *et al.*, 2002). Thus, increased levels of GM-CSF and NO in mice immunised with ACV + CyaA* could also enhance the uptake and presentation of antigens through the activation of antigen-presenting cells, such as peritoneal macrophages (Xing *et al.*, 1999) and dendritic cells (Bowne *et al.*, 1999; Wang *et al.*, 20000).

The HKC preparation was, except for IL-5, by far the best stimulant for cytokine production from spleen cells and NO production from macrophages. In some cases, cytokine levels from the spleen cells of PBS control mice were at levels similar to those from spleen cells from mice immunised with the antigen preparations, when stimulated with HKCs. This is most likely due to the high levels of LPS in the HKC preparation. LPS is a potent inducer of IL-12 in spleen cells (Mahon *et al.*, 1996). This cytokine was produced in large amounts by spleen cells from PBS control mice upon exposure to HKC. On the other hand, less IL-12 was released from cells from immunised mice upon antigen stimulation than from unstimulated cells. This may have been due to the presence of FHA

in the PAgs antigen mixture as FHA has been shown to suppress IL-12 production in mice (McGuirk and Mills, 2000a). CyaA* treatment of spleen cells also appeared to suppress IL-12 production compared to unstimulated cells. It was clear that the level of LPS contamination in the PAg mixture or in the CyaA* preparation was insignificant, as little or no cytokine release was seen in the cells from the PBS control group upon stimulation with these antigens.

Small amounts of IL-1β were also detected from antigen stimulated-peritoneal macrophages from mice immunised with ACV plus the different CyaA forms and CyaA* alone but not from the ACV alone or PBS control groups. Greater levels of TNFα were produced from the macrophages of mice immunised with ACV + CyaA or CyaA* compared with the other groups. Indeed, IL-1β and TNFα were both produced in greater amounts by the human monocyte cell line THP-1 after stimulation with WCV than with ACVs (Blood-Siegfried *et al.*, 1998) and the enhanced production of these two cytokines were believed to be attributed to LPS. Interestingly, in our study, HKC stimulation of macrophages did not induce IL-1β. However, the enhanced production of TNFα by macrophages of mice immunised with ACV + CyaA or CyaA* correlates with enhanced protection in these mice.

The data presented indicate that both CyaA and CyaA* have the ability to enhance the protective effects of an ACV, but they may act in different ways. AC enzymic activity and binding of a receptor by the toxin may have distinct modulatory effects on cells of the immune system. Bagley *et al.* (2002) reported that CyaA was a potent activator for maturation of human monocyte-derived dendritic cells and that this activity was dependent on the ability of CyaA to raise intracellular cAMP concentrations. Also, CyaA and non-acylated CyaA were reported to induce antigen-specific $CD4^+$ Th2 and Tr1 regulatory cells, but an acylated, non-enzymically active form of CyaA, equivalent to CyaA* used here, was unable to act in the same way as the enzymically-active forms (Ross *et al.*, 2004; Boyd *et al.*, 2005), indicating that these effects were dependent on raised cAMP levels. The lack of adjuvanticity observed by Boyd *et al.* (2005) for their CyaA*-equivalent toxin may be related to a different route of immunisation or to the concentration of toxin used (1 μg/dose). Enzymically-inactive mutants of the *E. coli* cAMP-elevating heat-labile toxin (LT) have no adjuvanticity at very low doses but their adjuvanticities are enhanced by increasing their doses (Pizza *et al.*, 2001).

The AC enzymic domain of CyaA does not need to be active for efficient antigen presentation, as CyaA lacking AC activity and with T-cell epitopes inserted within the N-terminal AC domain can act as an efficient delivery vehicle to stimulate both cell-mediated and humoral immunity to these epitopes (Simsova *et al.*, 2004). Delivery of viral and *M.*

tuberculosis epitopes by AC-deficient CyaA generated both $CD4^+$ and $CD8^+$ epitope-specific T cells (Simondon *et al.*, 1997; Mascarell *et al.*, 2005; Wilkinson *et al.*, 2005). The $CD4^+$ T cells were characterised as IFNγ-producing Th1 cells which indicated that induction of these T-cell responses was by a mechanism not involving increased intracellular cAMP production, such as cell-signalling events subsequent to receptor binding. Thus, whereas CyaA may favour induction of antigen-specific Th2-orientated responses via a mechanism dependent on increased intracellular cAMP, CyaA* appears to favour a more mixed Th1/Th2 response involving increased production of IL-5, IL-6, GM-CSF and IFNγ from spleen cells and IL-8, TNFα and NO from peritoneal macrophages.

Boyd *et al.* (2005) reported that non-acylated CyaA was able to act as an adjuvant to increase total IgG responses to a co-administered antigen and to boost antigen-specific IL-4, IL-5 and IL-10 production, *in vitro*, by popliteal lymph node cells obtained from immunised mice. This is presumably because proCyaA can intoxicate target cells with low avidity (Boyd *et al.*, 2005; Hewlett *et al.*, 2006; Sections 3.4.4 (inhibition of the oxidative burst) and 3.4.5.1 (apoptosis)). The fact that alum was a constituent of the ACV used here may have masked any potential adjuvant effect that either of the proCyaA forms may have had towards the ACV. In addition, it would be of interest to investigate whether the proCyaA and proCyaA* forms could enhance the protective properties of ACV in the aerosol challenge model. It would also be informative to investigate whether the different CyaA forms could reproducibly protect mice against aerosol challenge with *B. pertussis*. Ross *et al.* (2004) also demonstrated that LPS could synergise with CyaA to modulate the pro-inflammatory response of macrophages and dendritic cells. Therefore, even though the CyaA preparations used here contained very low levels of LPS, it cannot be ruled out that there may have been some synergism between CyaA and LPS at the concentration of toxin used. This problem could be addressed using CyaA expressed in *E. coli* BL21/DE3 *lpxM* that was shown to induce significantly less IL-6 secretion by MM6 cells compared with CyaA expressed from *E. coli* BL21/DE3. Urea could also be problematic for inclusion in vaccines. Some preliminary structural studies, using far UV CD, suggested that CyaA could be stably maintained in urea concentrations lower than 8 M urea (Fig. 29). A combination of both of these factors would allow the production of CyaA in a form more suitable for the inclusion in ACVs. However, further tests should be performed to evaluate the safety of CyaA* as a possible vaccine candidate as studies have shown that antibodies to CyaA can cross-react with mammalian AC (Monneron *et al.*, 1988). In addition, the *in vivo* bioassay used in this study may not have been sensitive enough to pick up the possible toxicity of the different CyaA forms (Prior *et al.*, 2005). Therefore, other more sensitive detection methods are required evaluate the possible toxicity properties of CyaA forms.

In summary, these results show, for the first time, that the presence of enzymically-inactive CyaA form, CyaA*, can significantly enhance protection afforded by ACV in mice. The adjuvant properties of the CyaA* derivative suggest that it has potential as a vaccine component through enhancement of both Th1 and Th2 immune responses.

Chapter 6

Discussion – Global gene responses in murine bone marrow-derived macrophages to different CyaA forms

The mechanisms by which CyaA mediates it cytotoxic effects on cells is largely unknown although cAMP-dependent signalling pathways are thought to play an important role. Microarray technology was used to provide more information on the mechanisms mediated by CyaA upon exposure to bone marrow-derived macrophages (BMMs). Since the BMMs express the CR3 receptor, these cell should be susceptible to CyaA intoxication. The gene responses in BMMs in response to CyaA may also help us more fully understand the ways that *B. pertussis* causes damage during infection. BMMs, treated with 20 ng/ml of CyaA for 24 h, showed over 1000 significant changes in gene transcription compared with treatment with the same concentration of CyaA*, proCyaA* or with urea alone. The alteration of so many genes in BMMs after CyaA treatment is most likely due to the increase of cAMP caused by the invasive function of CyaA; no genes were altered in BMMs after treatment with proCyaA*. Only two genes were significantly altered by CyaA* and not by CyaA. These genes coded for *Aoah* (acyloxyacyl hydrolase) and *Irg1* whose expressions were both up-regulated by 2.17 and 4.3-fold, respectively. AOAH is an enzyme that hydrolyses acyloxyacyl bonds in the lipid A region of LPS, thereby detoxifying the LPS (Munford and Hall, 1989). By contrast, little is known about Irg1 except that its expression is increased by LPS (Lee *et al.*, 1995) and that Irg1 has also been detected in murine macrophages upon *in vitro* stimulation with *Mycobacterium smegmatis* (Basler *et al.*, 2006b). The significance of up-regulation of these genes by CyaA* is unknown. It would be of interest to perform more microarray analyses with greater concentrations of CyaA* and proCyaA* to investigate the types of genes that are regulated by pore-formation and AC activity induced by CyaA, respectively.

6.1 CyaA causes up-regulation of genes involved in the activation of T cells

The transcription profile for BMMs treated with CyaA suggested an activated state as indicated by increased transcription of co-stimulatory molecules encoded by *CD80* (B7-1) and *CD86* (B7-2). APCs expressing CD80 and CD86 can both engage with CD28, expressed on T cells, which then helps to activate T cells in conjunction with the TCR/MHC class II complex (Section 1.6). Bagley *et al.* (2002) showed increased expression of both CD80 and CD86 in human monocyte-derived dendritic cells (DCs) upon exposure to CyaA for 20 h. By contrast, Boyd *et al.* (2005) failed to detect significant

enhancement of CD86 expression on the cell surface of murine DCs after treatment for 24 h with CyaA, although CyaA enhanced CD80 and MHC-II expression. According to our microarray data, MHC-II gene transcription was decreased after treatment for 24 h with CyaA and this agrees with a study by Kambayashi *et al.* (2001) who showed that cAMP-elevating agents suppressed the surface expression of MHC-II on murine bone marrow-derived DCs.

CD207, the mouse homologue of the human langerin receptor, was markedly up-regulated by 141-fold. CD207 is a C-type lectin restricted to a subset of dendritic cells called langerhans cells (Takahara *et al.*, 2002). It is thought to interact with CD1a to present non-peptide antigens, such as sugar-containing molecules found on bacterial membranes, to T cells (Hunger *et al.*, 2004). Surprisingly, CyaA induced the transcription of the integrin α_M gene, *Itgam*, which forms one half of the CR3 (CD11b/CD18) receptor although it was reported that CyaA was not able to increase CR3 expression on human neutrophil PMNLs *in vitro* (Mobberley-Schuman and Weiss, 2005). There was up-regulation (3.7-fold) of semaphorin 4a (Sema4A), a transmembrane protein which is preferentially expressed on bone marrow derived and splenic APCs, providing a co-stimulatory signal to activate T cells (Kumanogoh *et al.*, 2002). Sema4A is also expressed on activated T cells and may promote conditions favourable for Th1 differentiation in mice (Kumanogoh *et al.*, 2005). TREM-1, a member of the immunoglobulin superfamily, was up-regulated 50-fold. In the presence of bacteria or fungi, TREM-1 has been shown to be up-regulated on phagocytic cells *in vivo* and *in vitro* (Bouchon *et al.*, 2001). Moreover, human primary monocytes activated by TREM-1 have an improved ability to elicit T cell proliferation and production of IFNγ (Bleharski *et al.*, 2003).

6.2 CyaA induces inflammatory cytokine and chemokine transcription

6.2.1 IL-1β transcription

The genes encoding the pro-inflammatory cytokine, IL-1β (*Il1b*), and its corresponding receptor, *Il1r2*, and accessory protein, *Il1rap*, were significantly up-regulated. This is interesting because there was no IL-1β detected in the supernates of BMMs after treatment for 24 h with CyaA (data not shown). IL-1β is synthesised as a cytosolic 31 kDa precursor protein which must be cleaved by the IL-1β converting enzyme (ICE) to release the 17.5 kDa mature form of IL-1β (Wilson *et al.*, 1994). If IL-1β is not cleaved in BMMs, this would explain the lack of IL-1β detected in the supernates. A Western blot with monoclonal antibodies to full length or mature IL-1β could be performed on cell lysates to determine if this was the case. LPS is known to stimulate IL-

1β production and the possibility of residual LPS in the CyaA preparation inducing gene changes in this experiment is discussed in Section 6.6.

6.2.2 Chemokine transcription

CyaA altered the transcription of several types of chemokines in BMMs. The chemokine family is comprised of four subgroups: two important subgroups include the CXC chemokines have an amino acid 'X' located between the first two conserved cysteine residues and are particularly chemotactic for neutrophil PMNLs whereas CC type chemokines do not have an amino acid between the first two conserved cysteine residues and are chemotactic for T cells and monocytes. Interestingly, *Ccl4* (Mip-1β) and *Ccl3* (Mip-1α) transcription was down-regulated by CyaA. Mice deficient in Ccl3 are more susceptible to *Klebsiella pneumoniae* infection (Lindell *et al.*, 2001) as well as a number of viral and parasitic infections (Salazar-Mather *et al.*, 2000; Olszewski *et al.*, 2000). Thus, down-regulation of this chemokine by CyaA may facilitate *B. pertussis* infection. Other chemokines that were highly up-regulated included *Cxcl7* (305-fold) and *Cxcl5* (25-fold). Cxcl7 and Cxcl5 are both chemotactic and have been shown to promote adhesion and transmigration of neutrophil PMNLs *in vitro* (Schenk *et al.*, 2002; Chandrasekar *et al.*, 2003). However, Cxcl5 also has the ability to promote IL-1β mRNA transcription in rat cardiac-derived endothelial cells (Chandrasekar *et al.*, 2003). These chemokines are thus pro-inflammatory and their up-regulation by CyaA, which might encourage neutrophil influx, would seem to be counter-productive for the establishment of infection.

Sustained or excessive production of inflammatory cytokines, such as IL-1β, can have damaging consequences for the host such as enhancing the invasiveness of some bacteria by increasing tissue destruction (Sansonetti *et al.*, 1995). In response, the immune system has evolved systems to prevent excessive cytokine signalling. One group of cytokine-induced proteins, known as suppressors of cytokine signalling (SOCS) proteins alter cytokine signalling via feedback inhibition of the JAK/STAT signalling pathway (Alexander *et al.*, 1999; Kovanen and Leonard, 1999). Indeed, *Socs3* was up-regulated (*P* value = 0.0124) as well as the IL-1 receptor antagonist, *Il1rn*. IL-1rn binds to the same receptor as IL-1α and β (Hannum *et al.*, 1990) and does not activate signalling pathways, thereby, preventing the action of IL-1α and β. Furthermore, caveolin-1, a 22 - 24 kDa protein which has a multitude of functions which extend from the formation of caveolae on cell membranes to cell-signalling (Anderson, 1993), was up-regulated and has been shown to suppress IL-6 and TNFα inflammatory cytokine production by LPS-stimulated mouse macrophages *in vitro* (Wang *et al.*, 2006).

In summary, CyaA has a potent effect on the induction of inflammatory cytokine and chemokine transcription. This may be important for the host immune system as many phagocytic cell types will be recruited to fight against *B. pertussis*. The macrophages would, in turn, activate T cells to mount an adaptive immune response. However, *B. pertussis* may also take advantage of this situation by deactivating and killing infiltrating phagocytes by the action of CyaA after binding to CR3 receptors expressed on incoming phagocytes using FHA, PRN and CyaA (Ishibashi *et al.*, 1994; Everest *et al.*, 1996; El-Azami-El-Idrissi *et al.*, 2003)

6.3 The effect of CyaA-induced cAMP accumulation on gene transcription

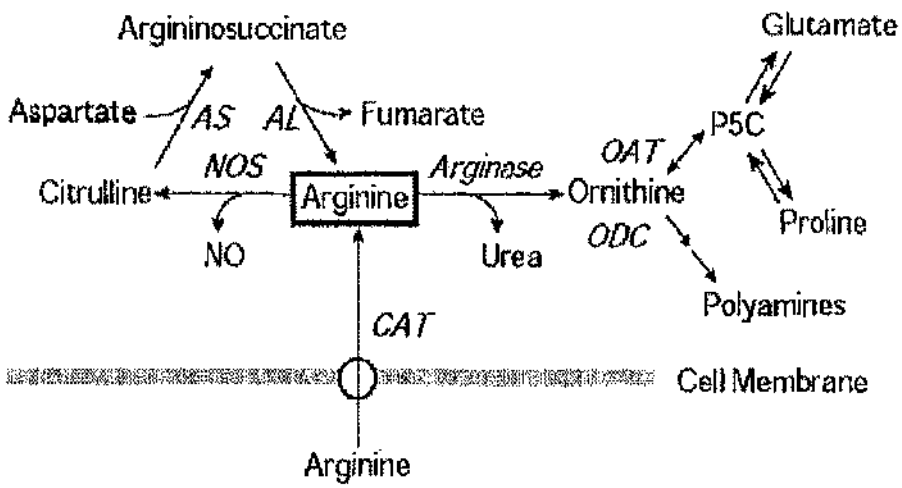
Increased levels of cAMP have been shown to down-regulate the production of lipoprotein lipase (*Lpl*) mRNA transcription in murine macrophages (Desanctis *et al.*, 1994). In agreement with this report, there was > 100-fold reduction in *Lpl* transcription in the present study. The expression of Lpl, which is involved in the transport of lipoprotein into the cell, is up-regulated when peripheral blood monocytes differentiate into tissue macrophages (Khoo *et al.*, 1981; Chait *et al.*, 1982). Syndecan-1, a proteoglycan that may serve to anchor cells to the extracellular matrix (ECM), is also regulated by cAMP in mouse peritoneal macrophages (Yeaman and Rapraeger, 1993). Increased expression of syndecan-1 (up-regulated 13-fold by CyaA) may be important for macrophages to adhere and migrate through the ECM. This may allow the phagocytes to infiltrate the site of infection. Interestingly, macrophages synthesise and release prostaglandins, which elevate intracellular levels of cAMP by interacting with cell membrane-associated G protein-coupled E-prostanoid receptors (Breyer *et al.*, 2001), in response to inflammatory stimuli (Humes *et al.*, 1977). Prostaglandins are synthesised from essential fatty acids, such as arachidonic acid by cyclooxygenases, such as Cox-1 (Ptgs1) and Cox-2, and prostaglandin (PGE) synthases and coincidently, *Ptgs1* expression was increased ~5.8-fold. Thus, in addition to cAMP being generated by CyaA, cAMP may also be generated by other mechanisms inside the cell.

6.3.1 CyaA induces arginase transcription which may alter NO production

cAMP also induces arginase production in murine macrophages (Corraliza *et al.*, 1997) which is known to down-regulate NO production (Gotoh and Mori, 1999). NO is synthesised from arginine by nitric oxide synthase (NOS) generating citrulline (Fig. 51). Arginine can be obtained from blood circulation, intracellular protein degradation, by regeneration from citrulline through the "citrulline-NO cycle" or by importing from the extracellular environment by protein transporters such as cationic amino acid transporters (Bogle *et al.*, 1992). As arginine is a common substrate for both NOS and arginase (Fig.

Figure 51 Diagram of arginine metabolism in eukaryotic cells

Arginine is transported from the extracellular space by CAT and is then used for the production of NO or for the synthesis of glutamate and proline.



Arginine metabolism. Citrulline-NO cycle is composed of nitric oxide synthase (NOS), argininosuccinate synthetase (AS) and argininosuccinate lyase (AL). CAT; cationic amino acid transporter; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; P5C, pyrroline-5-carboxylate.

Taken from Mori and Goto (2004).

51), arginase may compete with NOS for arginine, and down-regulate the production of NO. It has been postulated that arginase and NOS both regulate the production of NO (Mori and Gotoh, 2004). The 27-fold up-regulation of arginase mRNA by CyaA suggests that CyaA had induced arginase activity, which in turn may have diminished the capacity of the BMMs to produce NO. In addition, there was up-regulation of *Slc7a7* (3.23-fold) and *Slc7a11* (8-fold) which both code for cationic transporters that import L-arginine (Rotoli *et al.*, 2004) (Fig. 51) suggesting that CyaA-treated cells are importing large amounts of arginine. However, further tests need to be carried out to confirm the presence of arginase enzymic activity.

6.4 Signalling pathways

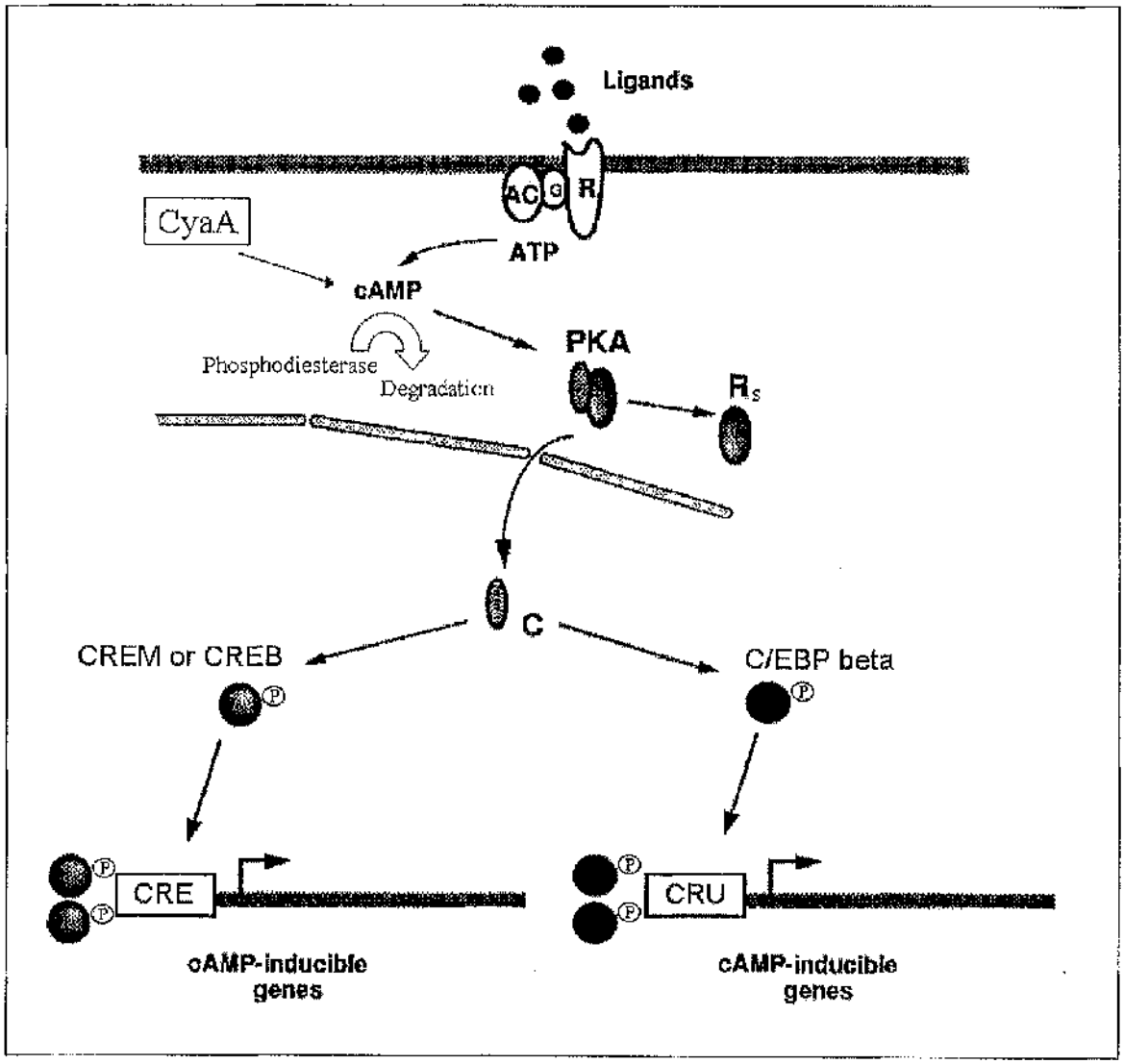
Little is known about the types of signalling pathways that CyaA can induce. However, as cAMP is a dominant feature of cell intoxication, it stands to reason that the gene changes observed in this study could be due to the activation of cAMP-dependent cell-signalling pathways.

6.4.1 The PKA signalling pathway activates CREB, CREM and C/EBP β

One of the best known cAMP-dependent signalling pathways is via protein kinase A (PKA) which is composed of two regulatory and two catalytic subunits. As shown in Figure 52, when cAMP binds to two PKA regulatory subunits, the two PKA catalytic subunits are released and, in turn, can phosphorylate a number of target proteins such as cAMP-response element binding protein (CREB), cAMP-responsive element modulator (CREM) and CCAAT/enhancer-binding protein β (C/EPB β) (Sassone-Corsi, 1998; Don and Stelzer, 2002). Phosphorylated CREB homodimers bind to a CREB binding protein which allows interaction with the specific DNA regions called cAMP-response elements (CREs) that are found upstream of genes involved in hormonal responses, differentiation or proliferation (Della Fazia *et al.*, 1997). CREM is highly analogous to CREB and has the same efficiency and specificity of binding to CREs as CREB. However, CREM acts as a suppressor of cAMP-induced transcription (Foulkes *et al.*, 1991). Similarly, when cAMP activates C/EPB β, it translocates into the nucleus (Pelletier *et al.*, 1998), binds to DNA sequences called cAMP responsive units (Wilson and Roesler, 2002) found upstream of genes associated with cell proliferation (Buck and Chojkier, 2003) and inflammation (Ramji and Foka, 2002) and induces transcription of these genes. Eukaryotic cells up-regulate phosphodiesterase gene expression in response to excessive cAMP accumulation (Ma *et al.*, 1999) in order to degrade signalling cyclic nucleotides, such as cAMP and cGMP and prevent their accumulation (Fig. 52).

Figure 52 The PKA signalling pathway

Ligand binding to receptors (R) activates coupled G-proteins (G) which, in turn, stimulate the activity of the membrane associated adenylyl cyclase (AC) converting ATP to cAMP. cAMP is also generated by invasive CyaA. cAMP causes the dissociation of the inactive tetrameric protein kinase A (PKA) complex into the active catalytic subunits and the regulatory subunits (R_s). Catalytic subunits (C) migrate into the nucleus, where they phosphorylate and thereby activate transcriptional activators such as CREB, CREM or CCAAT/enhancer-binding protein β (C/EPB β). CREB or CREM interact as a dimer with the cAMP response enhancer element (CRE) whereas C/EPB β binds to DNA sequences called cAMP responsive units (CRU). Genes found downstream of these sequences are then up-regulated. Phosphodiesterases regulate the PKA pathway by degrading intracellular cAMP.



Adapted from Sassone-Corsi (1998).

6.4.2 Mitogen-activated kinase signalling pathways

Mitogen-activated protein kinase (MAPK) signalling cascades are activated by a wide variety of receptors including receptor tyrosine kinases, integrins and ion channels (Fig. 53). MAPKs phosphorylate specific serines and threonines of target protein substrates, such as other protein kinases, phospholipases, transcription factors and cytoskeletal proteins, that regulate cellular activities including gene transcription, mitosis, movement, proliferation and apoptosis. MAPKs are part of a phosphorelay system composed of three sequentially-activated kinases and, like their substrates, MAPKs are regulated by phosphorylation. Thus, MAPKs are activated by MAPK kinases (MAPKKs) which are, in turn, activated by MAPK kinase kinases (MAPKKKs). Activation of these signalling pathways occurs when specific receptors detect the correct stimuli, e.g. cytokines, oxidative stress or binding of ligands to integrins. These activation signals are transduced to small GTP binding (G) proteins which in turn activate the MAPKKKs leading to the eventual activation of MAPKs (Fig. 53). In eukaryotes, there are three well characterised subfamilies of MAPKs and these are shown in Fig. 53. These MAPKs include the extracellular response kinase (ERK), c-Jun N-terminal Kinase (JNK) and p38 signalling pathways. The ERK pathway can be activated by growth factors, cytokines and bacterial infections (Chang and Karin, 2001). The JNK signalling pathway is activated by a variety of environmental stresses, inflammatory cytokines, growth factors and G-protein-coupled receptor agonists (Weston and Davis, 2002). The p38 signalling pathway can be activated by LPS as well as IL-1 and osmotic shock (Su and Karin, 1996). Phosphatases act as regulators of the MAPK signalling pathways by dephosphorylating the activated kinases.

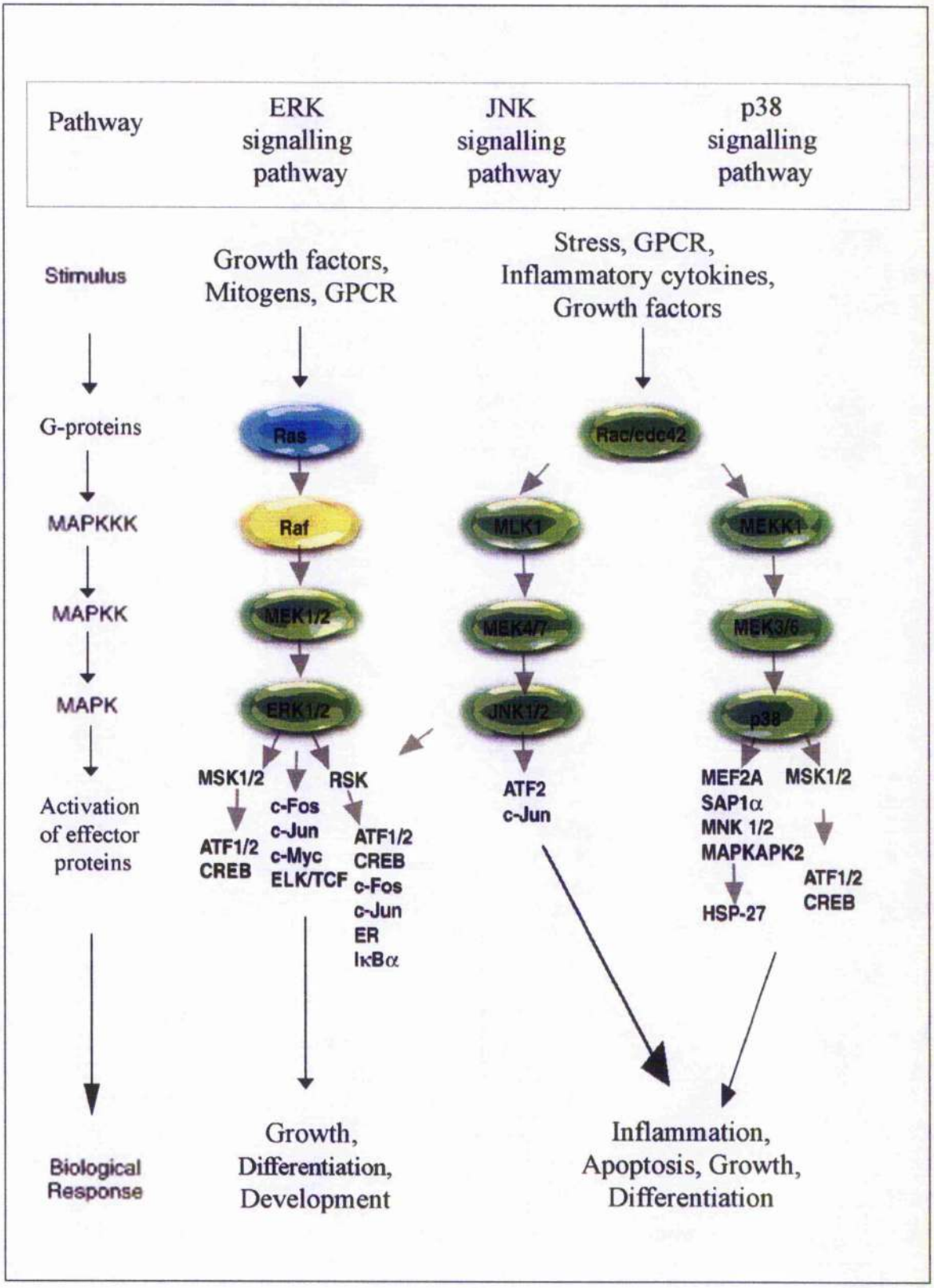
6.4.2.1 The AP-1 regulatory complex

The transcription factors activated by ERK and JNK MAPKs bind to regions of DNA called serum response elements (SRE) and TPA response elements (TRE), respectively. This can lead to the transcription of the *fos* and *jun* family of genes, respectively. The protein encoded by *c-fos* dimerises with proteins encoded by *c-jun* to form an active gene regulatory protein called AP-1 (Karin *et al.*, 1997). The AP-1 complex is not exclusively composed of heterodimers of Fos and Jun, rather, AP-1 can also be heterodimers of several other transcription factors (Angel and Karin, 1991). A further level of complexity is that there are several members within the Fos and Jun family of proteins; e.g. the Jun family is comprised of c-Jun and JunB. JunB, as part of the AP-1 transcription factor, arrests cell differentiation in hemapoietic cells and has a role in apoptosis (Passegue and Wagner, 2000; Liebermann and Hoffman, 2002). Thus, the levels of different Jun members can modulate cell proliferation and cell death (Shaulian and Karin, 2001). Moreover, the ERK

Figure 53 The ERK, JNK and p38 signalling pathways

The ERK, JNK and p38 MAPK signalling cascades are activated by a wide variety of stimuli such as activation of G-protein-coupled receptors (GPCR), growth factors, cytokines, stress and mitogens. A signal is transduced to small GTP binding (G) proteins (Ras, Rac/cdc42), which in turn activate a MAPKKK (Raf, MLK1, MEKK1), a MAPKK (MEK1/2, MEK4/7, MEK3/6) and MAPK (ERK1/2, JNK1/2, p38). The MAPKs activate effector proteins which function as dimers to regulate different cellular functions. Activation of the ERK and JNK MAPKs also leads to the transcription of *c-fos* and *c-jun*, respectively. c-jun can be activated by ERK 1/2 and JNK 1/2.

Adapted from Karin (1995),
http://www.biosource.com/content/literatureContent/Pathway2005/pdfs/MAPk.pdf and
http://www.cellsignal.com/reference/pathway/MAPK_Cascade.asp


Pathway
ERK signalling pathway
JNK signalling pathway
p38 signalling pathway
Stimulus
Growth factors, Mitogens, GPCR
Stress, GPCR, Inflammatory cytokines, Growth factors
G-proteins
Ras
Rac/cdc42
MAPKKK
Raf
MLK1
MEKK1
MAPKK
MEK1/2
MEK4/7
MEK3/6
MAPK
ERK1/2
JNK1/2
p38
Activation of effector proteins
MSK1/2
RSK
c-Fos
c-Jun
c-Myc
ELK/TCF
ATF1/2
CREB
ATF1/2
CREB
c-Fos
c-Jun
ER
IκBα
ATF2
c-Jun
MEF2A
SAP1α
MNK 1/2
MAPKAPK2
MSK1/2
ATF1/2
CREB
HSP-27
Biological Response
Growth, Differentiation, Development
Inflammation, Apoptosis, Growth, Differentiation

signalling pathway is important in the production of key cell cycle regulatory proteins such as cyclin A, cyclin D, cyclin dependent kinase 2 (Cdk2) and Cdc25A (L'Allemain *et al.*, 1997; Schmitt and Stork, 2002).

6.4.3 The cAMP/Epac-1/Rap1 signalling pathway

An increase of intracellular levels of cAMP could also activate Epac signalling pathway (Bos, 2003) as shown in the boxed area of Fig. 54. The cAMP/Epac-1/Rap1 pathway regulates re-organisation of the actin cytoskeleton and the resulting morphological changes are important for many cellular processes, including cell migration and adhesion (Kitayama *et al.*, 1989; Caron *et al.*, 2000; Sebzda *et al.*, 2002; Rangarajan *et al.*, 2003).

6.4.4 CyaA and cell-signalling

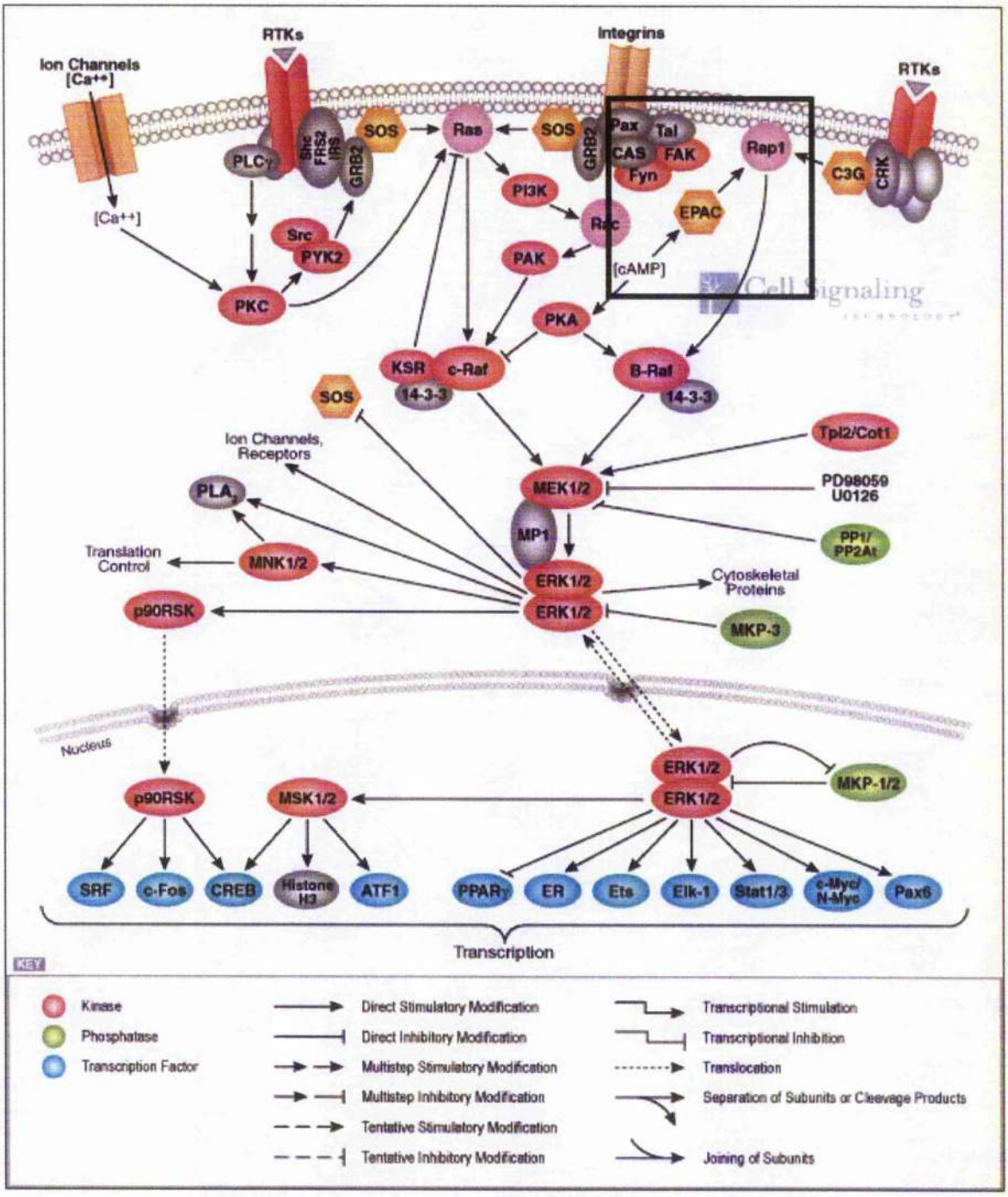
Studies have shown that cross-linking of the CD11b receptor with monoclonal antibodies activates the ERK signalling pathway in a human monocyte (THP-1) cell line (Reyes-Reyes *et al.*, 2002). Since CyaA also binds to CD11b (Guermonprez *et al.*, 2001), it may be that this MAP kinase pathway could be activated in this way. However, the fact that CyaA* and proCyaA* altered significantly few or no genes, respectively, suggests that CyaA, at 20 ng/ml, cannot induce cell-signalling via interaction with the CR3 receptor and its affects must be cAMP-mediated. The cytotoxicity assays indicated that CyaA* killed cells at concentrations greater than 0.5 μg/ml in the presence of calcium (Section 3.4.3). ProCyaA has been shown to intoxicate macrophages at higher toxin concentrations (Boyd *et al.*, 2005; Hewlett *et al.*, 2006; Basler *et al.*, 2006a). Therefore, it would be interesting to study global gene responses in BMMs to CyaA* and proCyaA at higher toxin concentrations.

The evidence presented above indicates that CyaA can induce the activation of different signalling cascades through cAMP by 'cross-talk' between the PKA, Epac1 and ERK signalling pathways (Fig. 54). So, it would not be surprising to observe several different signalling cascades being activated in BMMs after CyaA treatment. As discussed in Section 6.4.1, cAMP can activate the PKA signalling cascade. From the analyses, CyaA induced transcription of *Cebpb* and *Crem* by ~4-fold and ~18-fold, respectively. CEBP/β and CREM are activated by the PKA catalytic subunits. Interestingly, inhibiting the PKA signalling pathway with a chemical inhibitor prevented the activation of human monocyte-derived dendritic cells by CyaA, as shown by a decreased level of CD80 and CD86 expression (Bagley *et al.*, 2002). *JunB*, *Fos* and *Fosl2* transcription were also up-regulated 4.6, 4.4 and 10-fold suggesting that the JNK and ERK signalling pathways are activated (Section 6.4.2.1). These gene products combine to form the dimeric AP-1 regulatory complex.

Figure 54 Cross-talk between cAMP and the different signalling pathways

cAMP is an activator of several different signalling pathways in eukaryotic cells. cAMP activates the PKA and Epac signalling pathways (boxed area). Activated PKA can also indirectly activate the ERK signalling pathway through interaction with the Raf family of MAPKKKs by activating B-Raf. The inhibition of ERK by cAMP is linked to C-Raf inhibition, whereas ERK activation is linked to B-Raf. cAMP can uncouple C-Raf from Ras through direct phosphorylation of C-Raf by PKA.

Taken from http://www.cellsignal.com/reference/pathway/MAPKERK.asp


RTKs
Integrins
RTKs
Ion Channels [Ca++]
PLCγ
Shc FRS2 IRS
GRB2
SOS
Ras
SOS
GRB2
Pax
Tal
CAS
FAK
Fyn
Rap1
C3G
CRK
PI3K
Rac
EPAC
[cAMP]
[Ca++]
Src
PYK2
PAK
PKC
PKA
Cell Signaling
KSR
c-Raf
B-Raf
14-3-3
14-3-3
SOS
Ion Channels, Receptors
Tpl2/Cot1
MEK1/2
PD98059
U0126
PLA2
MP1
PP1/
PP2At
Translation Control
MNK1/2
ERK1/2
Cytoskeletal Proteins
p90RSK
ERK1/2
MKP-3
Nucleus
ERK1/2
MKP-1/2
p90RSK
MSK1/2
ERK1/2
SRF
c-Fos
CREB
Histone H3
ATF1
PPARγ
ER
Ets
Elk-1
Stat1/3
c-Myc/ N-Myc
Pax6
Transcription
KEY
Kinase
Phosphatase
Transcription Factor
Direct Stimulatory Modification
Direct Inhibitory Modification
Multistep Stimulatory Modification
Multistep Inhibitory Modification
Tentative Stimulatory Modification
Tentative Inhibitory Modification
Transcriptional Stimulation
Transcriptional Inhibition
Translocation
Separation of Subunits or Cleavage Products
Joining of Subunits

Elk3 (up-regulated 2.5-fold) is a member of the Ets family (Tamai *et al.*, 1995) and like Elk1 is also activated by ERK (Wasylyk *et al.*, 1998; Fig. 57). Interestingly, Elk3 has been shown to behave as a transcriptional repressor of nitric-oxide synthase 2 in a mouse macrophage cell line (Chen *et al.*, 2003). This may be another mechanism by which CyaA is able to down-regulate NO production in addition to up-regulating arginase transcription (Section 6.3.1). On the other hand, *Dusp6* transcription was up-regulated 3.6-fold. Dusp6 (Dual phosphatase 6) is an important protein for regulating the ERK signalling pathway because it dephosphorylates MAP kinase ERK2 on both thr-183 and tyr-185, thereby inhibiting the activity of ERK2 (Muda *et al.*, 1996). If the ERK pathway is being strongly activated by CyaA, the detection of Dusp6 would correlate with the cells trying to regulate the activation of this pathway. The lack of phosphorylated ERK 1/2 (Section 3.4.6) from J774.2 macrophages after incubation for 10 min with 20 ng/ml CyaA would suggest that the ERK pathway is not activated within this short time period. It would be interesting to investigate how soon *Dusp6*, *c-fos* and *c-jun* transcription occurs by using RT-PCR or by Western blotting. Moreover, the phosphodiesterase, *Pde4b*, was up-regulated by 16.5-fold. This suggests that cAMP is strongly activating cAMP-dependent signalling pathways and the BMMs are trying to regulate its activation by controlling the amount of cAMP in the cell.

The changes in cell morphology of mouse fibroblast cells by the oedema toxin of *B. anthracis* (EdTx), which includes cell rounding and the production of long thin processes, is PKA dependent (Hong *et al.*, 2005). Therefore, CyaA-induced morphological changes of BMMs and baby hamster kidney cells (Westrop *et al.*, 1994) are likely to be a consequence of cAMP-activated PKA signalling. The changes in cell morphology may be important for allowing bacterial dissemination through host tissues (Hong *et al.*, 2005). Alternatively, cAMP/PKA can also directly phosphorylate Rho proteins (such as Rhoe) causing their inability to interact with effector proteins (Dong *et al.*, 1998). Rho proteins are intimately connected with the regulation of the actin cytoskeleton (Hall, 1998). There is no evidence, from the microarray study, to suggest that the Epac1 pathway is activated in BMMs after CyaA treatment. However, Misra and Pizzo (2005) showed that this pathway could be activated in mouse peritoneal macrophages by using a cAMP-elevating agent, forskolin. It would be of interest to investigate if this pathway was activated by CyaA.

6.4.4.1 Cell-signalling by bacterial toxins

It was shown recently that the p38 signalling pathway can be activated by pore-forming toxins from Gram-positive and Gram-negative species, such as the pneumolysin of *Streptococcus pneumoniae* and the α-HlyA from *E. coli*, respectively (Ratner *et al.*, 2006). At 20 ng/ml, CyaA may not be able to produce pores (Chapter 4) which may explain the

lack of evidence from microarray analysis to suggest that the p38 signalling pathway was activated. Interestingly, the cytotoxic enterotoxin (Act) of *Aeromonas hydrophila* can activate the ERK pathway in murine macrophages (Galindo *et al.*, 2004b) as well as producing the AP-1 transcription factor through increasing cAMP levels by stimulating prostaglandin production *in vitro* in murine macrophages (Chopra *et al.*, 2000). Thus, there could be a high chance that CyaA can also promote AP-1 formation, especially when *c-fos* and *c-jun* transcription were increased. Galindo *et al.* (2003 and 2004a) also investigated the global gene responses in murine macrophages to Act. In the present study, it was very interesting to observe that CyaA also induced some of the same genes as Act, such as increased transcription of *Bcl2l11/Bimel*, *Cebpb*, *Socs3*, *Il1rn* and *Junb*. However, Act did not down-regulate large numbers of cell cycle-associated genes (Section 6.5). This ability of both toxins to increase intracellular cAMP levels in macrophages, albeit by different mechanisms (Confer and Eaton, 1982; Chopra *et al.*, 2000) most probably accounts for the similarity in gene changes observed in macrophages after treatment with these toxins. However, the macrophages were incubated for 12 h with Act (Galindo *et al.*, 2003) and for 24 h with CyaA which would create differences in the cAMP levels in the cells. These factors may explain the differences in gene transcription between CyaA and Act.

Another study investigated the gene responses of RAW 264.7 murine macrophages to the lethal toxin of *B. anthracis* (Comer *et al.*, 2005). Lethal toxin is comprised of protective antigen, which is responsible for binding and entry into host cells (Bradley *et al.*, 2001), and lethal factor, a zinc protease which cleaves MAPKKs (Duesbery *et al.*, 1998). Therefore, it was not surprising to see down-regulation of genes which are regulated by down-stream activated effector proteins of the ERK signalling pathway. For example, after treatment of macrophages for 1.5 h with lethal toxin, there was a decrease in *c-fos* and *c-jun* transcription by 3.4 and 2.2-fold, respectively. In addition, lethal toxin up-regulated prostaglandin E2 receptor, subtype 4 transcription, which has been shown to simulate cAMP production and activate the ERK1/2 via phosphatidylinositol 3-kinase (Fujino *et al.*, 2002), as well as Dusp6. The possible influence of LPS in the toxin preparations used, including CyaA, could have contributed to changes in gene transcription is discussed in Section 6.6.

It is evident from the gene lists that CyaA induces cell-signalling cascades as shown by increased transcription of several transcription factors. However, the activation of the ERK, PKA and Epac signalling pathways can only be speculative. Using a variety of different techniques, such as the use of chemical inhibitors and monoclonal antibodies against phosphorylated signalling proteins, the activation of any of these pathways can be elucidated. The activation of signalling cascades in macrophages, or indeed by other

immune effector cells, by bacterial toxins could be a way for the host to deal with the infiltrating pathogens. Little is known about the cell-signalling mechanisms induced by CyaA, but the microarray study produced some indication of the complex results of CyaA intoxication on cell-signalling pathways.

6.5 Cyclins and the cell cycle

The eukaryotic cell cycle is shown diagrammatically in Fig. 55A. Cells start division in the G1 phase then cycle through the S, G2 and M phases. Cells may either leave the G1 phase and become resting cells or continue replication by entering the G1 phase again. Several factors, such as cyclins, are involved in controlling the cell cycle. Cyclins are a family of proteins involved in the progression of cells through the cell cycle and normally form a complex with a specific cyclin-dependent kinase (Cdk) partner, a serine/threonine kinase, forming a cdk complex (Fung and Poon, 2005). Cylins are produced or degraded as needed in order to drive the cell through the different stages of the cell cycle. When its concentration in the cell is low, cyclin detaches from cdk, inhibiting the enzyme's activity (Bai *et al.*, 1994). There are several different cyclins which are active in different parts of the cell cycle (Fig. 55B) and cause Cdks to phosphorylate different substrates. The cyclin B: Cdk (Cdc2a - also known as Cdk1) complex is called M-phase promoting factor (MPF). MPF promotes the entrance into mitosis from the G2 phase (Labbé *et al.*, 1989, Draetta *et al.*, 1989; Gautier *et al.*, 1990; Fig. 55B) by phosphorylating multiple proteins needed during mitosis, including condensins, various microtubule-associated proteins, proteins involved in the degradation of the nuclear envelope. Other cyclins include cyclin E, which is required for the transition from G1 to S phase (Fig. 55B) and cyclin A that is required for the cell to progress through the S phase (Fig. 55B). A cyclin-Cdk complex can also be regulated by several kinases and phosphatases, including Wee, Cdk-activating kinases (Cak) and Cdc. Wee functions by phosphorylating Cdc2a and related Cdks on conserved tyrosine and threonine residues. This phosphorylation blocks the activity of the Cdc2a and prevents entry into mitosis. On the other hand, Cdc is a phosphatase that removes the inhibitor phosphate added by Wee, rendering the complex active. Cdk feeds back on Wee and Cdc to inhibit and enhance their respective activities, respectively (Mueller and Leise, 2005).

6.5.1 CyaA and cell proliferation

The microarray data showed that many genes whose products are involved in the cell cycle were down-regulated by CyaA, suggesting that a large population of the macrophages were not in a proliferative state. All the down-regulated genes that were involved in the cell cycle and their interactions with other proteins have been highlighted

Figure 55 The eukaryotic cell cycle

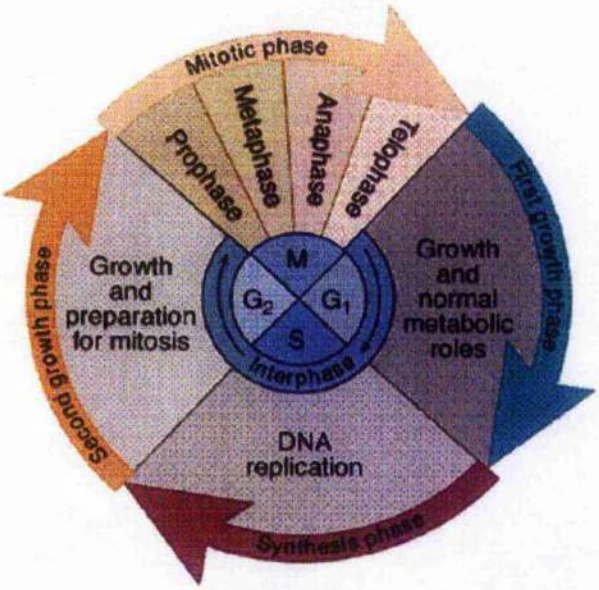
(A) Representation of the different stages of the eukaryotic cell cycle. Cells start division in the G1 phase then cycle through the S, G2 and M phases. Cells may either leave the G1 phase and become resting cells or continue replication by entering the G1 phase again. Several factors, such as cyclins, are involved in controlling the cell cycle.

Adapted from http://www.biologycorner.com/bio4/notes/mitosis.php,

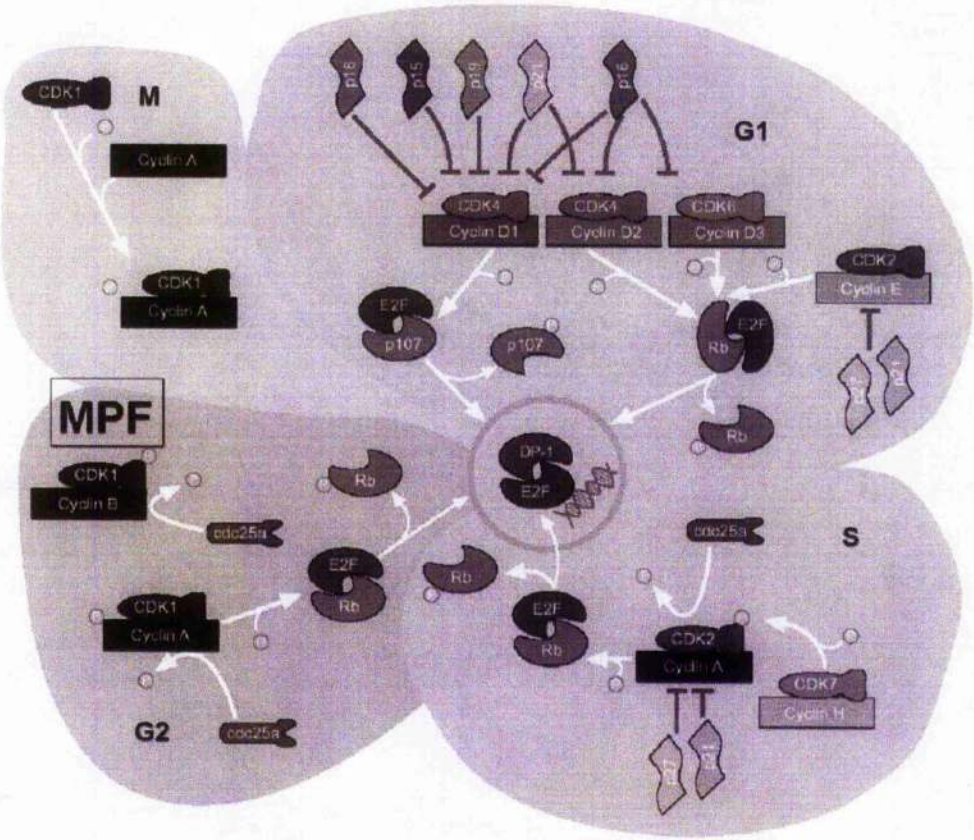
(B) The involvement of different cyclins and cyclin dependent kinases (cdks) in the eukaryotic cell cycle. The involvement of some of these proteins are described in Section 6.5.

Adapted from cell cycle methods on
http://www.biosource.com/content/literatureContent/methodPDFs/index.asp

A


Mitotic phase
Prophase
Metaphase
Anaphase
Telophase
First growth phase
Growth and normal metabolic roles
Synthesis phase
DNA replication
Second growth phase
Growth and preparation for mitosis
M
G2
G1
S
Interphase

B

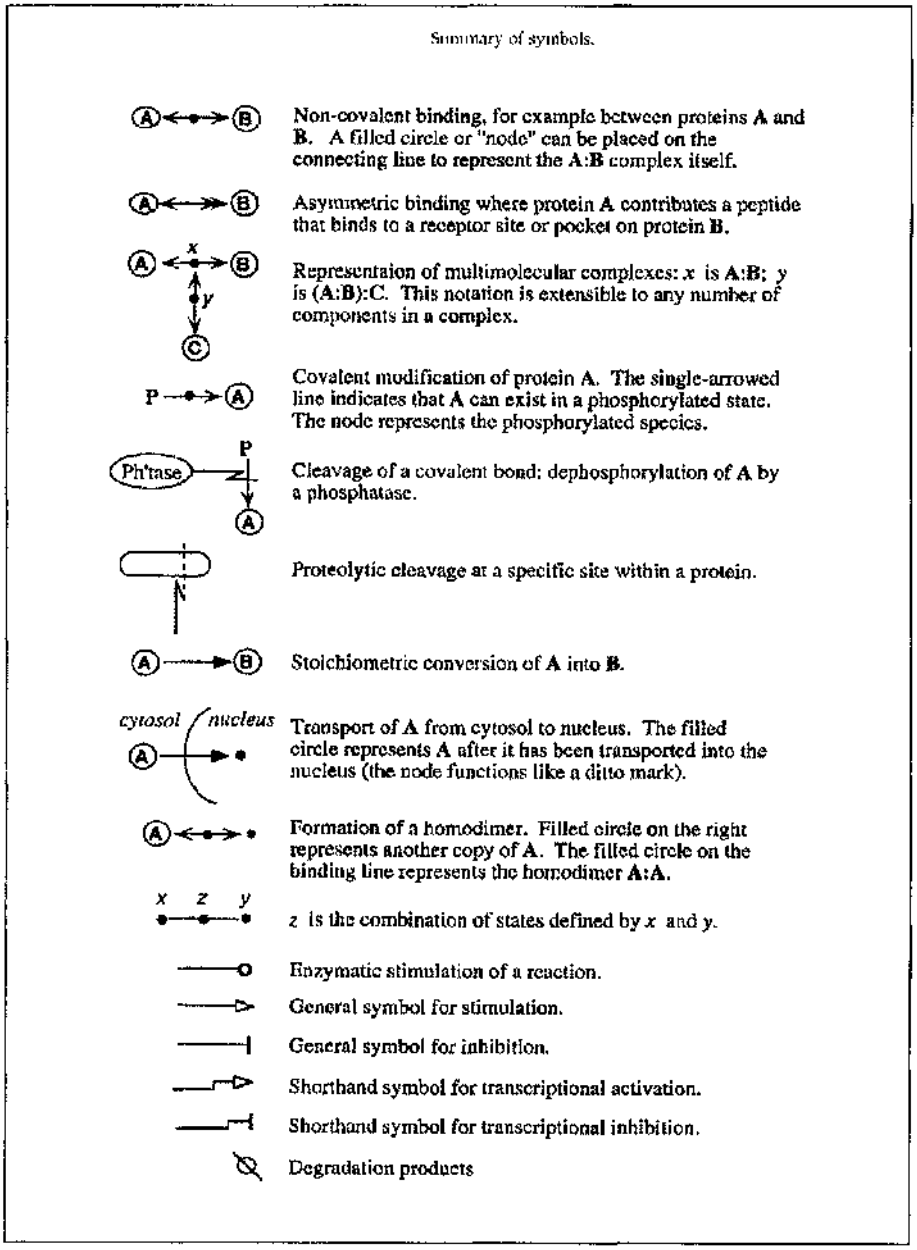

CDK1
M
Cyclin A
p16
p15
p19
p21
p18
G1
CDK4
Cyclin D1
CDK4
Cyclin D2
CDK6
Cyclin D3
CDK2
Cyclin E
E2F
p107
Rb
MPF
CDK1
Cyclin B
cdc25c
DP-1
E2F
S
CDK2
Cyclin A
CDK7
Cyclin H
G2
cdc25a

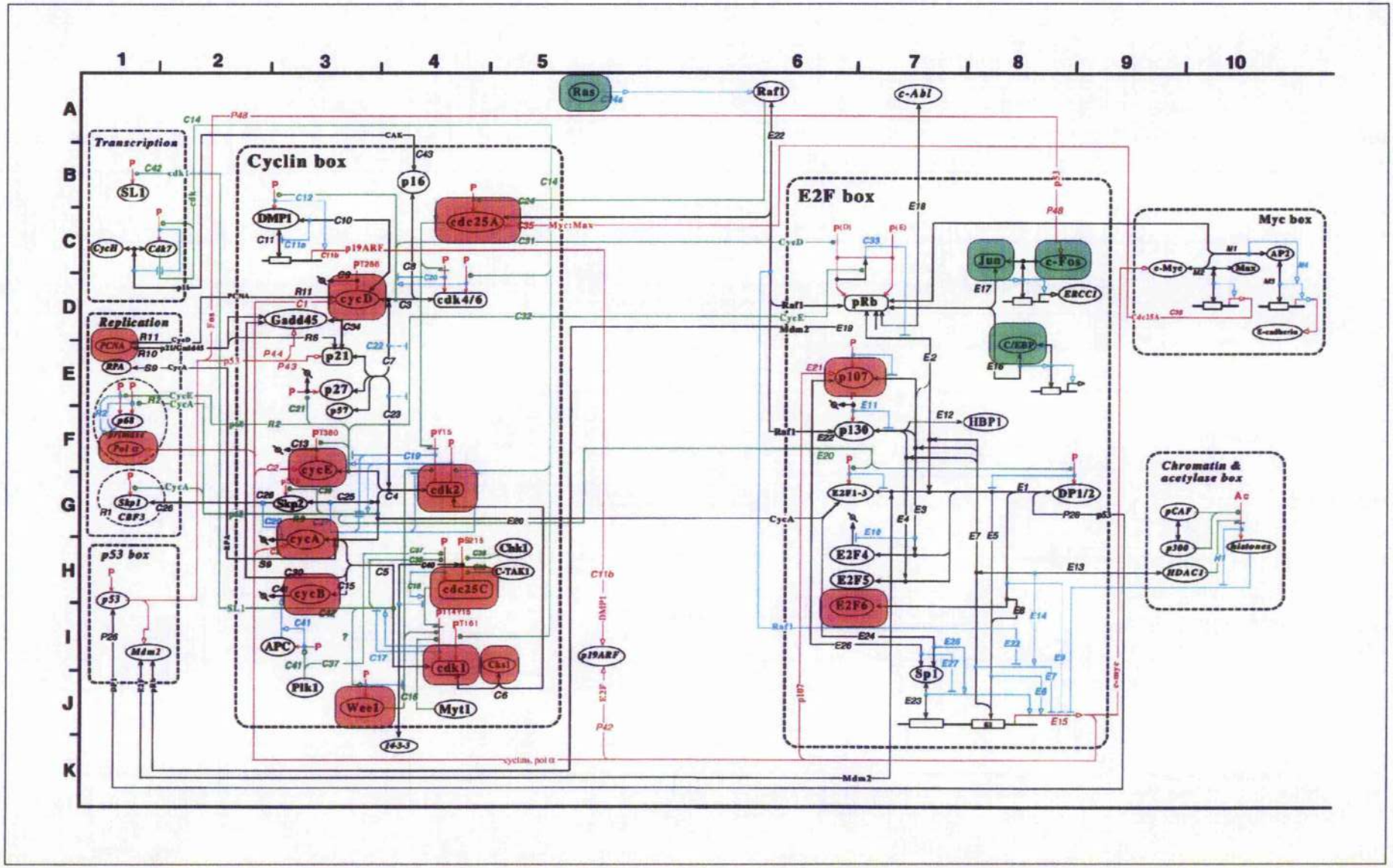
in red in Figure 56. These include *cyclins A*, *B*, *E* and *F* as well as many *Cdc* genes and *Wee1*, which is involved in regulating the cyclin/cdc complexes. Importantly, there was down-regulation of *CcnB1* (8.7-fold), *CcnB2* (7.9-fold) and *Cdc2a* (6-fold) whose gene products form cyclin B1, cyclin B2 and their Cdk partner Cdc2a (also known as Cdk1), respectively, which combine to make the M-phase promoting factor. Thus, lack of this complex would mean that the cells could not proceed into the mitotic phase (cell division) of the cell cycle.

CyaA also down-regulated several other genes whose functions are important for DNA replication such as several minichromosome maintenance deficient (*Mcm*) genes which have roles in DNA unwinding (Pacek *et al.*, 2006) and several kinesins (*Kif*) which have roles in spindle organisation (Kurasawa *et al.*, 2004) and in mitosis (Haraguchi *et al.*, 2006). There was decreased transcription of helicase (9.6-fold), topoisomerase (14.6-fold) and DNA polymerase (15-fold). These enzymes are required for the temporary separation, unknotting and the production of DNA, respectively. There was also decreased transcription of ribonucleotide reductase (35-fold) which is an enzyme that synthesises deoxyribonucleotides from ribonucleotides, providing the precursors necessary for DNA synthesis and repair (Eklund *et al.*, 2001). Finally, Rad51 and Rad54, down-regulated by 18 and 11.5-fold, respectively, functionally cooperate to mediate homologous recombination and the repair of damaged chromosomes by recombination (Raschle *et al.*, 2004). All these enzymes are central for DNA replication. There were a few genes involved in the cell cycle that were up-regulated by CyaA. These included *Cnnm2* (20-fold), *Vegfa* (8-fold) and *Cspg2* (232-fold). Cyclin M2 has yet to be characterised. On the other hand, Vascular endothelial growth factor-A (VEGFA) is a multifunctional cytokine that has roles in endothelial cell proliferation and the formation of lymphatic vessels from pre-existing lymphatic vessels (Dvorak *et al.*, 1995). Its expression is induced in the presence of macrophage colony-stimulating factor (which was used originally to culture the cells) and *Hif1a* expression (Varney *et al.*, 2005). Coincidentally, *Hif1a* expression was up-regulated by 4.4-fold. Thus, the expression of *Vegfa* may be correlated with *Hif1a* expression but the importance of Cyclin M2 and VEGFA in the macrophage response to CyaA function is unknown. Chondroitin sulphate proteoglycan (Cspg2) is expressed at high levels in human monocytes as they differentiate into macrophages (Kolset *et al.*, 1983). Chondroitin sulphate proteoglycan has also been shown to bind lipoprotein lipase (Edwards *et al.*, 1995). However, as discussed in Section 6.3, Lpl transcription was decreased by ~100-fold, presumably as a result of cAMP accumulation. Nevertheless, the high levels of Cspg2 transcription suggest that the macrophages are in a differentiated state as a result of CyaA action.

Figure 56 Molecular interactions of proteins in the eukaryotic cell cycle

Black arrows indicate binding interactions and stoichiometric conversions. Red arrows infer covalent modifications and gene expression. Green arrows show enzyme actions. Blue arrows indicate stimulations and inhibitions. Shaded boxes highlight the up (green) or down (red)-regulation of the genes in BMMs after CyaA treatment. This figure maps the interactions involving E2F, pRb, Cyclin, and Cdk family members, their activators and inhibitors, as well as some important interactions with other components. The E2F transcription factors regulate cell cycle progression by controlling gene expression of key cell cycle regulators. Retinoblastoma gene product (RB) family members are important regulators of E2F function. Figure modified from Kohn (1999).




Transcription
Cyclin box
E2F box
Myc box
Replication
p53 box
Chromatin & acetylase box
Ras
Raf1
c-Abl
p16
cdc25A
DMP1
p19ARF
cycD
cdk4/6
Gadd45
p21
p27
p57
cycE
cdk2
cycA
Skp2
cycB
cdc25C
Chk1
C-TAK1
APC
Plk1
Wee1
Myt1
cdk1
Cks1
14-3-3
SL1
CycH
Cdk7
PCNA
RPA
p68
Pol α
Skp1
CBF3
p53
Mdm2
Jun
c-Fos
ERCC1
pRb
C/EBP
p107
p130
HBP1
E2F1-3
DP1/2
E2F4
E2F5
E2F6
Sp1
c-Myc
Max
AP2
E-cadherin
pCAF
p300
HDAC1
Histones
Myc:Max
cyclins, pol α

CyaA has been shown to induce apoptosis of macrophages (Khelef *et al.*, 1995; Bachelet *et al.*, 2002). Therefore it was surprising to find transcriptional regulation of only a few apoptosis-associated genes. These included up-regulation of pro-apoptotic genes *Bcl2l11* (Gressner *et al.*, 2005; Kirschnek *et al.*, 2005), *Egln3* (Straub *et al.*, 2003) and *Stk17b* (Inbal *et al.*, 2000), and down-regulation of anti-apoptotic genes such as *Birc5*. Interestingly, Birc5 is an inhibitor of caspase-3 and caspase-7 (Shin *et al.*, 2001). Thrombospondin-1 (up-regulated 360-fold) is an adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions (Bornstein, 2001) which can promote chemotaxis of human peripheral blood monocytes (Mansfield and Suchard, 1994). However, thrombospondin, is thought to act as a bridge in the recognition of apoptotic cells by $\alpha_V\beta3$/CD36 expressed on macrophages (Friedl *et al.*, 2002).

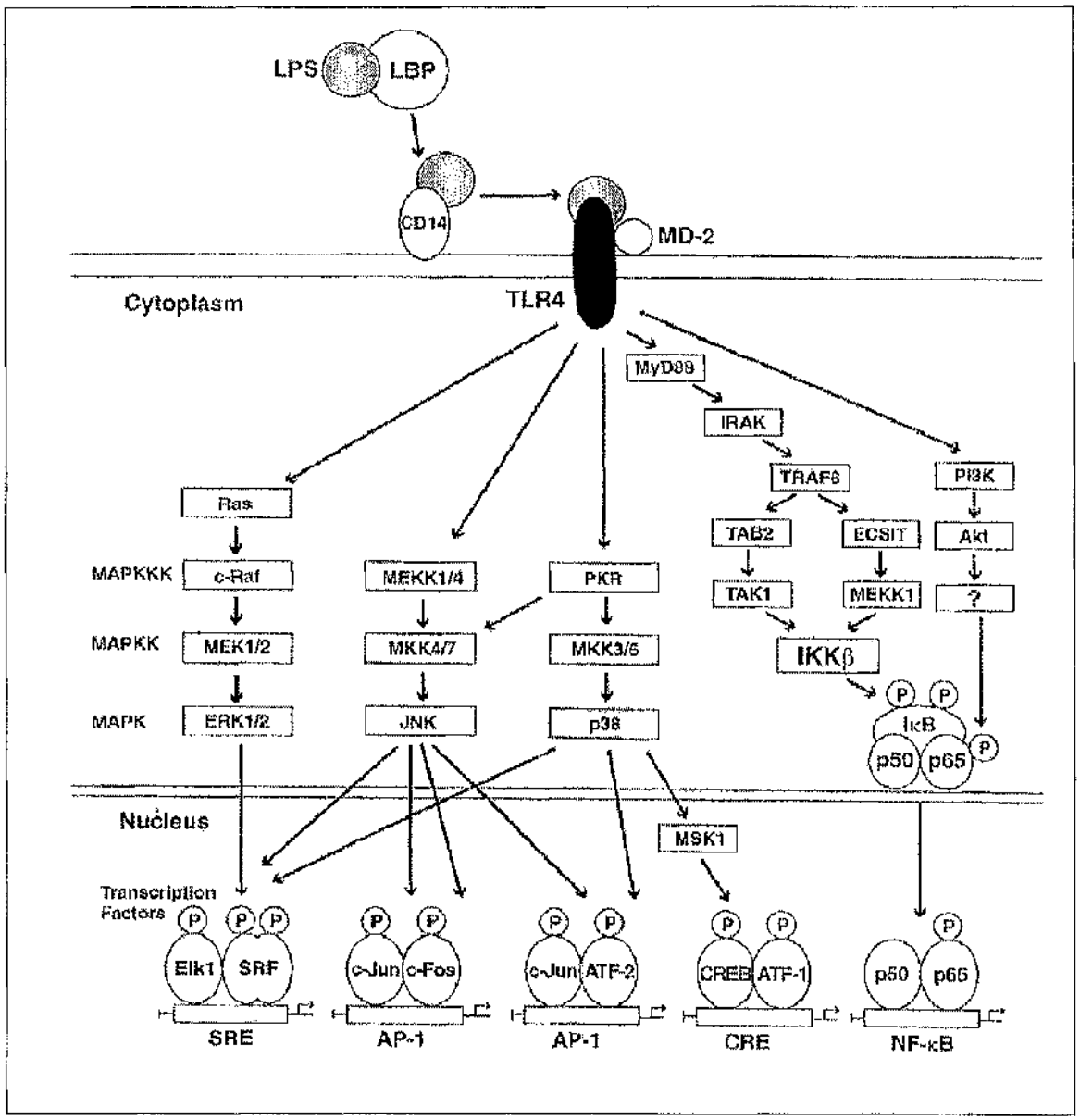
In summary, there is an overwhelming number of genes involved in cell proliferation which have been down-regulated in BMMs after treatment with CyaA suggesting that the cells are in a differentiated but in a non-proliferative state. Low levels of caspase 3/7 were detected at 20 ng/ml (Section 3.4.5.2) which would imply that at this concentration the macrophages were only slightly apoptotic. Nevertheless, the microarray data indicated by BMMs were undergoing apoptosis upon exposure to CyaA at this concentration. The process of apoptosis may not require the need for *de novo* production of caspases as these are activated from pre-existing pro-enzymes and this may explain why there was no caspase gene induction observed in this study.

6.6 Macrophage gene responses to LPS

The possibility that LPS in the final CyaA preparation may have contributed to some of the transcriptional changes needs to be considered. LPS, at concentrations down to 1 EU/ml can activate several signalling pathways (van der Bruggen *et al.*, 1999), such as the ERK pathway (Fig. 57), after binding to the TLR4/MD-2 complex (Guha and Mackman, 2001). In addition, LPS-induced IL-1β production has been shown to cause the production of IL-6 and matrix metalloproteinases (Dinarello, 1994). *Mmp9*, *Mmp19* and *Il-1b* were up-regulated by 6.08, 6.47 and 35-fold, respectively (Appendix L.7). Treatment of murine peritoneal macrophages with bacterial LPS can also increase expression of *c-fos* (Introna *et al.*, 1986) and can stimulate IL-1β, IL-6 and TNFα production in murine macrophages *in vitro* (Hirohashi and Morrison, 1996; Guha and Mackman, 2001). Gao *et al.* (2002) showed that 192 and 71 genes were significantly ($P < 0.05$) up- and down-regulated, respectively, more than 2-fold in RAW 264.7 murine macrophages when treated with 1000 EU/ml of *E. coli* LPS for 6 h. However, the transcription profile of LPS-stimulated RAW macrophages was different from CyaA-stimulated BMMs (Gao *et al.*,

Figure 57 Induction of cell-signalling cascades by LPS after binding to the TLR4/MD-2 complex

LPS binds with LPS binding protein (LPB) which helps facilitate interaction with the Toll-like receptor (TLR4)/MD-2 complex via surface bound CD14. A number of signalling cascades can be induced as a result of this interaction and are represented schematically below. These events lead to the induction of several types of transcription factors that regulate different aspects of cell function, such as cell proliferation and inflammatory responses.



Adapted from Guha and Mackman (2001).

2002). For example, some genes such as *Il10*, *Cxcl10* and *Tnfa* were up-regulated by LPS but these were down-regulated or not altered by CyaA. In a previous report, CyaA (containing 2.2 EU/ml LPS) was shown to stimulate low levels of IL-6 from J774 murine macrophages and this was enhanced in the presence of LPS (Ross *et al.*, 2004). However, no IL-6, IL-1β or TNFα were detected in the supernates after CyaA treatment of BMMs (data not shown) nor was there any alteration in IL-6 gene transcription. By contrast, genes that were altered in BMMs by CyaA but not by LPS (Gao *et al.*, 2002) included the down-regulation of many genes involved in cell proliferation (Section 6.5). In addition, treatment of BMMs with CyaA* or proCyaA*, which contained almost exactly the same amounts of residual LPS as CyaA, altered significantly fewer genes compared with CyaA. Thus, it seems reasonable to conclude that the gene transcription changes prompted by CyaA were due to CyaA alone and it can be argued that the LPS in the CyaA preparation (0.001 EU/ml LPS in 20 ng protein/ml), was not present in large enough quantities to influence gene responses in BMMs.

In studying the global gene responses of RAW 264.7 murine macrophage to the lethal toxin of *B. anthracis* (Comer *et al.*, 2005) LPS was used at 2 EU/ml as a control. This was equivalent to the level of LPS contamination in the lethal toxin preparation used for stimulation of macrophages. The levels of LPS in the final concentration of CyaA used (0.001 EU/ml) was 200-fold lower than that of Comer *et al.* (2005) and 10^6-fold lower than that of Gao *et al.* (2002). It cannot be ruled out, however, that CyaA and LPS can act synergistically to promote cell-signalling events. We observed significantly lower levels of IL-6 produced by MM6 cells after treatment with CyaA (containing 0.04 EU LPS/μg protein) expressed from *E. coli* BL21/DE3 compared with LPS from *E. coli* BL21/DE3 used at the same concentration. Moreover, increased cellular cAMP levels modulated the response to LPS in dendritic cells (Bagley *et al.*, 2002) and synergism was also detected between LPS and CyaA for dendritic cell activation (Ross *et al.*, 2004).

Microarray technology is not without its drawbacks; not all of the total RNA may be amplified to make cRNA. Thus, theoretically, some important genes may not have made it on to the final gene lists. In addition, the gene responses that took place in BMMs at intervals between 2 h and 24 h are unknown. The transcription of genes is not likely to remain static during the treatment period. For example, Comer *et al.* (2005) monitored gene transcription in macrophages after treatment for 1.5 h and 3 h with lethal toxin and found significant differences in gene transcription involved in intracellular signalling, energy production, and protein metabolism. Galindo *et al.* (2003) monitored gene transcription in macrophages after treatment for 2 h and 12 h with Act and found differences in the levels of cytokine transcription. Translating microarray data also requires

caution as mRNA levels are not representative of protein levels. Thus it is not possible to identify activation of signalling pathways, for example, by phosphorylation. Moreover, some proteins require post-translational modification before becoming active as suggested by the differences between IL-1β gene transcription and secretion of the cytokine by BMMs after CyaA stimulation.

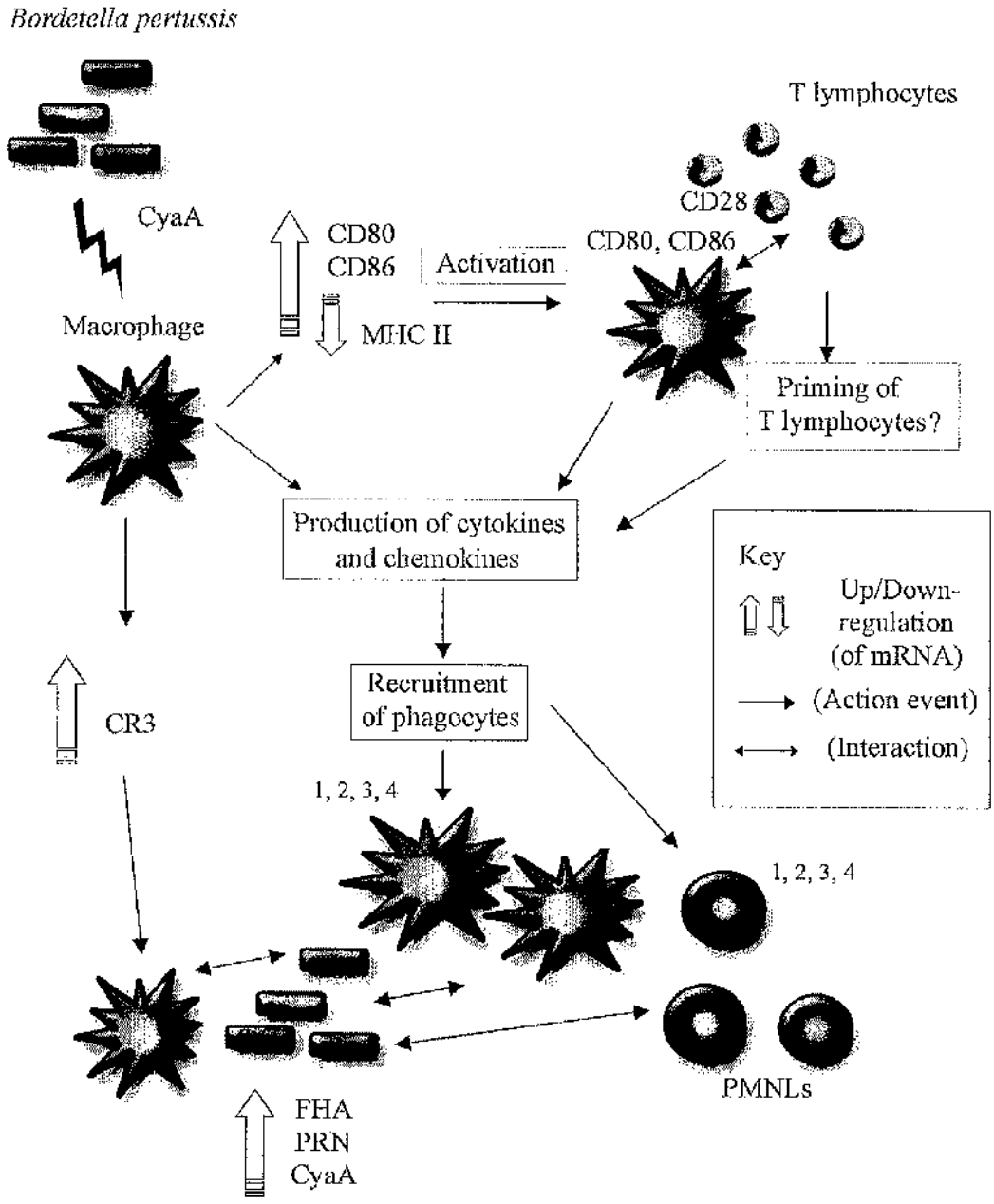
6.7 Conclusions

The microarray data have provided a valuable insight into the possible mechanisms of CyaA intoxication. A hypothetical situation is shown in Figure 58. Upon initial contact with the upper respiratory tract, *B. pertussis* switches on virulence-activated genes required for synthesis of adhesins, such as FHA and PRN, to adhere to ciliated cells, and toxins, such as CyaA which binds to macrophages via the CR3 receptor. Macrophages intoxicated by CyaA express greater levels of CD80, CD86, CD207 and TREM-1 which help activate T cells. As there is down-regulation of the MHC class II gene, the activation of T cells may be compromised. However, the macrophages may still be able to present antigen peptides to T cells using existing MHC class II molecules. The interaction between macrophages and T cells causes the induction of several different signalling pathways for both cell types. In the case of macrophages, cAMP accumulation and interaction with T cells induce signalling pathways that lead to the synthesis of IL-1β and chemokines that can help recruit macrophages and neutrophil PMNLs to the site of infection. CyaA-intoxicated BMMs would express cytokine regulators and regulators for the ERK and PKA signalling pathways to try to antagonise the effects mediated by the supraphysiological increase of cAMP levels. However, this may be problematic for the host as continual production of CyaA by *B. pertussis* could potentially deactivate arriving cells expressing the CR3 receptor by interfering with the presentation of antigens, thereby antagonising the ability of the host to mount an adaptive immune response towards *B. pertussis* infection. In addition, increased production of the integrin α_M (CD11b – part of CR3) receptor could potentiate the association of *B. pertussis*, via FHA and PRN, with macrophages to cause further neutralisation of host phagocytes by the induction of CyaA-mediated apoptosis and cAMP intoxication.

Up-regulation of cAMP in the BMMs by CyaA may activate several different pathways leading to increased transcription of *c-fos* and *jun* which encode transcription factors to form the AP-1 protein complex which regulates cell proliferation and cell arrest. The increased transcription of the anti-proliferative *JunB* may have effects on the down-regulation of cell cycle-associated genes and genes involved in DNA replication. Continual increases in cAMP ultimately leads to the induction of apoptosis by CyaA in a mechanism that still remains unclear although it is known that caspases 3/7 are activated and that

Figure 58 CyaA interaction with BMMs

A generalised view of how *B. pertussis* may utilise CyaA to deactivate cells of the immune system *in vivo*. The description of these events can be found in Section 6.7. Numbers: [1]impairment of phagocytosis, [2]induction of apoptosis, [3]inhibition of cell proliferation, [4]cell death.



mitochondria are involved (Bachelet *et al.*, 2002). The deactivation of the immune effector cells in the surrounding area of infection may give an opportunity for *B. pertussis* to proliferate to high numbers to cause disease by the production of several types of toxins.

Evidently, CyaA is an important mediator in disabling host defences. Microarray analyses of macrophages treated with CyaA have generated a great deal of data and allowed new insights as to how CyaA may function *in vitro* and *in vivo*. However, more detailed research is required to validate the hypotheses that have been formulated from the gene transcription data.

Future work

Further experiments are required to validate the gene changes in BMMs. RT-PCR should be used to confirm the up- and down-regulation of some of the key genes. Inhibition of NO production could be tested by stimulation of BMMs with LPS and then incubation with CyaA. NO can be detected using the Greiss assay. The production of the AP-1 complex could be determined by the gel-shift assay. Consequnces of the activation of signalling pathways induced by CyaA could be determined using chemical inhibitors or with monoclonal antibodies to phosphorylated signalling proteins. The effects on the cell cycle could be investigated in a similar manner using monoclonal antibodies to phosphorylated cyclins. Greater concentrations of proCyaA and CyaA* can intoxicate and kill cells, respectively. Thus, further microarray experiments could be done on BMMs using toxin concentrations greater than 20 ng/ml with proCyaA and CyaA*. This may help separate the types of gene changes induced specifically by intoxication and/or pore-formation by CyaA.

With regards to the *in vivo* experiments, further work may focus on the adjuvant effects of the different CyaA forms towards an ACV in mice after parenteral immunisation, but where mice are challenged by aerosol rather than intranasally. Preliminary experiments from this study showed that the different CyaA forms were able to protect mice after aerosol challenge with *B. pertussis*. Thus, the other non-acylated CyaA forms may be a more attractive alternative for inclusion in ACVs especially when CyaA* showed cytotoxicity towards murine macrophages *in vitro*.

Finally, it is unknown how CyaA molecules interact to form pores. Preliminary studies with analytical ultracentrifugation indicated that the conditions provided by simple dialysis were not optimal to study CyaA properly as judged by the extensive degradation. Thus, if conditions were optimised to minimise the degradation of CyaA, it may be possible to determine the molecular weight of a CyaA oligomeric complex in solution and to find out how many oligomers form a pore.

Appendices

Appendix A: Medium composition for bacterial growth

A.1 Luria-Bertani broth (LB)

Tryptone (Duchefa Biochemie)	10 g
Yeast extract (Duchefa Biochemie)	5 g
NaCl	5 g

Made up to 1000 ml in distilled water then autoclaved.

A.1.1 Luria-Bertani agar (LA)

For LA, add 1.2% (w/v) agar to LB prior to autoclaving.

A.2 Terrific broth (TB)

Media

Tryptone	12 g
Yeast extract	24 g
Glyercol	4 ml

Made up to 900 ml in distilled water

10× salt solution

KH_2PO_4	17 mM
K_2HPO_4	40 mM

Made up to 100 ml in distilled water

Autoclave separately and add 100 ml of 10× salt solution to 900 ml of medium before use.

A.3 Preparation of antibiotics

Antibiotic	Solvent	Stock concentration	Final concentration
Ampicillin	distilled water	50 mg/ml	50 μg/ml
Chloramphenicol	70% ethanol	12.5 mg/ml	12.5 μg/ml
Kanamycin	distilled water	50 mg/ml	50 μg/ml

All antibiotics were passed through a 0.2 μm filter and then stored at –20 °C.

A.4 10× Phosphate-buffered saline (PBS)

NaCl	1.7 M
KCl	100 mM
$Na_2HPO_4.12H_2O$	40 mM
KH_2PO_4	10 mM

Made up to 1000 ml in distilled water (pH 7.4). Dilute 1/10 before use.

Appendix B: DNA solutions

B.1 6× DNA loading buffer

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

B.2 10× Tris-Borate EDTA buffer (TBE)

Tris-base	0.9 M
Boric Acid	0.9 M
EDTA	32 mM

Made up to 2000 ml in distilled water. Dilute 1:10 before use.

Appendix C: Buffers for CyaA expression and purification

C.1 Competence solution

$CaCl_2.2H_2O$	100 mM
$MnCl_2.4H_2O$	70 mM
CH_3COONa	80 mM

Made up to 50 ml in distilled water (pH 5.5).

C.2 Buffers for urea extraction of CyaA from inclusion bodies

C.2.1 Solution A

Tris-HCl	50 mM
EDTA	5 mM

Adjust to pH 8.0.

C.2.2 Solution B

CHAPS*	1% (w/v) in Solution A

Adjust to pH 8.0. * Different detergents were compared as described in Section 2.4.4.1.1

C.2.3 Solution C

Tris-HCl	50 mM
EDTA	5 mM
Urea	2 M

Adjust to pH 8.0.

C.2.4 Solution D

Tris-HCl	50 mM
Urea	8 M

Adjust to pH 8.0.

C.3 Buffers for DEAE-Sepharose purification of CyaA

C.3.1 Solution 1

Tris-HCl	50 mM
NaCl	50 mM
Urea	8 M

Adjust to pH 8.0.

C.3.2 Solution 2

Tris-HCl	50 mM
NaCl	500 mM
Urea	8 M

Adjust to pH 8.0.

C.3.3 Solution 3

Tris-HCl	50 mM
Urea	8 M

Adjust to pH 8.0.

C.3.4 NaCl gradient

NaCl concentration	Volume solution 1	Volume solution 2	Volume solution 3
50 mM	70 ml		
100 mM		5 ml	20 ml
150 mM		7.5 ml	17.5 ml
200 mM		10 ml	15 ml
250 mM		12.5 ml	12.5 ml
500 mM		25 ml	

C.4 Buffers for Phenyl-Sepharose purification of CyaA

C.4.1 Buffer A

Tris-HCl	50 mM
NaCl	1 M

Adjust to pH 8.0.

C.4.2 Buffer B

Tris-HCl	50 mM
NaCl	100 mM
Urea	2 M

Adjust to pH 8.0.

Appendix D: Protein analysis solutions

D.1 5x loading buffer

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

D.2 Resolving gel

	% separation		
	7.5%	**12%**	**15%**
30% Acryl/Bis (37.5:1) (BioRad)	3.75 ml	6 ml	7.5 ml
1 M Tris-HCl (pH 8.8)	5.625 ml	5.625 ml	5.625 ml
dH_2O	5.7 ml	3.45 ml	1.95 ml
10% (w/v) SDS	150 µl	150 µl	150 µl
10% (w/v) Ammonium persulphate	150 µl	150 µl	150 µl
TEMED (add last)	15 µl	15 µl	15 µl

Enough solution for 4 gels of each.

D.3 Stacking solution

30% Acryl/Bis (37.5:1) (BioRad)	9 ml
1 M Tris-HCl (pH 6.8)	7.5 ml
dH_2O	42 ml
10% (w/v) SDS	50 µl

Store at 4 °C. To 3 ml of stacking solution, add 100 µl of 10% (w/v) APS and 8 µl TEMED.

D.4 10× Running buffer

Tris-Base	0.25 M
Glycine	1.92 M
SDS	0.035 M

Made up to 2000 ml in distilled water. Diluted 1 in 10 before use.

D.5 Coomassie Blue stain

Coomassie Blue	0.05% (w/v)
Methanol	500 ml
Glacial Acetic acid	100 ml
dH_2O	400 ml

For destain, Coomassie Blue was omitted.

D.6 Buffer A for Bradford's protein assay

1 in 4 dilution of 8 M urea, 50 mM Tris-HCl (pH 8.0) (Appendix C.2.4) with distilled water to make 2 M urea, 12.5 mM Tris-HCl (pH 8.0).

Appendix E: Silver staining solutions

E.1 Oxidising solution

Ethanol	400 ml
Glacial acetic acid	50 ml
dH_2O	550 ml
Periodic acid	0.7% (w/v)

E.2 Staining solution

Solution A		Solution B	
Ammonium hydroxide	2 ml	dH_2O	5 ml
0.1 M Sodium hydroxide	28 ml	Silver nitrate	1 g

Solution A and solution B were made separately and then mixed. A transient brown precipitate disappeared within seconds. 115 ml dH_2O was added to make 150 ml of staining reagent.

E.3 Development solution

Compound concentration	Stock required	Volume	Final concentration
Citric acid	10 mg/ml	1 ml	10 mg
Formaldehyde	37% (v/v)	0.1 ml	0.0185% (v/v)

Made up to 200 ml with distilled water.

E.4 Stop solution

10% (v/v) acetic acid.

Appendix F: Western blotting

F.1 10× Transfer buffer

Tris-Base	0.25 M
Glycine	1.92 M

Made up to 2000 ml in distilled water. Diluted 1 in 10 before use and made up with 20% (v/v) methanol.

F.2 PBST

1× PBS	Appendix A.4
Tween 20	0.2% (v/v)

Made up to 1000 ml in distilled water (pH 7.4).

F.3 Blocking buffer

PBST	Appendix F.2
Marvel	10% (w/v)

F.4 PBST diluent

PBST	Appendix F.2
Marvel	5% (w/v)

F.5 Substrate solution

3'3-Diaminobenzidine (DAB)	0.05 g
PBS	98 ml
1% (w/v) $CoCl_2$	2 ml
30% (v/v) H_2O_2	100 µl

Appendix G: *In vitro* characterisation of CyaA

G.1 Conductimetry

G.1.1 Buffer A

Imidazole	5 mM
EDTA	10 mM

Adjusted to pH 6.1.

G.1.2 Buffer B

Tris-HCl	10 mM
$CaCl_2$	1 mM

Adjusted to pH 8.0.

G.1.3 Bicine buffer

Bicine	10 mM
Magnesium acetate	1.5 mM
ATP	0.5 mM

Adjusted to pH 8.0.

G.2 Hanks HEPES buffer

NaCl	150 mM
KCl	5 mM
$MgCl_2$	1 mM
D-glucose	5 mM
HEPES	10 mM
Urea	200 mM
$CaCl_2$	0, 1, 2 or 3 mM (Section 2.6.2)

Made up with sterile distilled water at pH = 7.4 (Stored at RT).

G.3 Tissue culture

G.3.1 Maintenance medium for J774.2 macrophages

Foetal Bovine Serum (FBS) (Gibco)	50 ml
L-Glutamine (Gibco)	5 ml
Antibiotics/antimycotics (Gibco)	5 ml

Added to 500 ml media (RPMI or DMEM†)

†DMEM contains glutamax-1. Therefore, L-glutamine is not required.

G.3.2 Maintenance medium for MM6 monocyte/macrophages

HEPES (Gibco)	20 mM
Insulin (Sigma)	0.23 IU/ml
L-Glutamine (Gibco)	2 mM
Oxaloacetic acid (Sigma)	1 mM
Sodium pyruvate (Sigma)	1 mM
Non-essential amino acids (Gibco)	1×
Antibiotics/antimycotics (Gibco)	1×
FBS††	10% (v/v)

Added to 500 ml RPMI medium.

††MM6 cell assay medium contains 2% (v/v) FBS.

G.3.3 PBS wash buffer

PBS without calcium or magnesium (Difco)	500 ml
Antibiotics/antimycotics (Gibco)	5 ml
FBS	5% (v/v)

G.4 ERK lysis buffer

HEPES	25 mM
EDTA	2.5 mM
NaCl	50 mM
NaF	50 mM
Sodium pyrophosphate	30 mM
Glycerol	10% (v/v)
Triton-X100	1% (v/v)

Adjust to pH 7.5 and filter sterilised.

Appendix H: Immunological assays

H.1 Casamino acids solution

NaCl	0.6% (w/v)
Casamino acids (Difco)	1% (w/v)

Made up to 1000 ml in distilled water (pH 7.0).

H.2 Greiss reagent

N-[1-naphthyl] ethlyenadiamine dichloride	0.1% (w/v)
Sulphanilamide in 5% (v/v) H_3PO_4	1% (w/v)

Each solution was made separately and mixed (1:1 ratio) prior to performing an assay.

H.3 Enzyme Linked Immunosorbent Assay (ELISA)

H.3.1 Carbonate coating buffer

Na_2CO_3	15 mM
$NaHCO_3$	30 mM
NaN_3	7.7 mM

Made up to 1000 ml in distilled water (pH 9.5).

H.3.2.1 PBS-Tween

1× PBS	(Appendix G.2)
Tween 20	0.05% (v/v)

H.3.2.2 PBST diluent/PBST blocking buffer

10% (v/v) FBS was added to make PBST diluent/PBST blocking buffer.

H.3.3 10× acetate buffer

Sodium acetate-3-hydrate	0.5 M
Glacial acetic acid	816 µl

Made up to 500 ml in distilled water and adjusted to pH 6.0.

H.3.4 Peroxidase substrate solution

Acetate buffer (Appendix I.3.3)	12 ml
dH_2O	112 ml
Hydrogen peroxide	37 µl
1% (w/v) Tetramethyl benzidine in dimethylsulphoxide	1.25 ml

H.3.5 Fluorescence-Activated Cell Sorter (FACS) analysis

H.3.5.1 PBS Flow

PBS	100 ml
FBS	5 ml
10% (w/v) NaN_3	1 ml

H.3.5.2 FACS Fix

PBS	47.5 ml
dH_2O	47.5 ml
40% (v/v) formaldehyde	5 ml

Appendix I: Microarray studies

I.1 L929 DMEM growth medium

Foetal Calf Serum (Gibco)	10% (v/v)
Supernate from L929 cells	10% (v/v)
Penicillin/Streptomycin (Gibco)	100 U/ml

Added to 500 ml DMEM/ F-12 (Gibco) with Glutamax-1 and pyridoxine.

Appendix J: Amino acid sequence of CyaA* compared with CyaA

Predicted amino acid sequences of CyaA* (query) with CyaA (Sbjct) from nucleotide base sequences of the region from nucleotide 1438 to nucleotide 2103 in the 5' region of *cyaA* of pGW44/188 and pGW44, respectively. **LQ** is the dipeptide insertion. Letters in bold and underline indicate amino acid substitutions.

```
Query 699 RKTSDGRYAVQYRRKGGDDFEAVKVIGNAAGIPLTADLQIDMFAIMPHLSNFRDSACSSV 520
          ++TSDGRYAVQYRRKGGDDFEAVKVIGKAAGIPLTAD  IDMFAIMPHLSNFRDSA SSV
Sbjct 152 KETSDGRYAVQYRRKGGDDFEAVKVIGKAAGIPLTAD--IDMFAIMPHLSNFRDSARSSV 209

Query 519 TSGDSVTDYLARTRRAASEATGGLDRERIDLLWKIACAGARSAVGTEARRQFRYDGDMNI 340
          TSGDSVTDYLARTRRAASEATGGLDRERIDLLWKIA AGARSAVGTEARRQFRYDGDMNI
Sbjct 210 TSGDSVTDYLARTRRAASEATGGLDRERIDLLWKIARAGARSAVGTEARRQFRYDGDMNI 269

Query 339 GVITDFELEVRNALNRRAHAVGAQDVVQHGTEQNNPFPEADEKIFVVSATGESQMLTRGQ 160
          GVITDFELEVRNALNRRAHAVGAQDVVQIGTEQNNPFPEADEKIFVVSATGESQMLTRGQ
Sbjct 270 GVITDFELEVRNALNRRAHAVGAQDVVQHGTEQNNPFPEADEKIFVVSATGESQMLTRGQ 329
```

Appendix K: RNA quality and quantity

K.1 Quantification and qualification of total RNA isolated from BMMs after treatment with CyaA or urea after 2 h or 24 h

Incubation time	Sample	Replicate	$A_{260\ nm}$	$A_{280\ nm}$	$A_{260\ nm}/A_{280\ nm}$ ratio	RNA Conc (µg/µl)	Total RNA (µg)	RNA integrity number
2 h	Urea	1	0.23	0.19	1.2	4.6	41.4	9.3
		2	0.23	0.16	1.5	4.7	42.3	9.8
		3	0.34	0.23	1.5	6.8	61.2	9.5
		Mean	**0.26**	**0.20**	**1.4**	**5.4**	**48.3**	**9.53**
	CyaA	1	0.25	0.17	1.4	9.8	88.2	10
		2	0.28	0.19	1.5	11.2	100.8	10
		3	0.15	0.12	1.2	6	54.0	9.5
		Mean	**0.23**	**0.16**	**1.4**	**9**	**81.0**	**9.83**
24 h	Urea	1	0.19	0.13	1.4	3.7	33.3	9.2
		2	0.26	0.18	1.5	5.3	47.7	9.3
		3	0.24	0.16	1.4	4.7	42.3	9.8
		Mean	**0.23**	**0.16**	**1.4**	**4.6**	**41.1**	**9.43**
	CyaA	1	0.23	0.16	1.4	9.3	83.7	9.9
		2	0.24	0.17	1.5	9.8	88.2	10
		3	0.19	0.13	1.4	7.4	66.6	8.2
		Mean	**0.22**	**0.15**	**1.4**	**8.8**	**79.5**	**9.13**

K.2 Quantification and qualification of total RNA isolated from BMMs after treatment with different CyaA forms or urea after 2 h or 24 h

Treatment	Replicate	$A_{260\ nm}$	$A_{280\ nm}$	$A_{260\ nm}/A_{280\ nm}$ ratio	RNA Conc (µg/µl)	Total RNA (µg)	RNA integrity number
proCyaA* #1	1	0.87	0.56	1.56	2.2	19.7	
	2	0.87	0.56	1.56	2.2	19.7	
	3	0.87	0.56	1.56	2.2	19.7	
	Mean:	**0.87**	**0.56**	**1.56**	**2.2**	**19.7**	**9.3**
proCyaA* #2	1	0.92	0.57	1.61	2.3	20.7	
	2	0.92	0.57	1.61	2.3	20.7	
	3	0.92	0.57	1.61	2.3	20.8	
	Mean:	**0.92**	**0.57**	**1.61**	**2.3**	**20.7**	**8.9**
proCyaA* #3	1	1.22	0.76	1.61	3.1	27.5	
	2	1.22	0.76	1.61	3.1	27.6	
	3	1.22	0.76	1.60	3.1	27.6	
	Mean:	**1.22**	**0.76**	**1.61**	**3.1**	**27.6**	**8.8**
CyaA #1	1	0.55	0.36	1.52	1.4	12.5	
	2	0.56	0.37	1.52	1.4	12.6	
	3	0.56	0.37	1.52	1.4	12.7	
	Mean:	**0.56**	**0.37**	**1.52**	**1.4**	**12.6**	**8.9**
CyaA #2	1	0.38	0.25	1.52	0.96	8.6	
	2	0.38	0.25	1.52	0.96	8.7	
	3	0.38	0.25	1.52	0.96	8.7	
	Mean:	**0.38**	**0.25**	**1.52**	**0.96**	**8.7**	**7.1**
CyaA #3	1	0.52	0.33	1.57	1.3	11.9	
	2	0.52	0.33	1.57	1.3	11.9	
	3	0.52	0.33	1.57	1.3	11.9	
	Mean:	**0.52**	**0.33**	**1.57**	**1.3**	**11.9**	**8.6**

Treatment	Replicate	$A_{260\ nm}$	$A_{280\ nm}$	$A_{260\ nm}/A_{280\ nm}$ ratio	RNA Conc (μg/μl)	Total RNA (μg)	RNA integrity number
CyaA* #1	1	0.90	0.57	1.58	2.3	20.3	
	2	0.90	0.57	1.58	2.3	20.4	
	3	0.90	0.57	1.58	2.3	20.4	
	Mean:	**0.90**	**0.57**	**1.58**	**2.3**	**20.4**	**9.8**
CyaA* #2	1	1.00	0.63	1.60	2.5	22.6	
	2	1.01	0.63	1.60	2.5	22.7	
	3	1.01	0.63	1.60	2.5	22.8	
	Mean:	**1.01**	**0.63**	**1.60**	**2.5**	**22.7**	**9.8**
CyaA* #3	1	0.88	0.55	1.57	2.2	19.8	
	2	0.88	0.55	1.58	2.2	19.8	
	3	0.87	0.55	1.58	2.2	19.8	
	Mean:	**0.88**	**0.55**	**1.58**	**2.2**	**19.8**	**9.8**
Urea #1	1	0.72	0.45	1.59	1.8	16.4	
	2	0.73	0.46	1.59	1.8	16.5	
	3	0.73	0.46	1.58	1.8	16.6	
	Mean:	**0.73**	**0.46**	**1.59**	**1.8**	**16.5**	**9.6**
Urea #2	1	0.72	0.47	1.53	1.8	16.4	
	2	0.73	0.47	1.54	1.8	16.6	
	3	0.73	0.47	1.55	1.8	16.5	
	Mean:	**0.73**	**0.47**	**1.54**	**1.8**	**16.5**	**7.1**
Urea #3	1	1.06	0.67	1.56	2.7	23.9	
	2	1.07	0.68	1.56	2.7	24.1	
	3	1.08	0.68	1.56	2.7	24.3	
	Mean:	**1.07**	**0.68**	**1.56**	**2.7**	**24.0**	**8.7**

Appendix L: Gene lists

L.1 Up-regulated genes in BMMs after incubation for 2 h with CyaA – preliminary experiment

Affy ID	Gene Name	Fold change	Description
CD ANTIGENS			
99434_at	*Cd83*	3.39	CD83 antigen*
CYTOKINES AND CHEMOKINES			
103486_at	*Il1b*	16.52	interleukin 1 β
CELL SURFACE RECEPTORS INVOLVED IN IMMUNITY			
96912_s_at	*Ctla2a*	4.21	cytotoxic T lymphocyte-associated protein 2 alpha*
CELL SURFACE MOLECULES			
96553_at	*Gpcr25*	3.60	G-protein coupled receptor 25
97733_at	*Adora2b*	3.88	adenosine A2b receptor*
100064_f_at	*Gja1*	4.86	gap junction membrane channel protein α 1
APOPTOSIS			
160906_i_at	n/a	3.44	insert in the fas apoptosis gene of MRL-IPR/IPR
SIGNALLING			
93285_at	*Dusp6*	3.26	dual specificity phosphatase 6
97844_at	*Rgs2*	4.63	regulator of G-protein signalling 2
98018_at	*Procr*	5.28	protein C receptor, endothelial
160901_at	*Fos; c-fos*	5.39	c-fos oncogene
TRANSCRIPTION			
160495_at	*Ahr*	4.05	aryl hydrocarbon receptor
101727_at	*Nfkbie*	4.36	nuclear factor of kappa light polypeptide gene
101805_f_at	*Nfil3; E4BP4*	5.89	transcription factor mRNA*
92248_at	*Nr4a2*	10.08	nuclear hormone receptor*
CELL GROWTH/DIVISION/DIFFERENTIATION			
103520_at	*Vegfa*	3.11	vascular endothelial growth factor A
101583_at	*Btg2*	3.35	B-cell translocation gene 2, anti-proliferative
CELLULAR STRUCTURE			
95705_s_at	*Actb*	5.65	actin, β, cytoplasmic*
METABOLISM/BIOSYNTHESIS			
104371_at	*Dgat1*	5.61	diacylglycerol O-acyltransferase 1
MITCHONDRION			
93097_at	*Arg1*	25.50	arginase 1, liver
ADHESION			
160469_at	*Thbs1*	15.72	thrombospondin

An asterisk (*) indicates that the gene was detected by another probe (Section 1.10).
n/a – no gene name available.

L.2 Down-regulated genes in BMMs after incubation for 2 h with CyaA – preliminary experiment

Affy ID	Gene Name	Fold change	Description
CYTOKINES AND CHEMOKINES			
102629_at	*Tnfa*	-8.4	tumour necrosis factor alpha
CELL SURFACE MOLECULES			
102237_at	*Tbxa2r*	-3.49	thromboxane A2 receptor
93845_at	*Abcf2*	-8.12	ATP-binding cassette, sub-family F, member 2
APOPTOSIS			
98433_at	*Bid*	-3.09	BH3 interacting domain death agonist
92415_at	*CD137L*	-4.49	CD137L
102629_at	*Traf5*	-4.55	tnf receptor-associated factor 5
SIGNALLING			
99874_at	*Rap2b*	-3.23	RAS-related protein RAP-2B homologue (*Homo sapiens*)
103833_at	*Hipk2*	-3.59	homeodomain interacting protein kinase
297106_at	*Map3k8*	-15.14	mitogen activated protein kinase kinase kinase 8
TRANSCRIPTION			
95731_at	*Pa26*-pending	-4.1	SESTRIN 1 (P53-REGULATED)
99564_at	*Np95*	-6.48	nuclear protein 95
CELL GROWTH/DIVISION/DIFFERENTIATION			
92310_at	*Snk*	-3.23	serum-inducible kinase (polo-like kinase 2)
REGULATORY			
99603_g_at	*Tieg1*	-4.32	TGFB inducible early growth response 1

L.3 Up-regulated genes in BMMs after incubation for 24 h with CyaA – preliminary experiment

Affy ID	Gene Name	Fold change	Description
CD ANTIGENS			
102830_at	*Cd86*	3.75	CD86 antigen
CYTOKINES AND CHEMOKINES			
94142_at	*Csf3*	4.27	colony stimulating factor 3 (granulocyte)
94761_at	*Ccl7; Scya7*	4.42	cytokine gene
101160_at	*Cxcl2*	9.70	chemokine (C-X-C motif) ligand 2
95349_g_at	*Cxcl1*	10.45	chemokine (C-X-C motif) ligand 1
103486_at	*Il1b*	21.81	interleukin 1 β
CELL SURFACE RECEPTORS INVOLVED IN IMMUNITY			
161968_f_at	*Ccr5*	3.49	C-C chemokine receptor 5*
100906_at	*Itgb7*	4.55	integrin β 7
93061_at	*Itga7*	4.77	integrin α 7
99413_at	*Ccr1*	5.13	chemokine (C-C motif) receptor 1
103039_at	*Itga5*	14.71	integrin alpha 5 (fibronectin receptor alpha)
CELL SURFACE RECEPTORS			
100435_at	*Edg2*	4.92	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2
97733_at	*Adora2b*	8.09	adenosine A2b receptor*
103448_at	*S100a8*	8.52	S100 calcium binding protein A8 (calgranulin A)
100064_f_at	*Gja1*	16.12	gap junction membrane channel protein α 1
APOPTOSIS			
93836_at	*Bnip3*	3.41	BCL2/adenovirus E1B 19kDa-interacting protein
160829_at	*Phlda1*	4.77	pleckstrin homology-like domain, family A, member 1
SIGNALLING			
98628_f_at	*Hif1a*	3.01	hypoxia inducible factor 1, α subunit*
160280_at	*Cav*	5.30	caveolin, caveolae protein
TRANSCRIPTION			
92249_g_at	*Nr4a2*	4.56	nuclear hormonal binding receptor
CELL GROWTH/DIVISION/DIFFERENTIATION			
92504_at	*Hus1*	13.04	Hus1 homologue (*S. pombe*)
TRANSPORT			
93738_at	*Slc2a1*	4.12	solute carrier family 2, member 1
ADHESION			
104483_at	*Col9a1*	4.54	procollagen, type IX, alpha 1
160469_at	*Thbs1*	19.86	thrombospondin
OTHER			
93023_f_at	*Hist1h3f*	4.13	histone H3 (H3.2-221)

An asterisk (*) indicates that the gene was detected by another probe (Section 1.10).
n/a – no gene name available.

L.4 Down-regulated genes in BMMs after incubation for 24 h with CyaA – preliminary experiment

Affy ID	Gene Name	Fold change	Description
CD ANTIGENS			
93445_at	*Cd5l*	-5.25	CD5 antigen-like
CYTOKINES AND CHEMOKINES			
102424_at	*Ccl3*	-3.61	chemokine (C-C motif) ligand 3
94425_at	*Ly86*	-4.80	lymphocyte antigen 86
IMMUNE RESPONSE			
104696_at	*Ctse*	-3.04	cathepsin E gene
93583_s_at	*n/a*	-3.31	fragment for mu-immunoglobulin C-terminus
APOPTOSIS			
101521_at	*Birc5*	-4.99	baculoviral IAP repeat-containing 5
SIGNALLING			
92758_at	*Dusp2*	-3.06	tyrosine-threonine dual specificity phosphatase
94953_at	*Racgap1*	-3.75	Rac GTPase-activating protein 1
160862_at	*Ptp4a3*	-5.48	protein tyrosine phosphatase 4a3
TRANSCRIPTION			
96081_at	*Tk1*	-10.93	thymidine kinase
99564_at	*Np95*	-14.37	nuclear protein 95
CELL GROWTH/DIVISION/DIFFERENTIATION			
99632_at	*Mad2l1*	-3.05	mitotic arrest deficient, homologue-like 1 (yeast)
93356_at	*Mcm7*	-3.40	minichromosome maintenance deficient 7 (yeast)
100890_at	*Chaf1b*	-3.52	chromatin assembly factor 1, subunit B (p60)
99067_at	*Gas6*	-3.97	growth arrest specific 6
101957_f_at	*Adprt1*	-4.68	poly (ADP-ribose) polymerase (AA 1 - 1013)
93099_f_at	*Plk*	-6.08	polo-like kinase (*Drosophila*)*
100156_at	*Mcm5*	-6.91	minichromosome maintenance deficient 5 (yeast)
98469_at	*Aurkb*	-7.40	aurora kinase B
95546_g_at	*Igf1*	-12.50	insulin-like growth factor 1
102001_at	*Rrm2*	-12.91	ribonucleotide reductase M2
99578_at	*Top2a*	-18.76	topoisomerase (DNA) II α
CELLULAR STRUCTURE			
97909_at	*Stmn1*	-10.55	stathmin 1
METABOLIC/SYNTHETIC			
162341_r_at	*Akr1b3*	-5.52	aldose reductase
96295_at	*Psat1*	-5.71	phosphoserine aminotransferase 1
95611_at	*Lpl*	-84.68	lipoprotein lipase*
OTHER			
98398_s_at	*Apobec1*	-3.22	apolipoprotein B editing complex 1
92851_at	*Cp*	-3.73	ceruloplasmin

An asterisk (*) indicates that the gene was detected by another probe (Section 1.10).

L.5 Genes up-regulated in BMMs from 2 h to 24 h after incubation with CyaA – preliminary experiment

Affy ID	Gene name	Fold change	Description
CD ANTIGENS			
102237_at	*Cd28*	3.01	CD28 antigen
CYTOKINES AND CHEMOKINES			
92415_at	*Tnfsf9; 4-1BB-L*	5.36	4-tumor necrosis factor (ligand) superfamily, member 9
103255_at	*Traf5*	8.35	tnf receptor-associated factor 5
98772_at	*Cxcl5*	31.29	chemokine (C-X-C motif) ligand 5
CELL SURFACE RECEPTORS INVOLVED IN IMMUNITY			
98304_at	*Tlr6*	3.35	toll-like receptor 6
99413_at	*Ccr1*	3.45	chemokine (C-C motif) receptor 1
100906_at	*Itgb7*	3.50	integrin beta 7
CELL SURFACE MOLECULES			
92685_at	*Tbxa2r*	3.49	thromboxane A2 receptor
93845_at	*Abcf2*	3.67	ATP-binding cassette, sub-family F, member 2
APOPTOSIS			
93836_at	*Bnip3*	5.23	BCL2/adenovirus E1B 19kDa-interacting protein 1,
102921_s_at	*Tnfrsf6*	5.38	tumour necrosis factor receptor superfamily, member 6
SIGNALLING			
99953_at	*Rgl2*	3.10	ral guanine nucleotide dissociation stimulator-like 2
100064_f_at	*Gja1*	3.30	gap junction membrane channel protein α 1
94063_at	*Sema4a*	3.31	semaphorin 4A
93708_at	*Pias3*	3.65	protein inhibitor of activated STAT 3
102711_at	*Rgs14*	3.78	regulator of G-protein signalling 14
98600_at	*S100a11*	4.43	S100 calcium binding protein A11 (calizzarin)
160280_at	*Cav*	4.80	caveolin, caveolae protein
97106_at	*Map3k8*	28.94	mitogen activated protein kinase kinase kinase 8
TRANSCRIPTION			
93074_g_at	*Nfatc2*	3.71	nuclear factor of activated T-cells, cytoplasmic 2
103049_at	*Nmyc1*	10.39	neuroblastoma myc-related oncogene 1
CELL GROWTH/DIVISION/DIFFERENTIATION			
162204_r_at	*Notch1*	3.50	notch 1
95597_at	*Ptgs1*	4.18	prostaglandin-endoperoxide synthase 1
TRANSPORT			
93471_at	*Slc4a7*	3.05	solute carrier family 4, member 7
103510_at	*Slc6a12*	4.06	solute carrier family 6, member 12
OTHER			
96907_at	*Cherp*	3.31	calcium homeostasis endoplasmic reticulum protein
161969_f_at	*Capg*	6.85	capping protein (actin filament), gelsolin-like

L.6 Genes down-regulated in BMMs from 2 h to 24 h incubation with CyaA – preliminary experiment

Affy ID	Gene name	Fold change	Description
CD ANTIGENS			
93445_at	*Cd5l*	-5.22	CD5 antigen-like
CYTOKINES AND CHEMOKINES			
102310_at	*Ccl22*	-3.22	chemokine (C-C motif) ligand 22
104669_at	*Irf7*	-3.72	interferon regulatory factor 7
94146_at	*Mip1b; Ccl4*	-4.35	gene for macrophage inflammatory protein 1b.
103639_at	*Ifit2*	-21.81	IFN-induced protein with tetratricopeptide repeats 2
IMMUNE RESPONSE			
92689_at	*Il18bp*	-3.44	interleukin 18 binding protein
98822_at	*G1p2*	-4.39	interferon, alpha-inducible protein
160092_at	*Ifrd1*	-5.08	interferon-related developmental regulator 1
93584_at	*Igh-6*	-6.93	immunoglobulin heavy chain 6 (of IgM)
94425_at	*Ly86*	-7.21	lymphocyte antigen 86
CELL SURFACE RECEPTORS INVOLVED IN IMMUNITY			
93617_at	*Ccrl2*	-3.54	chemokine (C-C motif) receptor-like 2
100998_at	*H2-Ab1*	-3.74	histocompatibility 2, class II antigen A, β 1
CELL SURFACE MOLECULES			
98018_at	*Procr*	-3.28	protein C receptor, endothelial
APOPTOSIS			
101521_at	*Birc5*	-5.27	baculoviral IAP repeat-containing 5
SIGNALLING			
160532_at	*Tpm1*	-3.06	tropomyosin 1, α
96553_at	*Gpcr25*	-3.46	G-protein coupled receptor 25
97096_at	*Prkar2a*	-3.81	protein kinase, cAMP dependent regulatory, type II α
160082_s_at	*Arf4*	-3.94	ADP-ribosylation factor 4
160862_at	*Ptp4a3*	-4.11	protein tyrosine phosphatase 4a3
94953_at	*Racgap1*	-5.10	Rac GTPase-activating protein 1
97844_at	*Rgs2*	-7.16	regulator of G-protein signalling 2
103025_at	*Mov10*	-8.28	Moloney leukemia virus 10
TRANSCRIPTION			
101805_f_at	*Nfil3; E4BP4*	-3.61	transcription factor E4BP4
98977_at	*Tinf2*	-4.37	Terf1 (TRF1)-interacting nuclear factor 2
101727_at	*Nfkbie*	-5.15	nuclear factor of kappa enhancer in B-cells inhibitor ε
96081_at	*Tk1*	-7.08	thymidine kinase, exon 1 and 2, partial
100533_s_at	*Crem*	-11.94	cAMP responsive element modulator*
CELL GROWTH/DIVISION/DIFFERENTIATION			
96120_at	*Dnajb6*	-3.05	DnaJ (Hsp40) homologue, subfamily B, member 6
102779_at	*Gadd45b*	-3.25	growth arrest and DNA-damage-inducible 45 β
100156_at	*Mcm5*	-3.25	minichromosome maintenance deficient 5 (yeast)
93099_f_at	*Plk*	-3.86	polo-like kinase (*Drosophila*)
101979_at	*Gadd45g*	-7.12	growth arrest and DNA-damage-inducible 45 γ
102001_at	*Rrm2*	-7.81	ribonucleotide reductase M2
CELL DIVISION CYCLE GENES			
94036_at	*Cdc42ep4*	-3.23	CDC42 effector protein (Rho GTPase binding) 4
98067_at	*Cdkn1a*	-3.63	cyclin-dependent kinase inhibitor 1A (P21)*

MITOCHONDRIA			
94254_at	*Clic4*	-3.20	chloride intracellular channel 4*
99667_at	*Cox6a2*	-6.01	cytochrome c oxidase, subunit VIa, polypeptide 2
CELLULAR STRUCTURE			
101419_at	*Tubb4*	-3.27	tubulin, β 4
97909_at	*Stmn1*	-14.46	stathmin 1
DNA REPAIR/REPLICATION			
99578_at	*Top2a*	-12.15	topoisomerase (DNA) II α
TRANSPORT			
103065_at	*Slc20a1*	-3.17	solute carrier family 20, member 1
93506_at	*Slc25a3*	-3.65	solute carrier family 25, member 3
96202_at	*Slc1a2*	-4.86	solute carrier family 1, member 2
104221_at	*Slc7a5*	-4.93	solute carrier family 7, member 5
METABOLISM/BIOSYNTHESIS			
100046_at	*Mthfd2*	-3.64	methylenetetrahydrofolate dehydrogenase
95608_at	*Ctsb*	-3.66	cathepsin B*
104371_at	*Dgat1*	-4.85	diacylglycerol O-acyltransferase 1
95611_at	*Lpl*	-100.38	lipoprotein lipase*
CELL ADHESION			
93194_at	*Ly9*	-3.81	lymphocyte antigen 9
OTHER			
92851_at	*Cp*	-6.08	ceruloplasmin

An asterisk (*) indicates that the gene was detected by another probe (Section 1.10).

L.7 Up-regulated genes in BMMs after incubation for 24 h with CyaA

Affy ID	Gene name	*P* value	Fold change	Description
CD ANTIGENS				
1450513_at	*Cd33*	0.0015	3.57	CD33 antigen
1437025_at	*Cd28*	8.76E-05	4.29	CD28 antigen*,†
1451950_a_at	*Cd80*	0.000301	4.30	CD80 antigen*,†
1418394_a_at	*Cd97*	0.000321	5.28	CD97 antigen†
1449858_at	*Cd86*	0.0018	13.96	CD86 antigen†
1425243_at	*Cd207*	0.000212	141.20	CD207 antigen (langerin)
CYTOKINES AND CHEMOKINES				
1421228_at	*Ccl7*	1.92E-05	3.52	chemokine (C-C motif) ligand 7†
1417266_at	*Ccl6*	0.00114	3.59	chemokine (C-C motif) ligand 6
1449195_s_at	*Cxcl16*	0.00178	3.66	chemokine (C-X-C motif) ligand 16
1449984_at	*Cxcl2*	1.68E-05	4.95	chemokine (C-X-C motif) ligand 2†
1448995_at	*Cxcl4*	0.000543	6.25	chemokine (C-X-C motif) ligand 4
1419209_at	*Cxcl1*	0.0001	6.30	chemokine (C-X-C motif) ligand 1*,†
1419728_at	*Cxcl5*	0.00119	25.35	chemokine (C-X-C motif) ligand 5
1449399_a_at	*Il1b*	0.000605	34.86	interleukin 1 β†
1418480_at	*Cxcl7*	5.79E-05	305.80	chemokine (C-X-C motif) ligand 7
IMMUNE RESPONSE				
1438767_at	*Osm*	0.00254	3.13	oncostatin M
1426083_a_at	*Btg1*	9.17E-06	3.14	B-cell translocation gene 1, anti-proliferative
1415856_at	*Emb*	4.34E-05	3.42	embigin
1438934_x_at	*Sema4a*	0.00141	3.70	semaphorin B, mRNA sequence*
1453055_at	*Sema6d*	0.00443	5.19	Semaphorin transmembrane domain and cytoplasmic domain 6D
1424759_at	*Arrdc4*	0.000244	9.69	arrestin domain containing 4*
1429348_at	*Sema3c*	0.000314	13.69	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, 3C
CELL SURFACE RECEPTORS INVOLVED IN IMMUNITY				
1448731_at	*Il10ra*	0.00629	3.23	IL-10 receptor, α
1424595_at	*F11r*	3.94E-06	3.25	F11 receptor
1417460_at	*Ifitm2*	0.000151	3.34	IFN induced transmembrane protein 2
1421844_at	*Il1rap*	0.0036	3.74	IL-1 receptor accessory protein
1422190_at	*C5r1*	6.54E-05	4.03	complement component 5, receptor 1
1419132_at	*Tlr2*	0.000438	4.04	toll-like receptor 2
1422046_at	*Itgam*	9.66E-06	4.09	integrin α M†
1428018_a_at	*Igsf7*	7.68E-05	4.29	immunoglobulin superfamily, member 7
1449379_at	*Kdr*	2.47E-05	5.08	kinase insert domain protein receptor
1455660_at	*Csf2rb1*	6.18E-05	5.27	colony stimulating factor 2 receptor, β 1, low-affinity (granulocyte-macrophage)
1425225_at	*Fcrl3*	0.000275	5.31	Fc receptor-like 3
1448673_at	*Pvrl3*	0.00567	5.34	poliovirus receptor-related 3
1421034_a_at	*Il4ra*	0.00132	5.48	interleukin 4 receptor, α*,†
1435645_at	*Mmd*	0.00101	5.74	monocyte to macrophage differentiation-associated
1421304_at	*Klra2*	0.00117	6.57	killer cell lectin-like receptor, subfamily A, member 2
1418741_at	*Itgb7*	0.00141	7.66	integrin β 7†
1460271_at	*Trem3*	0.00404	8.10	triggering receptor expressed on myeloid cells 3
1451584_at	*Havcr2*	0.00031	8.82	hepatitis A virus cellular receptor
21425951_a_at	*Clecsf10*	0.000658	9.81	C-type lectin, superfamily member 10 (Ca^{2+} dependent, carbohydrate recognition domain)
1455660_at	*Csf2rb2*	6.78E-05	9.95	colony stimulating factor 2 receptor,

				β 2, low-affinity (granulocyte-macrophage)
1435560_at	*Itgal*	2.33E-05	11.01	integrin α L*
1422041_at	*Pilrb*	0.00156	11.06	paired immunoglobin-like type 2 receptor β
1440865_at	*Ifitm6*	8.78E-05	14.54	interferon induced transmembrane protein 6
1419609_at	*Ccr1*	0.000371	19.51	chemokine (C-C motif) receptor 1†
1424254_at	*Ifitm1*	0.000112	33.33	interferon induced transmembrane protein 1
1419532_at	*Il1r2*	0.000103	36.27	interleukin 1 receptor, type II†
1447284_at	*Trem1*	8.26E-05	53.32	triggering receptor expressed on myeloid cells 1*
1417625_s_at	*Cmkor1*	0.000565	55.78	chemokine orphan receptor 1
CELL SURFACE RECEPTORS				
1454966_at	*Itga8*	0.000151	3.08	integrin α 8*
1418826_at	*Ms4a6b*	0.000808	3.17	membrane-spanning 4-domains, subfamily A, member 6B
1417179_at	*Tm4sf9*	0.000407	3.22	transmembrane 4 superfamily member 9
1426794_at	*Ptprs*	0.00237	3.25	protein tyrosine phosphatase, receptor type, S
1428615_at	*P2y5*	3.54E-05	3.25	purinergic receptor (family A group 5)
1421839_at	*Abca1*	0.00389	3.48	ATP-binding cassette, sub-family A member 1
1428988_at	*Abcc3*	0.000774	3.82	ATP-binding cassette, sub-family C (CFTR/MRP), member 3
1419601_at	*Kcnj10*	0.00111	4.07	potassium inwardly-rectifying channel, subfamily J, member 10
1437363_at	*Homer1*	0.00246	4.13	homer homolog 1 (*Drosophila*)*
1450234_at	*Ms4a6c*	5.56E-05	4.40	membrane-spanning 4-domains, subfamily A, member 6C
1452521_a_at	*Plaur*	0.00149	5.67	urokinase plasminogen activator receptor
1420407_at	*Ltb4r1*	0.00524	5.78	leukotriene B4 receptor 1†
1417894_at	*Gpr97*	0.00562	6.45	G protein-coupled receptor 97
1450214_at	*Adora2b*	0.000755	6.57	adenosine A2b receptor†
1450868_at	*D8Ertd354e*	0.00085	7.14	DNA segment, Chr 8, ERATO Doi 354,
1419309_at	*Gp38*	0.00589	7.53	glycoprotein 38†
1426258_at	*Sorl1*	0.00293	7.70	sortilin-related receptor
1420603_s_at	*Raet1a*	0.000103	8.16	retinoic acid early transcript γ
1420401_a_at	*Ramp3*	0.00117	10.28	receptor (calcitonin) activity modifying protein 3
1429310_at	*Flrt3*	0.000679	11.05	fibronectin leucine rich transmembrane protein 3*
1419647_a_at	*Ier3*	0.000104	11.90	immediate early response 3†
1423467_at	*Ms4a4b*	0.00025	14.82	membrane-spanning 4-domains, subfamily A, member 4B
1420842_at	*Ptprf*	0.00125	16.87	protein tyrosine phosphatase, receptor type, F
1415800_at	*Gja1*	0.000141	19.46	gap junction membrane channel protein α 1*,†
1419759_at	*Abcb1a*	0.00021	33.24	ATP-binding cassette, sub-family B member 1A
1456601_x_at	*Fxyd2*	1.33E-05	42.68	FXYD domain-containing ion transport regulator 2*
APOPTOSIS				
1450731_s_at	*Tnfrsf21*	0.000328	3.24	tumor necrosis factor receptor superfamily, member 21
1456006_at	*Bcl2l11*	0.00013	4.81	BCL2-like 11*
1450997_at	*Stk17b*	4.97E-05	4.85	serine/threonine kinase 17b*
1418649_at	*Egln3*	0.00263	5.33	EGL nine homolog 3 (*C. elegans*)
1427127_x_at	*Hspa1b*	1.13E-05	13.34	heat shock protein 1A*
SIGNALLING				
1422818_at	*Nedd9*	0.00146	3.33	neural precursor cell expressed, developmentally down-regulated gene 9
1445539_at	*Pde7b*	0.00138	3.50	phosphodiesterase 7B
1415834_at	*Dusp6*	8.93E-05	3.59	dual specificity phosphatase 6†
1452202_at	*Pde2a*	0.000951	4.12	phosphodiesterase 2A

1438097_at	*Rab20*	0.000217	4.17	RAB20, member RAS oncogene family
1451715_at	*Mafb*	0.00152	4.36	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)
1428733_at	*Gngt2*	0.000417	5.25	guanine nucleotide binding, γ transducing activity polypeptide 2
1417694_at	*Gab1*	4.92E-05	5.81	growth factor receptor bound protein 2-associated protein 1
1455181_at	*Rasa2*	0.00119	6.02	RAS p21 protein activator 2*
1420664_s_at	*Procr*	0.000437	6.23	protein C receptor, endothelial[†]
1434261_at	*Sipa1l2*	0.00135	7.76	signal-induced proliferation-associated 1 like 2
1416701_at	*Rhoe*	0.000272	7.80	ras homolog gene family, member E*
1450808_at	*Fpr1*	0.00369	11.20	formyl peptide receptor 1[†]
1417143_at	*Edg2*	0.000262	13.27	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2*
1422474_at	*Pde4b*	1.50E-05	16.49	phosphodiesterase 4B*
1449145_a_at	*Cav*	0.00025	44.11	caveolin, caveolae protein[-]

TRANSCRIPTION

1417394_at	*Klf4*	0.00366	3.06	Kruppel-like factor 4 (gut)
1423176_at	*Tob1*	0.000105	3.46	transducer of ErbB-2.1
1449530_at	*Trps1*	2.60E-05	3.51	trichorhinophalangeal syndrome I (human)
1417662_at	*Elk3*	3.33E-06	3.55	member of ETS oncogene family*
1427844_a_at	*Cebpb*	0.000529	3.98	CCAAT/enhancer binding protein β*,[†]
1419354_at	*Klf7*	0.00356	4.04	Kruppel-like factor 7 (ubiquitous)
1421604_a_at	*Klf3*	0.000245	4.16	Kruppel-like factor 3 (basic)**
1423100_at	*Fos*	0.000568	4.44	FBJ osteosarcoma oncogene
1416035_at	*Hif1a*	0.000209	4.51	hypoxia inducible factor 1, α subunit*
1415899_at	*Junb*	0.000838	4.61	Jun-B oncogene[+]
1426464_at	*Nr1d1*	0.00203	4.61	nuclear receptor subfamily 1, group D, member 1
1418025_at	*Bhlhb2*	0.000411	4.83	basic helix-loop-helix domain, class B2
1451132_at	*Pbxip1*	3.68E-05	5.20	pre-B-cell leukemia transcription factor interacting protein 1
1422697_s_at	*Jarid2*	0.000388	5.48	jumonji, AT rich interactive domain 2
1420796_at	*Ahrr*	0.000668	5.99	aryl-hydrocarbon receptor repressor
1451255_at	*Lisch7*	0.000657	7.45	liver-specific bHLH-Zip transcription factor
1425732_a_at	*Mxi1*	0.00153	8.09	Max interacting protein 1* contains bhlh domain
1437247_at	*Fosl2*	0.000498	10.11	fos-like antigen 2
1440275_at	*Runx3*	0.00321	10.58	runt related transcription factor 3
1428306_at	*Ddit4*	0.000236	10.60	DNA-damage-inducible transcript 4
1448713_at	*Stat4*	0.000205	13.79	signal transducer and activator of transcription 4
1449037_at	*Crem*	0.000119	18.58	cAMP responsive element modulator[-]
1422631_at	*Ahr*	0.000259	24.16	aryl-hydrocarbon receptor[†]

CELL GROWTH/DIVISION/DIFFERENTIATION

1434496_at	*Plk3*	0.00385	4.10	polo-like kinase 3 (*Drosophila*)[+]
1436405_at	*Dock4*	0.000338	5.25	dedicator of cytokinesis 4
1418634_at	*Notch1*	0.00224	7.26	Notch gene homolog 1 (*Drosophila*)[+]
1420909_at	*Vegfa*	0.00397	8.04	vascular endothelial growth factor A[†]
1423475_at	*Cnnm2*	0.000291	19.49	cyclin M2
1427256_at	*Cspg2*	0.000125	232.80	chondroitin sulfate proteoglycan 2

MITOCHONDRION

1416381_a_at	*Prdx5*	1.51E-05	4.58	peroxiredoxin 5
1418847_at	*Arg2*	0.000442	27.75	arginase type II*,[†]

CELLULAR STRUCTURE

1421090_at	*Epb4.1l1*	0.00284	3.14	erythrocyte protein band 4.1-like 1*
1455405_at	*Pstpip2*	0.00156	3.74	proline-serine-threonine phosphatase-interacting protein 2*

1416298_at	*Mmp9*	0.00521	6.08	matrix metalloproteinase 9*
1421976_at	*Mmp19*	0.000268	6.47	matrix metalloproteinase 19*
1448990_a_at	*Myo1b*	0.000368	8.22	myosin IB***
1415943_at	*Sdc1*	0.00012	13.50	syndecan 1*
PLASMA MEMBRANE/SIGNALLING				
1421407_at	*F2rl2*	0.000625	23.96	coagulation factor II (thrombin) receptor-like2
TRANSPORT				
1453721_a_at	*Slc31a2*	0.00038	3.01	solute carrier family 31, member 2
1435009_at	*Slc9a6*	0.0017	3.11	solute carrier family 9 (sodium/hydrogen exchanger), isoform 6
1434773_a_at	*Slc2a1*	0.00336	3.23	solute carrier family 2 (facilitated glucose transporter), member 1†
1447181_s_at	*Slc7a7*	0.000523	3.23	solute carrier family 7 (cationic amino acid transporter, y+ system), member 7
1453915_a_at	Slc37a3	0.000456	3.69	solute carrier family 37 (glycerol-3-phosphate transporter), member 3
1420697_at	*Slc15a3*	3.08E-05	5.73	solute carrier family 15, member 3
1449005_at	*Slc16a3*	1.15E-05	6.95	solute carrier family 16 (monocarboxylic acid transporters), member 3
1457989_at	*Slc4a11*	0.00278	7.86	solute carrier family 4, sodium bicarbonate transporter-like, member 11
1420413_at	*Slc7a11*	0.00139	8.01	solute carrier family 7 (cationic amino acid transporter, y+ system), member 11
METABOLISM/BIOSYNTHESIS				
1449454_at	*Bst1*	0.00419	3.10	bone marrow stromal cell antigen 1
1449538_a_at	*Gcnt1*	0.000651	3.30	glucosaminyl (N-acetyl) transferase 1, core 2
1416086_at	*Tpst2*	5.83E-05	3.31	protein-tyrosine sulfotransferase 2
1418645_at	*Hal*	0.00015	3.36	histidine ammonia lyase
1448647_at	*Man2a1*	0.000193	3.43	mannosidase 2, α 1
1419692_a_at	*Ltc4s*	0.000375	3.72	leukotriene C4 synthase
1454666_at	*Sptlc2*	0.00494	3.80	serine palmitoyltransferase, long chain base subunit 2
1435133_at	*Ugcg*	0.00263	4.25	UDP-glucose ceramide glucosyltransferase*
1423414_at	*Ptgs1*	9.67E-05	5.78	prostaglandin-endoperoxide synthase 1*
1451843_a_at	*Ggta1*	0.00321	6.66	glycoprotein galactosyltransferase α 1, 3*
1430388_a_at	*Sulf2*	8.83E-05	6.68	sulfatase 2*
1420994_at	*B3gnt5*	0.00194	18.41	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5
1428547_at	*Nt5e; CD73*	4.08E-05	60.75	5' nucleotidase, ecto*,†
ADHESION				
1460302_at	*Thbs1*	1.21E-06	362.90	thrombospondin 1*,†
REGULATORY				
1451798_at	*Il1rn*	0.00769	2.65	interleukin 1 receptor antagonist*,†
1434891_at	4833445A08Rik	0.00331	4.53	prostaglandin F2 receptor negative regulator

* indicates the gene being recognised by a second probe (Section 1.10).

† indicates that the gene has been detected in the preliminary experiment (Section 3.10.3).

L.8 Down-regulated genes in BMMs after incubation for 24 h with CyaA

Affy ID	Gene name	*P* value	Fold change	Description
CD ANTIGENS				
1448182_a_at	*Cd24a*	0.00149	-3.57	CD24a antigen
1436346_at	*Cd109*	0.000699	-4.76	CD109 antigen
CYTOKINES AND CHEMOKINES				
1418930_at	*Cxcl10*	5.30E-05	-3.85	chemokine (C-X-C motif) ligand 10
1419561_at	*Ccl3*	4.23E-05	-5.62	chemokine (C-C motif) ligand 3[†]
1421578_at	*Ccl4*	6.70E-05	-4.63	chemokine (C-C motif) ligand 4[†]
IMMUNE RESPONSE				
1448736_a_at	*Hprt*	0.00105	-3.51	hypoxanthine guanine phosphoribosyl transferase
1441075_at	LOC329416	0.00248	-3.82	nitric oxide synthase trafficker
1421547_at	*Ly78*	0.00509	-4.35	lymphocyte antigen 78
1451905_a_at	*Mx1*	0.0011	-4.52	myxovirus (influenza virus) resistance 1
1419721_at	*Gpr109b*	0.00115	-4.88	interferon-γ inducible gene, Puma-g
1415855_at	*Kitl*	0.00152	-5.49	kit ligand
1427329_a_at	*Igh-6*	0.000116	-14.31	immunoglobulin heavy (of IgM) chain 6
CELL SURFACE RECEPTORS INVOLVED IN IMMUNITY				
1421596_s_at	*H28*	0.00169	-3.19	histocompatibility 28
1452431_s_at	*H2-Aa*	0.00138	-3.34	histocompatibility 2, class II antigen A α[*,†]
1450648_s_at	*H2-Ab1*	0.00144	-3.37	histocompatibility 2, class II antigen A β 1[*,†]
1422892_s_at	*H2-Ea*	0.00218	-4.03	histocompatibility 2, class II antigen E α[†]
1444040_at	*Lair1*	0.000927	-4.93	leukocyte-associated Ig-like receptor 1*
1416050_a_at	*Scarb1*	0.000857	-6.80	scavenger receptor class B, member 1[†]
1421792_s_at	*Trem2*	0.000438	-7.46	triggering receptor expressed on myeloid cells
CELL SURFACE RECEPTORS				
1420699_at	*Clecsf12*	0.00263	-3.33	C-type (calcium dependent, carbohydrate recognition domain) lectin, superfamily member 12
1440882_at	*Lrp8*	0.000421	-3.97	low density lipoprotein receptor-related protein 8
1419605_at	*Mgl1*	0.000695	-4.90	macrophage galactose N-acetyl-galactosamine specific lectin 1
1422430_at	*Fignl1*	0.00511	-7.69	fidgetin-like 1
1424208_at	*Ptger4*	0.00513	-9.26	prostaglandin E receptor 4
1437347_at	*Ednrb*	0.000471	-10.92	endothelin receptor type B
1422445_at	*Itga6*	0.00292	-12.58	integrin α 6
APOPTOSIS				
1429494_at	*Trim35*	0.00124	-3.18	tripartite motif-containing 35
1428842_a_at	*Ngfrap1*	0.0018	-3.28	nerve growth factor receptor (TNFRSF16) associated protein 1
1424278_a_at	*Birc5*	0.00344	-16.61	baculoviral IAP repeat-containing 5[†]
SIGNALLING				
1449175_at	*Gpr65*	0.00224	-3.69	G-protein coupled receptor 65
1434518_at	*Phka2*	5.60E-05	-5.10	phosphorylase kinase α 2
1437303_at	*Il6st*	0.00398	-4.48	interleukin 6 signal transducer*
1419247_at	*Rgs2*	0.000467	-8.06	regulator of G-protein signalling 2[†]
1451358_a_at	*Racgap1*	0.000104	-15.34	Rac GTPase-activating protein 1[*,†]
1448627_s_at	*Pbk*	3.88E-05	-29.59	PDZ binding kinase

TRANSCRIPTION

1418036_at	*Prim2*	0.00409	-3.13	DNA primase, p58 subunit
1439012_a_at	*Dck*	0.00103	-4.27	Deoxycytidine kinase, mRNA sequence
1435368_a_at	*Adprtl*	0.00203	-4.35	ADP-ribosyltransferase 1
1424629_at	*Brca1*	0.00103	-4.83	breast cancer 1
1448834_at	*Foxm1*	0.00272	-5.46	forkhead box M1
1424105_a_at	*Pttg1*	0.000218	-6.45	pituitary tumor-transforming 1
1420715_a_at	*Pparg*	0.000305	-6.94	peroxisome proliferator activated receptor γ
1437187_at	*E2f7*	0.000214	-8.20	E2F transcription factor 7
1449363_at	*Atf3*	0.00214	-8.77	activating transcription factor 3
1416544_at	*Ezh2*	0.00126	-11.64	enhancer of zeste homolog 2 (*Drosophila*)
1423809_at	*Tcf19*	0.000229	-15.77	transcription factor 19
1416258_at	*Tk1*	0.000547	-22.37	thymidine kinase 1[†]
1415810_at	*Uhrf1*	0.000199	-34.01	ubiquitin-like, containing PHD and RING finger domains, 1 (nuclear protein 95)[†]

CELL GROWTH/DIVISION/DIFFERENTIATION

1415775_at	*Rbbp7*	0.000919	-3.32	retinoblastoma binding protein 7
1450886_at	*Gsg2*	0.000745	-3.39	germ cell-specific gene 2
1416773_at	*Wee1*	0.00358	-3.55	wee 1 homolog (*S. pombe*)
1450414_at	*Pdgfb*	0.000691	-4.13	platelet derived growth factor, B
1427005_at	*Plk2*	0.000788	-4.39	polo-like kinase 2 (*Drosophila*)
1451567_a_at	*Ifi203*	0.00466	-4.65	interferon activated gene 203*[,†]
1424511_at	*Stk6*	0.000868	-4.76	serine/threonine kinase 6 (Aurora kinase A)
1424156_at	*Rbl1*	0.00364	-4.78	retinoblastoma-like 1 (p107)
1452954_at	*Ube2c*	0.000771	-5.92	ubiquitin-conjugating enzyme E2C
1416214_at	*Mcm4*	0.000439	-6.58	minichromosome maintenance deficient 4 homolog (*S. cerevisiae*)*
1448635_at	*Smc2l1*	0.000194	-7.75	structural maintenance of chromosomes 2-like 1 (yeast)*
1426653_at	*Mcm3*	0.00194	-8.13	minichromosome maintenance deficient 3 (*S. cerevisiae*)*[,†]
1424128_x_at	*Aurkb*	0.00261	-8.26	aurora kinase B[†]
1437580_s_at	*Nek2*	0.000962	-8.33	never in mitosis gene A-related expressed kinase 2
1419838_s_at	*Plk4*	0.00152	-8.33	polo-like kinase 4 (*Drosophila*)*[,†]
1423877_at	*Chaf1b*	0.0006	-8.47	chromatin assembly factor 1, subunit B (p60)[†]
1416746_at	*H2afx*	0.00136	-9.17	H2A histone family, member X
1422460_at	*Mad2l1*	0.00316	-9.17	mitotic arrest deficient, homolog-like 1 (yeast)
1423775_s_at	*Prc1*	2.13E-05	-9.17	protein regulator of cytokinesis 1
1447363_s_at	*Bub1b*	0.00246	-10.65	budding uninhibited by benzimidazoles 1 homolog, β (*S. cerevisiae*)*
1448777_at	*Mcm2*	0.000658	-13.95	minichromosome maintenance deficient 2 mitotin (*S. cerevisiae*)
1416030_a_at	*Mcm7*	0.002	-18.69	minichromosome maintenance deficient 7 (*S. cerevisiae*)[†]
1415945_at	*Mcm5*	0.000415	-19.01	minichromosome maintenance deficient 5, cell division cycle 46 (*S. cerevisiae*)*[,†]
1418293_at	*Ifit2*	0.000808	-19.72	interferon-induced protein with tetratricopeptide repeats 2[†]
1416309_at	*Nusap1*	0.00169	-22.22	nucleolar and spindle associated protein 1
1419519_at	*Igf1*	0.00159	-31.45	insulin-like growth factor 1*[,†]
1438852_x_at	*Mcm6*	0.00207	-32.05	minichromosome maintenance deficient 6 (MIS5 homolog, *S. pombe*)*

CELL DIVISION CYCLE GENES

1417131_at	*Cdc25a*	0.0019	-3.50	cell division cycle 25A, mRNA sequence
1454742_at	*Rasgef1b*	8.24E-05	-4.08	RasGEF domain family, member 1B
1426002_a_at	*Cdc7*	0.000302	-4.63	cell division cycle 7 (*S. cerevisiae*)
1439377_x_at	*Cdc20*	0.00277	-7.09	cell division cycle 20 homolog (*S. cerevisiae*)*[,†]
1456077_x_at	*Cdc25c*	0.00268	-5.32	cell division cycle 25 phosphatase mRNA
1416575_at	*Cdc45l*	0.00472	-5.92	cell division cycle 45 homolog (*S. cerevisiae*)-like

1448314_at	*Cdc2a*	5.90E-05	-5.99	cell division cycle 2 homolog A (*S. pombe*)†
1417458_s_at	*Cks2*	0.000293	-6.21	cell division cycle 28 protein kinase regulatory subunit 2
1417019_a_at	*Cdc6*	0.00148	-6.76	cell division cycle 6 homolog (*S. cerevisiae*)
1448441_at	*Cks1*	0.00089	-8.85	cell division cycle 28 protein kinase regulatory subunit 1
1430811_a_at	*Cdca1*	0.000484	-12.29	cell division cycle associated 1
CYCLINS				
1417420_at	*Ccnd1*	5.21E-05	-4.52	cyclin D1*,†
1416868_at	*Cdkn2c*	0.0019	-6.80	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)
1422513_at	*Ccnf*	0.00306	-6.99	cyclin F†
1450920_at	*Ccnb2*	0.000145	-7.94	cyclin B2
1419943_s_at	*Ccnb1*	0.000873	-8.70	cyclin B1*
1422535_at	*Ccne2*	0.000758	-10.36	cyclin E2
1417910_at	*Ccna2*	0.00332	-20.88	cyclin A2*
KINESINS				
1450692_at	*Kif4*	0.00115	-6.45	kinesin family member 4
1437611_x_at	*Kif2c*	0.00496	-9.52	kinesin family member 2C*,†
1452315_at	*Kif11*	7.96E-05	-11.29	kinesin family member 11*
1451128_s_at	*Kif22*	0.000237	-11.98	kinesin family member 22*,†
MITOCHONDRION				
1450048_a_at	*Idh2*	0.00318	-3.53	isocitrate dehydrogenase 2 (NADP+), mRNA
1416345_at	*Timm8a*	0.000238	-3.58	translocase of inner mitochondrial membrane 8 homolog a (yeast)
1423392_at	*Clic4*	0.000451	-4.52	chloride intracellular channel 4*,†
1422978_at	*Cybb*	0.00343	-6.54	cytochrome b-245, β polypeptide†
1426423_at	*Shmt2*	0.00449	-8.00	serine hydroxymethyl transferase 2
CELLULAR STRUCTURE				
1417144_at	*Tubg1*	0.00355	-3.57	tubulin, γ 1
1425476_at	*Col4a5*	0.00257	-3.97	procollagen, type IV, α 5
1451989_a_at	*Mapre2*	0.000223	-4.48	microtubule-associated protein, RP/EB family, member 2
1433892_at	*Spag5*	0.000783	-5.41	sperm associated antigen 5*
1428976_at	*Tmpo*	0.000568	-6.45	thymopoietin*
1417450_a_at	*Tacc3*	0.000138	-7.46	transforming, acidic coiled-coil containing protein 3*
1434748_at	*Ckap2*	0.004	-8.55	cytoskeleton associated protein 2†
1449153_at	*Mmp12*	0.000328	-8.62	matrix metalloproteinase 12
1417445_at	*Knte2*	0.000616	-8.85	kinetochore associated 2
DNA REPLICATION				
1417947_at	*Pcna*	0.000219	-4.27	proliferating cell nuclear antigen
1438130_at	*Taf15*	0.00252	-4.72	TATA box binding protein (TBP)-associated factor
1416641_at	*Lig1*	0.000829	-5.29	ligase I, DNA, ATP-dependent
1416915_at	*Msh6*	0.00365	-8.20	mutS homolog 6 (*E. coli*)
1452534_a_at	*Hmgb2*	0.000393	-8.33	high mobility group box 2*
1417541_at	*Hells*	0.000145	-9.62	helicase, lymphoid specific (Hells), mRNA*
1450862_at	*Rad54l*	3.96E-05	-11.52	RAD54 like (*S. cerevisiae*)
1454694_a_at	*Top2a*	0.000765	-14.62	topoisomerase (DNA) II α†
1419397_at	*Pola1*	4.74E-05	-15.06	polymerase (DNA directed), α 1
1418281_at	*Rad51*	0.000133	-18.08	RAD51 homolog (*S. cerevisiae*)†
1448226_at	*Rrm2*	0.00068	-35.97	ribonucleotide reductase M2*,†

TRANSPORT

1415775_at	*Rbbp7*	0.000919	-3.32	retinoblastoma binding protein 7
1451782_a_at	*Slc29a1*	0.00116	-3.06	solute carrier family 29 (nucleoside transporters), member 1
1417061_at	*Slc40a1*	0.00603	-3.08	solute carrier family 40 (iron-regulated transporter), member 1
1416954_at	*Slc25a10*	0.00283	-3.45	solute carrier family 25 (mitochondrial carrier; dicarboxylate transporter), member 10
1416629_at	*Slc1a5*	0.00536	-3.91	solute carrier family 1 (neutral amino acid transporter), member 5
1421129_a_at	*Atp2a3*	0.00216	-4.10	ATPase, Ca++ transporting, ubiquitous
1422788_at	*Slc43a3*	0.000493	-5.62	solute carrier family 43, member 3
1418326_at	*Slc7a5*	0.00106	-13.16	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5
1437052_s_at	*Slc2a3*	0.00166	-14.64	solute carrier family 2 (facilitated glucose transporter), member 3

METABOLISM/BIOSYNTHESIS

1415852_at	*Impdh2*	0.000321	-3.18	inosine 5'-phosphate dehydrogenase 2
1455106_a_at	*Ckb*	0.000544	-3.38	creatine kinase, brain
1415823_at	*Scd2*	0.00243	-3.70	stearoyl-Coenzyme A desaturase 2*
1416563_at	*Ctps*	0.0037	-3.86	cytidine 5'-triphosphate synthase
1452681_at	*Dtymk*	0.000449	-4.67	deoxythymidylate kinase*
1454843_at	*Prps2*	0.000572	-4.78	phosphoribosyl pyrophosphate synthetase 2
1423569_at	*Gatm*	0.00153	-5.29	L-arginine:glycine amidinotransferase
1423828_at	*Fasn*	0.000331	-5.32	fatty acid synthase
1437874_s_at	*Hexb*	0.000104	-5.81	hexosaminidase B
1437325_x_at	*Pycs*	0.000179	-6.58	pyrroline-5-carboxylate synthetase
1454607_s_at	*Psat1*	0.000347	-8.26	phosphoserine aminotransferase 1[†]
1419270_a_at	*Dutp*	0.00229	-10.11	deoxyuridine triphosphatase
1422479_at	*Acas2*	0.0015	-10.33	acetyl-Coenzyme A synthetase 2 (ADP forming)*
1424991_s_at	*Tyms*	0.0034	-12.21	thymidylate synthase
1433966_x_at	*Asns*	0.0019	-12.44	asparagine synthetase*
1431056_a_at	*Lpl*	0.000184	-102.04	lipoprotein lipase[†]

REGULATORY

1426065_a_at	*Trib3*	0.00319	-4.50	Tribbles homologue*

OTHER

1419513_a_at	*Ect2*	9.23E-05	-6.71	ect2 oncogene
1419254_at	*Mthfd2*	0.00201	-14.95	methylenetetrahydrofolate dehydrogenase (NAD+ dependent)

* indicates the gene being recognised by a second probe (Section 1.10).

[†] indicates that the gene has been detected in the preliminary experiment (Section 3.10.3).

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