ACTIVATION AND IMMUNOGENICITY OF *BORDETELLA PERTUSSIS* ADENYLATE CYCLASE TOXIN

E. KALANTAR HORMOZI

Activation and Immunogenicity of *Bordetella pertussis* Adenylate Cyclase Toxin

E. KALANTAR HORMOZI

Presented for the Degree of Doctorate of Philosophy in the Faculty of Medicine, University of Glasgow.

Division of Infection and Immunity **June, 1997**

DEDICATION

I should like to dedicate this thesis to my wife, Pourandokht, and my children, Afaf, Beesat, Olya and Rafat for all their love, mental support, happiness, encouragement and motivation throughout my research.

CONTENTS

2. MATERIALS AND METHODS

3. RESULTS

Declaration

This thesis is the original work of the author.

E. Kalantar Hormozi

Acknowledgements

I wish to express my sincere thanks to my supervisors Dr. John G. Cootc and Dr. Roger Parton for excellent guidance, encouragement, patience, useful comments and suggestions, continued supervision and constructive discussion throughout my research and during the writing of this thesis.

I am also grateful to Dr. Gareth Westrop for his useful suggestions, collaboration, kindness, help and providing plasmids during my work.

I greatly acknowledge Dr. R. Aitken for his guidance in computer handling and helping me in converting the Figs. into colour prints.

I am also thankful to Dr. Elizabet Hall and Dr. M. Roberts and Prof. Duncan Stewart-Tull for generous donation of antisera and adjuvant.

I greatly appreciate the help of Dr. Cathrine Lawrence in performing cytokine assays.

I also thank Berit Adams and Susan Campbell for their technical assistance.

I would like to thank Dr. M. Saadati, Dr. R. Hosseiniduost, Dr. A. Azad, C. Brotherston and Giamal Fellah for helpful discussions and friendliness.

I am thankful to all Microbiology staff members for their help in making this research so enjoyable.

I express my deepest gratitude to my wife, my children and the rest of my family for their help and encouragement.

Finally, I thank my financial support by the Ministry of Health and Medical Education, Islamic Republic of Iran for awarding me a scholarship and their financial support for completing my Ph.D.

PRESENTATIONS (ABSTRACT/PUBLICATION)

G. D. WESTROP, E. K. HORMOZI, M. SAADATI, R. PARTON AND J. G. COOTE. (1995). Studies of the adenylate cyclase toxin (CyaA) of *Bordetella pertussis* and the leukotoxin (LktA) of *Pasteurella haemolytica* in-vitro and trans-complemented by heterologous C proteins in vivo. Presented at the Seventh European Workshop Conference on Bacterial Protein Toxins, July 1995. Hindsgavl, Middlefart, Denmark.

E. KALANTAR HORMOZI, GARETH WESTROP, ROGER PARTON AND JOHN G. COOTE. (1996). Protective activities of active, inactive and hybrid adenylate cyclase toxins of *Bordetella pertussis*. Presented at the annual meeting of Society for General Microbiology (SGM), University of Essex, September 1996.

GARETH D. WESTROP, E. KALANTAR HORMOZI, NUNO A. DA COSTA, ROGER PARTON, JOHN G. COOTE. (1996). *Bordetella pertussis* adenylate cyclase toxin: ProCyaA and CyaC proteins synthesised separately in *Escherichia coli* produce active toxin in vitro. *Gene*, **180** : 91-99.

GARETH WESTROP, KALANTAR HORMOZI, NUNO DA COSTA, ROGER PARTON AND JOHN G. COOTE. (1997). Structure-function studies of the adenylate cyclase toxin of *Bordetella pertussis* and the leukotoxin of *Pasteurella haemolytica* by heterologous C protein activation and construction of hybrid proteins. *Journal of Bacteriology* , **179** : 871-879.

ABBREVIATIONS

Abbreviation	
A_{650}	Absorbance at 650 nm
aa	Amino acid (s)
Abs	Absorbance
AC	Adenylate cyclase
ACP	Acyl carrier protein
ACV	Acellular vaccine
ADP	Adenosine diphosphate
Ag	Antigen
AGG	Agglutinogen
Ap	Ampicillin
Ap^R	Gene encoding ampicillin resistance
ATP	Adenosine triphosphate
BG	Bordet-Gengou agar
BHK	Baby hamster kidney
BP	*Bordetella pertussis*
bp	Base pair (s)
BSA	Bovine serum albumin
bvg	*Bordetella* virulence regulatory gene
°C	Degrees Celsius
CAA	Casamino acids
CAF	Cytosolic activation factor
CaM	Calmodulin
cAMP	Cyclic-adenosine monophosphate
CAN CyaA	Crude active native adenylate cyclase toxin
CAR CyaA	Crude active recombinant adenylate cyclase toxin
CDL	Cyclodextrin liquid media

cfu	**Colony forming units**
CI	**Chemiluminescence inhibition**
CIN CyaA	**Crude inactive native adenylate cyclase toxin**
CIR CyaA	**Crude inactive recombinant adenylate cyclase toxin**
CL	**Chemiluminescence**
Cm	**Chloramphenicol**
cm	**Centimetre**
Cm^R	**Gene encoding chloramphenicol resistance**
Conc	**Concentration**
Cya	**Adenylate cyclase phenotype**
cya A	**Gene encoding CyaA**
CyaA	**Adenylate cyclase toxin**
CyaC	**CyaA protoxin activator protein**
cyaC	**Gene encoding CyaC**
DAB	**3-3'-diaminobenzidene tetrahydrochloride**
DMSO	**Dimethylsulphoxide**
DNA	**Deoxyribonucleic acid**
DNDH	**Dimethylamino-naphthalene-1,2-dicarbonicacid hydrazide**
DPT	**Diphtheria Pertussis Tetanus triple vaccine**
EDTA	**Ethylenediamine tetra-acetic acid**
EGTA	**Ethyleneglycol-bis N,N'-tetraacetic acid**
ELISA	**Enzyme linked immunosorbent assay**
FCS	**Foetal calf serum**
FHA	**Filamentous haemagglutinin**
fha	**Filamentous haemagglutinin gene**
fim	**Fimbrial gene**

g	**Grammes**
h	**Hour (s)**
^{3}H	**Radioisotope hydrogen**
HA	**Hamagglutination**
HEPES	**N-2-hydroxy ethylpiperazine-N'-2-ethanosulphonic acid**
HH	**Hanks Hepes**
HLT	**Heat labile toxin**
Hly	**Haemolysin phenotype**
hly	**Haemolysin gene**
HlyA	**Haemolysin protein**
hlyA	**Gene encoding HlyA**
HRP	**Horse radish peroxidase**
Hyb1	**Hybrid 1 toxin**
hyb1	**Gene encoding Hyb1**
Hyb2	**Hybrid 2 toxin**
hyb2	**Gene encoding Hyb2**
i/m	**Intramuscular**
Ig	**Immunoglobulin**
IL	**Interleukin**
INF	**Interferon**
IPTG	**Isopropyl β-D-thiogalactopyranoside**
kb	**Kilo base (s) or 1000 base**
kbp	**Kilo base pairs**
kDa	**Kilo Dalton**
Km	**Kanamycin**
l	**Litre**
lac	**Lactose operon gene**

Lkt	**Leukotoxin**
lkt	**Leukotoxin gene**
LktA	**Leukotoxin protein**
lktA	**Gene encoding LktA**
LktC	**LktA protoxin activator protein**
lktC	**Gene encoding LktC**
LPS	**Lipopolysaccharide**
M	**Molar**
MA	**Myristic acid**
mA	**Milliamps**
Mab	**Monoclonal antibody**
mCi	**Millicurie**
MeβCD	**Heptakis (2, 6-0-methyl) β-cyclodextrin**
μg	**Microgram**
mg	**Milligram (s)**
min	**Minute (s)**
μl	**Microlitre**
ml	**Millilitre (s)**
mM	**Millimolar**
μm	**Micrometre**
mm	**Millimetre (s)**
Mol. wt	**Molecular Weight**
NCIMB	**National Collection of Industrial and Marine Bacteria**
NCTC	**National Collection of Type Cultures**
nm	**Nanometre**
OA	**Oleic acid**

Oa	Ovalbumin
OD	Optical density
OM	Outer membrane
OMP	Outer membrane protein (s)
OPD	O-phenylene diamine
ORF	Open reading frame
ou	Opacity units
^{32}P	Radioisotope phosphorus
PA	Palmitic acid
PAGE	Polyacrylamide gel electrophoresis
PAN CyaA	Purified active native adenylate cyclase toxin
PAR CyaA	Purified active recombinant adenylate cyclase toxin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pg	Picogram
pH	Hydrogen ion concentration
PIN CyaA	Purified inactive native adenylate cyclase toxin
PIR CyaA	Purified inactive recombinant adenylate cyclase toxin
PLA	Palmitoleic acid
PMA	Phorbol myristate acetate
Pmol	Picomole
PRN	Pertactin
prn	Pertactin gene
pro CyaA	Adenylate cyclase protoxin
proHyb2	Hybrid 2 protoxin
proLktA	Leukotoxin protoxin
psi	Pounds per square inch

PT	**Pertussis toxin**
ptx	**Pertussis toxin gene**
RBC	**Red blood cell**
rcf	**Relative centrifugal force (g)**
RID	**Radial immuno diffusion test**
RNA	**Ribonucleic acid**
rpm	**Revolutions per minute**
RTX	**Repeats in toxin**
S100	**Cytosolic fraction prepared from *E. coli* BL21 by centrifugation at 100,000 g**
s/c	**Subcutaneously**
SDS	**Sodium dodecyl sulphate**
Sec	**Second (s)**
SEM	**Standard error of mean**
Tc	**Tetracycline**
TCT	**Tracheal cytotoxin**
TEMED	**N, N, N', N'-tetramethyl-ethylenediamine**
Tn	**Transposon**
Tris	**Tris (hydroxymethyl) aminomethane**
Tris-HCl	**Tris hydrochloride**
TSB	**Transformation and storage buffer**
V	**Volt (s)**
v/v	**Volume/volume ratio**
w/v	**Weight/volume ratio**
WCV	**Whole cell vaccine**
WHO	**World Health Organization**

LIST OF FIGURES

LIST OF FIGURES PAGE

LIST OF TABLES

LIST OF TABLES

SUMMARY

Adenylate cyclase toxin (CyaA) was produced and purified in the toxic CyaC-modified form and the unmodified non-toxic form from both *B. pertussis* and recombinant *E. coli* strains in sufficient quantity to allow large scale experimentation. Immunoblot analysis of crude and purified CyaA preparations revealed that the toxins were prepared as full length 200 kDa proteins with a small amount of degradation products of lower molecular weight protein in both toxic and non-toxic forms. The in vivo CyaC-modified CyaA toxin produced from recombinant *E. coli* showed comparable levels of cytotoxic and invasive activity to that produced from *B. pertussis* but its haemolytic activity was weaker.

In addition, the leukotoxin (LktA) from *Pasteurella haemolytica* was also produced by expression from recombinant plasmids in *E. coli*, in both the LktC-modified toxic form and unmodified non-toxic form. The A and C proteins of both toxins were produced separately in *E. coli* and each could be co-expressed on compatible plasmids. This allowed heterologous activation of CyaA by LktC and LktA by CyaC. The LktC- or CyaC-modified LktA of 105 kDa protein was produced and partially purified from recombinant *E. coli* strains, but the yield of LktA production was low compared to that of CyaA in the same T7 expression system. The LktC-modified CyaA toxin was also produced but showed no cytotoxic or haemolytic activity. Heterologous activation of LktA by CyaC was successful and the toxin was almost as active against bovine lymphoma (BL3) cells as the LktC-modified toxin. However, the heterologous activation by CyaC did not change its specificity for ruminant cells, because no cytotoxic activity against mouse J774.2 cells or haemolytic activity against horse red blood cells was detected. The CyaC-modified LktA showed a greater haemolytic to cytotoxic ratio than LktC-modified LktA. Thus, LktA modified by CyaC was more

haemolytic than the LktC-modified form, but nevertheless retained the specificity for ruminant cells which is a feature of the native toxin.

Two hybrid toxins derived from CyaA and LktA were also produced and purified. Hyb1 contained the N-terminal enzymic domain and the pore forming domain from CyaA (amino acids [aa] 1-687), with the remainder of the protein derived from the C-terminal end of LktA (aa 379-953). Hyb2 was created from Hyb1 by replacement of the LktA C-terminal domain of Hyb1 with the C-terminal domain of CyaA (aa 919-1706). Part of the region concerned with C-modification site of CyaA (aa 688-918) was therefore replaced with the equivalent region from LktA. The Hyb1 toxin of 150 kDa protein had normal AC enzymic activity, but showed no toxic activity when modified in vivo by CyaC or LktC. In contrast to CyaA, the 200 kDa Hyb2 protein was activated more efficiently by LktC than by CyaC, although the cytotoxic and haemolytic activity of Hyb2 modified with LktC or CyaC was lower compared to recombinant active CyaA modified by CyaC. However, LktC-modified Hyb2 showed more toxic activity against ruminant than against murine nucleated cells, whereas CyaC-activated Hyb2 displayed a similar, but lower, activity against both cell types, indicating that LktC and the region with which it interacted had an influence on the target cell specificity of the activated toxin.

An in vitro activation system was developed to investigate the nature of the modification of the protoxins (CyaA, LktA and Hyb2) by CyaC or LktC. ProCyaA, proLktA and proHyb2 and the activator proteins CyaC or LktC were overproduced separately in recombinant *E. coli* strains using an efficient T7 RNA polymerase expression system and then partially purified. Active CyaA, LktA or Hyb2 toxin were produced in vitro when the protoxins were mixed with C-proteins together with a cytosolic extract (CAF) derived from *E. coli*. However, although LktC was able to activate LktA or Hyb2 in vitro, it was unable to

activate proCyaA as found when the two proteins were expressed in vivo in *E. coli*. Hyb2 in vitro-activated by LktC was more active than Hyb2 in vitro-activated by CyaC. Thus, these results were similar to those found in vivo. Activation was assumed to occur by an acylation reaction which by analogy with previous data from *E. coli* haemolysin activation, was assumed to require acyl carrier protein (ACP) as a cofactor. The cytosolic activator factor for toxin activation was lost if the *E. coli* S100 extract was dialysed before use and the cytosolic factor could be replaced in the in vitro reaction by ACP charged separately in vitro with palmitic acid. A range of commercially available fatty acids was used to charge ACP, among which palmitic acid and to a lesser extent, myristic acid-charged ACP were able to activate CyaA, LktA or Hyb2 protoxins in vitro in the presence of CyaC or LktC.

The role of CyaA toxin as a protective antigen against bacterial colonization, in the intranasal mouse protection test was studied. Active CyaA or Hyb2 toxins were shown to have no obvious toxicity as measured by mouse weight gain. Active CyaA from *B. pertussis* or from recombinant *E. coli* strains and LktC-modified Hyb2 were protective against bacterial colonization, whereas non-toxic CyaA and non-modified Hyb2 toxin were not protective in mice.

The adjuvanticity of CyaA toxin on the antibody response to itself and to another protein antigen (ovalbumin) was also investigated. The antibody response to ovalbumin was enhanced when active CyaA toxin was co-administered with ovalbumin, whereas the inactive toxin did not enhance the antibody response. The antibody response to active CyaA alone was higher than that to inactive CyaA alone. Preliminary studies on the cytokine responses of spleen cells isolated from mice immunised with active CyaA indicated a predominantly Th2-type response.

OBJECT OF RESEARCH

The overall objectives of the research were to:

1. Purify CyaA in an active and inactive form from both *B. pertussis* and recombinant *E. coli* and to compare the enzymic, toxic and immunological properties of the toxins.

2. Express CyaA , LktA , CyaC and LktC on separate plasmids in *E. coli* and to assess the capacity of CyaC and LktC to trans-activate LktA and CyaA respectively.

3. Examine the properties of a hybrid derivative of CyaA where the region involved in activation had been replaced by that of LktA.

4. Develop an in vitro system for activation of CyaA and LktA to establish the nature of the activation process for these toxins, to investigate whether the protoxins could be charged with different fatty acids and to examine the biological properties of the toxins activated in vitro. In particular to assess if different acyl groups had an effect on toxicity and target cell specificity.

5. Compare the protective capacity of the activated and non-activated forms of CyaA and the CyaA hybrid in the mouse intranasal model of infection and to assess the toxicity of the active toxins.

6. Assess the adjuvant properties of active and inactive CyaA and CyaA hybrid by co-administration of these proteins with another antigen.

INTRODUCTION

1.1 THE GENUS *BORDETELLA*

There are six recognised species in the genus *Bordetella* which are responsible for respiratory infections of humans and animals. *B. pertussis* is responsible for a severe disease of child-hood called whooping cough or pertussis, mainly in unvaccinated communities. Pertussis is characterised by paroxysmal coughing, whooping and vomiting (reviewed by Parton, 1997). *B. parapertussis* causes a milder form of whooping cough in humans (Heininger *et al.*, 1994; Wirsing von Konig and Finger, 1994). The main difference between *B. pertussis* and *B. parapertussis* is due to pertussis toxin which is produced only by *B. pertussis*. *B. bronchiseptica* is an important respiratory pathogen in wild and domesticated animals but rarely in humans (Pittman and Wardlaw, 1981). It is implicated in atrophic rhinitis and bronchopneumonia in pigs (Rutter, 1985; Woolfrey and Moody, 1991) and kennel cough in dogs (Goodnow, 1980; Bemis, 1992). *B. avium* is a causative agent of the respiratory disease, turkey coryza or turkey rhinotracheitis in turkey poults or other birds (Kersters *et al.*, 1984). *B. hinzii* is found mainly in the respiratory tracts of chickens and turkeys where it is thought to be a non-pathogenic agent (Vandamme *et al.*, 1995) and has also been isolated from human sputum and the blood of an AIDS patient (Cookson *et al.*, 1994). Recently, the species *B. holmesii* has been isolated from blood cultures of human patients, especially in immunocompromised cases (Weyant *et al.*, 1995).

1.2 THE SPECIES BORDETELLA PERTUSSIS

1.2.1 Historical perspective

Bordetella pertussis was first isolated in 1906 by Bordet and Gengou on a blood-based potato glycerol agar without peptone. It was initially named *Haemophilus pertussis*, and later shown to be the causative agent of whooping cough. The growth of the organism in this liquid medium in the absence of X and V factors in blood (which is critical for growth of the genus *Haemophilus*) led to the conclusion that the organism had to be classified in another genus rather than *Haemophilus* (Hornibrook, 1940). However, *Bordetella pertussis* remained in the genus *Haemophilus* until Moreno-Lopez (1952) created the new genus *Bordetella* for the causative bacterium of pertussis and named it in honour of the initial work of Jules Bordet.

1.2.2 Characteristics

B. pertussis is a small gram negative coccobacillus, non-motile, strictly aerobic, nutritionally fastidious bacterium that is probably encapsulated and does not produce a spore. On BG agar plates containing 15-20% horse blood at 37 °C in a humid atmosphere, *B. pertussis* gives rise to tiny (0.5 mm diameter), smooth, convex, pearl-like colonies with entire edges and surrounded by a narrow zone of haemolysis (Pittman, 1974). A liquid defined medium was introduced by Stainer and Scholte (1971) and contained glutamic acid, proline, L-cysteine, ascorbic acid, nicotinic acid, glutathione, salts and Tris buffer. When supplemented with heptakis (2,6-O-dimethyl) β-cyclodextrin and

casamino acids, this not only improved the growth of *B. pertussis* but also increased the yield of major components such as PT, FHA and CyaA in culture supernatants (Imaizumi *et al.*, 1983). *B. pertussis* is a human pathogen that attaches and multiplies in the ciliated respiratory mucosa of the upper respiratory tract to cause pertussis infection (Bromberg, 1989; Sandros and Tuomanen, 1993), although it may invade the pulmonary macrophages and has been found inside cells (Bromberg *et al.*, 1991; Khelef *et al.*, 1994).

1.2.3 Genome organisation

A physical map of the *B. pertussis* chromosome was constructed by Stibitz and Galetts (1992) to demonstrate the location of the virulence-associated genes (*fha*, *ptx*, etc.) and other genes. The genome size of *B. pertussis* was about 3,750 kb which is approximately 80% of the genome size of *E. coli*. *Bordetella* virulence genes are scattered over the bacterial chromosome and some of them, such as the genes encoding pertussis toxin (*ptx* genes) and genes involved in secretion of pertussis toxin (*ptl* genes) are arranged in operons. There was no significant linkage between the virulence genes except for *fha*, *bvg* and some genes involved in fimbrial synthesis which were located close to each other. Insertion sequence (IS) elements and related repeated DNA sequences have been found in the genome of the *B. pertussis* but these elements are not thought to be involved in rearrangements of the genome or expression of virulence factors. IS481 is one of the specified insertion sequence elements which is scattered in 80 copies around the genome of *B. pertussis* and constitutes about 2% of the DNA (Stibitz and Galetts, 1992; Weiss, 1992).

1.3 PERTUSSIS, THE DISEASE

Whooping cough was first reported as an unusual type of coughing disease in Moulton's "The mirror of health" in 1540 (Lapin, 1943). Pertussis, a disease with world-wide endemicity, is an acute respiratory disease of young children with most death in infants under one year old. Although maternal antibody does pass to the foetus, protection is incomplete so that infections occur in early infancy (Preston, 1987). However, pertussis affects persons of all ages and adults with mild cases of whooping cough are the major reservoir of infection for children in an immunized population (Muller *et al.*, 1986). Whooping cough is transmitted from patients (usually children, sometimes adults) to non-immune healthy persons or non-immune child contacts at school by means of the secretions in the mouth and nose, to some extent through towels, hand-kerchiefs, hand to hand contact, but to a large extent by droplet infection (Long *et al.*, 1990). Mortality from whooping cough has been dramatically reduced over the past half century in developed countries with a combination of widespread effective vaccination, improved socio-economic conditions, intensive care in hospitals, and antibiotic therapy. The disease still occurs in epidemic waves at about 3 to 4 year intervals, presumably due to new susceptibles being exposed to the organism (Preston, 1987; Cherry *et al.*, 1989).

1.3.1 Steps in pertussis infection

Following inhalation of droplets, in the first step of infection the organisms attach to the ciliated epithelial cells of the bronchial tree. In the second step, the

bacteria proliferate on the surface of ciliated cells and colonize the mucosa of the respiratory tract between the entrance of the air way and the beginning of the lung. As a result of bacterial colonization and the production of toxic components by the bacteria, the ciliated cells will ultimately be damaged. In some uncommon cases of pertussis the bacteria move from the upper respiratory tract into the lungs and produce a form of pneumonia which can be complicated by the systemic effects of the disease (Wilson *et al.*, 1991).

1.3.2 Stages of pertussis disease

Typical clinical pertussis is divided for descriptive purpose into three separate stages, although the clinical features of illness may differ with age, general health and immune system status (Cherry *et al., 1988*; Walker, 1988). The first stage, called catarrhal or prodromal, almost always begins within 7-14 days after exposure to pertussis infection (Lapin, 1943). The catarrhal stage lasts from 1-2 weeks (Olson, 1975), presumably allowing active multiplication of bacteria in the respiratory epithelium. During this period of time, the patient exhibits only mild symptoms of an uncomplicated upper respiratory infection including sneezing, a watery nasal discharge, frequent cough, low grade fever and occasionally conjunctivitis . The second stage, called paroxysmal, usually lasts from 1-4 weeks and is characterized by progression to a paroxysmal cough that is a hallmark of the disease (Cherry *et al.*, 1988, Walker, 1988). A paroxysmal cough is characterized by uncontrollable coughing, up to 5 to 20 forcible hacking coughs in 15 to 20 seconds with no time for breathing between coughs (Preston, 1988). If prolonged, it may induce anoxia and cyanosis and often terminates with production of mucus or vomiting, followed

by a whoop which is a characteristic sound produced by an inspiratory breath through the narrowed glottis (Pittman, 1970). This symptom (whoop) gives the disease its name. Respiratory complications and central nervous system damage (irreversible neurological damage) can arise due to pressure effects and anoxia, both resulting from paroxysmal coughing (Cherry *et al.*, 1988). The third and final stage named convalescent stage, lasts several months and is characterized by a gradually decreasing number and frequency of paroxysmal coughing, and the disappearance of viable *B. pertussis* organisms from the respiratory tract until they are rarely found after the fourth week (Fine and Clarkson, 1982). The reason for persistence of the paroxsysmal cough long after the bacteria are no longer detectable in the respiratory tract is unexplained, but may possibly be due to some form of tissue sensitivity resulting from increasing histamine as observed in the infected mouse model (Pittman *et al.*, 1980).

1.3.3 Diagnosis

Unfortunately, the early stage (catarrhal) of the disease resembles a common cold and the true diagnosis of the disease may not be possible until the paroxysmal stage. Thus *B. pertussis* infection has to be diagnosed at the catarrhal stage paraclinically by recovery of the bacteria by culturing from a nasopharyngeal aspirate or cough plate or from nasal or oropharyngeal swabs (Onorato *et al.*, 1987). However, recovery of *B. pertussis* from previously immunised persons or patients treated with antibiotic is much more difficult than from un-treated or un-immunized persons (Baraff *et al.*, 1978). Because *B. pertussis* is very sensitive to drying, the specimen from the patient

must be either directly cultured on freshly prepared BG agar and incubated at 37 °C in a moist atmosphere for 7 days or the specimen placed in a suitable transport medium (Field and Parker, 1979). The biochemical reactions of *B. pertussis*, including positive oxidase, negative urease, negative motility or no growth on MacConkey agar can then be used for differentiation from other species of the genus *Bordetella*. In addition to the culture and biochemical reactions, serological identification tests of *B. pertussis* are available including fluorescent antibody, agglutination, complement fixation, ELISA and measurement of anti-pertussis toxin IgA that will confirm the isolation (reviewed by Friedman, 1988).

Measurement of cAMP produced from incubation of the clinical specimens with medium containing ATP and calmodulin has been used (Confer *et al.*, 1990). *B. pertussis* can also be identified in nasopharyngeal swabs by PCR amplification of suitable sequences in the genome (reviewed by Meade and Bollen, 1994).

1.3.4 Treatment

Treatment for pertussis is primarily with supportive care or nursing supervision of high risk people (children under one year of age) during the second stage when the disease is usually diagnosed. This might involve careful suction to remove the tenacious mucus secretion from the respiratory tract, oxygen therapy with increased humidity, hydration, nutrition and restoration of electrolyte balance (Altmeier and Ayoub, 1977). Antibiotic therapy has little clinical effect when the pertussis infection is well established and diagnosis occurs at the paroxysmal stage, although the bacterium is susceptible to

erythromycin and to a lesser extent to chloramphenicol or tetracycline. There is some evidence that if erythromycin is given early during the catarrhal stage, before the paroxysmal cough develops, and for at least 14 days, it may reduce and shorten the severity of whooping cough, presumably by elimination of bacteria from the nasopharynx. Antibiotic therapy also reduces the possibility of secondary infection such as bronchitis or pneumonia. Erythromycin may also be given to non-vaccinated infants or other high risk persons including hospital personnel who may be exposed to the pertussis patients or are in close contact with active cases, to reduce infectivity and to control or prevent the spread of infection (prophylaxis) (Bass, 1985). The efficacy of treatment with human pertussis hyper-immune serum has not been established, possibly because the serum was not checked for the presence of all three agglutinins (Preston, 1987).

1.3.5 Control and prevention

Neither antibiotics nor immunoglobulins are very effective for the prevention or control of pertussis in the population, even if given during the incubation period or the catarrhal stage. Control of the disease by quarantine is also unrealistic because patients typically distribute the organism for many weeks or months and children are infectious even before the most characteristic symptoms develop (Muller *et al.*, 1986). The best way for the prevention of whooping cough is by active immunization (vaccination).

1.4 PERTUSSIS VACCINES

1.4.1 Whole cell pertussis vaccine

An effective whole cell pertussis vaccine (WCV) was first developed in the 1940s (reviewed by Rappuoli *et al.*, 1991). There have been three generations of vaccines for induction of active protection against pertussis. The first generation was prepared from whole bacterial cells, whereas the second and third generations were prepared from purified protective components or toxoided virulence factors of *B. pertussis* and named acellular vaccines. The currently available WCV is usually given in a combined preparation (commonly called DPT vaccine) which contains diphtheria and tetanus toxoids and a suspension of whole *B. pertussis* cells, killed by formalin, heated at 56 °C for 30 min and absorbed on aluminium hydroxide as an adjuvant to enhance the immunogenicity and efficacy of the vaccine. This vaccine is still in widespread use except in Japan (Rappuoli *et al.*, 1991). In countries with a well-developed medical service, DPT vaccination is routinely begun at about two months of age unless pertussis is prevalent in the community in which case vaccination should be started earlier. DPT vaccine in three equal doses (4 protective units per dose) is given at 4-8 week intervals, with a booster vaccination at 12 to 18 months after first vaccination or prior to school. This schedule should give more than 90% protection (Preston, 1987).

Although the efficacy of pertussis vaccine is generally high, it may cause a local and occasionally systemic reaction in infants such as skin redness, pain, induration, fever, prolonged crying, convulsions and collapse. More serious systemic toxic reactions, including permanent brain damage and death may

occur at an estimated rate of 1 in 100,000 vaccinations. The concern about these reactogenic effects, especially the neurological damage, due to DPT vaccination led to a decrease in the acceptance of the vaccine in many western countries. For example in the U.K., vaccination fell from 90% to 30% of the population in the 1970s and as a consequence there was an increasing incidence of whooping cough. Because the risks associated with the disease are now recognised as greater than those associated with the side effects of vaccination, acceptance of vaccination in the U.K. has returned to the previous level of >80% (Parton, 1991). However, serious doubts about the safety of WCV have largely passed, and the World Health Organization recommends that vaccination with WCV has to be continued while at the same time the research is being focused on the development of new acellular vaccines with minimal side effects.

1.4.2 Acellular vaccine

The best solution to avoid the side effects arising from pertussis WCV is to produce a less toxic vaccine. A first step towards this goal is to analyse the composition of WCV and to identify the components that are immunogenic and important in the virulence of *B. pertussis*. As shown by Weiss *et al.* (1984), by construction of mutant *B. pertussis* defective in the expression of individual virulence factors, pertussis toxin (PT) is a major virulence factor and is also protective against challenge in the mouse model of infection (Rappuoli *et al.*, 1991). The first acellular vaccine was developed by Sato and contained formaldehyde toxoided PT and filamentous hemagglutinin (FHA) rather than intact bacteria, and this or similar vaccines have been used in Japan since 1981

(Sato *et al., 1984*). Because the Japanese do not vaccinate children until they are 2 years old, the incidence of pertussis in Japan is still high in children up to 2 years of age. In Sweden two acellular vaccines, one containing only formalin toxoided PT and the other vaccine containing formalin toxoided PT with FHA were used in field trials in 1986. Although both vaccines had lower protection efficacy (about 80%) than WCV and low reactogenicity, they still had some side-effects. There has been speculation that this reactogenicity was due to cross reactivity of anti-FHA with brain blood vessels or to incomplete chemical detoxification of PT (Kimura and Hikino, 1985; Ad Hoc group, 1988; Tuomanen *et al.*, 1993). On the basis of these observations, a number of other acellular vaccines have undergone field trials. All include chemically toxoided PT, alone or with one or more pertussis antigens such as FHA, pertactin (PRN) and agglutinogens (fimbrial antigens) (Storsaeter *et al.*, 1990). In order to achieve maximum safety and avoid the risk of side effects, a third generation of vaccines based on the construction of genetically detoxified pertussis toxin has been developed (Podda *et al.*, 1993; Rappuoli, 1993), because chemical detoxification of PT can not guarantee the absolute absence of active PT in acellular vaccine preparations. By substitution of two amino acids in the S1 subunit (Glu129 was replaced by Gly, Arg-9 with Lys) a non-toxic PT protein was produced which contains the immunodominant epitopes both for B- and T-lymphocytes and was highly immunogenic. Two candidate vaccines containing the double mutant PT-9K/129G molecule alone or associated with FHA and PRN were clinically tested in 250 infants. Preliminary results of these clinical trials showed that both vaccines were safe and more immunogenic than vaccines containing formalin toxoided PT (Rappuoli, 1996).

1.5 IMMUNITY IN PERTUSSIS

Recovery from whooping cough is accompanied by development of an immunity which lasts for many years. Second attacks occur only infrequently in children and then they are very mild but in older people second attacks are more severe. However, immunity acquired after infection with *B. pertussis* does not protect against the other *Bordetella* species. The antibody response following pertussis infection results in circulating antibody against *B. pertussis* antigens such as PT, FHA, PRN, AGGs and adenylate cyclase toxin (CyaA). These play a role in preventing bacterial attachment to the respiratory tract (Ashworth *et al., 1982*). The basis of immunity in pertussis is however incompletely understood. Because the titres of specific humoral antibodies may decrease over a period of years, effective immunity to whooping cough may not necessarily depend on the presence of circulating antibody.

Cell mediated immunity may be a crucial component of long-term immunity against *B. pertussis*. It has been reported that *B. pertussis* is not only an extracellar pathogen but also can invade and survive within eukaryotic cells, including macrophages, in which case cell mediated immunity would be necessary to prevent intracellular persistence of *B. pertussis* (Bromberg *et al.*, 1991). The cellular immune response to pertussis infection induces specific CD4+ T-cell clone proliferation in humans and mice (Gearing *et al.*, 1989). Adults who have had pertussis in childhood show T-cell responses to PT, FHA and PRN, but PT may not be a major target for T-cell responses in adults previously infected or vaccinated with DPT vaccine (De Magistris *et al*, 1988). *B. pertussis*-specific CD4+ T-cell clones from previously infected humans release IL-2 and INFγ, but little or no IL-4 which is characteristic of the Th1

response involved in cytotoxic functions for bacterial clearance from lungs (Peppoloni *et al.*, 1991). Redhead *et al.* (1993) showed that mice vaccinated with pertussis WCV produced a very good Th1 response and moderate antibody titre, whereas mice vaccinated with an acellular vaccine produced a strong Th2-antibody response (a characteristic of true helper T-cells for stimulating antibody response) and a low Th1 response. However, the antibody stimulated by Th2 cells in mice vaccinated with acellular vaccine did play a role in elimination of bacteria from the lungs in the early stages of infection which is compatible with involvement of the Th1 response in bacterial clearance.

Whatever the precise mechanism, recent studies in immunity to pertussis have concluded that while antibody responses do play a role in preventing bacterial colonization in the early stages of host defence against pertussis, a cell mediated response is required for complete bacterial elimination in the later stages of disease, presumably via stimulation of mechanisms for killing intracellular *B. pertussis* in infected macrophages (Redhead *et al.*, 1993).

1.6 VIRULENCE FACTORS

B. pertussis produces several potential virulence factors that may play a role in the pathogenesis of pertussis. These include adhesin factors that mediate attachment to cultured mammalian cells such as PT, FHA, PRN and fimbrial agglutinogens. In addition, toxins such as tracheal cytotoxin (TCT), heat labile toxin (HLT), endotoxin lipopolysaccharide (LPS) and adenylate cyclase toxin (CyaA) are produced as aggressins to combat the host's immune system (Weiss

and Hewlett, 1986; Friedman, 1988). The biological properties of these components are discussed in detail below.

1.6.1 Tracheal cytotoxin (TCT)

Tracheal cytotoxin is released extracellularly into the culture medium as a result of bacterial lysis or during the logarithmic growth phase of at least four species of *Bordetella*. This unusual toxin is a peptidoglycan fragment (1,6 anhydromuramic acid-N-acetyl-glucosamine tetra-peptide) with a low molecular weight (921 daltons) (Goldman, 1988). A recent study using human nasal biopsy specimens showed that TCT may play a role in pertussis infection, possibly by causing ciliostasis (destruction of ciliated cells) followed by ciliated cell extrusion from the epithelium of the respiratory tract (Heiss *et al.*, 1994). The ciliostatic effect of TCT was initially discovered by Goldman and Baseman (1980), in hamster tracheal organ cultures and inhibition of DNA synthesis in the hamster trachea epithelial (non-ciliated) cell cultures was also noted. TCT may cause these cytotoxic effects on ciliated and non-ciliated epithelial cells by causing synthesis of nitric oxide in response to stimulation of IL-1 secretion by Th1 cells (Heiss *et al.*, 1994). Such effects could well explain the cause of some of the pathological events in pertussis infection such as coughing, stimulation of excessive mucus production in the air ways, the persistence of respiratory tract abnormality in pertussis long after bacterial elimination from the lung and predisposition to secondary bacterial infections (Wardlaw and Parton, 1988). The immunogenicity of tracheal cytotoxin in pertussis is not known.

1.6.2 Heat-labile toxin (HLT)

Heat labile toxin or dermonecrotising toxin was one of the first toxins described in the genus *Bordetella* (Livey *et al.*, 1978). HLT is present mainly in the cytoplasm of *Bordetella* but may be partially located on the surface of the bacterial cells (Livey and Wardlaw, 1984; Walker and Weiss, 1994). Because of its instability, the purification of HLT in toxic form has proved very difficult (Nakase and Endoh, 1988). HLT is a 102 kDa protein reportedly consisting of two 24- and two 30-kDa subunits (Endoh *et al.*, 1986) which causes a dermonecrotic reaction (skin necrosis), loss of normal weight gain and spleen atrophy when injected subcutanously at low doses in mice or is responsible for mouse lethal toxicity when injected at high doses (Endoh *et al.*, 1990). The skin lesions resulting in mice after HLT injection could be due to a specific constrictive effect on vascular smooth muscle and such effects on the vascular smooth muscle tissues of the respiratory tract may cause a local inflammatory reaction and may be responsible for some of the pathological effects in pertussis infection (Nakase and Endoh, 1988). HLT has no role in protection induced by WCV because in the process of WCV preparation, HLT is inactivated by heating at 56°C for 10 min (Manclark and Cowell, 1984). A mutant strain deficient in HLT production was not impaired in its ability to cause a lethal infection in mice, indicating that HLT has no significant role in the disease process, at least in mice (Weiss *et al.*, 1989).

1.6.3 Lipopolysaccharide (LPS)

Endotoxin or LPS produced by all species of *Bordetella* comprises about 2% of the dry weight of bacterial cells. *B. pertussis* produces two distinct LPS molecules, LPS type I has a standard lipid A portion like the LPS of *E. coli*, whereas LPS type II has a distinctive lipid portion named lipid-X (2,3-diacylglucosamine 1-phosphate). The two types of LPS also have different polysaccharide portions. In LPS type I, the polysaccharide portion has an oligosaccharide core with 2-keto-3-octulosonic acid (non-phosphorylated KDO), and LPS type II contains an oligosaccharide core with phosphorylated KDO. Both LPS I and II lack the long chain polysaccharide O antigen as found in *E. coli* or other enterobacterial LPS (Brodeur *et al.*, 1993). Thus both types of *B. pertussis* LPS are generally referred to as a lipo-oligosaccharide (LOS), although they have the same kind of activities as classical LPS types such as that of *E. coli*, including toxicity, pyrogenicity and adjuvanticity as well as some unusual functions such as activation of the alternative complement pathway, stimulation of cytokine release, specific antiviral activity, B-cell mitogenicity, polyclonal B-cell and mononuclear phagocyte activation (Haeffner-Cavaillon *et al.*, 1984; Winters *et al., 1985*; Chaby and Caroff, 1988). However, the role of LPS in the pathogenesis of pertussis is not established, although it may be responsible for the mild fever in the early stages of pertussis and may cause much of the reactogenic symptoms of the commercial WCV. One of the main aims of developing an acellular vaccine was to eliminate this toxin and reduce significantly its reactogenic side effects (Cherry *et al.*, 1988). Although, LPS in WCV is not generally considered to be a protective antigen, it may have some role in immunity to pertussis infection

because boiled WCV still has heat stable LPS antigens and protects mice against intranasal challenge with *B. pertussis* (Standfast, 1958; Pittman, 1984). In addition, high titre of anti-LPS antibody have been reported in tracheobronchial washings from mice but not in serum (Wardlaw and Stevenson, 1984).

B. pertussis LPS was found to be a good immunological adjuvant for diphtheria toxoid in the guinea pig. It has the ability to stimulate antibody responses, and Lipid A was the most efficient adjuvant fragment (Munoz and Bergman, 1979; Ayme *et al., 1980*).

1 .6.4 Filamentous haemagglutinin (FHA)

FHA was initially observed as a component of the *B. pertussis* cell that agglutinated red blood cells, a feature that explained the name fimbrial haemagglutinin (Keogh *et al.*, 1947). Later work established that FHA is synthesised as a large precursor protein of 360 kDa which is exported to the bacterial surface where it is cleaved proteolytically to release the N-terminal 220 kDa mature protein that forms a rod shaped highly hydrophobic fine filamentous structure 2 nm in diameter and 40-100 nm in length on the surface of the cell (Arai and Sato, 1976; Domenighini *et al.*, 1990). The C-terminal portion remains in the outer membrane of the cell. The filamentous structures do not have the ordered structure characteristic of pili as previously thought, and thus seem to be a non-fimbrial surface protein (Ashworth *et al.*, 1982).

FHA acts as an adhesin, having binding activities which allow *B. pertussis* to adhere to ciliated cells or macrophages (Tuomanen and Weiss, 1985). The secondary infections in childhood pertussis may be explained by a piracy

phenomenon, whereby FHA in soluble form, and possibly in conjunction with PT, adheres either to *B. pertussis* itself or to unrelated bacteria such as *Haemophilus influenzae* or *Streptococcus pneumoniae*, which results in sticky bacterial cells that can attach to the cilia of respiratory ciliated cells (Tuomanen, 1988). The FHA binds to galactose residues of sulfated glycolipid in the membrane of ciliated cells as well as binding to a glycoprotein receptor (CR3) on polymorphonuclear neutrophils (PMN) or mononuclear cells (macrophage) by its Arg-Gly-Asp (RGD) domains. Such FHA-mediated binding of *B. pertussis* to ciliated cells or macrophages leads to entry of the organism into the lungs or enhances phagocytosis without triggering an oxidative burst which may be critical in the development of lethal pneumonia or intracellular survival of *B. pertussis* in macrophages respectively (Locht *et al.*, 1993; Mooi, 1994). Infection experiments in mice with a *B. pertussis* mutant unable to produce FHA have shown that the colonization in the lung and the persistence in the upper respiratory tract of mice were significantly reduced (Weiss and Goodwin , 1989).

In mice, immunization with FHA followed by intranasal challenge with *B. pertussis* showed protective activity of FHA antigen by a reduction of lung colonization (Kimura *et al.*, 1990). Passive immunization with antibodies to FHA have indicated that the adherence of *B. pertussis* to mammalian cells was inhibited (Sato *et al.*, 1981). FHA is one of the prime candidate antigens in the acellular pertussis vaccine (see section 1.4.2), because of its lack of toxicity and its protective activity in mice and induction of humoral and cellular immune responses in humans during natural disease.

1.6.5 Agglutinogens (AGGs)

Eldering *et al.* (1957) initially reported 14 agglutinogens as surface antigens which can induce production of bacterial cell agglutinating antibodies (agglutinins). The serotype of isolates in the genus *Bordetella* can be determined based on agglutination and agglutinin adsorption characteristics of agglutinogens (Preston *et al.*, 1982). *B. pertussis* strains produce three major agglutinogens, of which AGG 1 is species specific and AGG 2 and AGG 3 are strain specific fimbrial proteins (Irons *et al.*, 1985) , and 5 minor agglutinogens (AGG 4-7 and 13) of which AGG 4-6 are strain specific, and AGG 7 and AGG 13 are shared with *B. parapertussis* or with *B. bronchiseptica* respectively (Preston *et al.*, 1982). AGG2 and AGG3 fimbrial proteins in *B. pertussis* serotypes are mainly composed of protein subunits of 22.5 and 22 kDa respectively and both types also contain a minor component of 40 kDa (FimD). There are three serotypes such as 1,2,3; 1,2 and 1,3 (possessing at least two AGGs) which are recognized in *B. pertussis* infection in man (Preston, 1988). *B. pertussis* cells without fimbriae adhered well to human ciliated cells (Tuomanen and Weiss, 1985), but there is some evidence that AGGs may have a role as adhesins for establishment of pertussis infection by the initial adherence of *B. pertussis* to ciliated epithelial cells of the human respiratory tract (Gorringe *et al.*, 1985; Tuomanen, 1988). The fimbrial protein FimD may be located at the tip of the fimbrial structure and capable of binding *B. pertussis* to the VLA-5 cell surface integrin of human monocytes and thus may serve to facilitate phagocytosis (Mooi, 1994). Purified fimbrial proteins have been shown to protect mice against respiratory infection with different serotypes of *B. pertussis* (Robinson *et al.*, 1989). There is a suggestion that

WCV should contain AGGs 1, 2 and 3, in order to protect efficiently against all the serotypes of *B. pertussis* (Preston, 1988) and that fimbrial agglutinogens should be included in acellular vaccines as candidate antigens for induction of serospecific protection in children (reviewed by Cherry, 1993).

1.6.6 Pertactin (PRN)

Pertactin was first reported by Brennan *et al.* (1988) as a non- fimbrial AGG outer membrane protein (OMP). It is a surface associated protein and is produced by *B. pertussis, B. parapertussis* and *B. bronchiseptica* as a protein of MW 69, 70 and 68 kDa respectively as determined by SDS-PAGE analysis (Kobisch and Novotny, 1990). PRN from *B. pertussis* is first synthesized as a precursor polypeptide molecule of 93.5 kDa, which is then proteolytically cleaved to remove a 3 kDa N-terminal signal sequence and a 30 kDa C-terminal portion to produce a mature protein of 60 kDa. The descriptor P69 relates to the anomalous migration of the protein in SDS-PAGE with an apparent MW of 69 kDa. The cleaved C-terminal polypeptide remains as a protein (P30) in the outer membrane (Charles *et al.*, 1994). Both FHA and PRN have arginine-glycine-aspartic acid (RGD) integrin binding domains thus suggesting a role for both proteins in binding of *B. pertussis* to mammalian cells (Leininger *et al.*, 1991; Charles *et al.*, 1994). However PRN can mediate adhesion of *B. pertussis* to eukaryotic cells even in the absence of a functional RGD motif (Everest *et al.*, 1996). Pertactin has two proline-rich regions (PRRs) in its helix structure (Emsley *et al.*, 1996). One of the PRRs is a $(GGXXP)_5$ sequence which is located directly after the RGD sequence and may be associated with binding activity of pertactin to epithelial cells. The other C-terminal PRR contains the

motif (PQP)5, and is known to contain the immunoprotective epitope. In fact PRN in association with FHA may operate more efficiently than either alone in cell binding. In experimental *B. pertussis* infection, purified PRN (P69) alone or in association with FHA was protective in mice against aerosol challenge. PRN is now a candidate antigen in acellular vaccines to increase the potency of the vaccines in association with other protective antigens.

1.6.7 Other *B. pertussis* outer membrane proteins (OMPs)

Recently two other virulence-associated OMPs of *B. pertussis*, BrkA (*Bordetella* resistance to killing) and TCF (tracheal colonization factor), have been reported that have structural homology with PRN. The BrkA protein, like P69, is synthesised as a large precursor polypeptide molecule of 103 kDa, and proteolytically cleaved into two fragments, the N-terminal 73 kDa BrkA and a 30 kDa C-terminal fragment localized in the outer membrane. BrkA has two RGD domains and may potentiate the virulence of *B. pertussis* in respiratory tract infection by conferring adhesive properties and by resistance to complement dependent serum killing (Fernandez and Weiss, 1994). TCF is produced in the culture supernate of *B. pertussis* during growth. TCF protein of 68 kDa contains a RGD sequence and a proteolytic cleavage site which releases a 30 kDa C-terminal fragment. TCF probably has a role in colonisation of *B. pertussis* in mouse trachea (Finn and Stevens, 1995).

1.6.8 Pertussis toxin (PT)

Pertussis toxin was initially called histamine-sensitizing factor or mouse-protective antigen or heat labile adjuvant according to its activities (Levine and Pieroni, 1966) and also it was named leukocytosis and lymphocytosis promoting factor or islet activating protein (Yajima *et al.*, 1978). The term pertussigen was used for this toxin by Munoz and Bergman (1979) until it was named pertussis toxin with universal acceptance by Pittman (1979). *B. parapertussis* and *B. bronchiseptica* have transcriptionally silent genes for PT and only *B. pertussis* can produce it (Marchitto *et al.*, 1987).

PT is a complex protein exotoxin of 105 kDa, secreted during in vivo and in vitro bacterial growth and it consists of five different subunits called S1, S2, S3, S4 and S5. PT is a hexamer toxin molecule (5 binding subunits and 1 enzymatic subunit) because it contains two S4 subunits. PT is an A-B toxin like cholera toxin, except that the binding subunit of PT is more complex. The A subunit is a single polypeptide, with a disulfide bond, that carries the biological activity and the B oligomer subunit consists of two dimers, (S2-S4 and S3-S4) linked by S5, that binds the complex to the cell membrane of different cell types including T lymphocytes before transfer of the A subunit into the cytoplasm (Tamura *et al.*, 1982; Katada *et al.*, 1983). The S1 subunit acts as an ADP-ribosyl transferase and also as a NAD-glycohydrolase. After passage of the A subunit (S1 unit) into the cytoplasm by conformational changes of the binding B subunit, the disulfide bridge of the S1 subunit is reduced inside the cytoplasm to release the active form of the enzyme. This then catalyses the ADP-ribosylation of the G_i protein of the host cell which

promotes increased adenylate cyclase activity and ultimately increases the cAMP level in the host cell (Hildebrandt *et al.*, 1983; Ui, 1988).

Although many toxic activities of PT depend on the A subunit enzymatic activity, other activities, such as the T-cell mitogenic and haemagglutinating activities are due to the B subunit binding to cell surfaces. PT operates as an adhesin via its B subunit for binding of *B. pertussis* to ciliated cells and macrophages (Sandros and Tuomanen, 1993).

PT has been proposed to play a central role in the pathogenesis of pertussis (Pittman, 1984) but its actual role in disease progression in vivo is not known. PT could be responsible for increasing respiratory secretions and mucus production in pertussis, or in association with FHA, facilitate binding to human ciliated cells or promote *B. pertussis* uptake by macrophages. It may allow other bacteria such as *H. influenzae* to bind to human ciliated cells and thus may be responsible for the secondary infections associated with pertussis. PT probably is not responsible for paroxysmal coughing of pertussis, because *B. parapertussis* does not produce PT but causes the same type of cough (Tuomanen, 1988). PT is required for *B. pertussis* respiratory infection in the mouse model and this is probably due to the effect of PT on normal functions of circulating immune cells such as macrophages and lymphocytes (Weiss and Goodwin, 1989). Active immunization with formaldehyde-treated PTd or recombinant immunogenic detoxified PTd protects mice against intracerebral or intranasal *B. pertussis* infection.

1.6.9 Adenylate cylase toxin (CyaA)

This toxin is produced by *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* (Hewlett and Gordon, 1988) and is considered to be an important virulence factor. The CyaA of *B.pertussis* was first shown to be associated with toxic activity by its ability to attenuate neutrophil superoxide production and bactericidal capability. Subsequently, CyaA was shown to penetrate and intoxicate a wide range of cell types (Hewlett and Gordon, 1988). Immune effector cells such as neutrophils, monocytes, macrophages and natural killer cells are thought to be the primary targets, where those functions necessary for combating infection are inhibited. CyaA is dealt with in more detail in section 1.8.

1.7 Regulation of virulence factors

B. pertussis virulence genes can be regulated by both phenotypic (antigenic modulation) and genotypic (phase variation) changes. Antigenic modulation was first described by Lacey (1960) as a freely-reversible phenotypic change in response to enviromental changes like concentration of certain salts, organic acids and temperature. In the laboratory, expression of virulence genes is highest when *B. pertussis* is grown in medium containing NaCl at 37 °C, whereas at lower temperature (25 °C) or in the presence of magnesium sulphate or nicotinic acid, the expression of virulence genes is suppressed. Weiss *et al.* (1984) first identified by a single Tn5 insertion in the *B. pertussis* chromosome a virulence regulatory locus named *bvg* that activates the expression of *B. pertussis* virulence genes. The *bvg* locus contains two genes, *bvg*A and

*bvg*S, which constitute an operon and encode members of a two-component regulatory system (BvgA and BvgS) (Arico *et al.*, 1989; Scarlato *et al.*, 1991; Stibitz and Yang, 1991). BvgS is a histidine kinase with apparent molecular weight of 135 kDa that first senses environmental signals (temperature, Mg^{2+} and nicotinic acid) and autophosphorylates and then transfers the phosphoryl group to the transcriptional activator BvgA (23 kDa protein) which modulates responses to environmental signals via gene transcription (Steffen *et al.*, 1996; Zu *et al.*, 1996; Karimova *et al.*, 1996). Phosphorylated BvgA (BvgA~p) activates the expression of important *B. pertussis* virulence activated genes (*vag*) to produce virulence factors such as FHA, PRN, PT, AGGs and CyaA. Initially BvgA~p activates the *bvg* locus itself and the *fha* gene, the activation of the *ptx* and *cyaA* genes require higher levels of BvgA~p and it is thought that early transcription of genes for adherence, such as those encoding FHA and AGGs, allow initial colonisation of the host. This is followed later by transcription of genes for toxins which combat the host immune response to the initial infection (Scarlato *et al.*, 1991; Prugnola *et al.*, 1995). BvgA~p acts to repress other, virulence repressed genes (*vrg*). These genes may play a role in later stages of an adaptive process (for example bacterial survival once bacteria enter the phagocytic cells) as one *vrg* locus (*vrg*-6) contributes to colonization and survival in mice (Beattie *et al.*, 1992). A *B. pertussis* DNA fragment carrying the *bvg* locus together with the *fha* locus, when cloned in *E. coli*, expressed FHA antigen, but when a plasmid carrying the *fha* locus alone was cloned into *E. coli*, no FHA was expressed. This finding confirmed that trans-acting *bvg* locus gene products are important for the expression of the *fha* gene (Stibitz *et al.*, 1988; Domenighini *et al.*, 1990). The *bvg* locus of *B. brochiseptica* has also been characterised and genetic analysis has shown that

the loci of *B. pertussis* and *B. brochiseptica* are functionally interchangeable (Tejada *et al.*, 1996).

Phase variation from the virulent (Vir+) to the non-virulent phenotype (Vir^-) or vice versa occurs at a frequency of about 10^{-6} and for some strains has been shown to be caused by a frame shift mutation or irreversible deletion in the *bvg* locus (Stibitz and Miller, 1994).

1.8 ADENYLATE CYCLASE TOXIN (CyaA)

1.8.1 Historical perspective

Adenylate cyclase from *B. pertussis* was initially described in the supernate of a commercial whole-cell pertussis vaccine extracted by sodium chloride (Wolff and Cook, 1973). Subsequently, AC enzyme was purified from culture supernates of *B. pertussis* and whole-cell extracts (Hewlett and Wolff, 1976). Although *B. pertussis* CyaA was found in the culture supernate during log phase of bacterial growth, it was mainly cell-associated protein and localized on the external side of the cell membrane (Cowell *et al.*, 1979).

Hewlett *et al.* (1979 a) found that the enzyme was activated by a protein component present in commercial preparations of certain proteins such as haemoglobin and later identified as calmodulin (CaM) (Berkowitz *et al.*, 1980). Goldhammer and Wolff (1982) noted that the activation of enzymic activity was dependent on calmodulin concentration. Greenlee *et al.* (1982) observed that Ca^{2+} was not required for the majority of the enzymic CyaA activation by calmodulin. It stimulated activity at nanomolar levels of calmodulin but not when the calmodulin concentration was increased. Wolff and Cook (1982)

reported that phospholipids and some detergents activated CyaA enzyme activity and also increased calmodulin activation.

Utsumi *et al.* (1978) noticed that a urea extract of *B. pertussis* inhibited neutrophil functions such as chemotaxis and oxygen consumption. Confer and Eaton (1982) first demonstrated that this toxic activity was due to CyaA. Preparations of CyaA with a high specific activity generated a high level of intracellular cAMP from endogenous ATP in a time and dose dependent manner in human neutrophils and alveolar macrophages and impaired their abilities for chemotaxis, superoxide generation, phagocytosis and microbial killing. Leppla (1982) reported that *Bacillus anthracis*, the causative agent of anthrax also produced a calmodulin-dependent adenylate cyclase toxin or edema factor which could penetrate into target cells after binding to a cell surface receptor. In contrast to the *Bacillus anthracis* toxin, *B. pertussis* CyaA penetrates target cells without binding to a specific receptor (Gordon *et al.*, 1989; Mock and Ullmann, 1993).

1.8.2 Structure

The native CyaA of *B. pertussis* has been isolated in multiple forms with different molecular masses ranging from 43 to 630 kDa. The non-toxic smaller forms of the calmodulin activable CyaA (45-50 kDa) were probably formed by proteolytic cleavage of the high molecular weight toxic form of the enzyme (which has an apparent mass of 200-216 kDa in SDS-PAGE), and the larger forms (>200 kDa) were probably formed by aggregation of the catalytic products, because polyclonal antiserum raised against the smaller form of

CyaA cross-reacted with the larger form (Ladant, 1988; Rogel *et al.*, 1988; Bellalou *et al.*, 1990 a).

The toxic form of *B. pertussis* CyaA has an apparent molecular mass of 200 kDa, and is the form capable of penetrating eukaryotic cells and catalysing the formation of intracellular cAMP (Hanski, 1989). Masure and Storm (1989) have reported that the 45 kDa form of *B. pertussis* CyaA is also capable by itself or in the combination with other polypeptides of invading target cells. The 45 kDa protein is the major form of the enzyme which appears in the bacterial culture medium. It is possible that the toxic 200 kDa form of the enzyme is released into the medium but rapidly degraded and therefore its quantity is very small and barely detectable in the culture medium (Masure *et al.*, 1990). The extensive purification of the CyaA of 45 kDa from culture supernate yielded a CaM sensitive CyaA, which always lacked toxic activity (Bellalou et al., 1990 b). In the presence of proteolytic inhibitors, only the toxic form of 200 kDa CyaA could be detected in the culture medium (Bellalou *et al.*, 1990 a). However, more than 90% of the toxic form of CyaA is cell surface associated protein and localized on the outer surface of the bacterial cell membrane (Cowell *et al.*, 1979).

The first 450 amino acids at the N-terminal end of CyaA constitute the calmodulin-activated catalytic portion of the enzyme. Tryptic fragments of the N-terminal domain have established that the catalytic domain is located between amino acid residues 1-235/237 in which the ATP-binding site was located between amino acids 54-70. It contained lysine residues at positions 58 and 65 which were shown to be essential for the catalytic activity. The CaM activation domain was located between amino acids 235/237-399 in which the CaM binding site was between amino acids 235 and 254 around trp 242, a

region displaying an α helical structure (Glaser *et al*,. 1989; Ladant *et al.*, 1989). Further cleavage of the calmodulin-stimulated catalytic domain into two fragments produced a 28 kDa fragment corresponding to the N-terminal end which was shown to possess ATP-binding capacity, and a 19 kDa fragment which was reported to have the CaM-binding site (Munier *et al.*, 1991). The remaining 1250 carboxy terminal amino acids act as a haemolytic domain and share homology with a family of Ca^{2+}-dependent pore-forming cytolytic toxins (RTX toxins, see section 1.8.4). This portion consists of four structurally distinct regions that seem to be responsible for specific functions of the toxin: (1) Four hydrophobic potentially membrane-spanning regions, located between amino acids residues 500-700, and which are probably involved in insertion of the toxin into target cell membranes, in pore formation and translocation of the catalytic domain into target cells; (2) A CyaC-dependent activation region located between amino acids 710-1000; (3) A highly periodical structure consisting of glycine- and aspartate-rich repeats units located between residues 1000-1600 which represent the main Ca^{2+} binding sites of the protein; (4) The non-processed carboxy terminal secretion signal within the last 270 residues of CyaA which interacts with the membrane-located CyaB, CyaD and CyaE proteins (Sebo *et al.*, 1991; Gross *et al.*, 1992; Coote, 1992; Hewlett and Maloney , 1994). A simplified model for CyaA structure is given in Fig. 1 and for RTX toxins in general in Fig. 2B.

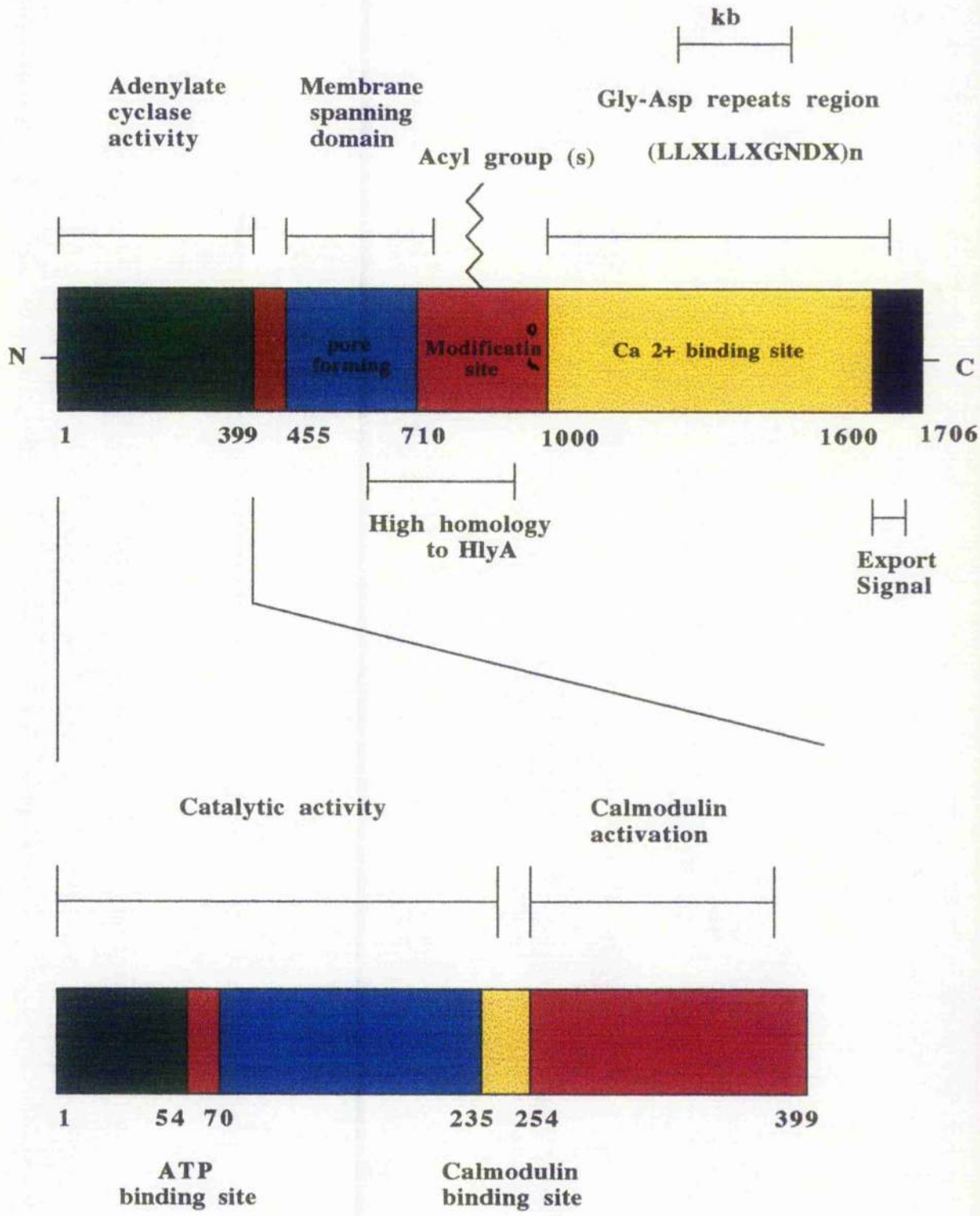
1.8.3 Genetic organization

The structural gene for the *Bordetella pertussis* adenylate cyclase toxin (*cyaA*) has been cloned (Brownlie *et al.*, 1988; Glaser *et al.*, 1988 a) and

Fig. 1

Schematic representation of the structural organization and functional domains within the CyaA toxin.

The adenylate cyclase enzymic domain is contained within the N-terminal 400 amino acid residue. The catalytic domain is located between amino acid residues 1-235/237, ATP binding site between amino acid residues 54-70 and the calmodulin activation site is located between amino acid residues 235-399. The C-terminal 1306 amino acids of CyaA contains hydrophobic membrane spanning regions between amino acid residues 500-700 (responsible for insertion, pore formation and translocation), CyaC-dependent activation region between amino acid residues 710-1000, glycine- and aspartate-rich repeat region located between amino acid residues 1000-1600 (responsible for Ca^{2+} binding sites) and secretion signal located within the last 270 amino acid residues.


kb
Adenylate cyclase activity
Membrane spanning domain
Acyl group (s)
Gly-Asp repeats region
(LLXLLXGNDX)n
N
pore forming
Modification site
Ca 2+ binding site
C
1
399
455
710
1000
1600
1706
High homology to HlyA
Export Signal
Catalytic activity
Calmodulin activation
1
54
70
235
254
399
ATP binding site
Calmodulin binding site

sequenced (Glaser *et al.*, 1988 a). The *cyaA* gene has an open reading frame of 1706 codons. Three additional genes downstream from the *cyaA* gene, *cyaB*, *cyaD* and *cyaE* have also been described, the gene products of which are required for secretion and transport of CyaA to the surface of the bacterial cell or to the culture medium (Glaser *et al.*, 1988 b; Coote, 1992). The *cyaC* gene whose product CyaC protein activates the CyaA protoxin, is located upstream of *cyaA* (Barry *et al.*, 1991).These genes exhibit extensive homology with similar operons encoding other RTX toxins (see section 1.8.4). For example, the *E. coli* α-haemolysin operon consists of four genes: *hlyA*, encoding α-haemolysin; *hlyC* required for the activation of HlyA; and *hlyB* and *hlyD* required for secretion of HlyA (Mackman *et al* ., 1988). These genes are expressed as a single operon which is activated by a cis-acting element *hly R* (Koronakis *et al.*, 1989). Similarly, the genes encoding the leukotoxin (LktA) of *P. haemolytica* are transcribed as a single operon (Strathdee and Lo, 1989). Highlander *et al.*,(1989) identified a region upstream of *lktC* which forms a DNA bend by virtue of four CAG (C/T) A repeats occurring at 10-base intervals. This may act as a regulatory sequence controlling the expression of the *lkt* operon. Analysis of the transcriptional organization of the *B. pertussis cya* locus has shown that *cyaA*, *B*, *D* and *E* are organized in a single operon and transcribed from the *cyaA* promoter adjacent to the *cyaA* gene (Laoide and Ullmann , 1990), and that the *cyaC* gene is transcribed in the opposite direction (Barry *et al.*, 1991). The *cyaC* gene is transcribed at a low level in a *bvg*-independent manner (Goyard and Ullmann 1991). In *B. pertussis*, the regulation of the expression of the other *cya* genes is achieved by the central regulatory *bvg* locus as described for regulation of virulence factors (section

1.7). A schematic model of gene arrangement and the direction of gene transcription of the RTX cytotoxin determinants is shown in Fig. 2A.

1.8.4 Homology among RTX toxins

A number of Gram negative bacterial pathogens produce Ca^{2+}-dependent cytolytic toxins. These include CyaA of *B. pertussis*, LktA of *P. haemolytica* HlyA of *E. coli*, haemolysin (ApxA) of *Actinobacillus pleuropneumoniae* and leukotoxin (AktA) of *Actinobacillus actinomycetemcomitans*. These form the RTX family (repeats in toxin) because of a common series of glycine-rich nonapeptide repeats (L-X-G-G-X-G-N-D-X), located close to the C-terminal end of each toxin (Strathdee and Lo, 1987; Welch, 1991; Coote, 1992). The gene clusters of the *hly*, *lkt*, *cya*, *akt* and *apx* determinants (Fig. 2A) share remarkable sequence homology. LktB and HlyB differ in length by only one amino acid residue and share 573 identical amino acids and have 81% identity, whereas LktD and HlyD are the same length and share 287 identical residues and have 60% identity (Strathdee and Lo, 1987). Similar degrees of homology have been reported between the *hly* gene products and those of the *akt*, *apx* and *cya* determinants, and thus it is likely that the toxins have marked similarities in their structure, the manner of secretion and pore forming activity on target cells (Lally *et al.*, 1989; Kraig *et al.*, 1990; Coote, 1992).

In spite of remarkable similarities between the products of the RTX operons, the target cell specifities of the toxins vary. LktA is toxic for ruminant leukocytes or platelets (Shewen and Wilkie, 1982; Clinkenbeard and Upton, 1991), whereas AktA is only toxic for human or primate leukocytes (Taichman *et al.*, 1987). CyaA and HlyA have little target cell specifity (Hanski and

Fig. 2

A. Gene arrangements of the RTX cytotoxin determinants

Four genes constitute the RTX determinants for the RTX cytotoxins. The A gene is the toxin structural gene, the *C* gene is requried for toxin activation and the B and D gene products are required for toxin secretion. For the CyaA toxin an additional gene, *cyaE*, is also required for toxin secretion. Vertical bars represent the regions encoding the repeat units and the arrows indicate the direction of transcription. The figure is adopted from Cootc (1992).

1 *Bordetella pertussis* adenylate cyclase (Cya A)

2 *Escherichia coli* haemolysin (HlyA)

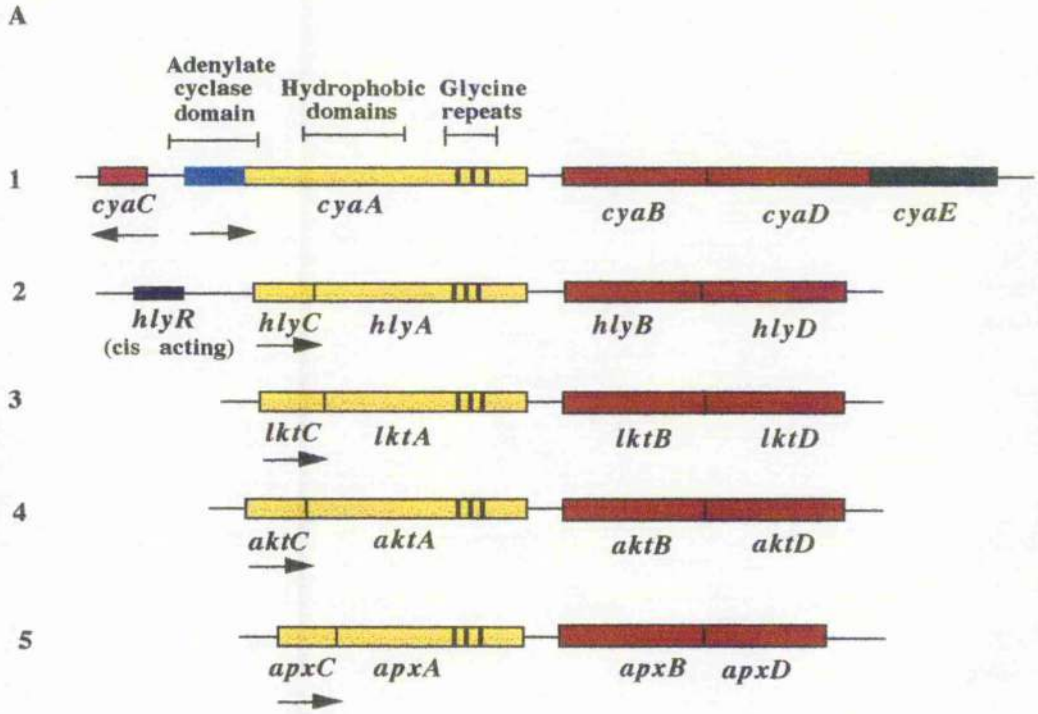
3 *Pasteurella haemolytica* leukotoxin (LktA)

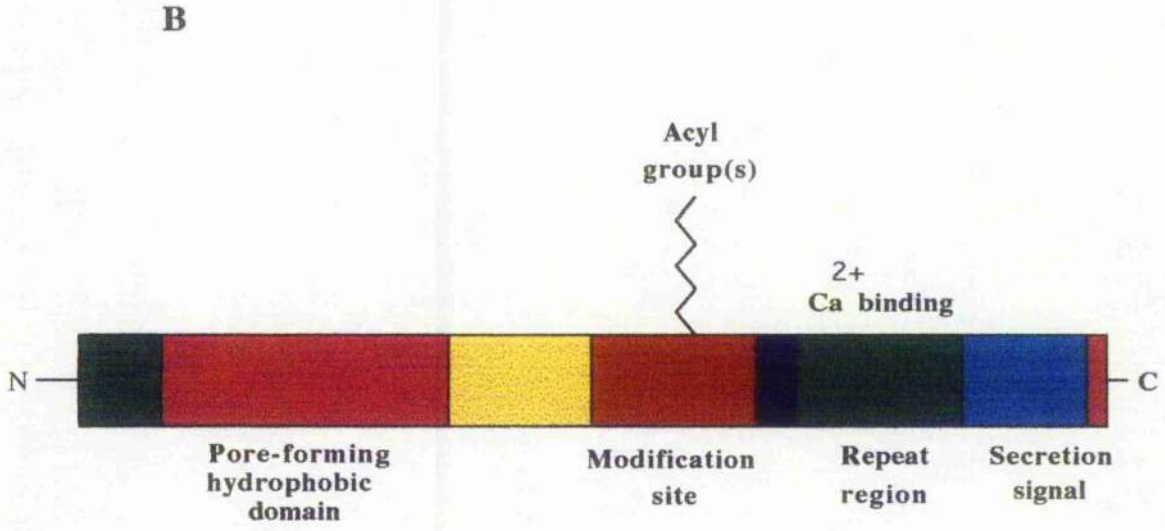
4 *Actinobacillus actinomycetemcomitans* leukotoxin (AktA)

5 *Actinobacillus pleuropneumoniae* haemolysin (ApxA)

B. Schematic representation of the RTX toxin structure

Four functional domains have been recognized in the structure of all RTX toxins. The hydrophobic domains are responsible for pore formation. The acylation site and adjacent repeat region are assumed to be responsible for conformational changes of the toxins necessary for interaction of the toxins with target cell membranes. The extreme C-teminal region functions as a secretion signal. The figure is adopted from Coote (1996).


A
Adenylate cyclase domain
Hydrophobic domains
Glycine repeats
1
cyaC
cyaA
cyaB
cyaD
cyaE
2
hlyR
(cis acting)
hlyC
hlyA
hlyB
hlyD
3
lktC
lktA
lktB
lktD
4
aktC
aktA
aktB
aktD
5
apxC
apxA
apxB
apxD


B
Acyl group(s)
2+
Ca binding
N
C
Pore-forming hydrophobic domain
Modification site
Repeat region
Secretion signal

Farfel, 1985; Coote, 1992) and will attack a wide variety of mammalian cells from different species. What influences target cell specificity is not known but it may be related to the folding of the toxin which influences its interaction with target cells and its ability to create pores in the cell membrane of different compositions (Coote, 1992). Specific receptors for RTX toxins have not been identified and binding may be non-specific. Variation in acylation by the attachment of different fatty acyl groups to each toxin may have an influence on the interaction of the proteins with membrane and their cell specificity (Szabo *et al.*, 1994) and this aspect was investigated in the work reported here.

1.8.5 Interaction of CyaA with target cells

Bordetella pertussis adenylate cyclase toxin binds to the target cells through a Ca^{2+} dependent process and directly penetrates the host cell plasma membrane. Two distinct steps for penetration of CyaA into target cells have been identified, namely insertion and translocation (Hanski, 1994). The Gly-Asp rich repeats of CyaA toxin are the target of Ca^{2+} binding which is involved primarily in the interaction of the toxin with the target cell membrane and the process of insertion and translocation. Acylation of the toxin by CyaC also favours interaction of the toxin with the target cell membrane. Ca^{2+} binding causes a conformational change of the CyaA toxin, corresponding to a transition from a globular structure to an elongated form (Hewlett *et al.*, 1991). It is proposed that the toxin first inserts into the plasma membrane in the presence of low concentrations of exogenous Ca^{2+} (μM) at a temperature ranging from 4 to 37 °C, through its hydrophobic domain, which is located between amino acids 500-700, and then in the second step, the N-terminal 45

kDa catalytically active fragment of the toxin in the presence of a high concentration of exogenous Ca2+ (mM) and above 20°C, is partially unfolded and translocated through a channel created by the hydrophobic domain of the toxin (Rogel and Hanski, 1992 a). The cleavage of the N-terminal fragment is not required for the translocation step or cAMP generation within the target cell stimulated by endogenous CaM, but it does take place in sheep erythrocytes (Friedman *et al.*, 1987 a; Hanski, 1994). A model for the interaction of CyaA toxin with target cells is given in Fig. 3. A and B.

1.8.6 Haemolytic activity

The toxic form of 200 kDa CyaA also possesses haemolytic activity. The pore-forming capacity of the CyaA molecule is responsible for haemolytic activity against sheep erythrocytes and is entirely independent of the presence and activities of the catalytic domain (Bellalou *et al.*, 1990 b; Rogel *et al.*, 1991; Szabo *et al.*, 1994). However, the main function of the haemolytic domain is probably not to lyse erythrocytes but to form channels for translocation of the N-terminal catalytic domain through the target cell membrane, and thus the pore forming capacity of CyaA is required for both haemolytic and toxic activities of CyaA (Bellalou *et al.*, 1990 b).

However, cAMP generation induced by CyaA toxin in sheep erythrocytes is immediate whereas the haemolysis of erythrocytes is delayed by about 1 hour and requires a higher level of adenylate cyclase toxin activity (Rogel and Hanski, 1992). Addition of exogenous calmodulin to sheep erythrocytes enhanced the haemolytic activity of the toxin, but blocked cAMP generation. Exogenous CaM appeared to inhibit the translocation of the catalytic domain

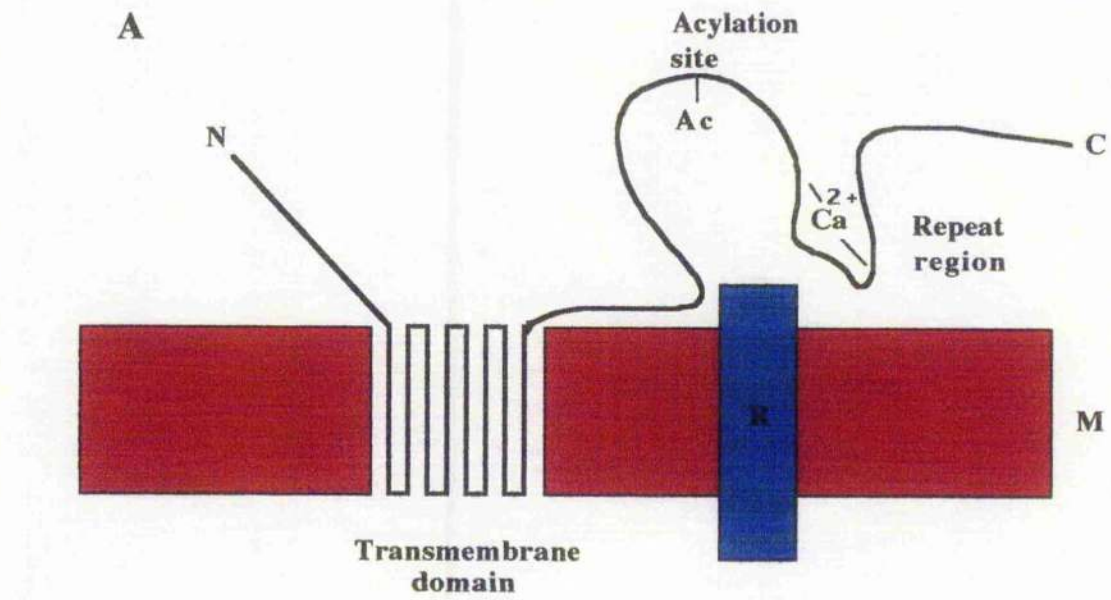
Fig. 3

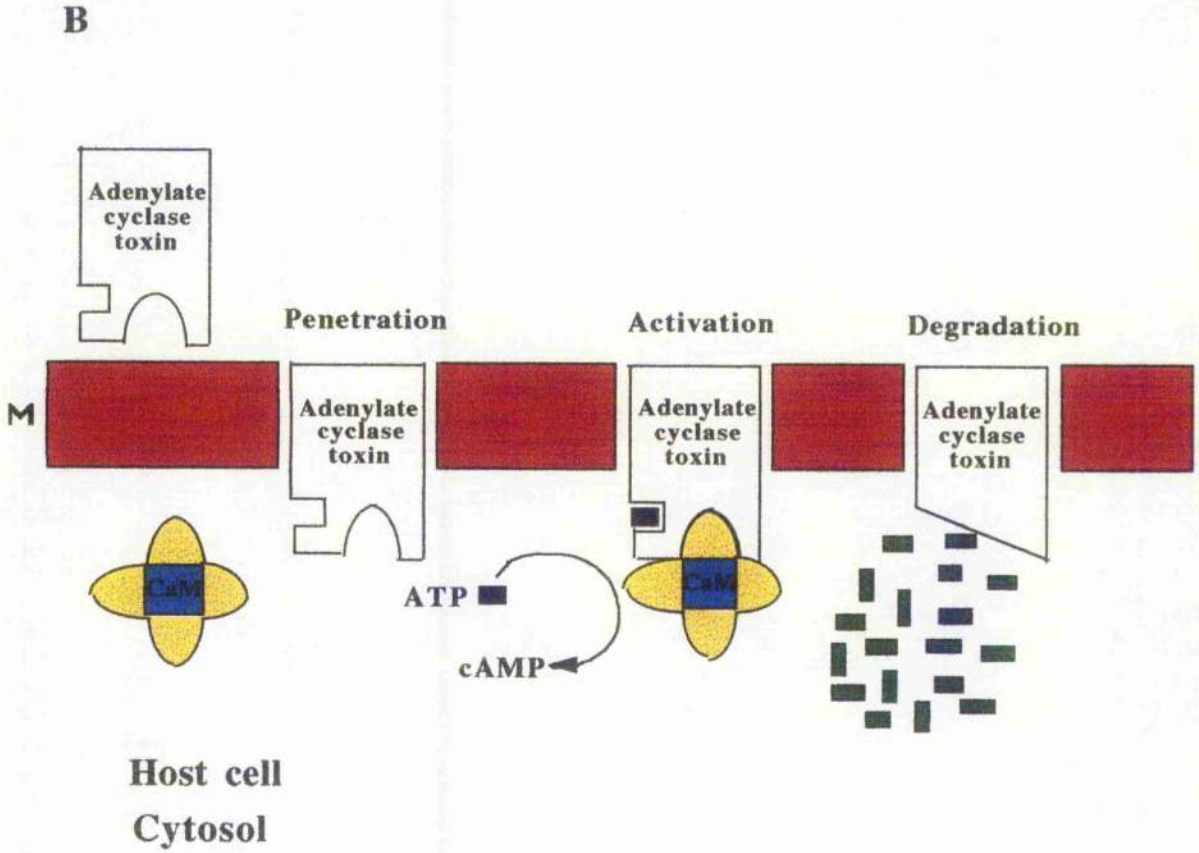
A Simplified model for the binding, insertion and translocation of CyaA toxin in the target cell membrane

The interaction of the acylation site and the repeat region of the toxin may create a conformational change in the toxin which allows it to bind to the receptor of the target cell membrane and insert the toxin into the plamsa membrane through its hydrophobic domain. The amino terminal portion of the toxin is assumed to be translocated into the target cells through a channel created by the hydrophobic domain of the toxin in the presence of Ca^{2+}. Abbreviations: R, receptor; Ac, acyl group; M, cell membrane; N, N-terminal and C, C-terminal end of the toxin. The figure adopted from Coote (1992).

B A simplified model for invasion of a target cell by *B. pertussis* CyaA toxin

B. pertussis CyaA penetrates the host cell plasma membrane with its hydrophobic domain, exposing the catalytic and CaM binding sites to the cytosol. The enzyme is activated upon interaction with CaM and converts intracellular ATP to cAMP. The enzyme within the cell is degraded and inactivated by a protease. The figure is derived from the model proposed by Rogel and Hanski (1989).


A
Acylation site
Ac
N
C
2+
Ca
Repeat region
R
M
Transmembrane domain


B
Adenylate cyclase toxin
Penetration
Activation
Degradation
M
Adenylate cyclase toxin
Adenylate cyclase toxin
Adenylate cyclase toxin
CaM
CaM
ATP
cAMP
Host cell
Cytosol

into erythrocytes but pore formation still took place and caused haemolysis (Rogel *et al.*, 1991). These data suggest that the toxic and haemolytic functions of *B. pertussis* CyaA toxin are separable and probably mediated by different domains on the CyaA toxin polypeptide (Sebo *et al.*, 1991; Rogel *et al.*, 1991). Szabo *et al.* (1994) demonstrated that CyaA toxin produces ion-permeable, cation-selective pores in phospholipid bilayers. However, the resulting membrane conductance was absolutely calcium dependent, as were the intoxication and haemolytic activities, and it was strongly affected by the polarity and magnitude of the membrane potential and enhanced by the presence of negatively charged phospholipid. The inactive CyaA toxin produced little or no change in conductance. Evaluation of the current-voltage relationships and the concentration dependence of pore formation or haemolysis suggested that a multimer of CyaA toxin, probably consisting of three or more holotoxin molecules, is involved in pore formation and haemolysis.

1.8.7 In vivo activation

In vivo activation of CyaA takes place intracellularly by transfer of a fatty acyl group to the protoxin from acyl carrier protein in the presence of CyaC. Two well-known types of protein acylation are palymitoylation through an oxy or thioester linkage to serine, threonine or cysteine residues and myristoylation through an amide linkage to an N-terminal glycine (Schlesinger *et al.*, 1993). Hackett *et al.* (1994) reported that proCyaA was modified in vivo by amide-linked palmitoylation of the ϵ-amino group of lysine 983, because the toxic effect of CyaA was resistant to hydroxylamine treatment and therefore no

ester-linked modification was implicated. Palmitoylated Lys-983 is not present in inactive AC toxin produced by a mutant strain BP DE386 in which the accessory gene, *cya*C has been disrupted. The palmitoyl-Lys-983 in CyaA lies between the hydrophobic domain and the calcium-binding domain (Fig. 3). A lysine residue at the equivalent position is conserved among some of the other RTX toxins.

Two kinds of peptides, composing the Lys-983 residue, were synthesized with and without the palmitoyl group and tested for their effects on toxin and haemolytic activities of CyaA. The non-palmitoylated peptide had no inhibitory effect on CyaA toxic activity. In contrast, the palmitoylated peptide reduced cAMP accumulation in a concentration dependent manner. These data suggest that palmitoylation at this site represents the modification that is required for the toxicity and haemolytic activity of CyaA toxin (Hackett *et al.*, 1994). Palmitoylation has been reported to mediate both protein-protein and protein-lipid interaction (Wedegaertner *et al.*, 1993). The work of Szabo *et al.* (1994), which indicated that the ability of CyaA toxin to create a transmembrane pore in an artificial lipid bilayer system was dependent on oligomerization of the toxin, suggested that the palmitoyl groups may be involved in protein-protein interactions to facilitate oligomerization. This thought is supported by the results from experiments in which the addition of palmitoyated peptide to CyaA toxin before addition of the mixture to the target cells was much more inhibitory for toxic effects than if the peptide was added to target cells before addition of the toxin (Hackett *et al.*, 1994).

For HlyA, the internal residues Lys-564 and Lys-690 are the modification sites in vivo (Ludwig *et al.*, 1996). Lys-690 of HlyA corresponds in location to Lys-983 of CyaA. Interestingly, when CyaA was isolated from recombinant *E.*

coli expressing CyaA and CyaC, it was found to be palmitoylated at Lys-860 as well as Lys-983 (Hackett *et al.*, 1995). Lys-860 corresponds to Lys-564 of HlyA. The proximal Lys residue is strongly conserved in all RTX toxins, but the distal site is not. For example, the equivalent of Lys-690 in HlyA or Lys-983 in CyaA is absent from LktA of *P. haemolytica* (Stanley *et al.*, 1994).

1.8.8 In vitro activation

Hardie *et al.* (1991) have demonstrated that in vitro post-translational activation of *E. coli* proHlyA was possible in a defined in vitro reaction containing purified proHlyA, HlyC and cytosolic activating factor (CAF). ProHlyA and HlyC proteins were produced separately by expression of recombinant plasmids in *E. coli* and CAF was found in the cytosol of enterobacteria such as *E. coli*, *Proteus vulgaris*, *Proteus mirabilis* and *Salmonella typhimurium*, but not in *Bacteroides fragilis* or *Enterococcus faecalis* or eukaryotic cells. CAF behaved as a 10 kDa polypeptide which was protease-sensitive and found to be identical to the acyl carrier protein. Protoxin was modified to mature protein by transfer of a fatty acyl group from acyl carrier protein to proHlyA and required HlyC to act as an acyl transferase. Only acyl carrier protein could promote HlyC-directed proHlyA acylation, but a range of acyl groups were effective (Issartel *et al.*, 1991). Acylation of one or more amino groups in proHlyA would explain the increased negative charge of the activated HlyA compared with proHlyA (Nicaud *et al.*, 1985). In vitro activation of proHlyA is calcium-independent and effective over a pH range of 6 to 9 and at temperatures from 4 °C to 42 °C. The HlyC of 20 kDa was present physiologically in a multimeric form (20-40 kDa) (Hardie *et al.*, 1991).

Although acyl transferase activity must presumably reside in HlyC, comparison of the HlyC sequence with known acyl transferases produced no identity. Sequence similarity between all the C-proteins of the RTX toxin family suggests that all the protoxins are modified in a similar manner. Thus CyaA protoxin has to be post-translationally modified by CyaC and is palmitoylated at Lys-983. It has not been established yet that CyaC has catalytic acyl chain transfer activity, but it is likely that, by analogy with HlyC activator of HlyA, the fatty acylation of proCyaA involves transfer of a fatty acyl moiety to proCyaA in a process involving association of the negatively-charged acyl ACP with the positively-charged CyaC, the latter providing recognition and interaction with specific proCyaA sequence.

Recently, Hoveker *et al.* (1994) have demonstrated chemical fatty acylation of CyaA by using a water soluble compound, acylpyrophosphate. They showed that undirected transfer of lauric, myristic or palmitic acid chains to the CyaA protoxin was able to confer weak haemolytic and toxic activities to CyaA. The chemically-modified protoxin showed a specific requirement for Ca^{2+} ions for toxic activity like the wild type toxin. The toxic and haemolytic activities of the chemically-modified CyaA were low in comparison to CyaA modified in vivo, suggesting that in vitro fatty acylation of the protoxin involved random modification of nucleophilic residues present in the toxin in contrast to the in vivo modification of specific sites.

1.8.9 Role in pathogenesis of pertussis

A clear contribution of *B. pertussis* CyaA toxin to virulence has been demonstrated and it is assumed to be an important colonization factor in *B.*

pertussis infection. Mutants of *B. pertussis* deficient in active CyaA production were shown to be essentially avirulent after intranasal infection in infant mice and the bacteria were cleared more rapidly from the lungs (Weiss *et al.*, 1984; Goodwin and Weiss, 1990; Khelef *et al.*, 1994). CyaA is required for the bacteria to initiate infection and colonize the mouse and although CyaA and PT both elevate intracellular cAMP, it has been proposed that CyaA may be responsible for the initial rapid increase in cAMP in immune effector cells while PT amplifies the cAMP signal thereafter (Masure *et al.*, 1987; Hanski, 1989; Goodwin and Weiss, 1990). The increased intracellular concentration of cAMP caused by CyaA activity inside cells inhibits the normal physiological processes of phagocytic leukocytes such as chemotaxis and oxidative responses and it is supposed that impairment of immune effector cell function is the major contribution of CyaA to the pathogenesis of *B. pertussis* (Confer and Eaton, 1985 ; Pearson *et al.*, 1987; Cootc, 1996). Both invasive and enzyme activities of CyaA are required during initial colonization of the mouse respiratory tract, as mutant strains lacking either one of these functions but retaining the other are severely impaired in colonization (Khelef *et al.*, 1994).

1.8.10 Immunogenicity

Early experiments suggesting a protective role for CyaA include those of Brezin *et al.* (1987) who showed that CyaA enzymic activity neutralizing and non-neutralizing antibodies prevented the lethal effects of the haemorrhagic alveolitis in mice infected with *B. pertussis*. CyaA from *B. pertussis* of 45 or 200 kDa stimulated a protective response that prevented death and bacterial colonization when mice were challenged intranasally with *B. pertussis* 14 days

after the second injection with CyaA (Guiso *et al.*, 1991). Passive and active immunization with CyaA significantly shortened the period of colonization of the mouse respiratory tract. Immunogenicity of CyaA depends on the CyaC-mediated post-translational modification of the toxin (Betsou *et al.*, 1993) which is required for presentation of CyaA protective epitopes located in the C-terminal portion of the toxin to the immune system (Betsou *et al.*, 1995). Experiments are required to test whether N-terminal or C-terminal portion of CyaA alone induces protection in mice.

Arciniega *et al.*, (1991) reported that pertussis infection in man leads to the production of high titres of CyaA antibodies, and such antibodies also are produced after pertussis vaccination. However, it was reported that the anti-CyaA antibodies found in human serum during pertussis infection did not neutralise the catalytic and penetrative activities of the enzyme (Farfel *et al.*, 1990). The lack of neutralizing capacity does not indicate that these antibodies are not important for the host immunity because they may interfere directly with the penetration of the invasive CyaA into immune effector cells and thus may be important for local defence against the CyaA toxin in the respiratory tract (Hewlett *et al.*, 1987; Betsou *et al.*, 1993).

The above work suggests that CyaA is a major toxin in the pathogenesis of pertussis and may be responsible for the local cytopathic syndrome in the respiratory tract. CyaA will act as a protective antigen against *B. pertussis* colonization in the murine model, but the efficacy of protection by CyaA was reported to be lower than the efficacy of the whole cell vaccine (Guiso *et al.*, 1991). The results suggest that CyaA could be a useful addition to an acellular vaccine, although this has been questioned because of its possible toxic effect and because of a reported immunological cross-reaction between the catalytic

domain of CyaA and rat brain adenylate cyclase (Monneron *et al.*, 1988; Guiso *et al.*, 1991).

1.8.11 Adjuvant effects on antibody response

Investigations on the suppressive or stimulatory immunological effects on antibody response and sensitization for anaphylaxis of various components of *B. pertussis* demonstrated that PT is the main adjuvant component of *B. pertussis* (Munoz and Peacock, 1990; Samore and Siber, 1996). PT markedly increased lymphocytosis due to inhibitory effects on the migration of circulatory lymphocytes into the lymphoid organ, suppressed chemotaxis and phagocytosis by neutrophils and macrophages, stimulated a delayed type hypersensitivity (DTH) mediated by a Th1 response and mediated passive cutaneous anaphylaxis due to enhancement of a specific IgE antibody response. It also enhanced class and subclass-specific antibody responses to co-administered protein antigens such as tetanus, diphtheria toxoid or ovalbumin by stimulation of a Th2 response associated with lymphokine secretion (Samore and Siber, 1996; Barnard *et al.*, 1996). The functional similarity between CyaA and PT in elevating intracellular cAMP suggests that some of the immunomodulatory effects such as adjuvanticity on antibody responses could be common to both toxin antigens. This feature was investigated for CyaA in this work.

MATERIALS AND METHODS

2.1 BACTERIAL STRAINS

2.1.1 *Bordetella pertussis*

The *B. pertussis* strains used in this study are given in Table 1. Strain BPDE386 is an insertion mutant derived from the Tohama strain of *B. pertussis*. It contains an oligonucleotide insertion which inactivates the expression of the activator gene (*cya*C) but does not affect the expression of *cyaA*. Therefore this strain produces a full-length CyaA protoxin which has enzymic activity but no invasive or cytolytic activity (Barry *et al.*, 1991). Strain BP348 (pRMB1) is a Tn5 insertion *cyaA* mutant of *B. pertussis* Tohama strain containing a recombinant plasmid (pRMB1) encoding the entire *cya* locus. This strain produces active CyaA toxin which has both AC enzymic and cytotoxic activities (Rogel *et al.*, 1989). *B. pertussis* 18323 strain is the reference strain of *B. pertussis* and was also used for mouse intranasal challenge and vaccine potency tests.

2.1.2 *Escherichia coli*

E. coli host strains for expression of pT7 vectors used in this study were two lysogens of bacteriophage λDE3 (BL21/DE3 and HMS174/DE3) (Novagen). Strain DH5αFIQ (Gibco-BRL) was also used. Strains BL21/ DE3 and HMS174/DE3 both contain λDNA expressing the T7 gene encoding T7 RNA polymerase under the control of the inducible *lacUV*5 promoter. Addition of IPTG to a growing culture induces the polymerase, which in turn transcribes the target DNA in the plasmid. *E. coli* strain BL21/DE3 lacks the *lon* protease

and *ompT* outer membrane protease that can degrade some recombinant proteins. Strain HMS174/ DE3 carries the *recA* mutation and has a markedly higher transformation efficiency than the BL21/DE3 strain. Strain DH5αF'IQ, in which the T7 promoter is transcriptionally silent, is more suitable for long term maintenance of plasmid preparations as they are more stable in this strain. Recombinant *E. coli* strains used in this study and their relevant characteristics are listed in Table 1.

The stock of the above bacterial strains and competent bacterial cells for transformation of plasmids were stored frozen at -70 °C in 50%(v/v) glycerol.

2.2 PLASMIDS

Plasmids used in this study and their relevant characteristics are listed in Table 1 and the structures are shown in Figs 4-6. These plasmids containing *cya*, *lkt* and *hyb* genes were constructed and kindly provided by Dr. Gareth Westrop.

2.3 CULTURE CONDITIONS

Frozen cultures of *B. pertussis* cells were grown on Bordet Gengou (BG) medium (Gibco-BRL, Appendix I. A) containing 20 % (v/v) defibrinated horse blood (Becton-Dickinson), 1% (v/v) glycerol and relevant antibiotics for 72 h at 37 °C in a humidified box. The bacterial cells harvested from plates were subcultured in cyclodextrin liquid medium (CDL, Appendix I. B) with 10% (v/v) bovine serum albumin (Sigma) in the presence of relevant antibiotics. Recombinant *E. coli* strains were grown at 37 °C for 24 h on 2xYT agar and

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Relevant Phenotype	Source or reference
Bacterial strains:			
B. pertussis :			
BP348	*cyaA* ::Tn5	CyaC, Km^R	1
BP348 (pRMB1)	*cyaA* ::Tn5 (*cyaA*$^+$, *cyaC*$^+$)	CyaA, CyaC, Tc^R	2
BPDE386	Δ*cyaC*	CyaA	3
BP 18323	wild type	CyaA, CyaC	4
E. coli:			
BL21	F$^-$*ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm*	non-expression host	5
BL21/DE3	F$^-$*ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (λDE3 lysogen)	T7 RNA polymerase	5
DH5α F'IQ**	F'Ø80d*lac* ZDM15 Δ *hsd* R17 (r_k-, m_k-) *gal* -	IPTG inducible	6
HMS174/DE3	F$^-$ *recA hsd* R (r_{k12}^- mk_{12}^+) Rif^R (DE3)	T7 RNA polymerase	5
P. haemolytica (P30)	wild type; A1 serotype	LktA, LktC	7
Plasmids:			
pRMB1	*cya*A,*cya*C	CyaA,CyaC	2
pGW44	*cya*A	CyaA	8
pGW14	*cya*C	CyaC	8
pGW54	*cya*C	CyaC	8
pGW64	*lkt*A	LktA	9
pGW78	*lkt*C	LktC	9
pGW80	*hyb*1	Hyb1	9
pGW88	*hyb*2	Hyb2	9
pCACT3	*cya*A,*cya*C	CyaA,CyaC	10

*All the plasmids encode Amp^R except pGW54 and pGW78 which are Cm^R and pRMB1 which encodes Tc^R.

** Full Genotype: F'Ø80d*lac* ZDM15 Δ(*lac* ZYA-*arg* F)U169 *deo* R *rec* A1 *end* A1 *hsd* R17 (r_k-, m_k-) *gal* - *pho* A *sup* E44l - *thi* -1 *gyr* A96 *rel* Al.

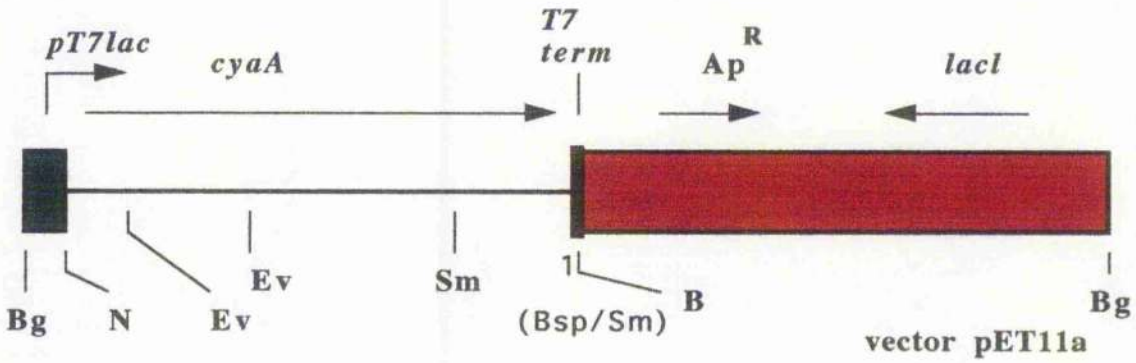
1. Weiss *et al*..,1983 2. Brownlie *et al* .,1988 3. Barry *et al* ., 1990 4. Glasgow University, Laboratory collection 5. Novagen 6. Gibco-BRL 7. Saadati *et al* ., 1997 8. Westrop et al ., 1996 9. Westrop et al ., 1997 10. Betson et al., 1993.

Fig. 4

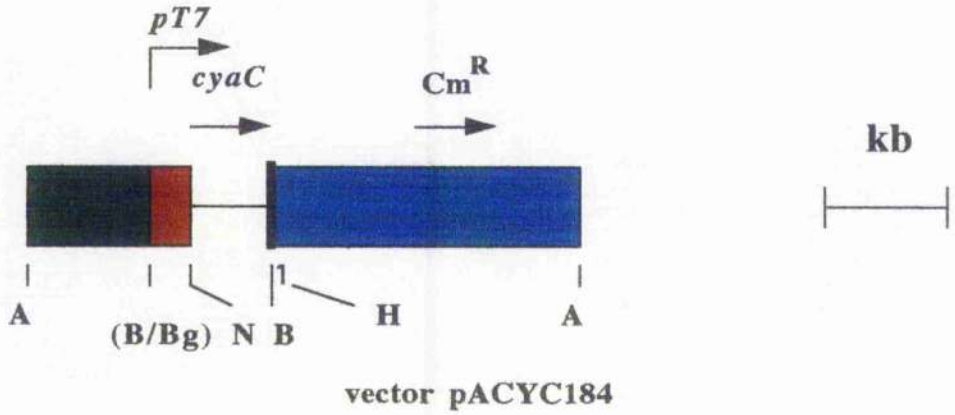
The structure of recombinant plasmids

Plasmid pGW14 was derived from T7 vector pT7-7 (obtained from I. Hunter, Strathclyde University) and contains the *cyaC* ORF linked directly to the translation initiation codon of the vector. The region of pGW14 including the T7 promoter , the gene leader sequence and the *cyaC* ORF was subcloned into vector pACYC184 (Chang and Cohen, 1978) to create pGW54. The orientation of the *cyaC* sequence of pGW54 precludes the possibility of constitutive expression from the *tet* promoter of the vector. Plasmid pGW44 was derived from vector pET11a (Novagen) and contained the *cyaA* ORF linked directly to the translation initiation codon of the vector. Transcription of *cyaA* is controlled by the T7*lac* promoter. Promoters indicated by PT7 and PT7*lac*. Arrows shows orientation of promoters and ORFs. Restriction endonuclease site shown by Bg, *Bgl*II; Bsp, *Bsp*Hi; Ev, *Eco*RV; Sm, *Sma*I; H, *Hind*III; N,*Nde*I; A, *Aat*II and B, *Bam*HI. Restriction endonuclease site in parentheses were destroyed by ligation. The figure is based on data of Westrop *et al.* (1996).

pGW44



pGW54



pGW14

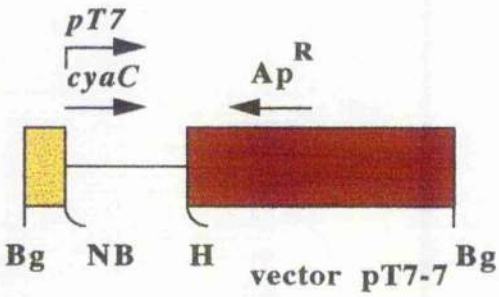


Fig. 5

The structure of recombinant plasmids (continued)

The construction of pGW44 (encoding *cyaA*), pW54 (*cyaC*), pGW64 (*lktA*) and pGW78 (*lktC*) involved direct linkage of the cya (shown in red or blue bars) or lkt (shown in green or yellow bars) gene sequence to the leader sequences of T7 vectors pET11a or pT7-7. The pGW80 (encoding *hyb1*) and pGW80 (encoding *hyb2*) were constructed using PCR mutagenesis together with conventional subcloning procedures to link *cyaA* and *lktA* gene sequences. Plasmids pGW54 and pGW78 derived from pACYC184 are compatible with plasmids pGW64, pGW44, pGW80 and pGW88 derived from pET11a, allowing co-expression of A and C genes in the same *E. coli* strain. Solid lines represent the T7 vectors pT7-7 (in pG54) or pET11a (in all other constructs) and broken lines the vector pACYC184. PT7 lac indicates the hybrid promoter composed of the T7 gene 10 promoter and the lac operator sequence from pET11a and pT7 indicates the T7 gene 10 promoter from pT7-7, Ap^R, gene for ampicillin resistance; Cm^R, gene for chloramphenicol resistance; *lacI*, gene for *lac* repressor. Arrows show the orientation of promoters and ORFs. Restriction endonuclease sites used to construct recombinant plasmids are indicated by symbols; A, *Aat*II; H, *Hind*III: M, *Mlu*I: N, *Nde*I; Nc, *Nco*I; Nr, *Nru*I; P, *Pvu*I; Ps, *Pst*I and X, *Xho*I.

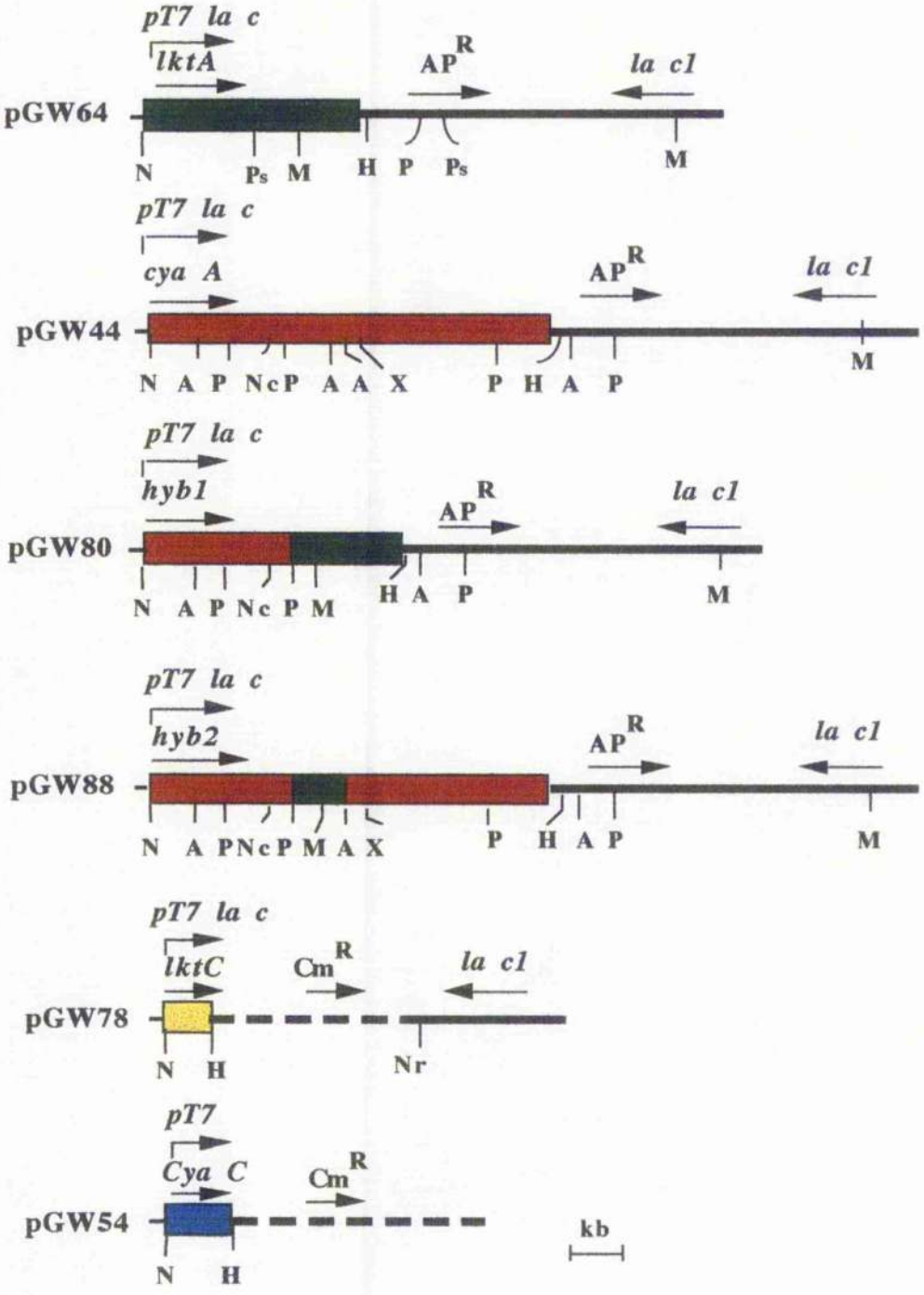
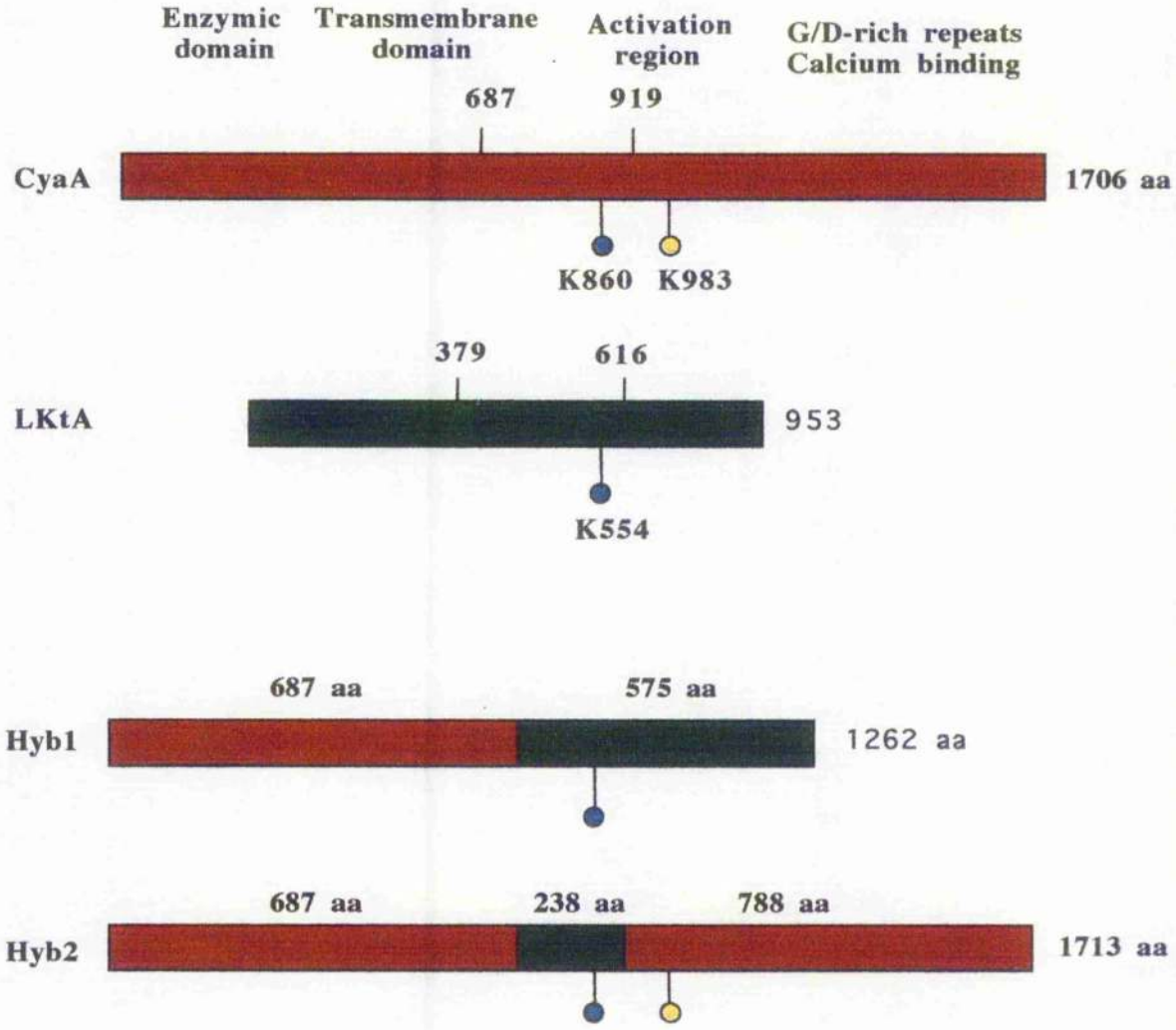

pT7 la c
lktA
AP R
la cl
pGW64
N Ps M H P Ps M
pT7 la c
cya A
AP R
la cl
pGW44
N A P NcP A A X P H A P M
pT7 la c
hyb1
AP R
la cl
pGW80
N A P Nc P M H A P M
pT7 la c
hyb2
AP R
la cl
pGW88
N A PNcP M A X P H A P M
pT7 la c
lktC
Cm R
la cl
pGW78
N H Nr
pT7
Cya C
Cm R
pGW54
N H
kb

Fig. 6

Schematic representation of the predicted structural organization and functional relationships in the CyaA , LktA and hybrid toxins derived from CyaA and LktA.

The positions of the domains in CyaA , LktA or hybrid toxins are shown. Numbers refer to amino acid residues. The position of lysine (K) residues that may be modified by C-mediated acylation are indicated. Residues 687 and 919 of CyaA and 379 and 616 of LktA indicate the junction codons at which crossovers were made for the construction of hybrid toxins. The figure is based on data of Westrop *et al.* (1997).


Enzymic domain
Transmembrane domain
Activation region
G/D-rich repeats Calcium binding
687
919
CyaA
1706 aa
K860
K983
379
616
LKtA
953
K554
687 aa
575 aa
Hyb1
1262 aa
687 aa
238 aa
788 aa
Hyb2
1713 aa

then transferred to 2xYT broth media (Sambrook *et al.*, 1989, Appendix I. D and I. E) with relevant antibiotics.

2.4 PREPARATION OF CyaA UREA EXTRACTS

2.4.1 Native CyaA from *B. pertussis*

B. pertussis BP348 (pRMB1) and BPDE386 were lawned on BG agar in the presence of tetracycline (10 mg/ml) and kanamycin (20 mg/ml) for strain BP348 (pRMB1). Bacteria were harvested from two lawn plates, and transferred to 1 litre of CDL medium containing relevant antibiotics in a 2 litre dimpled flask. Flasks were incubated in an orbital incubator at 150 rpm for 48 h at 37 °C. The morphology of cultured cells was checked by Gram stain for evidence of contamination. Cells were harvested by centrifugation at 9000 g for 30 min at 4 °C (Sorvall RC5B). Cell pellets (1 g wet weight) were resuspended in 8 ml of UE buffer (pH 8.0) (Appendix II. F) containing 1mM phenyl methyl sulphonyl fluoride (PMSF, Sigma) as a protease inhibitor and 8M urea (BDH). Suspensions were stirred for 2 h at 4 °C and then centrifuged at 15000 g for 30 min at 4 °C. The supernatants were then cleared by further centrifugation at 160,000 g in an OTD-combi/A 148561 centrifuge for 1 h and the resulting urea extracts were stored at -20 °C in 1 ml aliquots.

2.4.2 Recombinant CyaA from *E. coli*

Recombinant *E. coli* strains were constructed by transformation of *E. coli* BL21(for plasmids derived from vectors containing the *lac* promoter) or *E. coli* BL21/DE3 (for plasmids derived from T7 vectors).

2.4.2.1 Preparation of competent bacterial cells

The *E. coli* host strain was subcultured from a glycerol stock on to 2xYT agar and was incubated overnight at 37 °C. A cell suspension from an overnight plate was prepared in 2xYT broth and colony morphology was checked for evidence of contamination. The cell suspension was inoculated into 50 ml 2xYT broth in a 250 ml dimpled flask to give a starting A_{650} of 0.02. The flask was incubated with shaking at 130 rpm at 37 °C. When the culture had reached A_{650} of 0.3-0.6, 20 ml of the culture in a Falcon centrifuge tube was left on ice for 10 min before the cells were harvested by centrifugation at 9000 g for 10 min at 4 °C in a MSE-6L centrifuge. The cells were then resuspended in 2 ml of ice-cold TSB pH 8.0 (Appendix II. K) and the cell suspension left on ice for 10 min. The competent cells could be stored in 200 µl aliquots at -20 °C until required.

2.4.2.2 Preparation of plasmid DNA

Plasmid DNA was prepared according to the modified method of Ish-Horowicz and Burke (1981). A single colony from an overnight culture plate of recombinant *E. coli* strain was incubated overnight in 2 ml of 2xYT medium with relevant antibiotic at 37 °C with vigorous shaking. The resulting bacterial cells were centrifuged at 12000 g for 30 s at 4 °C. The pellet was aspirated and left to dry inverted on tissue paper and the cells then resuspended in 100 µl of ice-cold GTE buffer (Appendix II. L) by vigorous vortexing, and then 200 µl of ice-cold, freshly prepared NSD (Appendix II. M) was added and mixed by inverting five times. The suspension was mixed by gently vortexing after

addition of 1 ml of KAC (Appendix II. N), left for 10 min on ice and then centrifuged at 12000 g for 5 min at 4 °C and the supernatant was stored at -20 °C.

2.4.2.3 Transformation of *E. coli* host cells

Plasmid DNA (2 µl or 200 ng) was added to 200µl of competent cells and left on ice for 20 min before 800 µl TSB (Appendix II. K) containing 20 mM D-glucose was added. The cell suspension was incubated at 37 °C for 1 h with shaking (130 rpm). The transformed cells (100 µl) were spread on selective 2xYT agar containing relevant antibiotics and incubated overnight at 37 °C.

2.4.2.4 Growth of recombinant *E. coli* strains

Isolated colonies from primary transformants were subcultured on to selective 2xYT agar and incubated overnight at 37 °C. Cell suspensions in 2xYT broth from an overnight plate of recombinant *E. coli* strains were inoculated into 250 ml 2xYT broth containing relevant antibiotics in a 2 litre dimpled flask to give an OD at 650 nm of 0.02 and incubated at 37 °C with shaking, When the OD_{650} reached 0.6, isopropyl β-D-thiogalactopyranoside (IPTG,Sigma) was added at a final concentration of 1 mM to induce expression of the recombinant proteins. The growth was continued at 37 °C for 4 h. The cells were harvested by centrifugation at 9000 g for 10 min at 4 °C and the cell pellets were stored at -20 °C.

2.4.2.5 Preparation of urea extracts

For preparation of urea extracts of inclusion bodies from recombinant strains, the cell pellets were suspended in 25 ml (1/10 th volume of original culture) of TEN buffer (pH 8.0) (Appendix II. I) containing 1 mg/ml lysozyme and 5% (v/v) glycerol. After 1 h at 4 °C the cell suspension was sonicated using a sonicator (Vibra cell sonicator, Jencons Scientific) for 20 s at half maximal output to reduce viscosity and then centrifuged at 17,000 g for 30 min. The cell pellet was resuspended in 5 ml of 8 M urea in TC buffer (pH 8.0) (Appendix II. J) and rotated for 4 h at 4 °C. The suspension was then cleared by centrifugation at 160,000 g for 1 h. The supernatants containing recombinant proteins were stored in 1ml aliquots at -20 °C.

2.5 PURIFICATION OF CyaA

2.5.1 DEAE sepharose column chromatography

The method is based on electrostatic attraction between Diethyl-aminoethyl (DEAE) Sepharose and CyaA protein acting as an anion. The protein could be eluted from the column efficiently by increasing the ionic strength at a constant pH. The purification method was modified from that of Sakamoto *et al.* (1992). DEAE Sepharose CL4B (Pharmacia) (1.4 ml) was poured into a column (Econocolumn, 10 x 2.5 cm) and equilibrated with 10 ml DEQ buffer (Appendix II. F). Sodium chloride was added to 2 ml of urea extract to a final concentration of 150 mM, before loading the urea extract on to the equilibrated column. The column was washed with 10 ml of DEQ buffer

and the flow through collected, before fractions were eluted with 4 x 1 ml DEL buffer (Appendix II. F).

2.5.2 Calmodulin-agarose affinity chromatography

The procedure was essentially that of Hewlett *et al.* (1991) and is based on the affinity of calmodulin for CyaA. CyaA urea extract was diluted with an equal volume of buffer A containing 3 mM $CaCl_2$ (pH 8.0) (Appendix II. F) to give a final concentration of 4 M urea. The diluted urea extract (20 ml) was added to 2 ml calmodulin agarose (Sigma) previously washed with buffer A containing 3 mM $CaCl_2$. The mixture was rotated gently for 4 h at 4 °C and loaded onto a Econocolumn (10 x 2.5 cm). The column was washed with 3 bed volumes of buffer B (Appendix II. F) followed by 3 bed volumes of buffer A with 10% (v/v) BSA. In the final step, CyaA was eluted with 10 x 1ml of buffer A containing 8 M urea and 2% (v/v) Tween 20 (Sigma). The fractions were collected and assayed for protein content using absorbance at 280 nm and the degree of purity of the preparation was assessed by SDS-PAGE. Purified CyaA was stored at -70 °C in 1 ml aliquots.

2.5.3 Gel filtration column chromatography

A Sephadex G-75 column (Pharmacia), bed volume of 2 ml, was poured in an Econocolumn (10 x 2.5 cm) and equilibrated with 10 ml of equilibrating buffer (pH 7.5) (Appendix II. F). The column was calibrated by measuring the elution volume of an excluded sugar (Blue Dextran, 2000 KDa) and an included dye (Phenol red, 1 KDa). Urea extract (2 ml) was diluted with an

equal volume of equilibrating buffer, loaded onto the column and washed with 3 ml equilibrating buffer. The CyaA was eluted with 5 ml of equilibrating buffer containing 150 mM Na Cl.

2.5.4 Electroelution of CyaA from polyacrylamide gel slices

The method used was that of Leppard *et al.* (1983). The urea extract of CyaA was subjected to SDS-PAGE in 10% polyacrylamide gel (Appendix II. A) and stained with coomassie blue for 10 min, before cutting the 200 kDa protein band from the gel. The gel slice was transferred to dialysis tubing containing 5 ml of electroeluting buffer (pH7.4) (Appendix II. F) and positioned in a horizontal electrophoresis chamber with electroeluting running buffer (pH 7.4) (Appendix II. F) before electroelution at 100 V for 3 h. The purified CyaA solution was removed from the dialysis tube and stored at -70 °C in aliquots.

2.5.5 Concentration of purified CyaA by polyethylene glycol

The fractions of purified CyaA were mixed and placed in a dialysis tubing in a tray. The dialysis tubing with sample was surrounded completely by polyethylene glycol (PEG, Sigma, mol. wt 15,000-20,000) at 4 °C . When the volume of the sample was reduced to approximately 1/10 th of the original volume, the sample was removed from the dialysis tube and stored at -70 °C in 1 ml aliquots.

2.5.6 Concentration of purified CyaA by centrifugal concentrator

The mixed fractions of purified protein were poured into a centrifugal concentrator (Omega) with a 100 KDa cut-off membrane and centrifuged at 4000 g for 15 min. The concentrated protein was removed from the portion of tube above the membrane.

2.6 PURIFICATION OF OTHER PROTEINS BY UREA WASHING OF INCLUSION BODIES

Insoluble recombinant protein after expression and induction of protein by IPTG in *E. coli* strain BL21/DE3 was accumulated mainly in the form of inclusion bodies. For proteins such as LktA, LktC and CyaC which could not be purified by affinity chromatography, partial purification was achieved by washing soluble protein from inclusion bodies of the expressed proteins. The cells were lysed by treatment with lysozyme followed by sonication as described previously (section 2.4.2.5), then the cell lysate was centrifuged at 9000 g for 15 min at 4 °C. The pellet was suspended in 1 ml of deionized water and centrifuged at 27,000 g for 15 min at 4 °C. The pellet was then resuspended in 1 ml of 100 mM Tris-HCl (pH 8.5) containing 0.5 M urea and centrifuged at 27,000 x g for 15 min at 4 °C. The washing step was repeated with 1 M and 2 M urea. Hyb1 toxin was also partially purified by this method.

2.7 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-PAGE was done according to the method of Laemmli (1970) in a vertical slab gel apparatus (Protean; BioRad). Gel plates were assembled following the manufacturer's instructions. The polyacrylamide solution for the separating gel, containing 7.5% or 10% acrylamide (w/v) in resolving gel buffer (Appendix II. A), was poured between the plates about 4.5 cm below the top of the notched plate before overlaying the acrylamide solution with isobutanol. The gel was placed in a vertical position at room temperature until the gel had set (about 1 h), and then the overlay was poured off. The top of the separating gel was washed with distilled water and dried with the edge of a filter paper. The acrylamide solution for the stacking gel, containing 4.5 % acrylamide (w/v) in stacking gel buffer (Appendix II. A), was poured directly onto the polymerized separating gel and combs were placed into the gel solution. The gel was placed in a vertical position for 1 h until the stacking gel had set at room temperature and the comb was then carefully removed. The wells were immediately washed with distilled water and the gel was placed in a gel tank with running buffer (Appendix II. A). The samples and molecular weight standard (SDS-6H or SDS-7H, Sigma) were heated in solubilising buffer (Appendix II. A) at 85 °C for 10 min and loaded into the bottom of each well using a microlitre syringe or with a micropipetter fitted with a long narrow tip. Electrophoresis was started at 20 mA until the tracking dye had moved into the separating gel and then increased to 30 mA for about 5 h. When the tracking dye had reached the bottom of the gel, the power was turned off and the gel plates removed. The gel was stained with coomassie blue R-250

(Sigma) staining solution (Appendix II. A) for 2 h and destained by several changes of destaining solution (Appendix II. A).

2.8 IMMUNOBLOTTING (WESTERN BLOTTING)

Immunoblotting was carried out according to the method of Towbin *et al.* (1979) in a Bio Rad Transblot apparatus. The proteins were separated in a 10% SDS-PAGE gel and then the gel was soaked in transfer buffer (Appendix II. D) for 10-20 min. Nitrocellulose membrane (Hybond-C super, Amersham) and absorbent paper (Whatman 3 MM) were cut to the size of the gel and soaked in transfer buffer for 2 min. The gel was placed between the membrane and the absorbant paper with one Scotch bright pad on each side and rolled over the gel-paper surface to create a good contact. The complete assembly was placed in a tank of transfer buffer with the Hybond membrane facing the anode. Proteins were transferred overnight with cooling at 20 mA or 2 h at 400 mA. The membrane was removed and washed with PBS (Appendix II. B) before staining with 0.5% Ponceau S in 0.1% (v/v) acetic acid (Sigma, Appendix II. D) for 3 min or until the protein bands were visualized. The position of the transferred proteins and the molecular weight standards were marked and then the membrane was destained completely with PBS. The membrane was blocked with blocking buffer (Appendix II. D) containing 0.1 % (v/v) Tween 20 (Sigma) and 5% (w/v) dried skimmed milk in PBS with shaking at room temperature to prevent non-specific adsorption of the immunological reagents. The membrane was then washed twice for 5 min with 0.1% (v/v) Tween in PBS at room temperature with shaking. Primary antibody was diluted 1/1000 in PBS and incubated with the blot for 1 h at room temperature with shaking.

The membrane was then washed twice with PBS containing 0.1% Tween. HRP-conjugated secondary antibody (Scottish antibody production unit [SAPU]) was diluted 1/1000 in PBS and incubated with the membrane at room temperature. The membrane was then washed twice with PBS containing 0.1% Tween and developed with DAB (diaminobenzidine, Aldrich) solution (Appendix II. D) containing 0.03% (w/v) DAB, 98 ml PBS, 2 ml of aqueous 1%(w/v) $COCl_2$ and 100 µl of 30% hydrogen peroxide. The reaction was stopped after 3 min by dilution with distilled water and the membrane was stored at room temperature in the dark.

2.9 PROTEIN ESTIMATION

The protein concentration of preparations was determined according to the method of Bradford (1976). A concentration series (50, 100, 200, 400, 800 µg/ml) of bovine serum albumin (BSA, Sigma) was prepared by diluting a stock of BSA (1 mg/ml) in 150 mM NaCl. Three dilutions of sample (1/10, 1/50 and 1/100) were prepared in the same diluent. Each dilution from both standards and samples (100 µl) were transferred to duplicate tubes (16 x100 mm) before adding 5 ml of protein assay reagent (Appendix II. E) into each tube. Absorbance values of standards, samples and blank (diluent) were read at 595 nm. A standard curve was prepared by plotting the average A595 nm for each BSA concentration. The absorbance values of samples were then read off against the standard curve to determine the protein concentration.

2.10 DIALYSIS

This was done according to the method of Brownlie *et al.* (1988). Urea extracts of CyaA were diluted 1/20 in dialysis buffer (Appendix II. O) before dialysis to avoid protein aggregation. Diluted urea extract (1/20) was transferred into a dialysis membrane (size 1-8/32) and dialysed at 4 °C for 2 h with continuous stirring against dialysis buffer (Appendix II. O) or 25 mM HEPES (Sigma, pH 7.5) for production of toxin in the absence of calcium and chloride ions. The dialysis buffer was changed twice with fresh buffer during dialysis. After 2 h, the sample was removed from the dialysis tube and kept on ice for assay.

2.11 ADENYLATE CYCLASE (AC) ENZYMIC ASSAY

AC enzymic activity assay was measured according to the method of Salomon *et al.* (1974). CyaA samples were diluted 1/10000 and 10 µl of sample with 80 µl of reaction mix (Appendix II. G) were preincubated in a water bath at 30 °C for 10 min. Substrate mix (10 µl) (Appendix II. G) containing 20 mM ATP and 3000 Ci/mmol ^{32}P-ATP (Amersham) was added and incubation continued for 15 min at 30 °C. The reaction was stopped by the addition of 200 µl of 0.5 M HCl before neutralizing with 200µl of 1.5 M imidazole (Sigma). The neutralised solution (0.5 ml) was loaded onto a polypropylene column (Bio Rad) containing alumina powder (Merck) and ^{32}P-cyclic AMP eluted with 3 ml of 10 mM imidazole pH 7.6. Eluted solution was added to scintillation solvent and ^{32}P radioactivity counted in a Packard scintillation counter. In this assay ^{32}P-ATP is converted to ^{32}P-cyclic AMP.

The latter is separated by elution from the alumina column (^{32}P-ATP is retained on the column).

2.12 HAEMOLYSIS ASSAY

Whole fresh heparinized sheep blood (Becton-Dickinson) was diluted 1/2 in HH buffer (Appendix II. C) and layered on top of percoll (Sigma) with a density of 1.092 before centrifugation at 1200 g for 10 min. Percoll, plasma and leukocytes were removed and the pellet containing RBCs was washed in HH buffer until the supernate was clear. The RBCs were resuspended in HH buffer with 1 mM $CaCl_2$. CyaA was diluted in serial two-fold dilutions in HH buffer starting with 1/20 dilution before transferring (50 µl) to a 96-well flat bottom microtitre plate (Nunclon Delta). The RBCs (50 µl of $5x10^8$ cells) were added to the microtitre plate and mixed gently with the toxin. HH buffer alone was used as a negative control and saponin 1% (v/v) as a positive control. The plate was then incubated overnight at 37 °C before centrifugation at 1500 g for 15 min. The supernate after centrifugation was carefully transferred to a clean microtitre plate and the absorbance at 540 nm was read on an Anthos 2001 ELISA reader. The assays were done in duplicate and the mean value of percentage haemolysis for each dilution was calculated.

2.13 PREPARATION OF CULTURED MAMMALIAN CELLS

Baby hamster kidney (BHK 21) cells were prepared by the method of Westrop *et al.* (1994). The cells were passaged in tissue culture medium (pH 7.4) (Appendix I. H) for 48 h at 37 °C. Trypsinized cells were collected by

centrifugation at 5000 g and resuspensed in HH buffer to give a concentration of 10^4 cells/ml. Bovine lymphoma-derived (BL3) cells and mouse macrophage-derived (J774.2) cells were grown in tissue culture medium (Appendix I. I and J) for 48 h at 37 °C. The cells were pelleted at 5000 g and resuspended in fresh growth medium to give a concentration of 10^6 cells/ml .

2.14 BHK CELL SPREADING (SHAPE CHANGE) ASSAY

Invasive CyaA activities were determined for BHK cells using a cell spreading (shape change) assay performed according to the method of Westrop *et al.* (1994). The wells of 24-well plastic tissue culture plates were coated with 0.5 ml of fibronectin (25 μg/ml, Sigma) in HH buffer for 15 min. The wells were then coated with 0.5 ml of haemoglobin (0.5 mg/ml, Sigma) for 15 min. Urea extracts were diluted 1/20 in HH buffer containing 3 mM $CaCl_2$ and serial 3-fold dilutions were prepared. Urea extract dilutions (250 μl) were mixed with an equal volume of BHK21($2x10^4$cells/ml) and transferred to coated wells. The plate was incubated at 37 °C for 2 h and the cells were fixed with 4% (v/v) formaldehyde (250 μl/well) for 15 min. The fixed cells were then stained with 1% coomassie blue and examined with the 40x objective under brightfield microscopy for stellation, either by eye or image analysis using a Leitz Diavert microscope equipped with a Hamamatsu Vidicon C1000 camera and an Archimedes digitizer connected with an Archimedes 310 microcomputer. This assay is based on the induction of a stellated morphology in BHK cells which results from intracellular cAMP elevation (Westrop *et al.*, 1994).

2.15 CHEMILUMINESCENCE INHIBITION (CL) ASSAY

The chemiluminescence assay was done according to Chang *et al.* (1986) and is based on measuring the inhibition of the response of bovine or rabbit neutrophils to PMA stimulation. Freshly heparinised bovine or citrated rabbit blood (15 ml) was layered carefully on top of 7 ml Histopaque (Sigma) and centrifuged at 830 g for 30 min at room temperature. Supernatant containing mononuclear cells, Histopaque and plasma were removed carefully. RBCs in the pellet were lysed by the addition of 10 ml of ice-cold lysis buffer containing 155 mM NH_4Cl, 10 mM $KHCO_3$ and 1mM EDTA. The suspension was then centrifuged at 400 g for 5 min at 4 °C and the pellet containing neutrophils was suspended in HH buffer (pH 7.4). The viability of neutrophils was checked before CL assay by staining with 0.1% trypan blue dye. The CL assay was performed by mixing 1/20 dilution of CyaA urea extracts (50 µl) with 750 µl HH buffer containing neutrophils ($5x10^5$cells) and 100 µl of 10^{-5} M 7-dimethylamino-naphthalene-1,2-dicarbonic acid hydrazide (DNDH, Boehringer Mannheim GmbH, Germany). Following incubation for 20 min at 38 °C, the neutrophils were stimulated with 100 µl of 0.5 mg/ml opsonised zymosan (OZ) (Sigma) or 1 µg phorbol myristate acetate (PMA, Sigma). The CL emission was then measured in mV at 38 °C with a Wallac luminometer connected to an IBM-PC computer with multi-use software (Bio-Orbit). The samples were assayed in duplicate and the mean value of percentage inhibition was calculated.

2.16 MTT DYE REDUCTION ASSAY

Cytotoxic activity was determined by the MTT assay (Mosmann, 1983) using the cell titre assay (Promega) kit. This assay is based on the reduction of a yellow tetrazolium dye (3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl tetrazolium bromide, MTT) into insoluble purple formazan by dehydrogenases in active mitochondria of living cells. The activity of toxin on cells inhibits this reaction. Mouse macrophage-derived (J774.2) cells and bovine lymphoma derived (BL3) cells ($3x10^5$ cells/ml) were prepared in RPMI medium (section 2.13). Urea extracts were diluted in RPMI tissue culture medium in serial two-fold dilutions starting with 1/20 dilution and 50 µl of each dilution transferred to the wells of 96-well flat bottom microtitre plates (Nunclon), before the addition of 50 µl/well of cell supension ($1x10^6$ cells/ml). The plate was incubated at 37 °C in a humidified box for 2 h before addition of 15 µl/well of MTT dye (Promega) and incubation continued at 37 °C in the humidified box for 4 h. The solubilization / stop solution (100 µl/well) (Promega) was then added and incubation continued at 37 °C in the humidified box overnight. The A_{540} nm was measured by ELISA reader.

2.17 IN VIVO ACTIVATION OF PROTOXINS

E. coli strain BL21(pCACT3) was constructed by transformation of pCACT3(*cyaA*,*cyaC*) plasmid into competent *E. coli* BL21 cells. *E. coli* BL21/DE3 (pGW44) was constructed by transformation of pGW44 (*cyaA*) plasmid into competent *E. coli* BL21/DE3 cells and then the *E. coli* BL21/DE3 (pGW44, pGW54) strain was constructed by transformation of pGW54 (*cyaC*)

plasmid into competent *E. coli* BL21/DE3 (pGW44) cells. The transformed *E. coli* strains were grown and induced by addition of IPTG (section 2.4.2.4). The recombinant proteins were then extracted with urea from inclusion bodies from induced cell lysates (see section 2.4.2.5). Hyb1, Hyb2 and LktA protoxins alone or activated in vivo by CyaC or LktC were also produced by this method, using BL 21/DE3 competent cells.

2.18 IN VITRO ACTIVATION OF PROTOXINS

2.18.1 Preparation of A and C toxin proteins and the activation component

ProCyaA, proLktA, proHyb2, CyaC and LktC were prepared from *E. coli* strains BL21/DE3 (pGW44), (pGW64), (pGW88), (pGW54) and (pGW78) respectively. Bacterial cells were grown in 2xYT broth containing ampicillin at 37 °C to an A_{650} nm of 0.6 and recombinant protein induced by addition of IPTG. Induced cultures were centrifuged at 9000 g for 10 min and cell pellets were resuspended in 25 mM HEPES (pH 7.5) and lysed by sonication using a Micro processor sonicator for 3 x 20 sec with 40 sec intervals for cooling. The cell lysates were centrifuged at 17,000 g for 30 min at 4 °C and pellets were resuspended in 25 mM HEPES (pH 7.5), containing 8.0 M urea. Proteins were extracted by rotation at 4 °C for about 4 h and the urea extracts cleared by centrifugation at 160,000 g for 1.5 h. The urea extract supernates were stored at -20 °C.

The cytoplasmic activator factor (CAF) was prepared from *E. coli* BL21 cells. *E. coli* BL21 was grown in 2xYT broth at 37 °C to late exponential phase

(A_{650} nm of 1.5) and cells were harvested by centrifugation at 9000 g for 15 min. The cell pellet was resuspended (10% w/v) in 25 mM HEPES (pH 7.5) and lysed by sonication as above. The cell lysate after sonication was centrifuged at 100,000 g and the supernate stored in 1ml aliquots at -70 °C. CAF was also prepared from cultures of *B. pertussis* BP348 and *Pasteurella haemolytica* (Ph30) strains in the same way.

2.18.2 In vitro activation reaction

The in vitro activation components (protoxin+C protein+CAF) were each diluted 1/20 in 25 mM Hepes (pH 7.5) and preincubated separately at 4 °C for 30 min. The reaction was set up by mixing 50 µl of each component followed by incubation for 30 min at 4 °C. Aliquots were removed and assayed immediately for CyaA toxic activity. A number of reactions were done in this way, varying different factors and reaction conditions such as incubation time and temperature, effect of different ions and EGTA, and alteration of in vitro activation components.

2.18.3 In vitro acylation of acyl carrier protein (ACP)

The reaction for enzymatic synthesis of Acyl-Acyl Carrier Protein (Acyl-ACP) was carried out according to the method of Rock *et al.* (1981). All the materials used in the acylation of ACP reaction were purchased from Sigma. The acylation reaction mix (Appendix II. P) (1 ml) contained 5 mM ATP, 2 mM dithiothreitol (DTT), 2% (v/v) Triton X-100, 100 mM Tris-HCl (pH 8.0), 400 mM LiCl, 160 µM fatty acid (sodium salt), 155 µM ACP-SH,

10 mM $MgCl_2$ and 3.0 units of acyl-ACP synthetase. It was incubated initially in a water bath at 37 °C for 3 h and then incubated for another 16 h at 30 °C. The acylated ACP was diluted 1/200 before use in in vitro activation in place of CAF.

2.19 ANTIBODIES

2.19.1 Production of anti-rCyaA polyclonal antibody in rabbits

Urea extracts of active and inactive recombinant CyaA proteins were separated on 10% SDS-PAGE gels and stained with Coomassie blue R 250 for 5 min until the 200 KDa protein band was visualized. The protein band at the 200 KDa position was carefully cut out in 5mm strips. The strips were washed with deionized water and then destained for 20 min. The gel bands were crushed after washing in sterile PBS and then suspended in 2 ml of sterile PBS. The suspensions were centrifuged at 15,000 g for 30 min at 4 °C and the supernatants was stored at -20 °C. Before use, protein antigens were dialysed against PBS at 4 °C for 24 h and the supernatant from the dialysis sac emulsified in Freund's incomplete adjuvant (Sigma) in a 1:1 ratio. Two female New Zealand Albino rabbits were injected intramuscularly with 2 ml of CyaA antigen after bleeding for pre-immune sera. The immune sera were collected 1 week after 4 booster injections at 4 week intervals. The immune sera were tested for specificity by immunoblotting and used as a reference serum in ELISA experiments. An immune serum against CyaC was prepared by the same method.

2.19.2 Preparation of mouse reference antiserum raised against CyaA or ovalbumin

A reference antiserum for anti-CyaA and anti-Oa in ELISA was produced by immunizing mouse with the purified CyaA (PAR CyaA) or ovalbumin (Oa) according to the immunization schedule (section 2.21). Sera with high titres of anti-CyaA or anti-Oa for each specific immunoglobulin were pooled to make the reference sera.

2.19.3 Other antibodies

Monoclonal antibody (9D4) (IgG2a) raised against *B. pertussis* CyaA toxin was kindly provided by Dr. E. Hewlett, University of Virginia, USA. This MAb was used in immunoblots and reacted with CyaA. Monoclonal antibody 2-5 (IgG) raised against CyaA toxin was kindly provided by Dr. N. Heveker, Pasteur Institute, Paris. This MAb reacted with the repeat region of LktA as well as that of CyaA. Rabbit polyclonal antibody raised against the 100-110 KDa bands of rLktA from 7.5 % acrylamide gels were kindly provided by Dr. M. Saadati, Division of Infection and Immunity, Glasgow University. The convalescent serum from a rat infected by *B. pertussis* 18323, hyper immune serum from mice vaccinated with *B. pertussis*, anti-FHA and anti-PT polyclonal antibody raised against purified FHA and PT were kindly provided by Dr. Elizabeth Hall, Division of Infection and Immunity, Glasgow University. Polyclonal antibody raised against PRN in mice was kindly provided by Dr. M. Roberts, Veterinary Pathology Department, Glasgow

University. These antibodies were used for screening for the presence of other *B. pertussis* components in purified preparations of CyaA by western blotting and ELISA. Anti-cytokine monoclonal antibodies were purchased from Pharmingen.

2.20 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Purified CyaA antigen was diluted with coating buffer (containing 50 mM sodium carbonate (Appendix II. H) to a concentration of 50 μg/ml and 100 μl portions of diluted CyaA dispensed by a multichannel pipette into wells of a microtitre plate (Immulon-2, Dynatech). The plates were covered and incubated at 4 °C overnight in a humidified box, then washed carefully three times with washing buffer (Appendix II. H) containing 0.05% Tween 20 in PBS (pH 7.4). For each wash, wells were filled with washing buffer and allowed to stand for 1 min prior to draining. The plates were pounded on paper towels as a final step. The wells were then blocked by addition of 100 μl/well of blocking buffer (Appendix II. H) containing 0.05 % (v/v) Tween 20 and 2% (v/v) BSA in PBS (pH 7.4) and incubated for 1 h at 37 °C. The plates were washed three times and dried as above. The serum samples were diluted 1/1000 in washing buffer and then 100 μl/well dispensed, except for the two last columns which were used for reference serum and buffer (as negative control). The plates were then incubated for 1 h at 37 °C before washing three times as above. The anti-mouse or anti-rabbit IgG-HRP-enzyme-conjugate (SAPU) at 1/2000 dilution in washing buffer (100 μl/well) was added and then incubated for 1 h at 37 °C. The substrate solution (Appendix II. H) for HRP-conjugate was prepared by dissolving 34 mg of ortho-phenylene diamine (OPD, Sigma)

in 100 ml of citrate-phosphate buffer (Appendix II. H). The substrate solution (100µl /well) plus 20 µl H_2O_2 was added and incubated at room temperature in the dark for 20 min. The reaction was stopped by addition of 50 µl/well of 12% H_2SO_4 and the absorbance values were measured at A492 nm in the Anthos 20001 ELISA reader. The above ELISA method was used for measuring IgG, IgG1, IgG2a, IgG2b and IgE antibodies to CyaA or Oa. HRP-sheep anti-mouse IgG, IgG1, IgG2a, IgG2b and IgE were the conjugates used in ELISA, respectively. The conjugates and standard reference sera were purchased from Serotec (22 Oxford OX5 1JE, England).

2.21 INTRANASAL MOUSE PROTECTION TEST

The experimental protocol was performed essentially according to the method of Betsou *et al.* (1995).

2.21.1 Preparation of CyaA and hybrid toxin vaccines

The crude and purified CyaA or hybrid toxins were prepared as active and inactive preparations depending on whether they had been made in the presence of C-protein or not (see sections 2.4 and 2.5). The vaccines were prepared by mixing gently 75 µg/ml CyaA or hybrid preparation with an equal volume of alhydrogel (2.5 mg/ml) kindly provided by Prof. D. Stewart-Tull, Division of Infection and Immunity, Glasgow University.

2.21.2 Active immunization

Male 3-4 week old HAM ICR (CD-1) mice randomized in groups of ten were anaesthetized with halothane and vaccinated subcutaneously with 15μg per mouse of crude or purified CyaA or hybrid vaccines or 1/5 human dose (HD) of adsorbed diphteria-tetanus-pertussis (DPT)-vaccine containing killed whole *B. pertussis* (Welcome Trivax-AD) in PBS using two doses at a two week interval. One group of mice was vaccinated only with alhydrogel in PBS and one group of mice was left unvaccinated as controls.

2.21.3 Mouse intranasal challenge

B. pertussis 18323 was grown as a lawn on two BG plates for 24 h at 37 °C in a humidified box. The growth was suspended in 1% (w/v) casamino acid (Oxoid) in PBS (pH 7.3) and was made up to 10 opacity units (2×10^9 cfu/ml) by comparison with the 5th International Reference of Opacity (Perkins et al., 1973). The challenge suspension was prepared by diluting 1/100 to give 2×10^7 cfu/ml and a sublethal dose of the challenge suspension (1×10^6 cfu per mouse) was instilled intranasally in 5 mice of each group one week after the second vaccination. The weight of the mice before and at intervals up to 7 days after challenge were recorded. Mice were sacrificed 7 days after challenge and lung pathology and lung weight as % of body weight were determined. The lungs were removed aseptically and homogenized in a sterile Jencons homogenizer in 1% (w/v) casamino acids in PBS. Neat, 10^{-2} and 10^{-4} dilutions of lung homogenates were cultured on BG agar at 37 °C in a humidified box and colonies were counted after 72 h.

2.21.4 Preparation of immune sera

The 5 mice of each group which had not been challenged were anaesthetized with halothane and bled one week after the second vaccination, i.e. at the time that the other mice were challenged. The blood was allowed to clot at 37 °C for 1 h. The clot was then separated from the inside wall of the collection tube using a pasteur pipette and the samples placed at 4 °C overnight before collection of sera. The sera were cleared by centrifugation at 10,000 g for 10 min and stored at -20 °C in 1ml aliquots.

2.22 Adjuvanticity of CyaA

A group of 20 mice were injected with ovalbumin (Sigma) 20 μg/mouse alone or with 15 μg/mouse of purified active or inactive recombinant CyaA. Two weeks after the first injection, 10 mice of each group were given a second injection. Sera were taken from 5 mice of each group at one week intervals after the first injection.

2.23 PREPARATION OF CYTOKINES

The cytokine assay was carried out according to the method of Redhead *et al* (1993). The spleens were removed aseptically from the mice vaccinated with purified active and inactive CyaA 7 days after the second immunization and a suspension of spleen cells prepared in RPMI 1640 medium (Gibco-BRL) with 10% (v/v) FCS, 1% (w/v) penicillin and 1% (w/v) fungizone by gentle homogenisation in a Jencons homogeniser. The suspension was layered over

3 ml of heat-inactivated FCS for 10 min on ice to allow large debris to settle and then washed twice by centrifugation in RPMI medium at 350 g for 10 min at 4 °C. The cells were resuspended in 10 ml RPMI medium and counted. RPMI medium alone or ConA (20 μg/ml, Sigma) or CyaA (15 μg/ml) in RPMI medium was added to 1 ml of spleen cell suspension ($1x10^7$cells) in the wells of a 24 well tissue culture plate and incubated at 37 °C for 24 or 48 h in a humidified CO_2 incubator. The cell cultures were centrifuged at 5000 g for 10 min and the supernates collected and stored at -20 °C.

2.24 CYTOKINE ELISA

Cytokines were assayed with a Pharmingen kit. (Cambridge Bioscience) Microtitre plates (Dynatech Immunlon 4) were coated with 50 μl /well of anti-cytokine monoclonal antibody (2 μg/ml) in coating buffer (0.1M $NaHCO_3$, pH 8.0) and incubated overnight at 4 °C. The plates were washed twice and blocked as described in section 2.15. The standard cytokines (Pharmingen) were diluted in 10% FCS in PBS (pH 7.4), according to kit instructions (10-2000 pg/ml). The standards and samples were added at 100 μl/well and incubated at 37 °C for 3 h before washing four times as before. Biotinylated anti-cytokine detecting monoclonal antibodies (Pharmingen) were diluted (0.5-4 pg/ml) in 10%FCS in PBS and added (100 μl/well) before incubation at 37 °C for 1h. The plates were washed six times and then extravidin-peroxidase (2 μg/ml, Sigma) was added before incubation at 37 °C for 1 h. The plates were washed eight times and TMB substrate (Dynatech) at 100 μl/well was added before incubation for 30 min in the dark at room temperature. The plates were read at 630 nm in an ELISA reader with reference filter at 405nm.

2.25 RADIAL IMMUNODIFFUSION ASSAY (RID)

RID for the measurement of the concentration of total IgG and IgG subclasses of serum was performed according to the method of Fahey and McKelvey (1965). It is based on the radial diffusion of an antigen or antibody from a cylindrical well through an agarose gel containing a suitable mono-specific antibody or anti-antibody. Therefore an antigen-antibody complex or a complex of antibody and anti-antibody is formed as a precipitin ring. The RID kits were purchased from the Binding Site (Birmingham, U.K). Three dilutions of IgG and IgG subclasses standard calibrators (neat, 10% and 60%) and 1/10 sera dilutions (5 µl/well) were added to the wells of an agarose gel plate and incubated at room temperature in a humidified box for at least 5 days until complete precipitin rings were formed. The square of the diameter of the precipitin rings were plotted against their corresponding calibrator concentration. The concentration of antibody was read from the calibration curve and multiplied by 10 (dilution factor).

3.1 PRODUCTION OF NATIVE CyaA FROM *B. pertussis*

In order to produce high levels of CyaA toxin from *B. pertussis* 348 (pRMB1) or BP DE386, the effect of growth conditions was investigated by altering the growth media, incubation time, temperature and aeration conditions. The maximum yield of CyaA toxin was obtained from cultures in a modified CDL medium supplemented with various amino acids, ions, vitamins and 500 μg/ml of methyl β cyclodextrin (Appendix I. B and C) (section 2.3). The bacterial cultures were heavily inoculated from BG agar into 1 litre of modified CDL medium in a 2-litre dimpled flask and incubated at 37 °C for 24 , 48 or 72 h with shaking at 150 rpm for high aeration or 120 h in static culture. Under these various conditions, the yield of CyaA presented in Table 2 was obtained.

CyaA extracted from *B. pertussis* 348 (pRMB1) or BPDE386 grown for 48 h with shaking led to a maximum production of CyaA crude extract as shown in Table 2 and this procedure was adapted for routine production of CyaA. Purification of the CyaA by DEAE-sepharose column chromatography also gave maximum production of CyaA from the 48 h culture. Less CyaA was obtained from other cultures or cultures grown statically. The protein profiles of crude and purified preparations, as revealed by SDS-PAGE, are shown in Fig. 7.

Fig. 7

SDS-PAGE analysis of crude and purified CyaA preparations from *B. pertussis* and *E. coli* strains

Crude urea extract (25 μg protein/lane) and purified CyaA (4 μg protein /lane) preparations were subjected to electrophoresis on 10% polyacrylamide gels and stained with coomassie blue (section 2.7). Arrows indicate the positions of molecular weight standards.

Lane 1 : 6H mol wt marker proteins

Lane 2 : CAN CyaA: Crude CyaA from *B. pertussis* 348 pRMB1

Lane 3 : PAN CyaA: Purified CyaA from *B. pertussis* 348 pRMB1

Lane 4 : CIN CyaA: Crude CyaA from *B. pertussis* DE386

Lane 5 : PIN CyaA: Purified CyaA from *B. pertussis* DE386

Lane 6 : CAR CyaA: Crude recombinant CyaA from *E. coli* BL/DE3 (pGW44,pGW54)

Lane 7 : PAR CyaA: Purified recombinant CyaA from *E. coli* BL/DE3 (pGW44,pGW54)

Lane 8 : CIR CyaA: Crude recombinant CyaA from *E. coli* BL/DE3 (pGW44)

Lane 9 : PIR CyaA: Purified recombinant CyaA from *E. coli* BL/DE3 (pGW44)

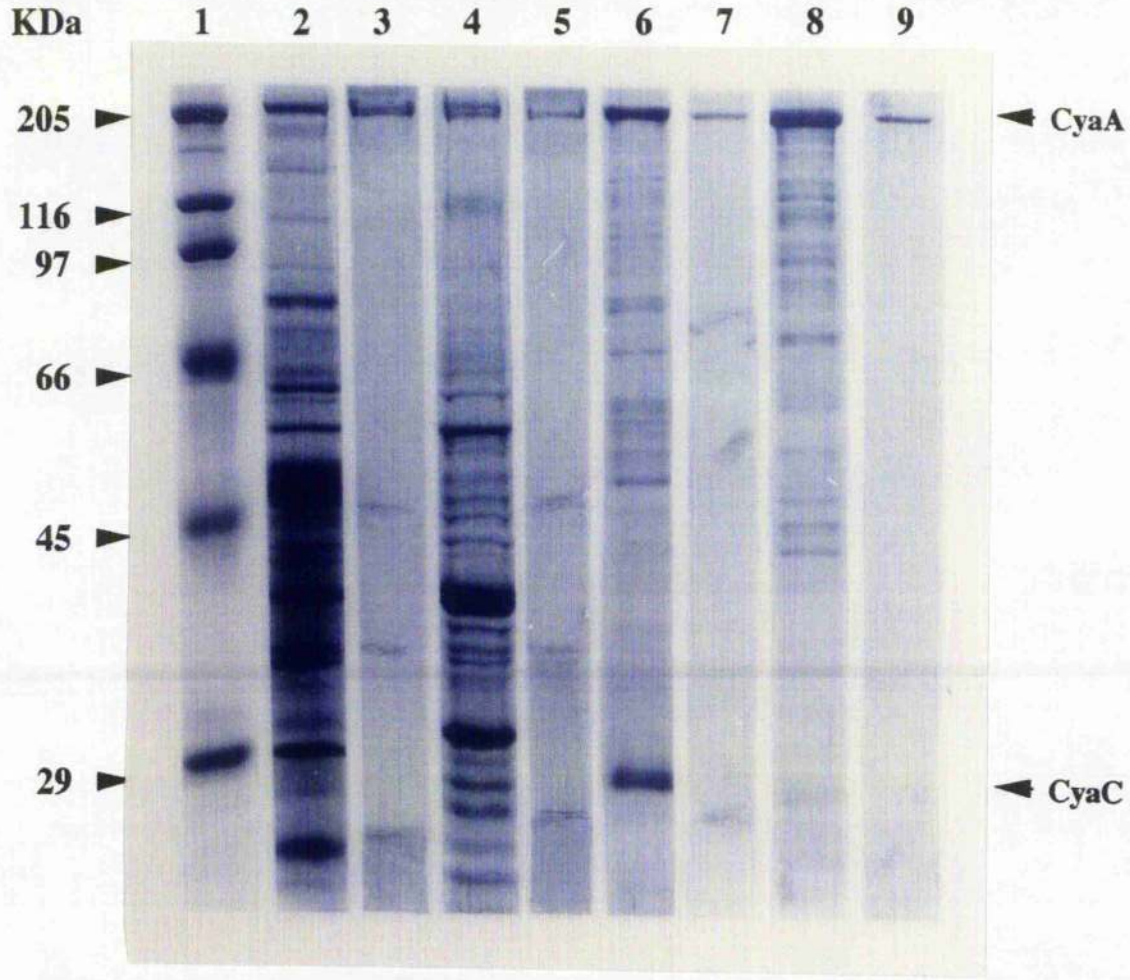

KDa
1
2
3
4
5
6
7
8
9
205
116
97
66
45
29
CyaA
CyaC

Table 2.

Effect of cultural conditions on the yield of CyaA from *B. pertussis*

B. pertussis strains	Cultural conditions	Yield of CyaA (μg protein/ml) ±SEM[a]	
		Crude urea extract[b]	Purified by DEAE sepharose[c]
1. Bp DE386	CDL (shaken, 24h)	1.36±0.31	0.17±0.06
2. Bp DE386	CDL (shaken, 36h)	4.34±0.49	0.35±0.14
3. Bp DE386	CDL (shaken, 48h)	6.10±1.72	0.46±0.17
4. Bp DE386	CDL (shaken, 72h)	2.87±0.35	0.27±0.11
5. Bp DE386	CDL (static, 120h)	0.83±0.09	0.09±0.04
6. Bp 348 pRMB1	CDL (shaken, 24h)	1.01±0.52	0.12±0.07
7. Bp 348 pRMB1	CDL (shaken, 36h)	3.52±0.86	0.29±0.13
8. Bp 348 pRMB1	CDL (shaken, 48h)	5.61±1.29	0.45±0.19
9. Bp 348 pRMB1	CDL (shaken, 72h)	1.94±0.54	0.18±0.02
10. Bp 348 pRMB1	CDL (static, 120h)	1.27±0.29	0.11±0.06

a. Results are the mean values ± SEM of 5 determinations. b. 1-5 = CIN CyaA (crude inactive native CyaA) ; b. 6-10 = CAN CyaA (crude active native CyaA); c. 1-5 = PIN CyaA (purified inactive native CyaA) ; c. 6-10 = PAN CyaA (purified active native CyaA).

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

Active recombinant CyaA toxin was produced in *E. coli* to a high level using the T7 RNA polymerase expression system. Co-expression of *cya*A and *cya*C in the presence of 1mM IPTG in *E. coli* BL21/DE3 harbouring two separate compatible plasmids pGW44 and pGW54 produced biologically active CyaA protein comparable to native CyaA produced from *B. pertussis*. Strain BL21/DE3 (pGW44, pGW54) synthesised CyaA in an amount comparable to that expressed from the individual plasmid pGW44 in *E. coli* as shown in Fig.7. This indicated that the expression of one gene did not interfere with the expression of the other gene.

Large amounts of CyaA were extracted from induced cultures of strain BL21/DE3 (pGW44, pGW54), presumably as inclusion bodies which could be solublized with 8 urea. The resulting urea extracts of strain BL21/DE3 (pGW44, pGW54) contained a 200 kDa protein which reacted with anti-CyaA polyclonal antibody in western blots (Fig. 8). The data in Figs. 7 and 8 indicated that expression of the *cyaA* gene in the absence of the *cyaC* gene (BL21/DE3 (pGW44)) produced a CyaA protein with the same apparent molecular weight (200 kDa) as the active form of CyaA synthesised in strain BL21/DE3 (pGW44, pGW54).

The effect of cultural conditions on the yield of CyaA preparations from BL21/DE3 (pGW44) or BL21/DE3 (pGW44, pGW54)) is shown in Table 3. Maximum yield of crude and purified CyaA protein was obtained after induction of the cultures with IPTG for 4 h. Without IPTG addition, even at 8 h, the yield of CyaA was greatly reduced.

Fig. 8

Western blot analysis of crude and purified CyaA preparations with rabbit anti-200 kDa CyaA polyclonal antibody.

Crude urea extract (20 μg/lane) and purified CyaA by DEAE sepharose (4 μg protein/lane) preparations were subjected to electrophoresis on 10% polyacrylamide gels and transferred to a Hybond C-super membrane (section 2.8). The nitrocellulose blots were probed with rabbit anti-200 kDa CyaA polyclonal antibody (section 2.19.1). Arrows indicate the positions of molecular weight standards.

Lane 1 :	CAN CyaA	(Crude active native CyaA)
Lane 2 :	CIN CyaA	(Crude inactive native CyaA)
Lane 3:	CAR CyaA	(Crude active recombinant CyaA)
Lane 4 :	CIR CyaA	(Crude inactive recombinant CyaA)
Lane 5 :	PAN CyaA	(Purified active native CyaA)
Lane 6 :	PIN CyaA	(Purified inactive native CyaA)
Lane 7 :	PAR CyaA	(Purified active recombinant CyaA)
Lane 8 :	PIN CyaA	(Purified inactive recombinant CyaA)

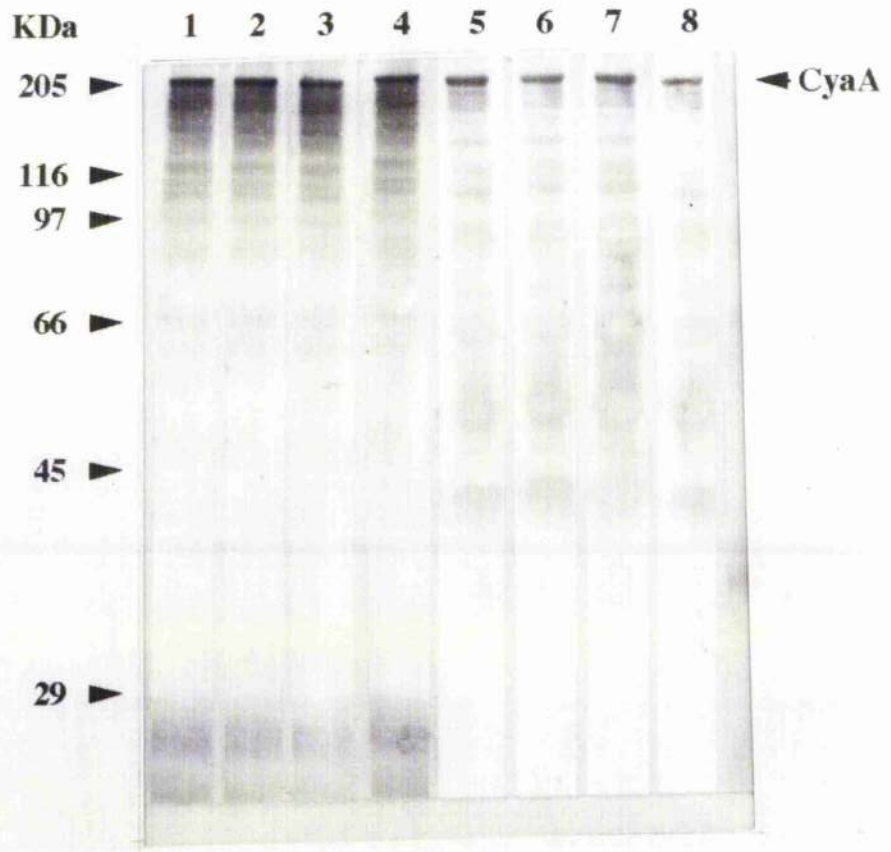

KDa
1
2
3
4
5
6
7
8
205
116
97
66
45
29
CyaA

Table 3.

Effect of cultural conditions on the yield of CyaA from recombinant *E. coli* strains

E. coli BL21/DE3 containing plasmid:	Cultural conditions[a]	Yield of CyaA (µg protein/ml) ±SEM[b]	
		Crude urea extract[c]	Purified by DEAE sepharose[d]
1. pGW44	2xYT+IPTG (shaken, 1h)	2.15±0.88	0.27±0.08
2. pGW44	2xYT+IPTG (shaken, 2h)	4.36±1.13	0.41±0.14
3. pGW44	2xYT+ IPTG (shaken, 3h)	6.51±1.37	0.56±0.21
4. pGW44	2xYT+ IPTG (shaken, 4h)	7.51±1.65	0.68±0.17
5. pGW44	2xYT (shaken, 8h)	0.68±0.11	0.05±0.02
6. pGW44,pGW54	2xYT+ IPTG (shaken, 1h)	1.81±0.61	0.15±0.09
7. pGW44,pGW54	2xYT+ IPTG (shaken, 2h)	3.19±1.06	0.28±0.11
8. pGW44,pGW54	2xYT+ IPTG (shaken, 3h)	4.70±1.2 1	0.48±0.19
9. pGW44,pGW54	2xYT+ IPTG (shaken, 4h)	6.23±1.75	0.52±0.26
10. pGW44,pGW54	2xYT (shaken, 8h)	0.96±0.09	0.08±0.05

a. Results are the mean values ± SEM of 5 determinations. b. Cells were grown to mid-exponential phase before addition of IPTG (section 2.4.2.4) c. 1-5 = CIR CyaA (crude inactive recombinant CyaA) ; c. 6-10 = CAR CyaA (crude active recombinant CyaA); d. 1-5 = PIR CyaA (purified inactive recombinant CyaA) ; d. 6-10 = PAR CyaA (purified active recombinant CyaA).

3.3 PREPARATION OF HYBRID TOXINS FROM RECOMBINANT *E. coli* STRAINS

Two hybrid toxin genes were constructed from *lkt*A and *cya*A genes (Westrop *et al.*, 1997). The *hyb*1 gene (on plasmid pGW80) was derived from the *cya*A and *lkt*A genes by a cross-over located between codons 687 (Ala) and 688 (Ala) of CyaA and codon 378 (Ala) and 379 (Gly) of LktA. Hyb1 was expressed in *E. coli* BL21/DE3 harbouring pGW80 as a stable protein of 1262 amino acids with enzymic AC activity. The N-terminal amino acid residues (1-687) were derived from CyaA and the C-terminal residues (688-1262) derived from LktA (residues 379-953). The *hyb*2 gene was derived from *hyb*1 by a second cross over located between codon 616 (Asp) and 617 (Asn) of LktA and codons 918 (Asp) and 919 (Val) of CyaA. Hyb2 was expressed in strain *E. coli* BL21/DE3 (pGW88) as a stable protein of 1713 amino acids with enzymic AC activity. In this construct, a highly conserved domain of CyaA (amino acid residues 688-918) had been replaced with amino acid residues 379-616 of LktA (Figs 5 and 6).

The cultures were grown in 2x YT medium to an A_{650} of 0.7. Hybrid toxins as inclusion bodies were induced by addition of IPTG to the mid-exponential cultures and incubation for a further 4 h. Urea extracts were prepared from cells expressing *hyb*1 or *hyb*2 together with *cya*C or *lkt*C genes as shown in Fig. 9. It is clear that the recombinant proteins were expressed in large amounts in *E. coli* and with the expected mol. wts of 200 kDa for CyaA and Hyb 2, 170 kDa for Hyb1, 105 kDa for LktA, 21 kDa for CyaC and 19kDa for LktC (Fig. 10).

Fig. 9

SDS-PAGE analysis of crude and purified hybrid toxin preparations from *E. coli* strains

Crude urea extract (25 μg protein/lane) and purified hybrid (6 μg protein/lane) preparations from *E. coli* strains were fractionated on 7.5% polyacrylamide gels and stained with coomassie blue. Arrows indicate the positions of molecular weight standards.

Lane 1 : 6H Mol wt marker proteins

Lane 2 : Crude Hyb 2 from *E. coli* BL /DE3 (pGW88)

Lane 3 : Purified Hyb 2 from *E. coli* BL /DE3 (pGW88)

Lane 4 : Crude Hyb 2/LktC from *E. coli* BL /DE3 (pGW88,pGW78)

Lane 5 : Purified Hyb 2/LktC from *E. coli* BL /DE3 (pGW88,pGW78)

Lane 6 : Crude Hyb 2/CyaC from *E. coli* BL /DE3 (pGW88,pGW54)

Lane 7 : Purified Hyb 2/CyaC from *E. coli* BL /DE3 (pGW88,pGW54)

Lane 8 : Crude Hyb 1/CyaC from *E. coli* BL /DE3 (pGW80,pGW54)

Lane 9 : Purified Hyb 1/CyaC from *E. coli* BL /DE3 (pGW80,pGW54)

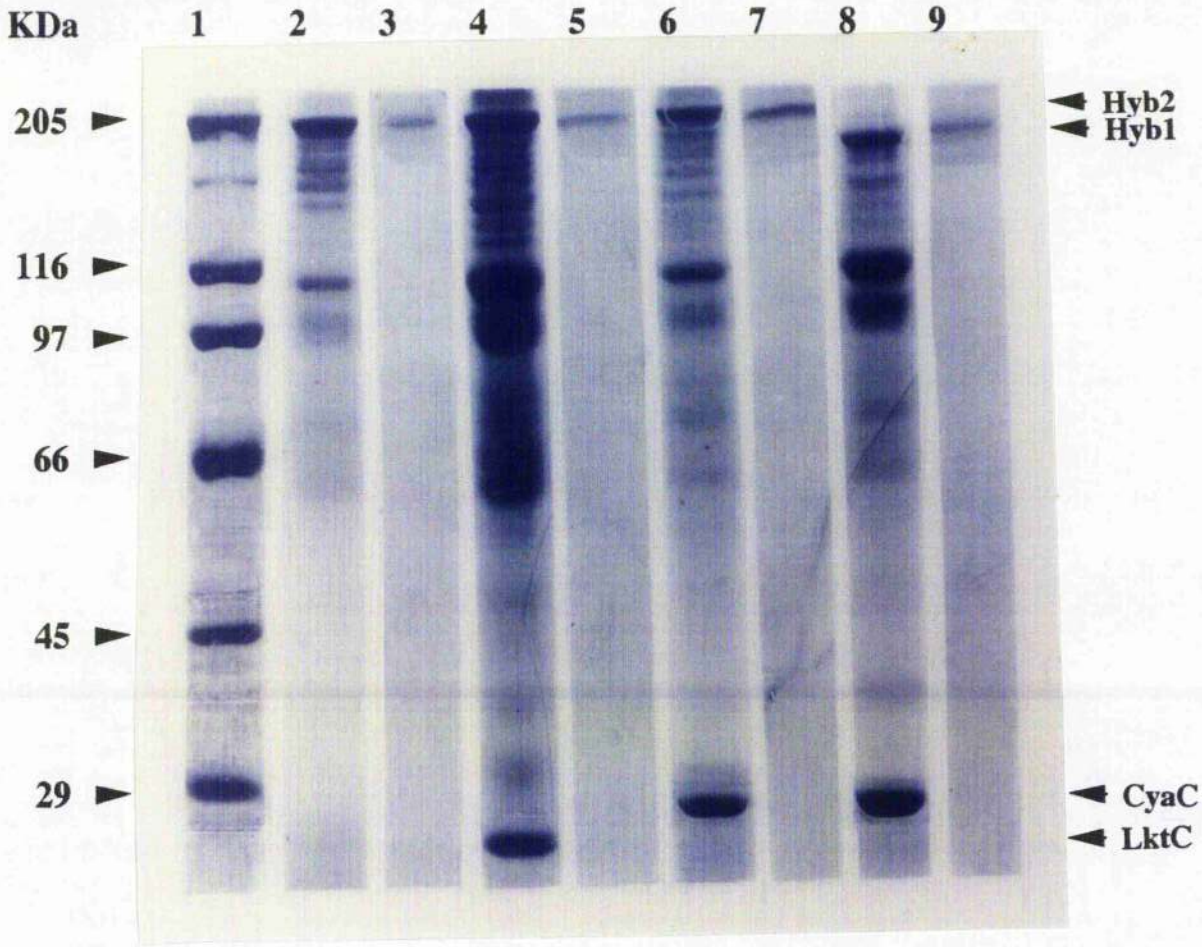
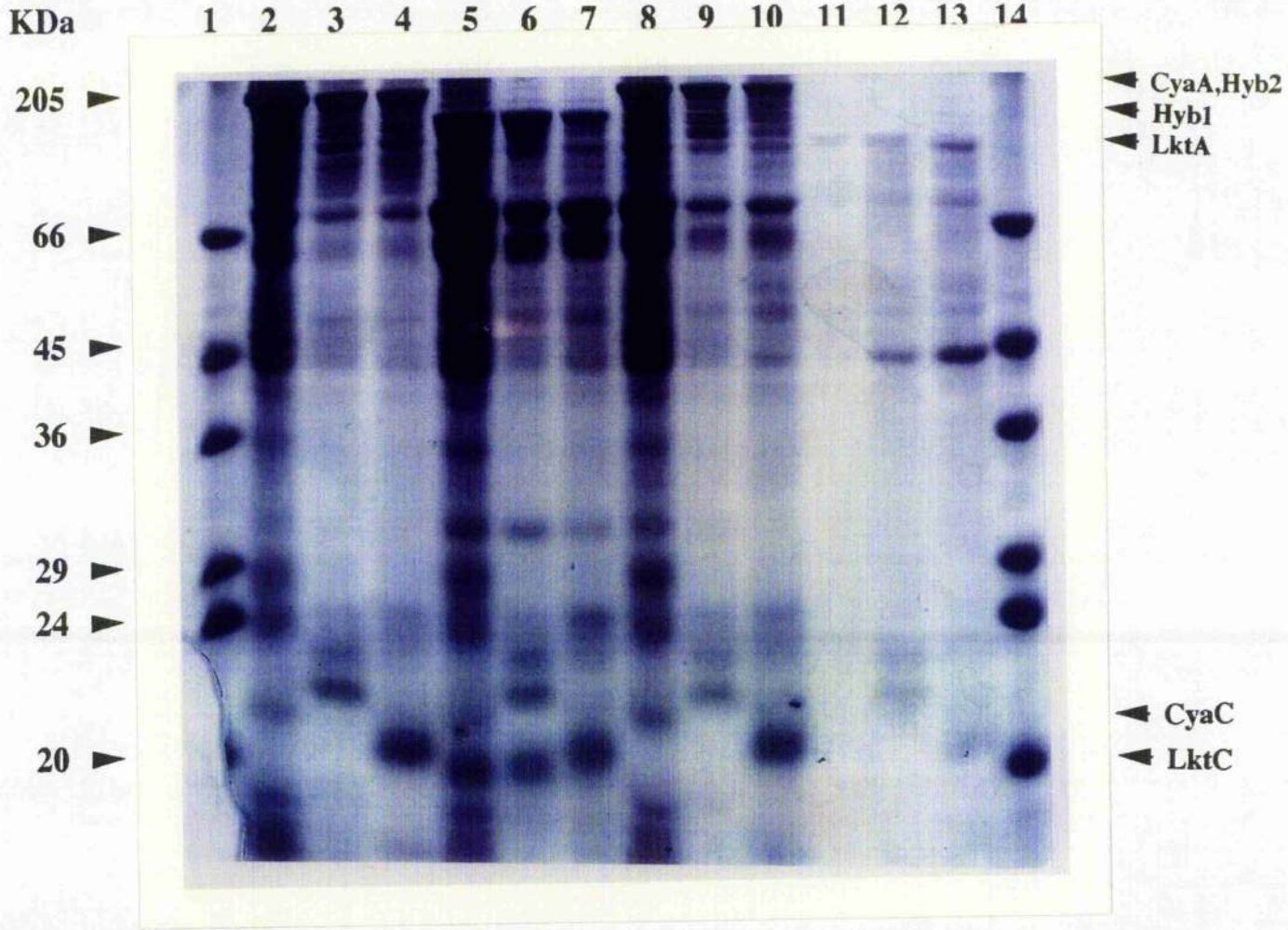

KDa
1
2
3
4
5
6
7
8
9
205
116
97
66
45
29
Hyb2
Hyb1
CyaC
LktC

Fig. 10

SDS-PAGE analysis of crude urea extracts of CyaA, Hyb2, Hyb1 and LktA preparations activated in vivo in *E. coli* by CyaC or LktC .

Crude urea extracts (50 µl/lane) were separated on a 7.5% polyacrylamide gel and stained with coomassie blue. Arrows indicate the positions of molecular weight standards.

Lane 1 : 7H Mol wt marker proteins
Lane 2 : CyaA from *E. coli* BL /DE3 (pGW44)
Lane 3 : CyaA/CyaC from *E. coli* BL /DE3 (pGW44,pGW54)
Lane 4 : CyaA/LktC from *E. coli* BL /DE3 (pGW44,pGW78)
Lane 5 : Hyb 1 from *E. coli* BL /DE3 (pGW80)
Lane 6 : Hyb 1/CyaC from *E. coli* BL /DE3 (pGW80, pGW54)
Lane 7 : Hyb 1/LktC from *E. coli* BL /DE3 (pGW80, pGW78)
Lane 8 : Hyb2 from *E. coli* BL /DE3 (pGW88)
Lane 9 : Hyb 2/CyaC from *E. coli* BL /DE3 (pGW88, pGW54)
Lane 10 : Hyb 2/LktC from *E. coli* BL /DE3 (pGW88, pGW78)
Lane 11 : LktA from *E. coli* BL /DE3 (pGW64)
Lane 12 : LktA/CyaC from *E. coli* BL /DE3 (pGW64, pGW54)
Lane 13 : LktA/LktC from *E. coli* BL /DE3 (pGW64, pGW78)
Lane 14 : 7H Mol wt marker proteins


KDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14
205
66
45
36
29
24
20
CyaA,Hyb2
Hyb1
LktA
CyaC
LktC

3.4 PURIFICATION OF CyaA FROM CRUDE EXTRACTS

Crude urea extracts from *B. pertussis* or recombinant *E. coli* strains were partially purified by DEAE-sepharose or calmodulin-agarose column chromatography (section 2.5). SDS-PAGE analysis and western blotting of crude and purified CyaA with monoclonal antibody 9D4 or rabbit polyclonal antiserum raised against the 200-kDa band indicated that the 200 kDa protein was present, but many other proteins and degradation products of CyaA of lower molecular weight were present in crude samples as shown in Figs 8 and 10. These were removed by the various purification procedures (section 2. 5), to give a 9-26 fold increase in enzymic activity (Fig. 11 and 12, Table 4).

3.5 EFFECT OF DIALYSIS AND OTHER TREATMENTS ON INVASIVE ACTIVITY OF PAN CyaA

The purified active native CyaA (PAN CyaA) preparation from *B. pertussis* 348 (pRMB1) in 8 M urea was dialysed against dialysis buffer (Appendix II. O) for 2 h or 24 h at 4 °C before measuring its invasive CyaA activity for BHK cells. The results showed a 12 and 19 % decrease in the invasive activity of CyaA at 2 and 24 h respectively in comparison with the invasive activity of CyaA after dilution to 0.2 M urea (Table 5). The loss of activity could be due to aggregation of CyaA toxin. The PAN CyaA was also stored at -70 °C for up to 12 months and assayed for its effect on BHK cells. After 2, 6 and 12 months, the loss of activity was about 5%, 8% and 12% respectively (Table 5). Three different treatments (BSA 10%w/v, 1mM PMSF and Triton X-100 5% v/v) were included in the preparation and purification

Fig. 11

SDS-PAGE analysis of crude urea extract and eluted fractions of purified CyaA prepared by DEAE sepharose column chromatography.

A crude urea extract of recombinant CyaA was purified with DEAE sepharose (section 2.5.1). Crude CyaA (25 μg protein/ml), the column flowthrough (10 μg protein/ml) and the first three elution fractions (2.5 μg protein/lane) were subjected to electrophoresis on a 10% polyacrylamide gel and stained with coomassie blue. Arrows indicate the positions of molecular weight standards with masses in kDa.

Lane 1 : Molecular weight marker proteins

Lane 2 : Crude CyaA from *E. coli* BL /DE3 (pGW44)

Lane 3 : Flow-through before elution

Lane 4 : First elution fraction

Lane 5: Second elution fraction

Lane 6 : Third elution fraction

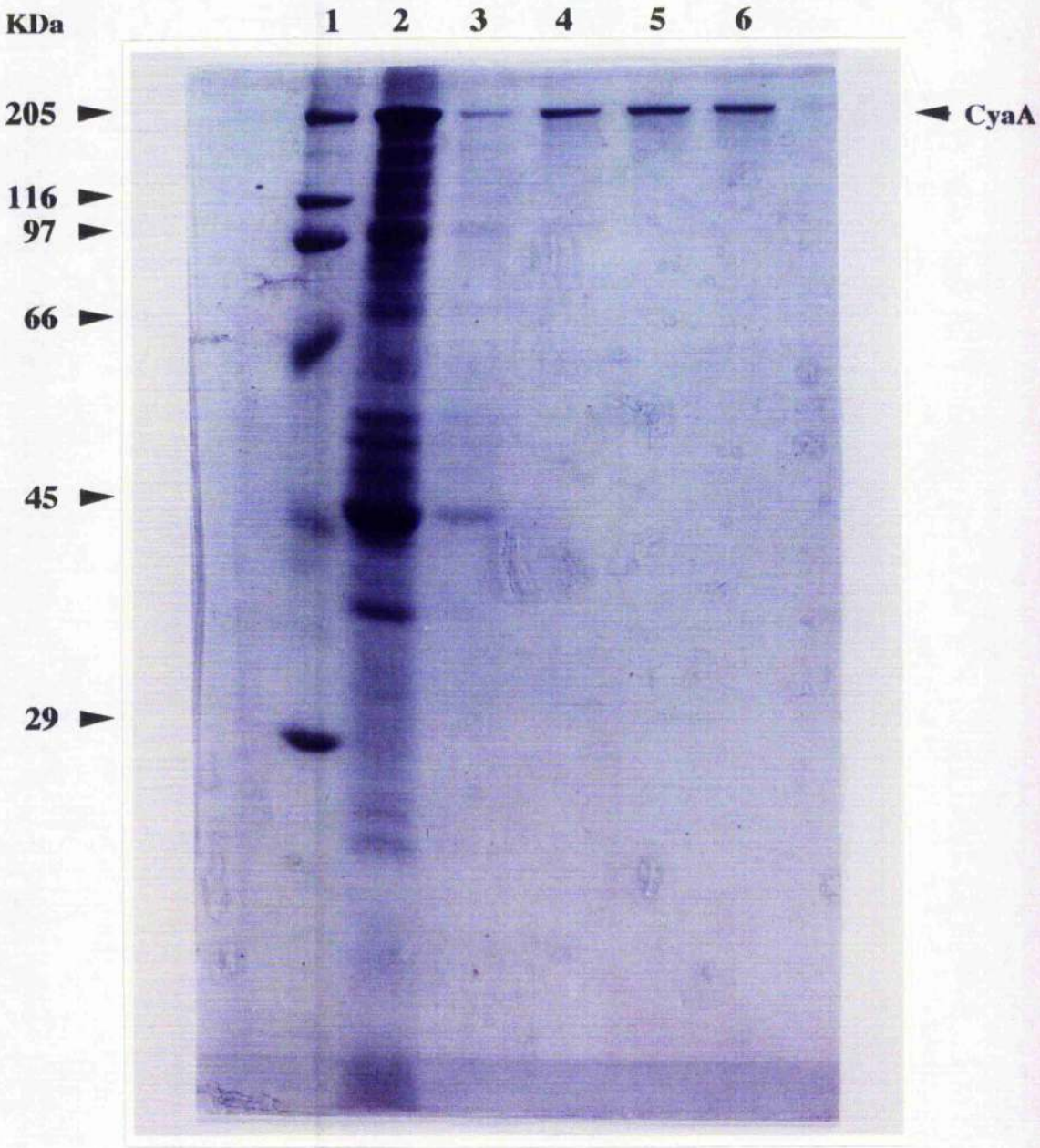

KDa
1
2
3
4
5
6
205
116
97
66
45
29
CyaA

Fig. 12

SDS-PAGE analysis of eluted fractions of purified CyaA prepared by calmodulin agarose column chromatography.

PAN CyaA was purified by calmodulin agarose column chromatography (section 2.5.2). Elution fractions (50 µl/lane .) were subjected to electrophoresis on a 7.5 % polyacrylamide gel and stained with coomassie blue. Arrows indicate the positions of molecular weight standards.

Lane 1-7 : First to seventh eluted (purified) fractions

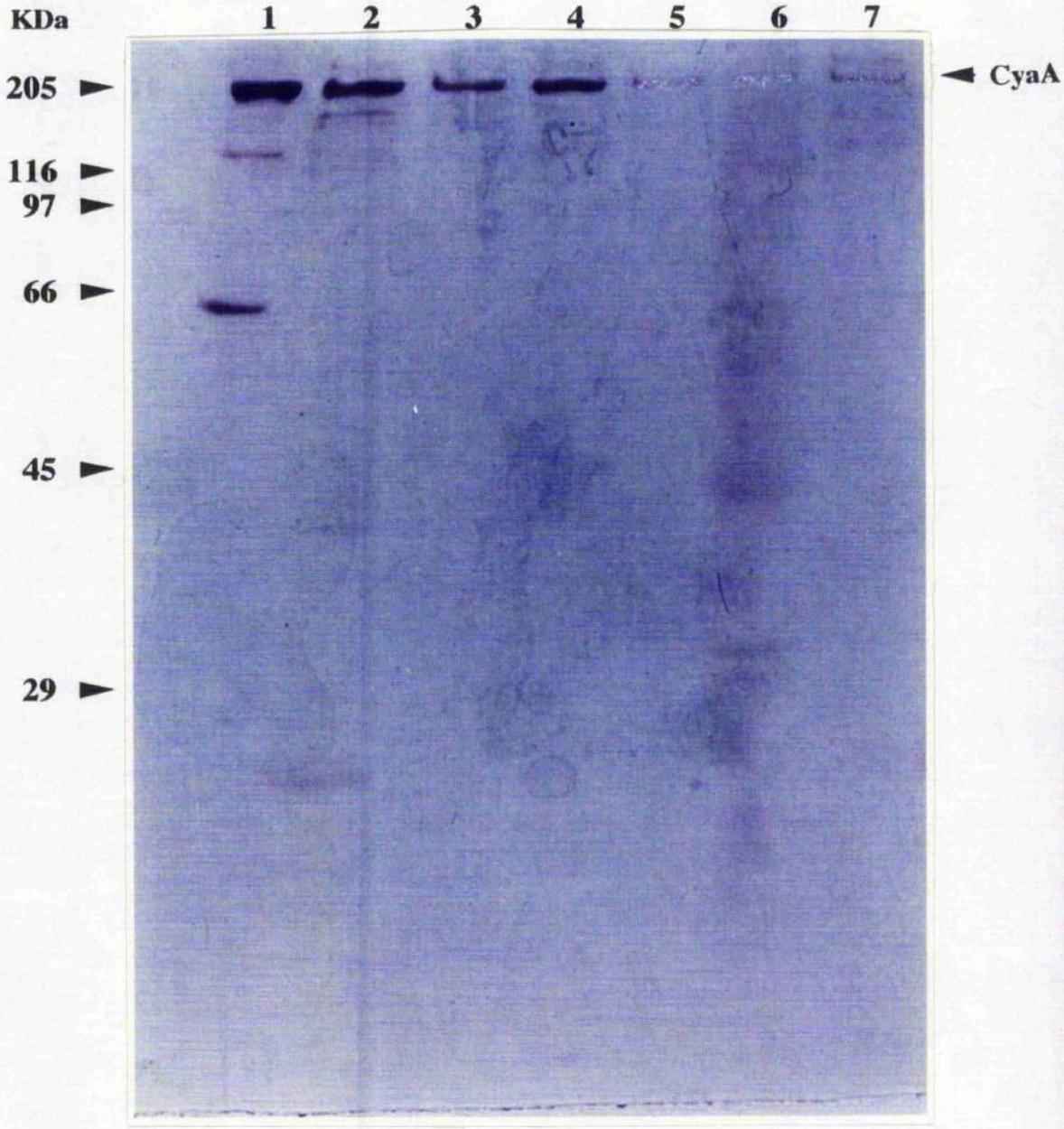

KDa
1
2
3
4
5
6
7
205
116
97
66
45
29
CyaA

Table 4.

Purification of CyaA

Procedure	Protein concentration (μg/ml)	Enzymic activity (μmol cAMP/min /mg protein)	Purification factor
Urea extraction	3261.91	42.69	0
Chromatography			
DEAE sepharose	319	396.47	9.29
Calmodulin-affinity	238	530.18	12.42
Calmodulin affinity and DEAE sepharose	165	765.62	17.93
Electroelution of 200 kDa band	116	1083.25	25.37

Table 5.

Effect of dialysis, storage time at -70 °C and other conditions on invasive CyaA activity.

Conditions	Concentration of PAN CyaA (μg protein/ml) required for 50%stellation of BHK cells [a]
Dialysis time (h) at 4°C	
2	4.15±0.78
24	4.42±0.42
purification in the presence of:	
PMSF	3.98±0.71
BSA	3.81±0.36
Triton X-100	3.90±0.83
Storage time (months) at -70 °C	
0	3.72±0.89
2	3.91±0.93
6	4.02±0.65
12	4.18±0.52

[a]Data are the mean values ± SEM of 4 determinations.

process of CyaA by addition to whole cells before extraction and then the samples were assayed for invasive activity. The inhibitors were removed before assay by dilution followed by dialysis against dialysis buffer for 2 h at 4 °C. The data given in Table 5 show that cytotoxicity was decreased about 7% by the presence of PMSF, 2% by BSA and 5% by Triton X-100, indicating that these substances, which were used in an attempt to reduce the degradation of CyaA protein, did not improve the yield or activities of the CyaA preparation.

3.6 PURIFICATION OF HYBRID TOXINS FROM CRUDE UREA EXTRACTS

The Hyb2 crude extracts were purified by the same purification steps as used for purification of CyaA, first by calmodulin agarose and then by DEAE -sepharose chromatography. As demonstrated in Figs 9 and 10 by SDS-PAGE, many proteins, including a 200 kDa protein and smaller proteins possibly resulting from degradation of the 200 kDa protein were present in crude extracts containing Hyb2 toxin. The purification of Hyb1 crude urea extracts was mainly achieved with calmodulin affinity chromatography. As shown in Fig. 9, the Hyb1 and Hyb2 proteins could be purified free of these smaller degradation products.

3.7 PREPARATION AND PARTIAL PURIFICATION OF LktA, LktC AND CyaC FROM RECOMBINANT *E. COLI* STRAINS

Partially purified LktA was obtained by expression of the recombinant protein as inclusion bodies in recombinant *E. coli* strain HMS174 harbouring

plasmids pGW64 and pGW78. These carried the *lktA* and *lktC* genes respectively and produced the toxic form of LktA. Soluble contaminant proteins were removed by washing in 2M urea, and then the partially purified LktA was extracted from the inclusion bodies in 8M urea (Fig. 13). The LktC or CyaC proteins were partially purified by the same procedure, as shown by SDS-PAGE analysis (Fig. 13).

3.8 SUMMARY OF SDS-PAGE ANALYSIS OF A AND C PROTEIN PREPARATIONS

CyaA and Hyb2 proteins migrated in gels at around 200 kDa, Hyb1 at 170 kDa, LktA at 105 kDa, CyaC at 21 kDa and LktC at 19 kDa positions (Figs 7, 9 and 10). The major band differences associated with contaminating proteins between the profiles of *B. pertussis* pRMB1 (CAN CyaA) and *B. pertussis* DE386 (CIN CyaA) urea extracts were seen at 29 kDa and 14 kDa (Fig 7 , lane 2 and 4), whereas the major band differences visualized in recombinant urea extracts represented expression of C proteins (CyaC at around 21 kDa or LktC at about 19 kDa) with the A proteins.

3.9 SUMMARY OF IMMUNOBLOTTING OF CyaA AND HYBRID PREPARATIONS

Generally, for crude preparations probed with anti-CyaA antibody, a profile with a band of 200 kDa and smaller degradation products were observed as previously reported (Rogel *et al.*, 1989). A cross-reaction with degradation products was reduced by purification of CyaA (Fig. 8). Western

Fig. 13

SDS-PAGE analysis of CIR CyaA, CIR LktA, CyaC and LktC used for in vitro activation of protoxins.

Inclusion bodies containing insoluble proteins were washed with 0.5 M and 2M urea as described in Material and Methods (section 2.6). Urea extracts prepared after washing of inclusion bodies of CyaA (20 μg/lane), LktA (10 μg) ,CyaC and LktC (10 μg/lane) were separated on a 10% polyacrylamide gel and stained with Coomassie blue. Arrows indicate the positions of molecular weight standards .

Lane 1 : 6H Mol wt marker proteins

Lane 2 : Urea extract of CyaA from *E. coli* BL 21/DE3 (pGW44) after washing with 2M urea

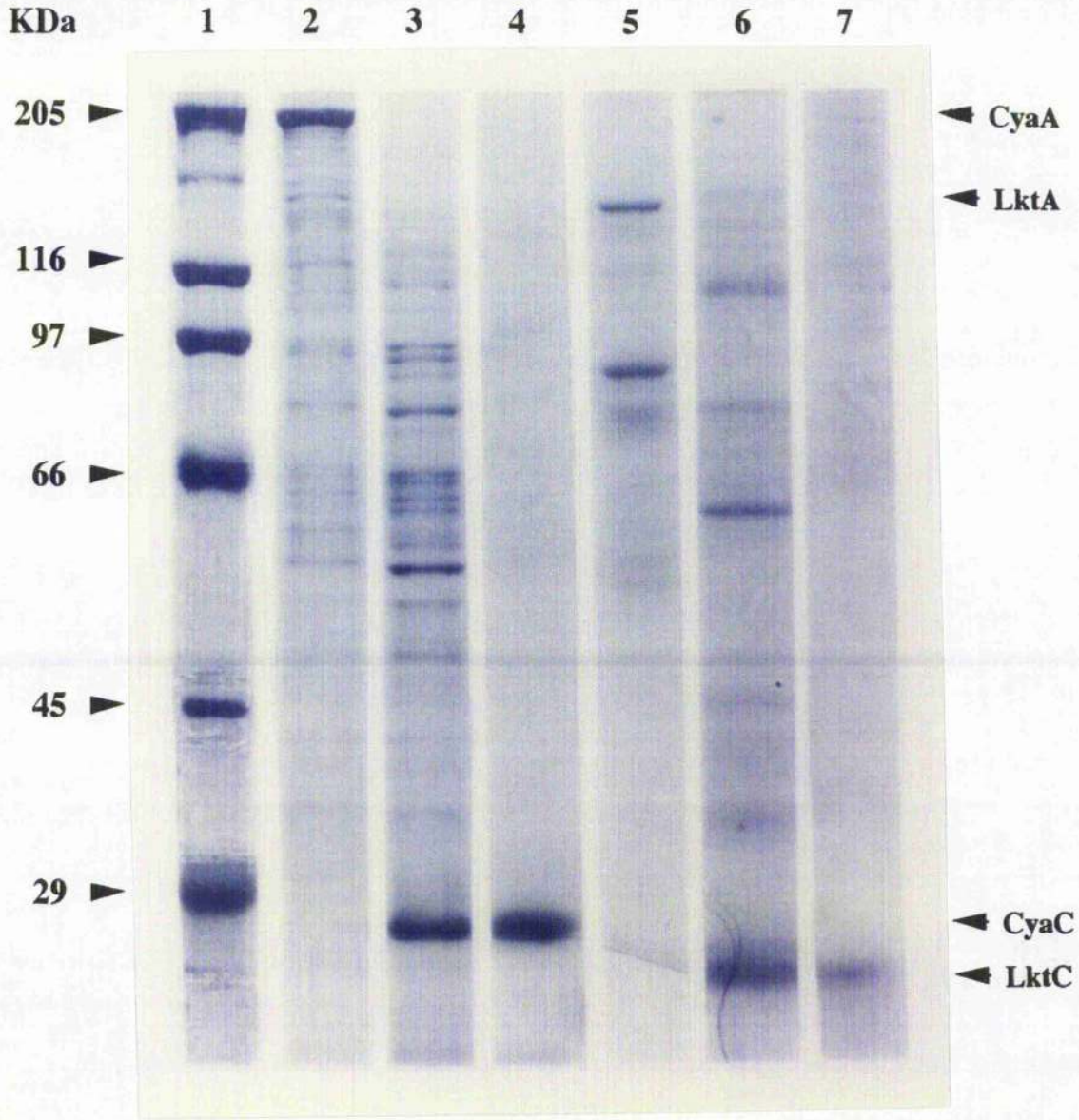
Lane 3 : Urea extract of CyaC from *E. coli* BL 21/DE3 (pGW14) after washing with 0.5 M urea

Lane 4 : Urea extract of CyaC from *E. coli* BL 21/DE3 (pGW14) after washing with 2M urea

Lane 5 : Urea extract of LktA from *E. coli* BL 21/DE3 (pGW64) after washing with 2M urea

Lane 6 : Urea extract of LktC from *E. coli* BL 21/DE3 (pGW78) after washing with 0.5M urea

Lane 7 : Urea extract of LktC from *E. coli* BL 21/DE3 (pGW78) after washing with 2M urea


KDa
1
2
3
4
5
6
7
205
116
97
66
45
29
CyaA
LktA
CyaC
LktC

blot of crude and purified CyaA from *B. pertussis* pRMB1 was also probed with antiserum raised in rats against whole cell sonicate antigen prepared from the *B. pertussis* strain 18323, or with polyclonal antiserum raised in mice against purified FHA, PT or PRN or with monoclonal 9D4 antibody to CyaA. A major band of 200 kDa and some minor bands, presumably of degradation products with smaller molecular weight, were visualized in profiles probed with anti-CyaA antibody (Fig. 14, lanes 3 and 4) or anti-whole cell antibody (Fig. 14, lanes 1 and 2). The finding that antisera to *B. pertussis* whole cells cross-reacts strongly with a band at a 200 kDa suggests that CyaA is a major immunogen of the whole cell preparation. However, there was no obvious cross reaction of the other antibodies at the molecular weights expected for FHA (210 kDa), PT (105 kDa) or PRN (69 kDa) (Fig. 14, lanes 6, 7 and 8), indicating that the purified CyaA was free of these antigens. Hyb2, Hyb1, CyaA and LktA were immunoblotted with rabbit anti-CyaA polyclonal antiserum or rabbit anti-LktA polyclonal antiserum. The data shown in Fig. 15 indicated that anti-CyaA polyclonal antibody recognised Hyb2 in Western blots but failed to react with Hyb1. Interestingly, the anti-LktA rec ognised Hyb1, but not Hyb2. These data indicated that the immunodominant region of CyaA or LktA was in the C-terminal region.

Fig. 14

Western blot analysis of CAN CyaA and PAN CyaA preparations using sera raised against *B. pertussis* 18323 whole cells, CyaA, PT, FHA and PRN polyclonal antibodies.

Crude urea extract of CyaA (25 μg/lane) and purified CyaA (4.5 μg) preparations form *B. pertussis* 348 pRMB1 were fractionated on a 10% polyacrylamide gel and transferred to nitrocellulose membrane. The nitrocellulose strips were probed with sera collected from rats vaccinated with *B. pertussis* 18323 whole cells (lane1-2), anti-CyaA polyclonal (lane 3-4), anti-CyaA monoclonal 9D4 (lane 5), anti-PT (lane 6), anti-FHA (Lane 7) and anti-PRN polyclonal antibodies (Lane 8). The numbers on the left of the blot indicate molecular weight markers in kDa.

Lane 1 : Crude CyaA from *B. pertussis* 348 pRMB1

Lane 2-8 : Purified CyaA from *B. pertussis* 348 pRMB1

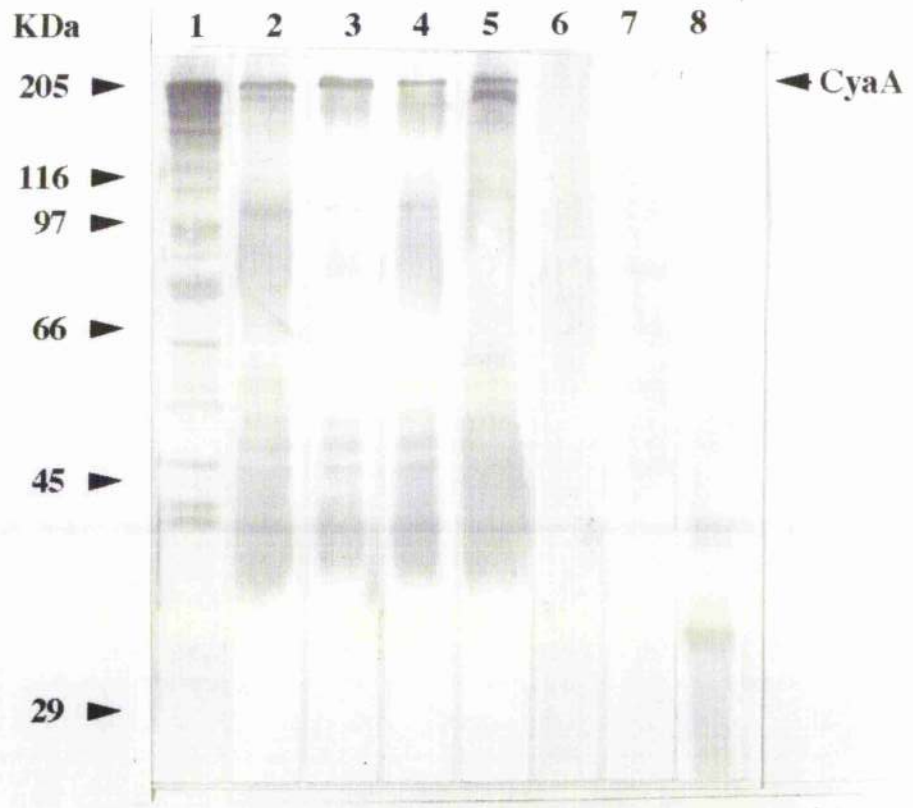

KDa
1
2
3
4
5
6
7
8
205
116
97
66
45
29
CyaA

Fig. 15

Western blot analysis of crude CyaA, LktA and hybrid preparations using anti-CyaA polyclonal antiserum (Panel A) or anti-LktA polyclonal antiserum (Panel B).

Crude urea extract (30 μg protein/lane) of CyaA, LktA and hybrid preparations from recombinant *E. coli* strains were separated on a 7.5% polyacrylamide gel (Panel A) or 10% polyacrylamide gel (Panel B). The resolved proteins were immunoblotted with anti-CyaA polyclonal antiserum (Panel A) or with anti-LktA polyclonal antiserum (Panel B). The numbers on the left of the blot indicate molecular weight.

PanelA: Blotted with anti-CyaA polyclonal

Lane 1 : CyaA

Lane 2 : CyaA+CyaC

Lane 3 : CyaA+LktC

Lane 4 : Hyb 1

Lane 5: Hyb 1+CyaC

Lane 6 : Hyb 1+LktC

Lane 7 : Hyb2

Lane 8 : Hyb2+CyaC

Lane 9 : Hyb 2 +LktC

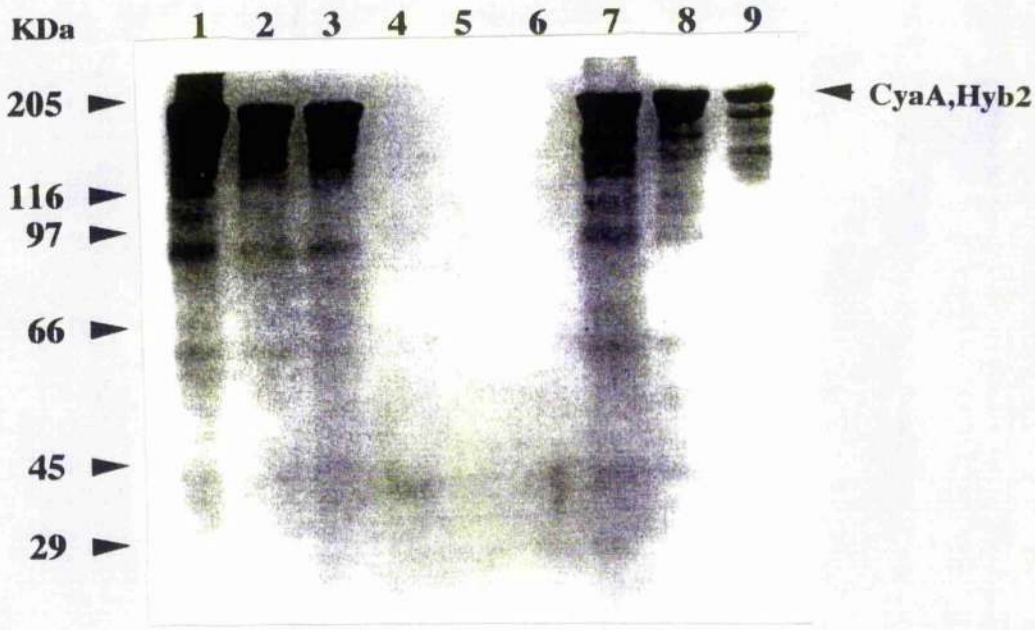
Panel B: Lane 1-9 : in the same order as above, blotted with anti-LktA

Lane 10: LktA

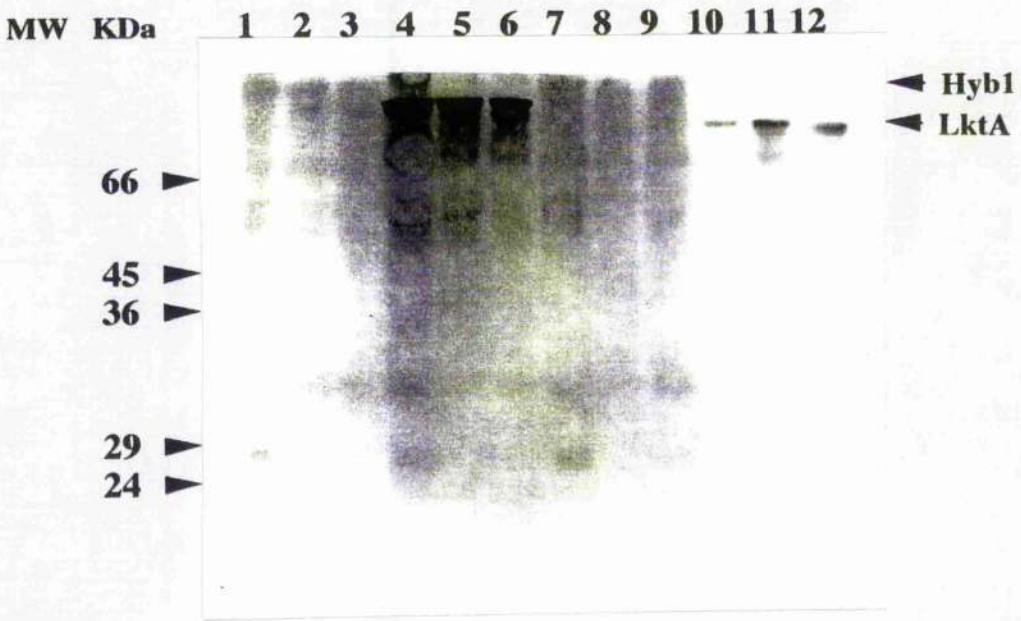
Lane 11: LktA+CyaC

Lane 12: LktA +LktC

A


KDa
1 2 3 4 5 6 7 8 9
205
116
97
66
45
29
CyaA,Hyb2

B


MW KDa
1 2 3 4 5 6 7 8 9 10 11 12
66
45
36
29
24
Hyb1
LktA

3.10 CHARACTERIZATION OF CyaA, LktA AND HYBRID TOXINS

3.10.1 Enzymic AC activity

AC activity of crude urea extract (CAN CyaA) and purified (PAN CyaA) from *B. pertussis* (pRMB1), crude urea extract (CIN CyaA) and purified (PIN CyaA) from *B. pertussis* BPDE386, crude urea extract (CAR CyaA) and purified (PAR CyaA) from *E. coli* BL21/DE3 (pGW44, pGW54) or crude urea extract (CIR CyaA) and purified (PIR CyaA) from *E. coli* BL21/DE3 (pGW44) preparations was determined (section 2.11). The levels of enzymic activity of various preparations were in the range of 66-76 μmol cAMP/min/mg protein for urea extracts and 304-674 for purified samples (Table 6). It should be noted that smaller molecular weight forms of these proteins would still possess AC enzymic activity if derived from the N-terminal end of the proteins. The amount of AC active peptides could be gauged from the activity of the 200 kDa protein eluted from a gel slice. This showed a 1.4-fold increase in AC enzymic activity over protein purified from calmodulin-sepharose chromatography, a 2-fold increase over the calmodulin affinity chromatography purified protein and 2.7-fold increase over DEAE sepharose purified protein (Table 4).

3.10.2 Invasive activity of CyaA for BHK cells

Invasive activity of crude urea extracts and purified CyaA preparations from *B. pertussis* and recombinant *E. coli* strains were measured by a cell spreading assay (section 2.14). The assay is based on the morphological effect

Table 6.
Properties of CyaA toxins from *B. pertussis* and recombinant *E. coli* strains

CyaA preparation	Enzymic AC activity [a] (μmol cAMP/min/mg protein)	Invasive activity [a] μg protein/ml for 50% stellation of BHK 21 cells	Haemolytic activity [a] μg protein/ml for 20% haemolysis of sheep RBC	Cytotoxic activity [a] μg protein/ml for 50% cell killing of J774.2	BL3
PAN CyaA	618.91±41.63	2.26±0.58	5.86±1.17	2.68±0.29	2.61±0.54
CAN CyaA	67.52±3.17	12.35±2.19	11.34±1.91	22.53±2.43	25.43±1.16
PIN CyaA	603.35±27.4	>58	>63	>60	>51
CIN CyaA	75.54±4.91	>591	>259	>220	>391
PAR CyaA	625.62±36.75	2.87±0.37	9.42±1.51	3.27±0.36	3.34±1.14
CAR CyaA	68.94±2.63	20.83±2.25	21.82±2.73	24.83±1.19	27.91±2.38
PIR CyaA	674.43±53.2	>72	>89	>72	>57
CIR CyaA	66.15±3.56	>725	>293	>277	>292
PAR CyaA(pCACT3)	304.56±21.36	NT[b]	8.83±1.27	3.27±0.71	3.46±0.85

[a]Data are the mean values ± SEM of 4 determinations, b. Not tested.

of the toxin on BHK21 cells. Untreated BHK cells or cells treated with CIN CyaA, PIN CyaA, CIR CyaA, PIR CyaA or LktC-modified CyaA, even at high concentrations showed bipolar fibroblast morphology (non-stellated cells), but after treatment with CAN CyaA, PAN CyaA, CAR CyaA, PAN CyaA or 1mM dibutyryl cAMP with 0.1mM IBMX, the cell morphology was changed from a fibroblast shape to a stellated or arborized shape as shown in Fig. 16. The stellation of BHK cells was thus related to the elevation of intracellular cAMP after entry of the mature, cell invasive CyaA toxins.

The dose responses of BHK cells to various CyaA preparations are shown in Fig. 17. The protein concentrations of these preparations required for half maximal stellation of BHK cells are shown in Table 6. These results also show that the invasive activity of recombinant CyaA was comparable to that of native CyaA toxin, although the native toxin was reproducibly slightly more active.

3.10.3 Haemolytic activity of CyaA preparations for sheep erythrocytes

Haemolytic activity of crude and purified CyaA toxin preparations from *B. pertussis* and recombinant *E. coli* strains for sheep erythrocytes was determined (section 2. 12). The results indicated that haemolytic activity of CAN CyaA and PAN CyaA were approximately 1.9-fold and 2-fold greater than CAR and PAR CyaA (Table 6, Fig. 18). PAR CyaA (pCACT3) showed slightly higher haemolytic activity than PAR CyaA (Table 6). These differences in haemolytic activity may be related to differences in maturation of the toxins by the C proteins in the different bacterial cells. Even at high concentration, no haemolysis was detected for CIN CyaA or PIN CyaA.

Fig. 16

Typical morphology of BHK cells after treatment with PAR CyaA and PIR CyaA.

Baby hamster kidney (BHK 21) cells were spread on fibronectin in HH buffer for 90 min at 37 °C, then fixed and stained with coomassie blue.The morphology of BHK cells after treatment with PAR CyaA and PIR CyaA was viewed in a bright field microscope with a 40x objective.

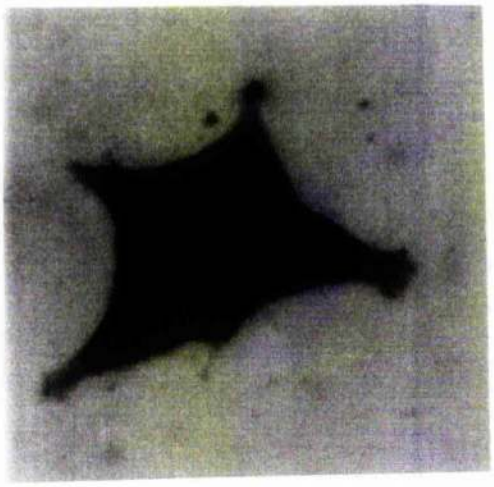
A : Normal BHK cell in HH buffer as negative control

B : BHK cell in presence of 1mM dibutyryl cAMP and 0.1 mM IBMX

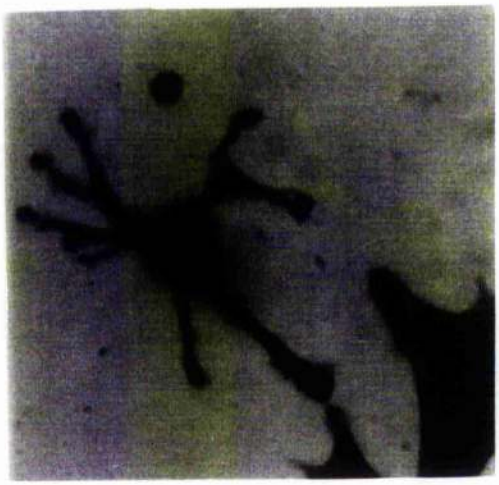
C : BHK cell after treatment with inactive recombinant CyaA

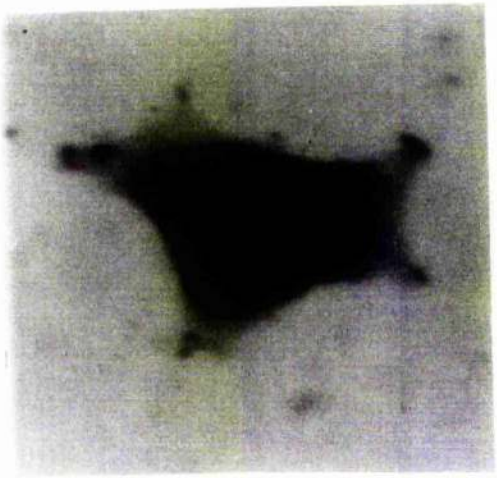
D : BHK cell after treatment with active recombinant CyaA

A



B





C

D

Fig. 17

Dose response of BHK cell stellation for CyaA preparations from *B. pertussis* and recombinant *E. coli* strains.

The invasive activity of various dilutions of crude and purified CyaA preparations from *B. pertussis* and recombinant *E. coli* strains was determined for BHK 21 cells using the cell spreading assay. A visual scoring system of 30 randomly picked cells was used to measure % stellation. The results represent the mean of four determinations ± SEM.

Panel A :

1 CAN CyaA

2 CIN CyaA

3 CAR CyaA

4 CIR CyaA

Panel B :

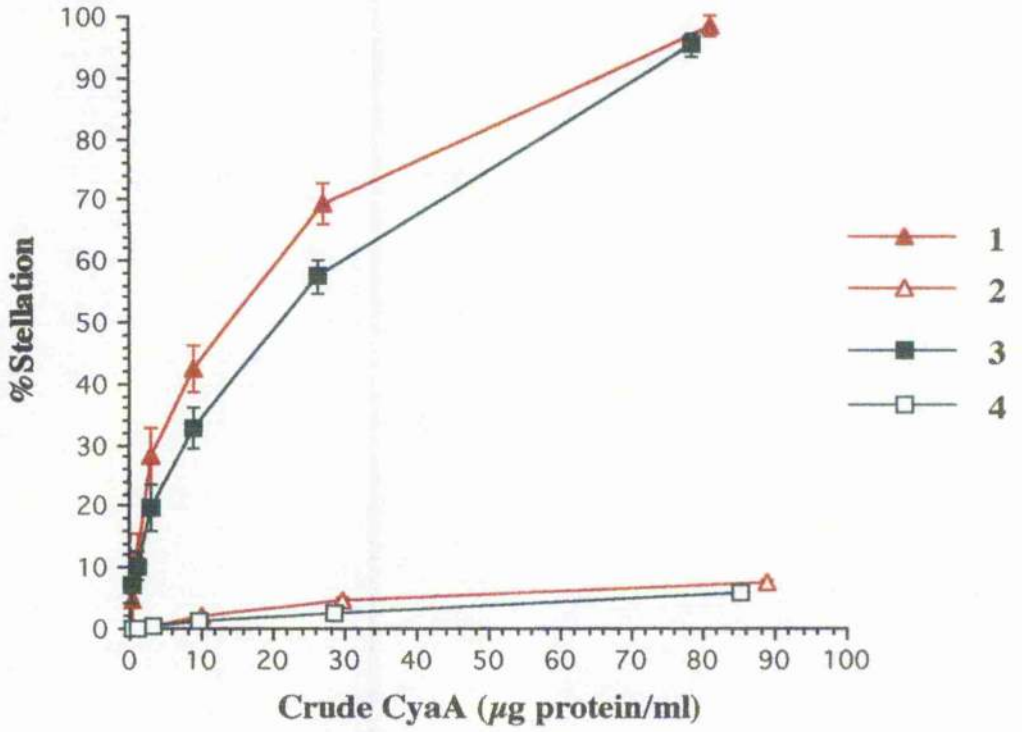
1 PAN CyaA

2 PIN CyaA

3 PAR CyaA

4 PIR CyaA

A


%Stellation
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
Crude CyaA (μg protein/ml)
1
2
3
4

B

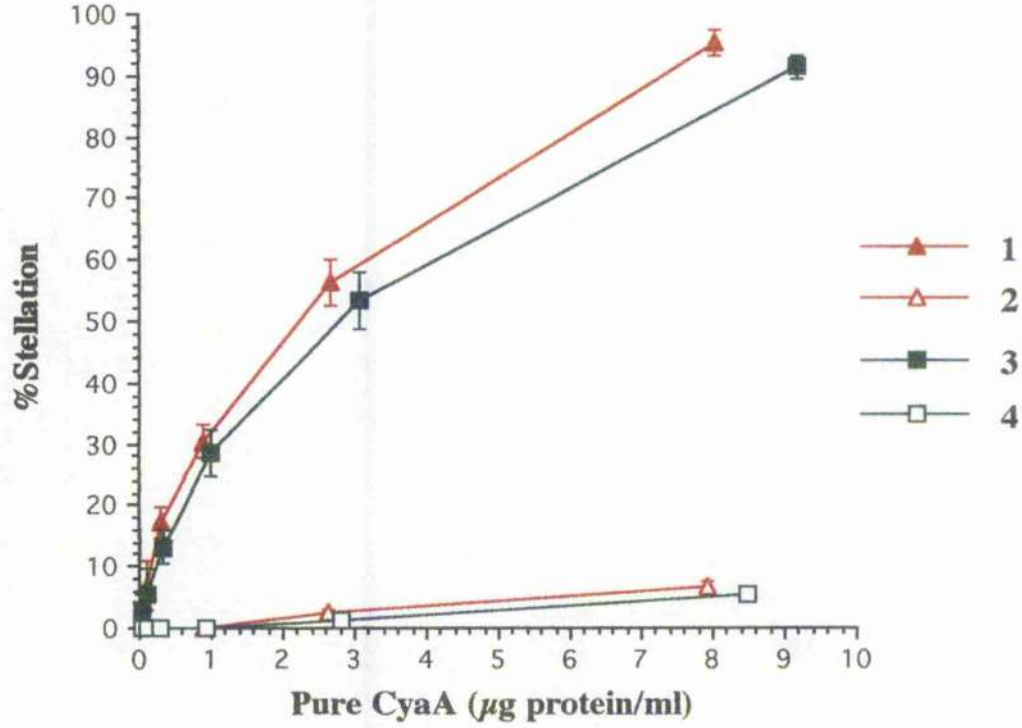

%Stellation
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
Pure CyaA (μg protein/ml)
1
2
3
4

Fig. 18

Dose response of sheep erythrocytes to haemolysis by CyaA preparations from *B. pertussis* and recombinant *E. coli* strains.

The haemolytic activity of various dilutions of crude and purified CyaA preparations were determined in 2-fold serial dilutions using sheep erythrocytes. Percentage haemolysis was measured in duplicate for each dilution of sample. The results represent the mean of four determinations ±SEM.

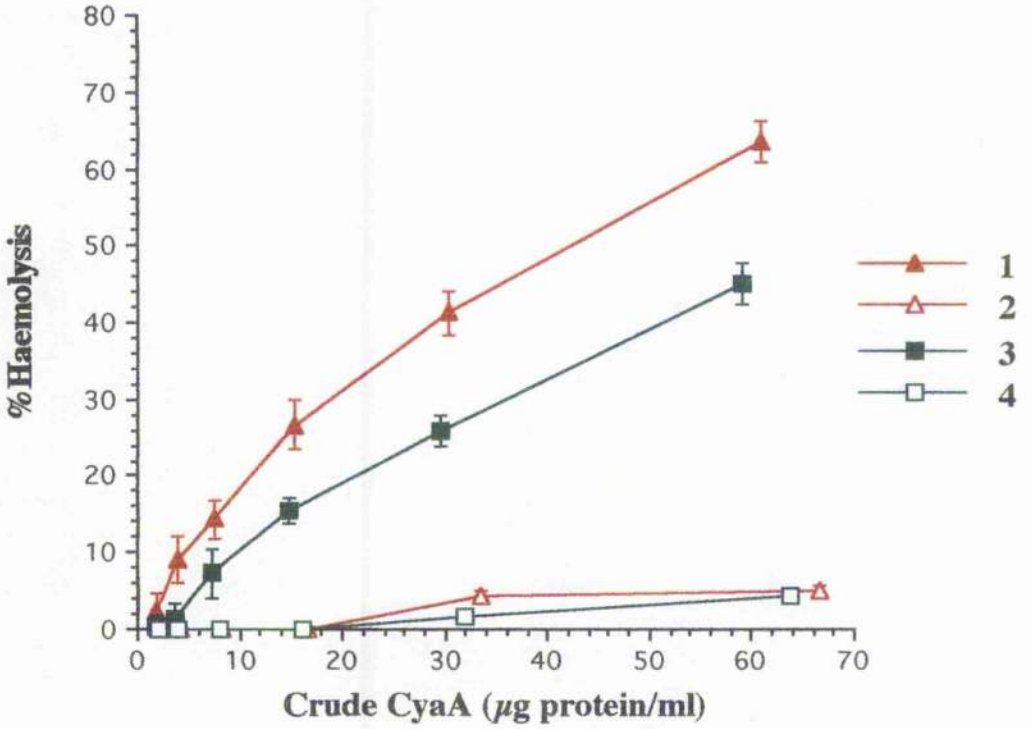
Panel A :

1 CAN CyaA
2 CIN CyaA
3 CAR CyaA
4 CIR CyaA

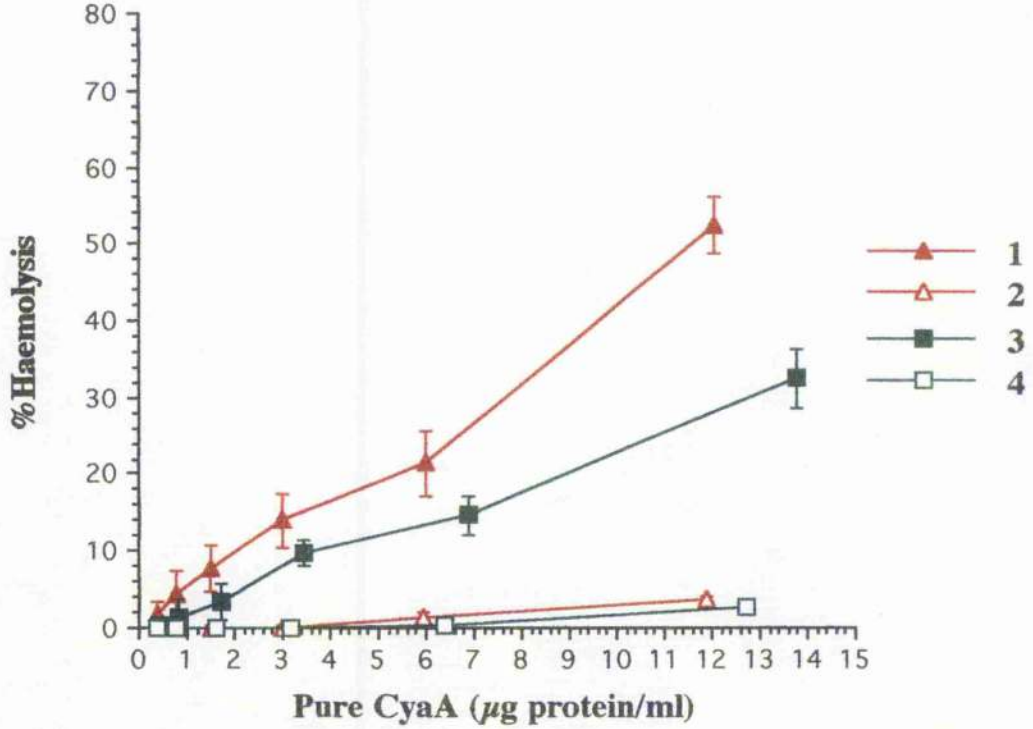
Panel B :

1 PAN CyaA
2 PIN CyaA
3 PAR CyaA
4 PIR CyaA

A


% Haemolysis
0
10
20
30
40
50
60
70
80
Crude CyaA (μg protein/ml)
1
2
3
4

B


% Haemolysis
0
10
20
30
40
50
60
70
80
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Pure CyaA (μg protein/ml)
1
2
3
4

3.10.4 Haemolytic activity of recombinant LktA and hybrid toxins

The haemolytic activity of recombinant LktA modified by LktC was 1.9- and LktA modified by CyaC was 1.4-fold lower than CyaA modified by CyaC according to the protein concentrations required for 20% haemolysis (Tables 6 and 7). Thus CyaC modification enhanced the haemolytic activity of LktA for sheep erythrocytes. In contrast, the CyaC-modified Hyb 2 showed 2.4-fold lower haemolytic activity than the LktC-modified Hyb2, suggesting that LktC modification was conferring some enhancement in haemolytic activity to Hyb2 (Fig. 19, Table 7). The haemolytic activity of LktC-modified Hyb2 was 4-fold higher than that of LktC-modified LktA (Table 7). No haemolytic activity was detected for PIR Hyb1 and PIR Hyb2 or PIRHyb1 activated by either CyaC or LktC, non-modified LktA or the urea extraction buffer diluted in the same way as the toxin dilutions in the assay.

3.10.5 Development of the MTT assay

Cytotoxicity or cell killing ability of CyaA preparations on eukaryotic cells was determined by the MTT assay based on the inhibition of the ability of active mitochondria in living cells to reduce tetrazolium dye solution. In order to obtain a significant A_{540} absorbance reading the effect of a constant amount of toxin on different cell numbers of J774.2 or BL3 cells was assayed after incubation for two hours with toxin. It was found that $3x10^5$ cells would be necessary to give sufficient metabolic activity in order to convert enough tetrazolium dye into formazan product (data not shown). The assay was also set up for various incubation times for both CyaA and LktA with $3x10^5$ cells at

Fig. 19

Dose response of sheep erythrocytes to haemolysis by hybrid toxin preparations from recombinant *E. coli* strains.

The haemolytic activity of 2-fold serial dilutions of purified hybrid preparations from recombinant *E. coli* strains was assayed for sheep erythrocytes. Percentage haemolysis was determined in duplicate for each dilution of sample. The results represent the mean of four determinations ± SEM.

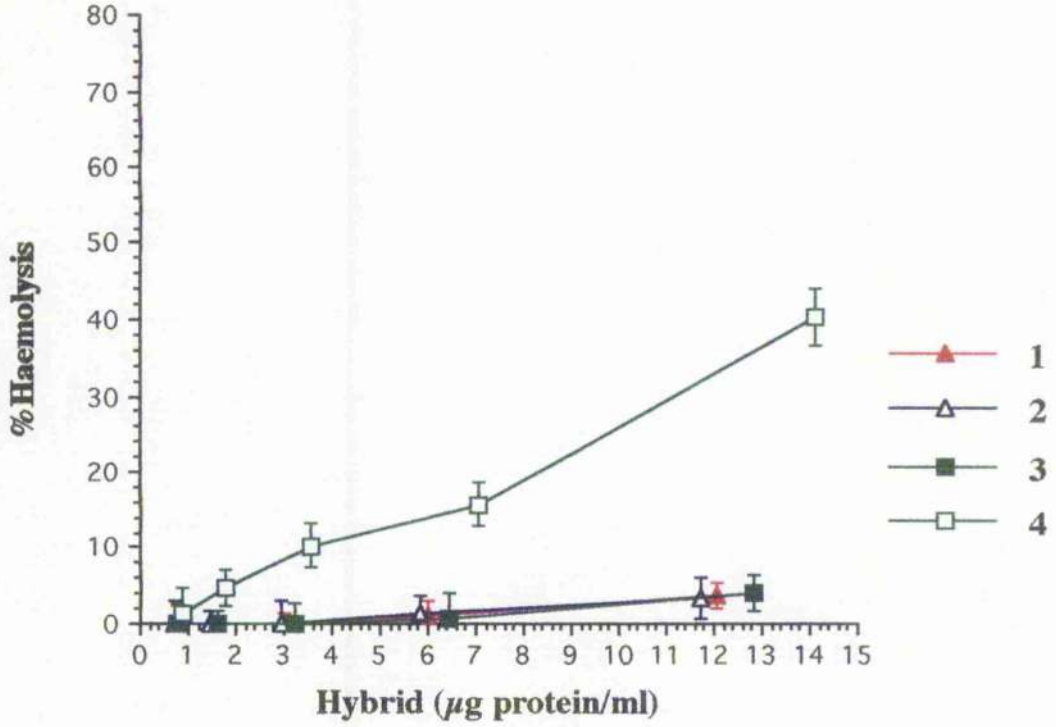
Panel A : Purified hybrid preparations from

1 Hyb1: Recombinant *E. coli* BL21/DE3 (pGW80)
2 Hyb1/CyaC: Recombinant *E. coli* BL21/DE3 (pGW80, pGW54)
3 Hyb1/LktC: Recombinant *E. coli* BL21/DE3 (pGW80. pGW78)
4 PAR CyaA (pCACT3): Recombinant *E. coli* BL21(pCACT3)

Panel B : Purified hybrid preparations from

1 Hyb2: Recombinant *E. coli* BL21/DE3 (pGW88)
2 Hyb2/CyaC: Recombinant *E. coli* BL21/DE3 (pGW88, pGW54)
3 Hyb2/LktC: Recombinant *E. coli* BL21/DE3 (pGW88. pGW78)

A


% Haemolysis
0
10
20
30
40
50
60
70
80
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Hybrid (μg protein/ml)
1
2
3
4

B

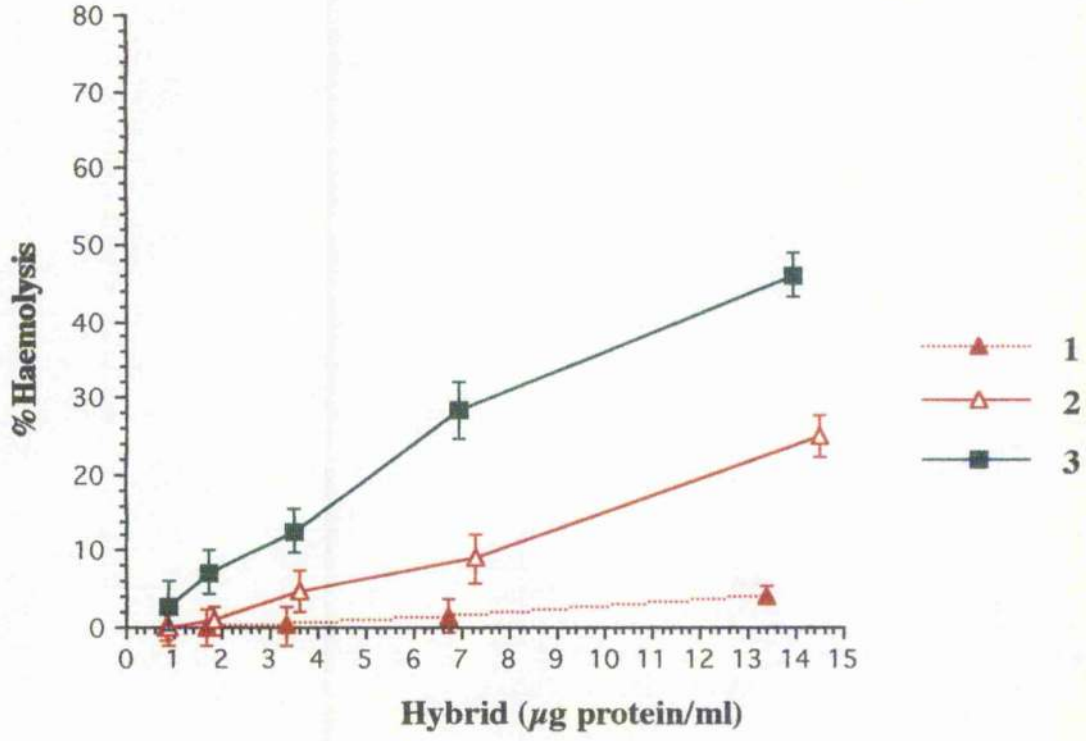

% Haemolysis
0
10
20
30
40
50
60
70
80
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Hybrid (μg protein/ml)
1
2
3

Table 7.

In vivo activation of LktA or hybrid toxins by C proteins

Preparation	Enzymic activity [a] (μmol cAMP/min/mg protein)	Haemolytic activity [a] μg protein/ml for 20% haemolysis of sheep RBC	Cytotoxic activity [a] μg protein/ml for 50% cell killing of	
			J774.2	BL3
LktA/CyaC	ND[b]	28.74±2.36	>208	16.27±2.05
LktA/LktC	ND	41.30±5.19	>331	27.81±4.63
CyaA /LktC	164.83±48.2	>51	>142	>130
PIR Hyb1	441.83±27.59	>64	>83	>117
PIR Hyb1/CyaC	425.31±24.65	>72	>62	>50
Hyb1/LktC	289.5±39.20	>86	>274	>72
PIR Hyb2	407.83±27.59	>64	>83	>117
PAR Hyb2/CyaC	291.16±53.21	24.38±1.96	15.61±1.74	19.18±2.61
PAR Hyb2/LktC	384.37±32.14	10.27±1.43	14.36±3.57	5.48±0.53

[a] Data are the mean values ± SEM of 4 determinations. [b] Not done

37 °C. The results indicated that incubation of cells with toxin for 2 h was sufficient for maximum activity against BL3 cells as shown in Fig. 20. J774.2 cells were very much less susceptible to attack by LktA.

3.10.6 Cytotoxic activity of various CyaA and hybrid toxin preparations on J774.2 or BL3 cells

The effect of CyaA preparations from *B. pertussis* or recombinant *E. coli* strains and hybrid toxins on cell killing of mouse macrophage-derived (J774.2) cells or bovine lymphoma-derived (BL3) cells were determined by the MTT assay. The cytoxicity of CAN CyaA or PAN CyaA preparations on both cell types was slightly higher than that of CAR CyaA or PAR CyaA (Table 6, Figs. 21 and 22). PARCyaA showed 5 or 6 fold higher cytoxicity on J774.2 cells than Hyb2 modified either by LktC or CyaC (Tables 6 and 7), However, it was noticeable that Hyb2 modified by LktC had noticeably increased activity against BL3 cells compared to J774.2 cells whereas Hyb2 modified by CyaC had roughly comparable activities in both cell types (Figs 23 and 24, Table 7) In addition LktC-modified LktA showed comparable cytotoxicity for BL3 cells compared to CyaC-modified CyaA (CAR CyaA), but no obvious cytotoxicity was detected for J774.2 cells, indicating the specificity of LktA for ruminant cells (Tables 6 and 7). It should be noted that LktA modified by CyaC showed greater toxic activity towards BL3 cells than LktA modified by LktC. This was in keeping with its greater haemolytic activity. No cytotoxic activity on both cell types was detected for PIR Hyb1, Hyb1 modified by CyaC or LktC, PIR Hyb2, non-modified LktA or urea extraction buffer diluted in the same way as the toxin dilutions in the assay.

Fig. 20

Effect of incubation time on cell killing of CyaA and LktA preparations from recombinant *E. coli* strains .

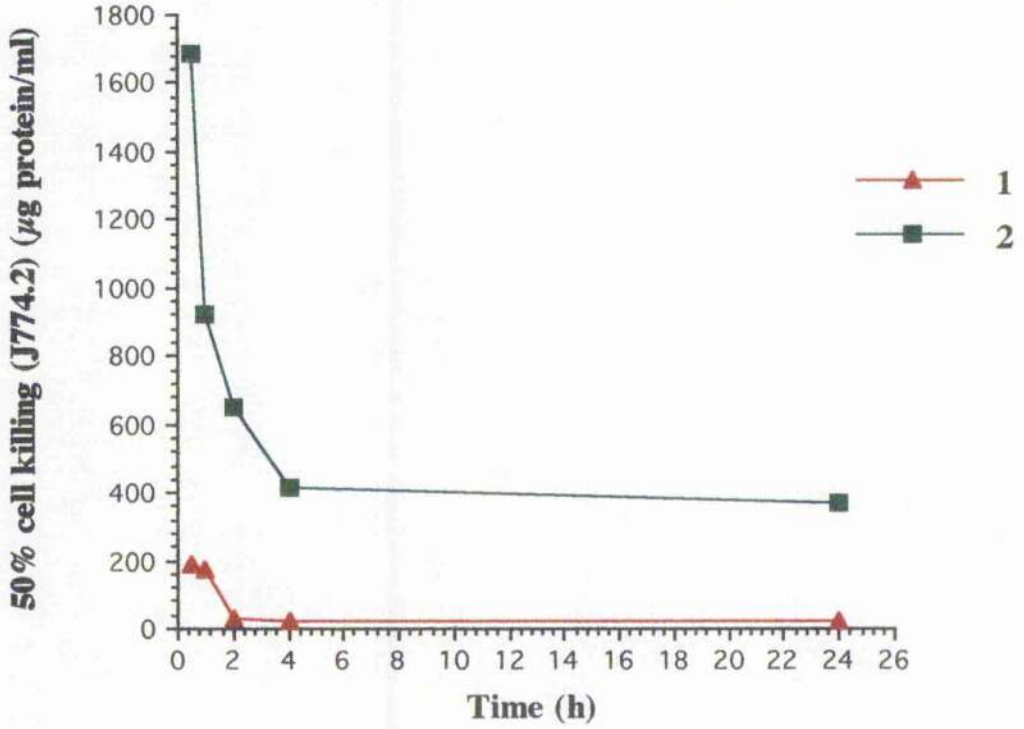
The cytotoxicity of crude recombinant CyaA and LktA preparations was determined for mouse macrophage-derived (J774.2) (Panel A) and bovine lymphoma-derived (BL3) cells (Panel B) by the MTT assay (section. 2.16). The results represent mean of duplicate determinations.

Panel A,B:

1 CAR CyaA

2 LktA/LktC

A


50% cell killing (J774.2) (µg protein/ml)
1800
1600
1400
1200
1000
800
600
400
200
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Time (h)
1
2

B

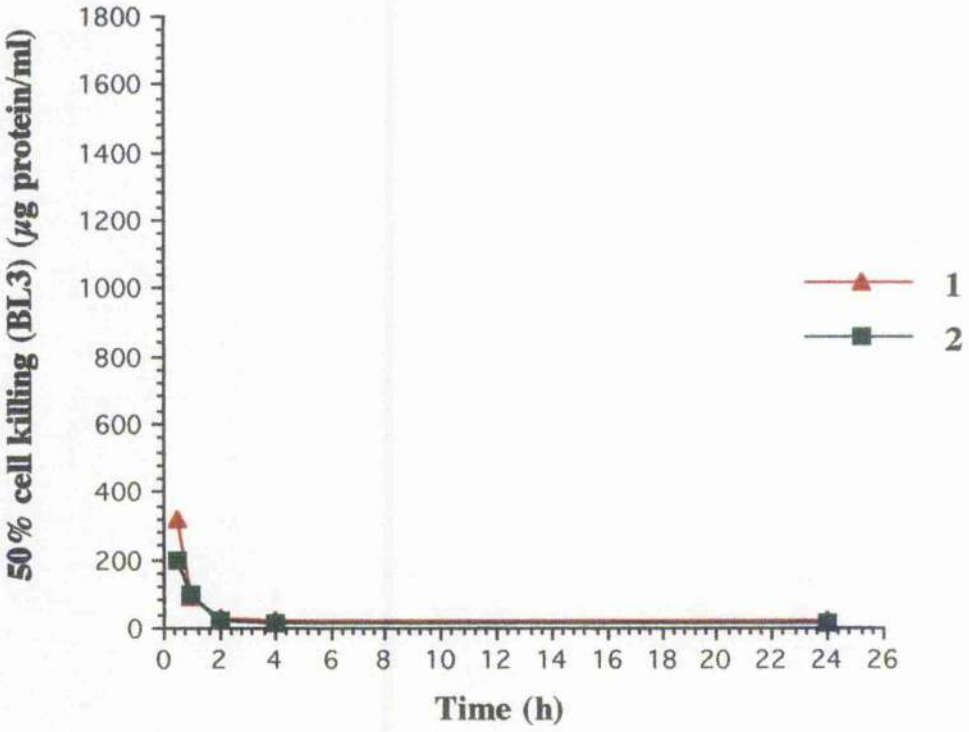

50% cell killing (BL3) (µg protein/ml)
1800
1600
1400
1200
1000
800
600
400
200
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Time (h)
1
2

Fig. 21

Cytotoxicity of CyaA preparations from *B. pertussis* and recombinant *E. coli* strains on mouse macrophage-derived (J774.2) cells

The cytoxicity of various dilutions of crude and purified CyaA preparations were determined for mouse macrophage-derived (J774.2) by the MTT assay (section 2.16). The samples were diluted in 2-fold serial dilutions in growth medium and incubated with cells for 2h at 37 °C. The results are the mean values of four determinations ±SEM.

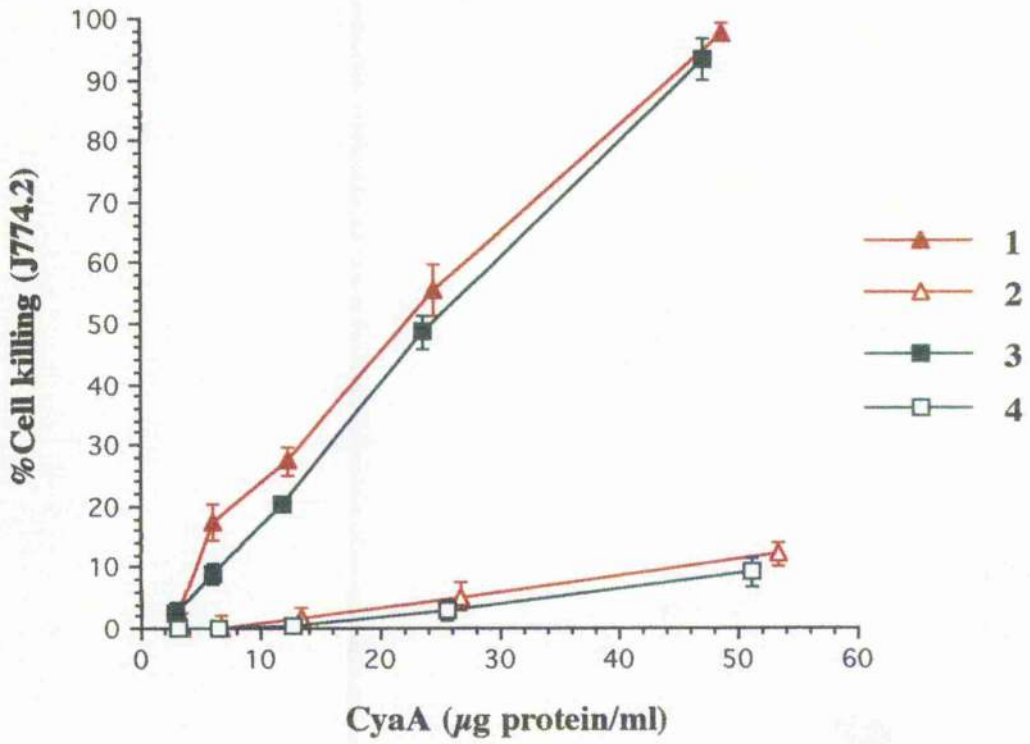
Panel A :

1 CAN CyaA
2 CIN CyaA
3 CAR CyaA
4 CIR CyaA

Panel B :

1 PAN CyaA
2 PIN CyaA
3 PAR CyaA
4 PIR CyaA

A


%Cell killing (J774.2)
CyaA (μg protein/ml)
1
2
3
4

B

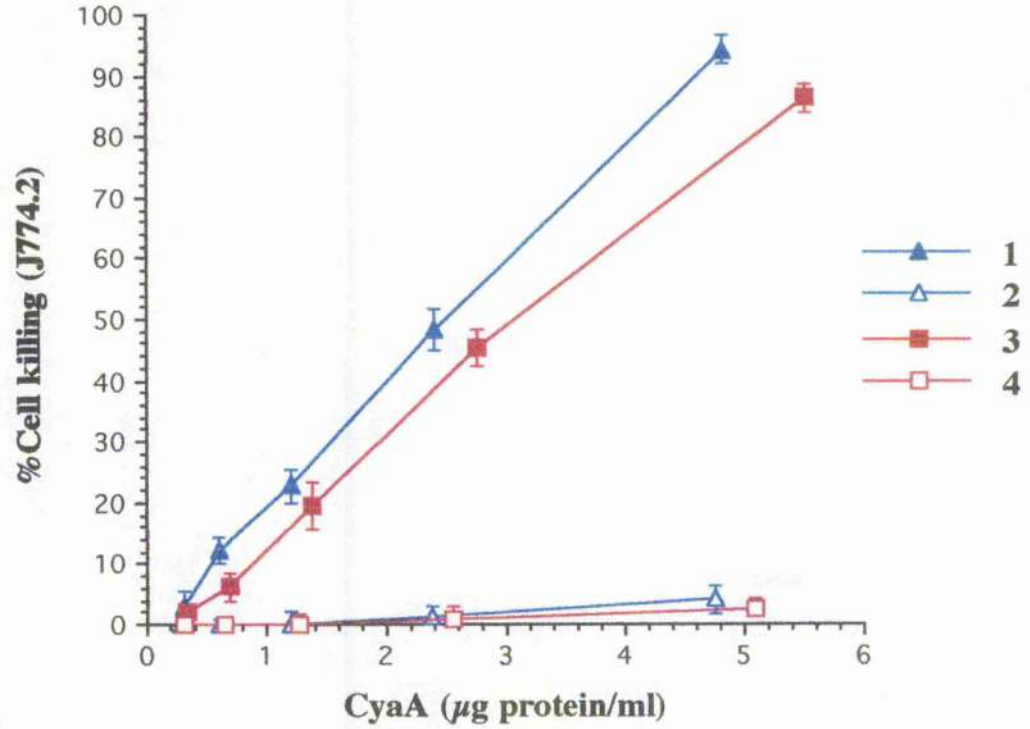

%Cell killing (J774.2)
CyaA (μg protein/ml)
1
2
3
4

Fig. 22

Cytotoxicity of CyaA preparations from *B. pertussis* and recombinant *E. coli* strains on bovine lymphoma-derived (BL3) cells

The cytoxicity of various dilutions of crude and puified CyaA preparations were determined for bovine lymphoma-derived (BL3) cells by the MTT assay The samples were diluted in 2-fold serial dilutions in growth medium and incubated with cells for 2h at 37 °C. The results represent the mean values of four determinations ±SEM.

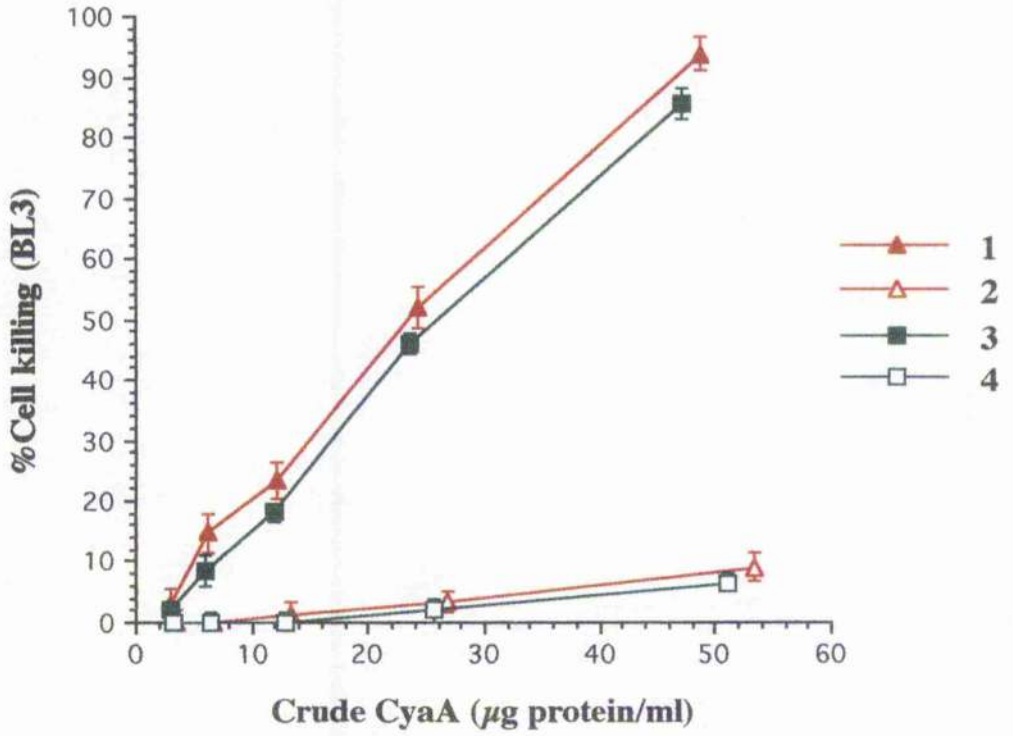
Panel A :

1 CAN CyaA
2 CIN CyaA
3 CAR CyaA
4 CIR CyaA

Panel B :

1 PAN CyaA
2 PIN CyaA
3 PAR CyaA
4 PIR CyaA

A


%Cell killing (BL3)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
Crude CyaA (μg protein/ml)
1
2
3
4

B

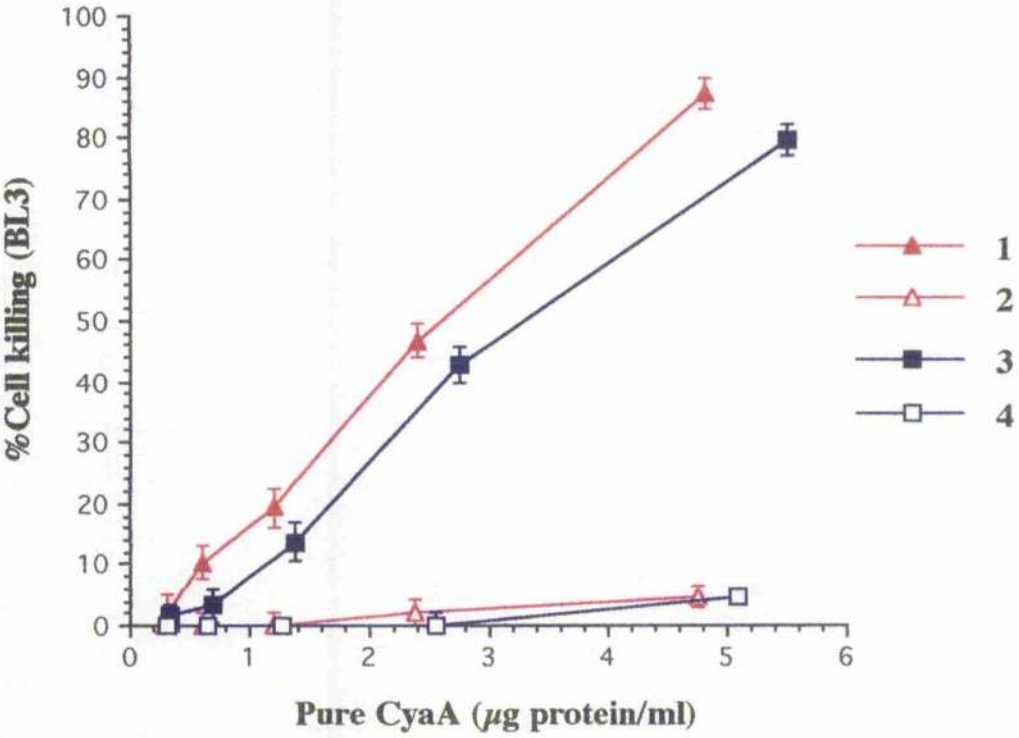

%Cell killing (BL3)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
Pure CyaA (μg protein/ml)
1
2
3
4

Fig. 23

Cytotoxicity of hybrid toxin preparations from recombinant *E. coli* strains on mouse macrophage-derived (J774.2) cells

The cytoxicity of various dilutions of purified hybrid toxin preparations were determined for mouse macrophage-derived (J774.2) cells by the MTT assay. The samples were diluted in 2-fold serial dilutions in growth medium and incubated with cells for 2h at 37 °C. The results represent the mean values of four determinations ±SEM. Panel A shows Hyb1 preparations with active CyaA as a control and panel B shows Hyb 2 preparations.

Panel A :

1 Hyb1

2 Hyb1/CyaC

3 Hyb1/LktC

4 PAR CyaA: from recombinant *E.coli* BL21(pCACT3)

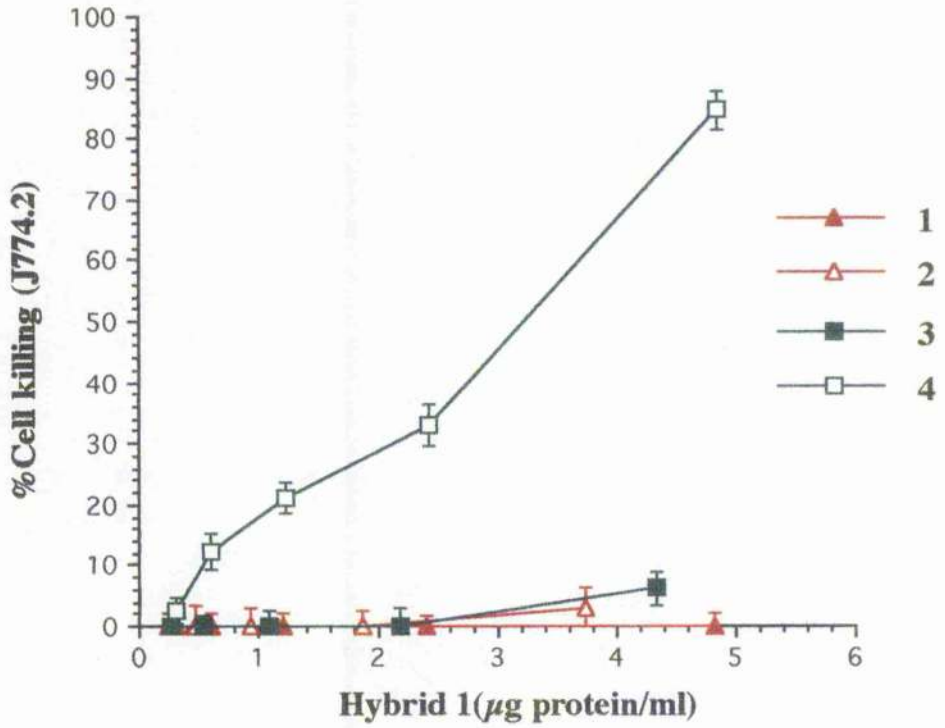
Panel B :

1 Hyb 2

2 Hyb 2/CyaC

3 Hyb 2/LktC

A


100
90
80
70
60
50
40
30
20
10
0
%Cell killing (J774.2)
0
1
2
3
4
5
6
Hybrid 1(μg protein/ml)
1
2
3
4

B

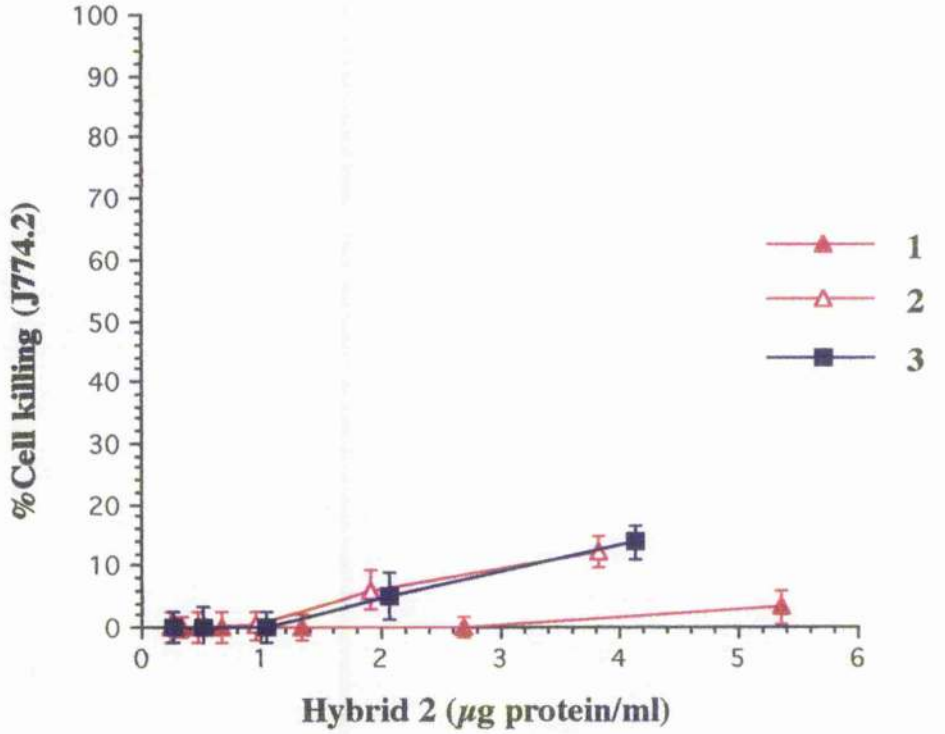

100
90
80
70
60
50
40
30
20
10
0
%Cell killing (J774.2)
0
1
2
3
4
5
6
Hybrid 2 (μg protein/ml)
1
2
3

Fig. 24

Cytotoxicity of hybrid toxin preparations from recombinant *E. coli* strains on bovine lymphoma-derived (BL3) cells

The cytoxicity of various dilutions of purified hybrid toxin preparations were determined for bovine lymphoma-derived (BL3) cells by the MTT assay. The samples were diluted in 2-fold serial dilutions in growth medium and incubated with cells for 2h at 37 °C. The results are the mean values of four determinations ±SEM.

Panel A :

1 Hyb1

2 Hyb1/CyaC

3 Hyb1/LktC

4 PAR CyaA: from recombinant *E. coli* BL21(pCACT3)

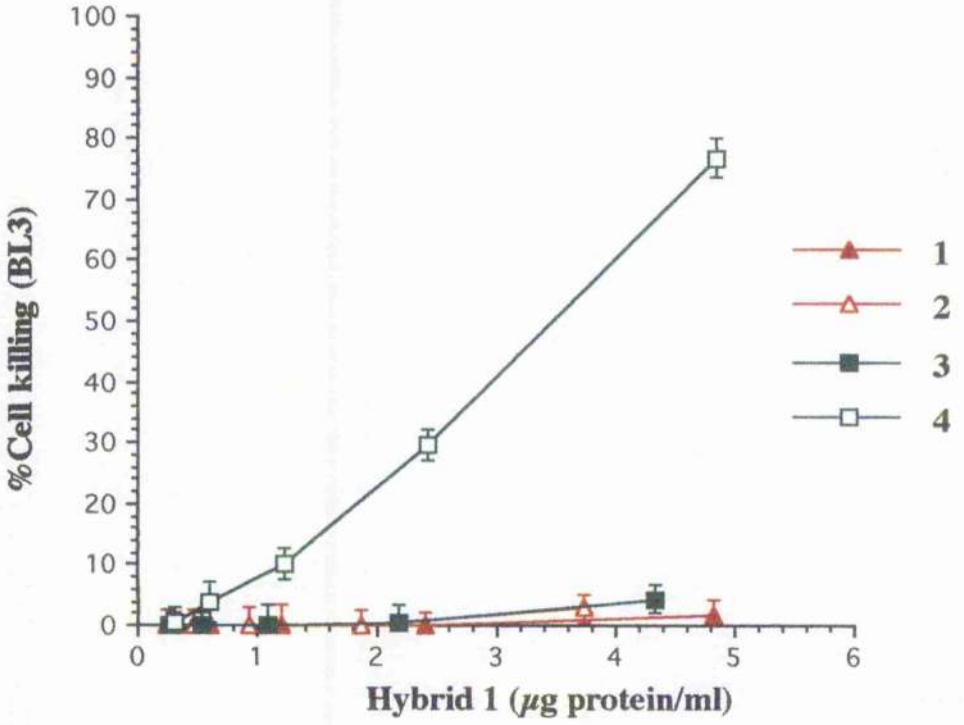
Panel B :

1 Hyb2

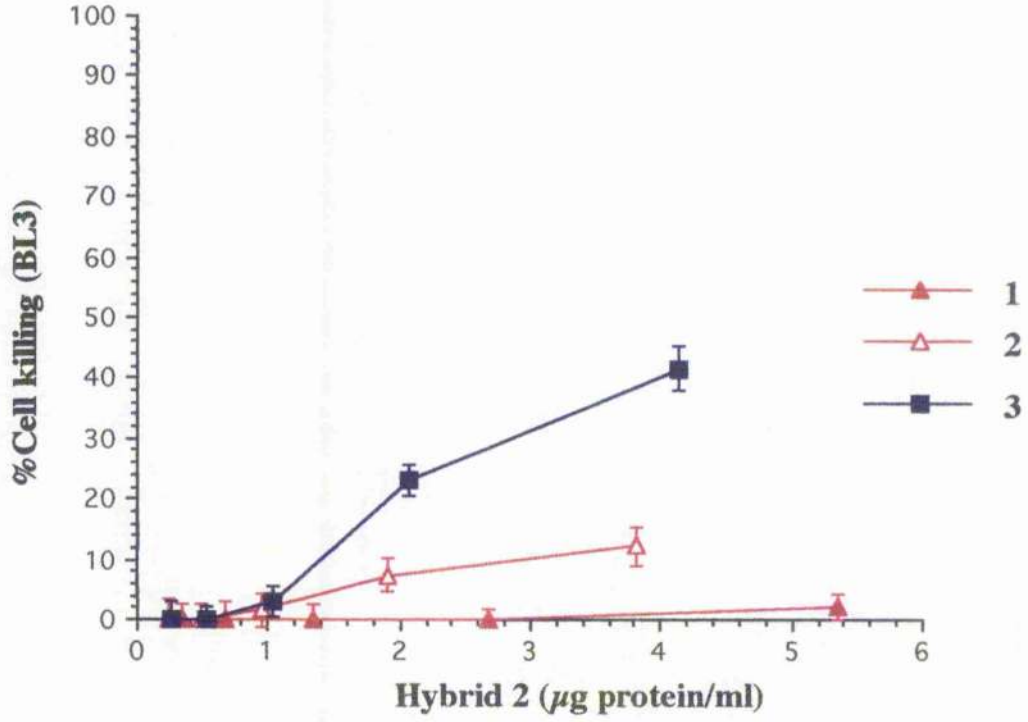
2 Hyb2/CyaC

3 Hyb2/LktC

A


100
90
80
70
60
50
40
30
20
10
0
%Cell killing (BL3)
0
1
2
3
4
5
6
Hybrid 1 (μg protein/ml)
1
2
3
4

B


100
90
80
70
60
50
40
30
20
10
0
%Cell killing (BL3)
0
1
2
3
4
5
6
Hybrid 2 (μg protein/ml)
1
2
3

3.10.7 Chemiluminescence inhibition (CI) by CyaA preparations

The chemiluminescence inhibition by CyaA preparations for bovine and rabbit neutrophil responses to opsonised zymosan stimulation were determined (section 2. 15). The chemiluminescence response of bovine and rabbit neutrophils to zymosan stimulation after treatment of the neutrophils for 20 min with PAN CyaA or PAR CyaA was shown to be > 98% inhibited, whereas PIN CyaA or PIR CyaA possessed only low CI activity at the highest concentration tested (Figs. 25 and 26). It should be noted that the residual urea (20 mM) in the preparations had little inhibitory effect on the CI response.

3.11 SUMMARY OF IN VIVO ACTIVATION OF CyaA, LktA, and HYBRID TOXINS BY C PROTEINS

Active recombinant CyaA (PAR CyaA) produced in vivo by co-expression of *cya*A and *cya*C in *E. coli* BL21/DE3 (pGW44, pGW54) showed comparable cytotoxicity on J774.2 and BL3 cells to *B. pertussis* pRMB1 CyaA (PAN CyaA), but slightly weaker haemolytic activity. An extract prepared from *E. coli* BL21/DE3 (pGW44, pGW78) strain, expressing *cya*A and *lkt*C, showed no haemolytic or cytotoxic activity on J774.2 or BL3 cells. These results clearly demonstrated the specificity of LktC (Tables 6 and 7). An extract prepared from *E. coli* HMS174/DE3 strain harbouring plasmids pGW64, pGW78, expressing *lkt*A and *lkt*C, was cytotoxic for BL3 cells, but not for J774.2 cells, and showed a low haemolytic activity with sheep erythrocytes. The cytotoxicity on BL3 cells and haemolysis with sheep erythrocyte of an extract from *E. coli* HMS174/ DE3 (pGW64, pGW54), co-expressing *lkt*A and

Fig. 25

Effect of CyaA preparations from *B. pertussis* and recombinant *E. coli* strains on chemiluminescence (CL) response of rabbit neutrophils to opsonised zymosan.

The chemiluminescence inhibition of 1/40 dilution of purified CyaA preparations was determined for rabbit neutrophils using the CL inhibition assay (section 2.15). Rabbit neutrophils ($5x10^5$/ml) were incubated with samples for 30 min at 38 °C before addition of opsonised zymosan. CL emission was measured in mV at 38 °C in a Wallac 1251 luminometer running on multi-use software. The results represent the mean values of four determinations ±SEM. 20 mM urea was used a control as this was the final concentration in samples containing toxin.

Panel A :

1 PAN CyaA

2 PIN CyaA

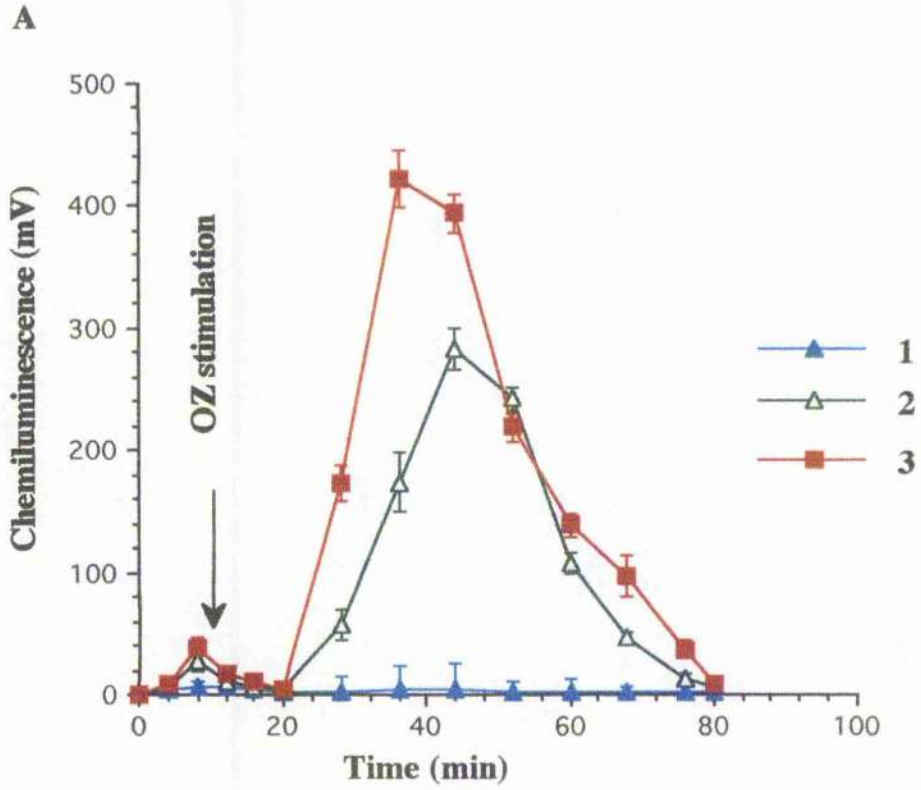
3 Cells in 20 mM Urea

Panel B :

1 PAR CyaA

2 PIR CyaA

3 Cells in Hanks HEPES (HH)


A
Chemiluminescence (mV)
OZ stimulation
Time (min)
1
2
3

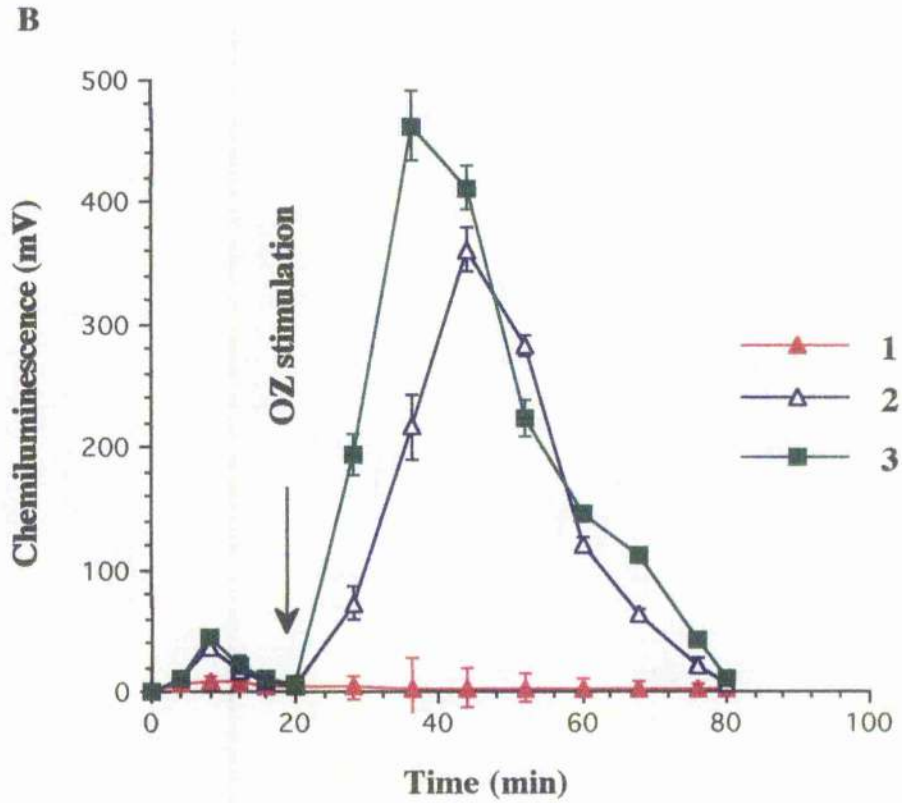

B
Chemiluminescence (mV)
OZ stimulation
Time (min)
1
2
3

Fig. 26

Effect of CyaA preparations from *B. pertussis* and recombinant *E. coli* strains on chemiluminescence (CL) response of bovine neutrophils to opsonised zymosan.

The chemiluminescence (CL) inhibition of 1/40 dilution of purified CyaA preparations were determined for bovine neutrophils using the CL inhibition assay. Bovine neutrophils ($5x10^5$/ml) were incubated with samples for 30 min at 38 °C before addition of opsonised zymosan. CL emission was measured in mV at 38 °C in a Wallac 1251 luminometer running on multi-use software. The results are the mean values of four determinations ±SEM.

Panel A :

1 PAN CyaA

2 PIN CyaA

3 Cells in 20 mM Urea

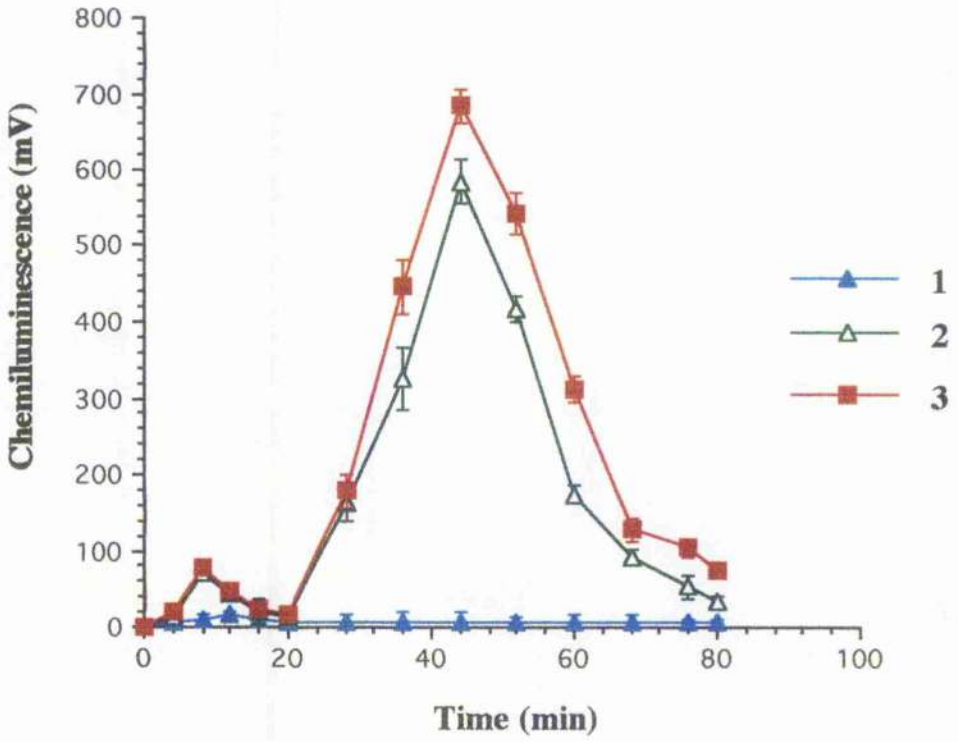
Panel B :

1 PAR CyaA

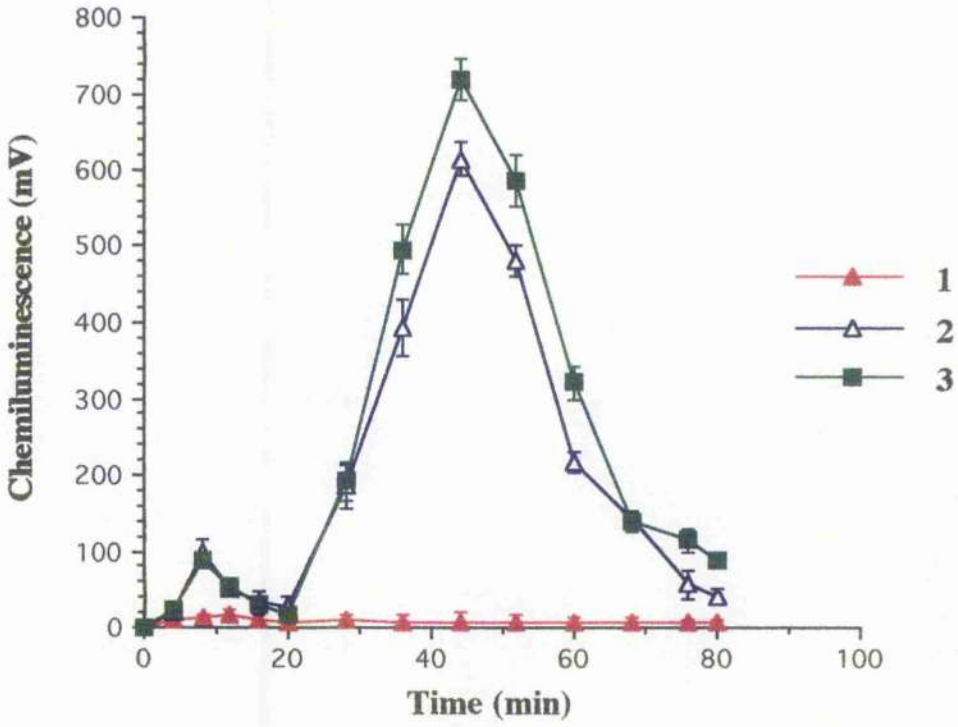
2 PIR CyaA

3 Cells in Hanks HEPES (HH)

A


Chemiluminescence (mV)
800
700
600
500
400
300
200
100
0
0
20
40
60
80
100
Time (min)
1
2
3

B


Chemiluminescence (mV)
800
700
600
500
400
300
200
100
0
0
20
40
60
80
100
Time (min)
1
2
3

*cya*C were slightly higher than that of LktA/LktC but there was no activity against J774.2 cells. These results showed two things. First, that CyaC was non-specific in its activation of A proteins, and was equally active against CyaA and LktA and second, that LktA modified by CyaC still retained its specificity for ruminant cells.

The hybrid toxin genes derived from *lkt*A and *cya*A genes showed a slightly different pattern of activation by C proteins. Extracts from *E. coli* BL21/ DE3 expressing Hyb1, Hyb1/CyaC, Hyb1/LktC, Hyb2, Hyb2/CyaC or Hyb2/LktC all had enzymic activity, but only Hyb2/LktC or Hyb2/CyaC showed haemolytic or cytotoxic activity. These activities, however were lower than those of active CyaA toxin. Interestingly, whereas LktC was unable to activate CyaA, it was able to activate Hyb2 where the region believed to be involved in the activation process in CyaA had been replaced by that of LktA. Indeed, activation by LktC was greater than that by CyaC as evidenced by greater haemolytic activity against sheep erythrocytes and greater toxicity to BL3 cells. Toxicity towards J774.2 cells was roughly the same for LktC or CyaC activated Hyb2, suggesting LktC activation bestowed some ruminant-specific target cell specificity on Hyb2.

3.12 IN VITRO ACTIVATION OF CyaA, LktA AND HYBRID TOXINS BY C-PROTEINS

3.12.1 In vitro activation components

The activation components used in activation reactions were inactive CyaA (proCyaA), inactive LktA (proLktA), inactive Hyb2 (proHyb2), C proteins (CyaC or LktC) and cytosolic activating factor (CAF). ProCyaA, CyaC, proLktA, LktC and proHyb2 were prepared in 8M urea, 25 mM HEPES pH 7.5 from cellular extracts of induced cultures of *E. coli* strains BL21/DE3 (pGW44) expressing *cyaA*, BL21/DE3 (pGW14) expressing *cyaC*, HMS174/ DE3 (pGW64) expressing *lktA*, HMS174/ DE3 (pGW78) expressing *lktC* and BL21/DE3 (pGW88) expressing *hyb2* (section 2.17). All proteins expressed as inclusion bodies were partially purified by washing of the inclusion bodies with 0.5 M and 2 M urea rather than by calmodulin affinity or DEAE sepharose chromatography (section 2.6). The reasons for this were two-fold. Firstly, LktA and the C proteins could not be purified by the procedures used for CyaA and it was thought best to prepare all the proteins in the same way. Secondly, purified CyaA was time-consuming to prepare and it was considered best to retain this for immunological studies where quite large amounts were required. The SDS-PAGE profiles of some of these partially purified proteins are shown in Fig. 13. CAF was prepared from cultures of *E. coli* BL21, *B. pertussis* 348 lacking an active *cya*A gene and *P. haemolytica* (Ph 30) which does not express LktA (Saadati *et al.*, 1997). Urea extracts of partially purified proteins were diluted and used directly in the activation reaction instead of attempting to remove urea by dialysis, as dialysis of urea extracts resulted in

aggregation of CyaC protein (data not shown) and therefore the loss of its activation ability.

3.12.2 Kinetic study of in vitro activation of proCyaA by CyaC

The length of incubation time and temperature dependence of the in vitro activation were examined for CyaC activation of CyaA (Fig. 27). It was shown that obvious erythrocyte haemolysis or BHK cell stellation in the presence of Ca^{2+} was observed rapidly within 10 min. Later time points indicated that the activation reaction occurred most rapidly at 37 °C, but the activity declined markedly after 10 min. Although the initial rate of reaction was slower at 25 °C and 4 °C, the activated CyaA appeared to be more stable at these temperatures, particularly at 4 °C. The lowest extent of activation was achieved at 42°C. These data indicated the extreme lability of the activated toxin.

3.12.3 Activity of in vitro-activated proCyaA, LktA and Hyb2

Pro CyaA, CyaC and CAF, 20-fold dilutions of each, were mixed and incubated at 4 °C and after 30 min the reaction mix was assayed for invasive, haemolytic and cytotoxic activities and chemiluminescence inhibition (CI) of rabbit or bovine neutrophils. As described in the previous section, proCyaA incubated with CyaC and CAF became activated and was active in the invasive, haemolytic, cytotoxic and CI assays (Table 8). The in vitro activated CyaA showed a high level of CI of bovine and rabbit neutrophils. The dose responses of in vitro-activated CyaA are shown in Figs 28-33. With an incomplete in vitro activation mix lacking CAF, there was little stellation with BHK cells, a background level of haemolysis with sheep erythrocytes, a low

Fig. 27

Temperature and time dependence of in vitro activation of CyaA by CyaC.

In vitro activation reactions were performed in 150 μl of 25mM HEPES (pH 7.5) containing CyaA from *E. coli* BL21/DE3 (pGW44),CyaC from *E. coli* BL21/DE3 (pGW14) and *E. coli* BL21 S100 cytosolic activating factor (CAF) (section 2.18.1). Replicate activation reaction mixes, one reaction for each time-point were incubated at 4 °C, 25 °C, 37 °C and 42 °C and then used in the haemolysis and BHK cell spreading assays (section 2.14). The results represent the mean values of duplicate determinations.

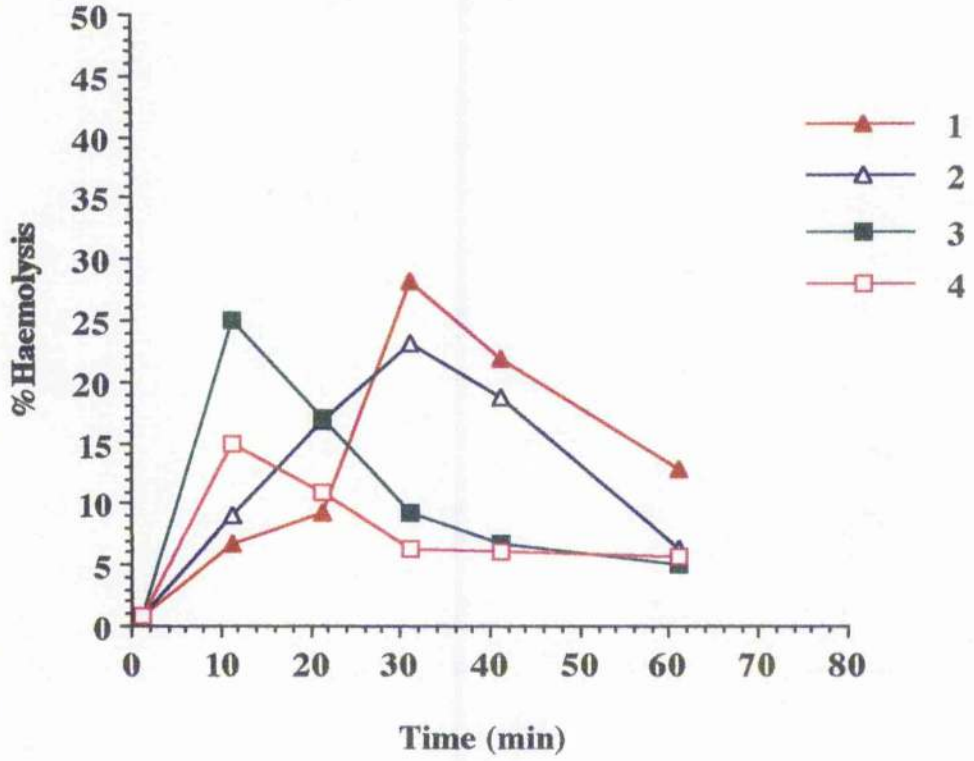
Panel A : Haemolytic activity of in vitro-activated CyaA

1 4 °C
2 25 °C
3 37 °C
4 42 °C

Panel B : BHK cell stellation by in vitro-activated CyaA

1 4 °C
2 25 °C
3 37 °C
4 42 °C

A


50
45
40
35
30
25
20
15
10
5
0
% Haemolysis
0
10
20
30
40
50
60
70
80
Time (min)
1
2
3
4

B

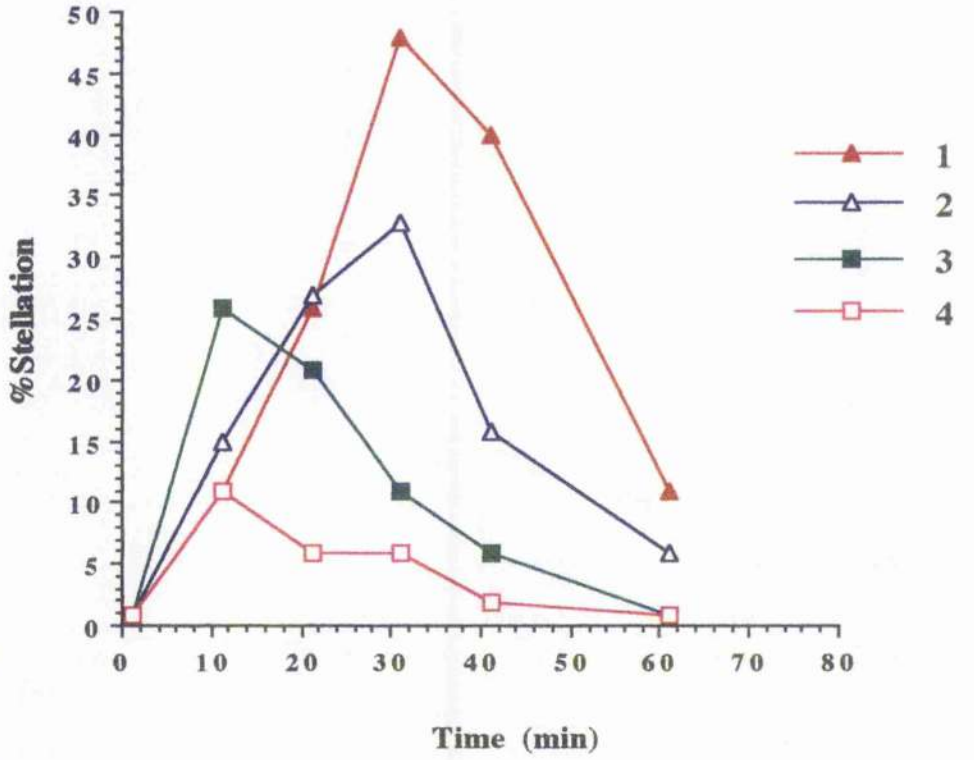

50
45
40
35
30
25
20
15
10
5
0
% Stellation
0
10
20
30
40
50
60
70
80
Time (min)
1
2
3
4

Fig. 28

Dose response of BHK cell stellation by in vitro-activated CyaA.

In vitro activation reactions were set up in 150 μl of 25mM HEPES (pH 7.5) containing increasing concentrations of CyaA from *E. coli* BL21/DE3 (pGW44) with constant concentrations of CyaC from *E. coli* BL21/DE3 (pGW14) or LktC from *E. coli* BL21/DE3 (pGW78) and *E. coli* BL 21 S100 cytosolic activating factor (CAF) at 4 °C for 30 min and then serial two fold dilutions of in vitro-activated CyaA were assayed using the BHK cell spreading assay. The results represent the mean values of 8 determinations (four separate experiments determined in duplicate).

Activation reactions:

1 CyaA + CyaC+CAF

2 CyaA + LktC+CAF

3 CyaA+HEPES buffer pH 7.5

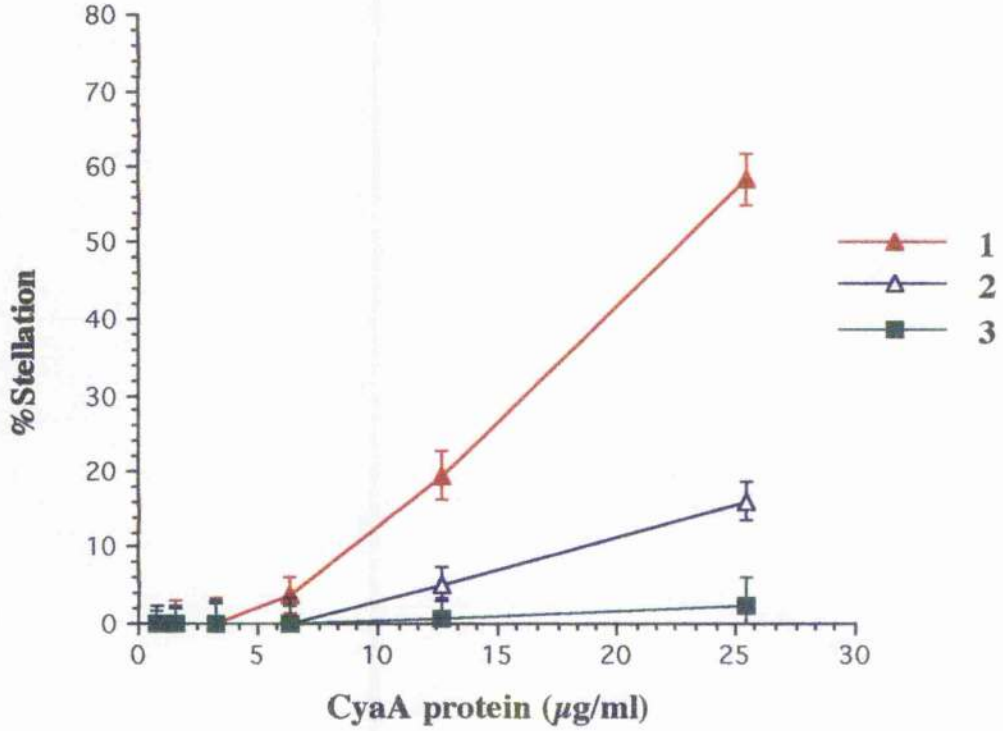

80
70
60
50
40
30
20
10
0
%Stellation
0
5
10
15
20
25
30
CyaA protein (μg/ml)
1
2
3

Fig. 29

Dose response of haemolysis of sheep erythrocytes by in vitro-activated CyaA, LktA and Hyb2 preparations.

In vitro activation reactions were carried out as described in the legend to Fig. 28 and then various dilutions of in vitro activated CyaA were assayed using the haemolysis assay (section 2.12). The results represent the mean values of 4 determinations ± SEM.

Panel A :

1 CyaA + CyaC+CAF

2 CyaA + LktC+CAF

3 CyaA + HEPES buffer pH 7.5

Panel B :

1 LktA + CyaC+CAF

2 LktA + LktC+CAF

3 LktA + HEPES buffer pH 7.5

Panel C :

1 Hyb2+CyaC+CAF

2 Hyb2+LktC+CAF

3 Hyb2+HEPES buffer pH 7.5

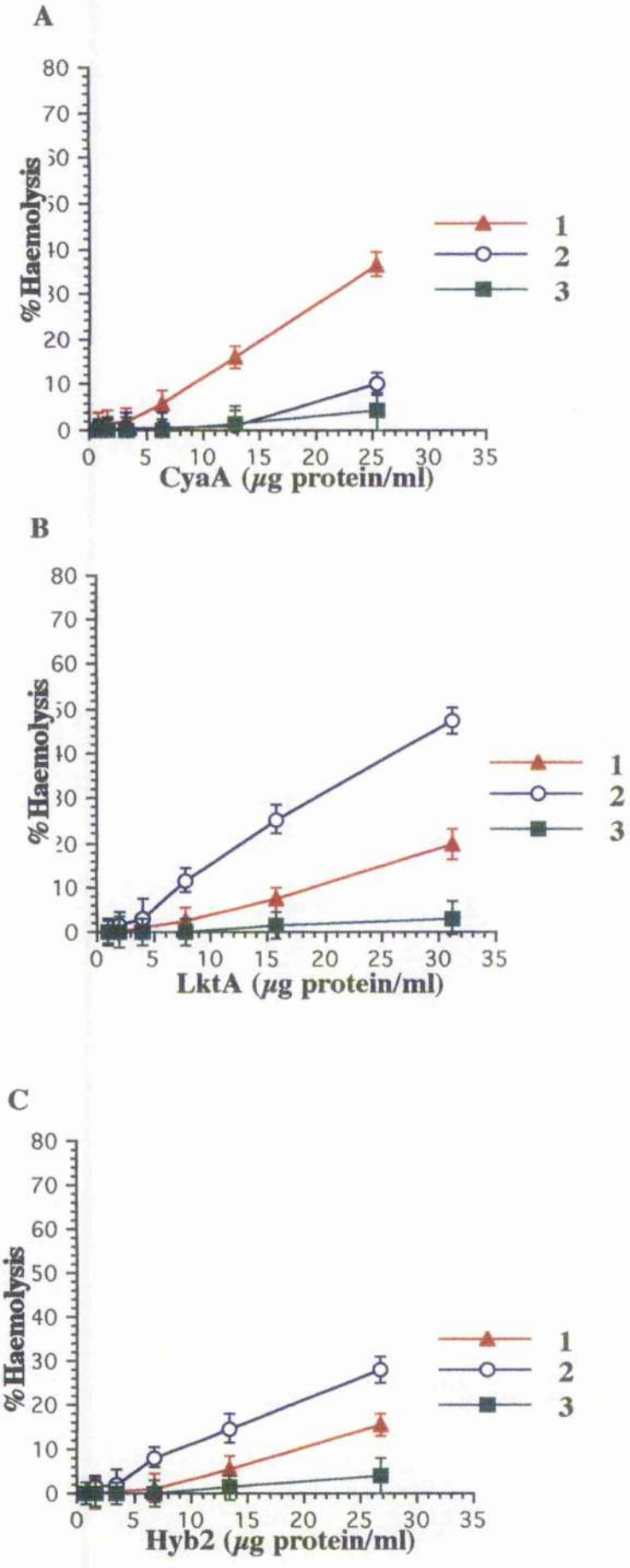

A
% Haemolysis
CyaA (μg protein/ml)
1
2
3
B
% Haemolysis
LktA (μg protein/ml)
1
2
3
C
% Haemolysis
Hyb2 (μg protein/ml)
1
2
3

Fig. 30

Dose response of J774.2 cell toxicity by in vitro-activated CyaA, LktA and Hyb2 preparations.

In vitro activation reactions of CyaA, LktA or Hyb2 by C proteins were performed as described in legend to Fig. 28. Serial two fold dilutions were tested in the MTT assay with J774.2 cells (section 2.16). The results represent the mean values of 4 determinations ± SEM.

Panel A :

1 CyaA+CyaC+CAF

2 CyaA+LktC+CAF

3 CyaA+HEPES buffer pH 7.5

Panel B :

1 LktA+CyaC+CAF

2 LktA+LktC+CAF

3 LktA+HEPES buffer pH 7.5

Panel C :

1 Hyb2+CyaC+CAF

2 Hyb2+LktC+CAF

3 Hyb2+HEPES buffer pH 7.5

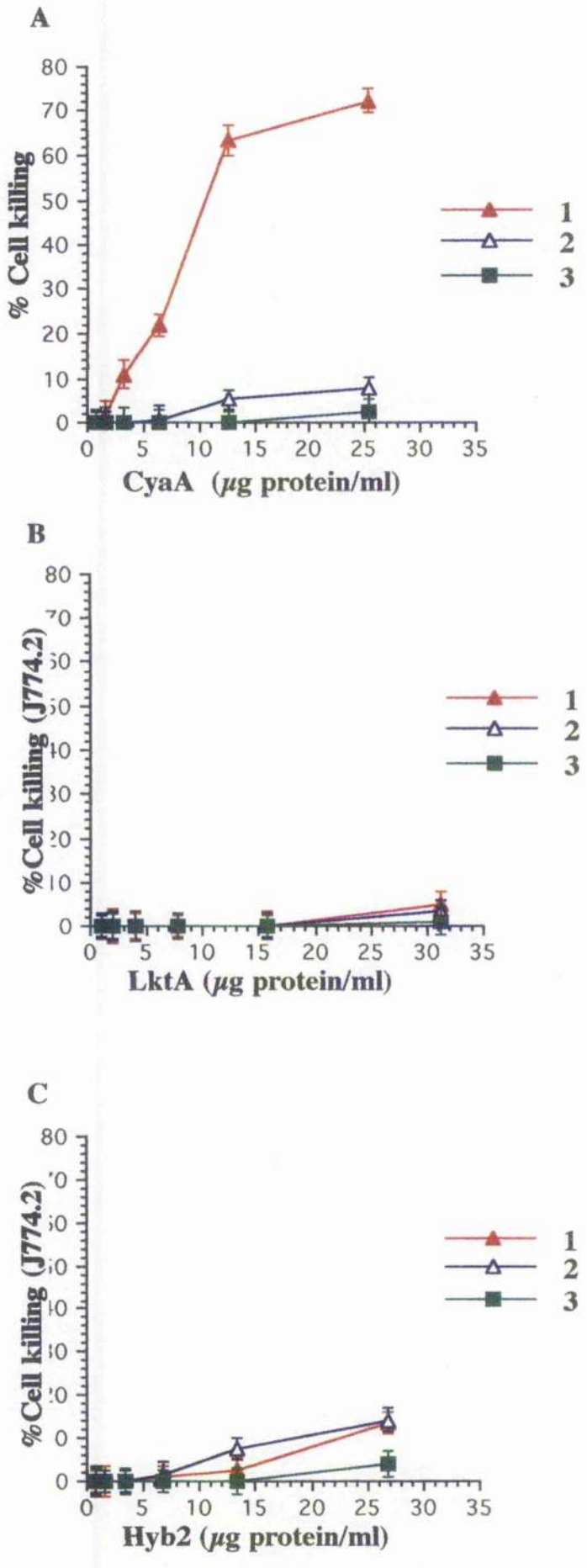

A
% Cell killing
CyaA (μg protein/ml)
1
2
3
B
%Cell killing (J774.2)
LktA (μg protein/ml)
1
2
3
C
%Cell killing (J774.2)
Hyb2 (μg protein/ml)
1
2
3

Fig. 31

Dose response of BL3 cell toxicity by in vitro-activated CyaA, LktA and Hyb2 preparations.

In vitro activation reactions were performed as described in the legend to Fig. 28 and then various dilutions were assayed with BL3 cells in the MTT assay (section 2.16). The results represent the mean values of 4 determinations (two separate experiments performed in duplicate).

Panel A :

1 CyaA+CyaC+CAF

2 CyaA+LktC+CAF

3 CyaA+HEPES buffer pH 7.5

Panel B :

1 LktA+CyaC+CAF

2 LktA+LktC+CAF

3 LktA+HEPES buffer pH 7.5

Panel C :

1 Hyb2+CyaC+CAF

2 Hyb2+LktC+CAF

3 Hyb2+HEPES buffer pH 7.5

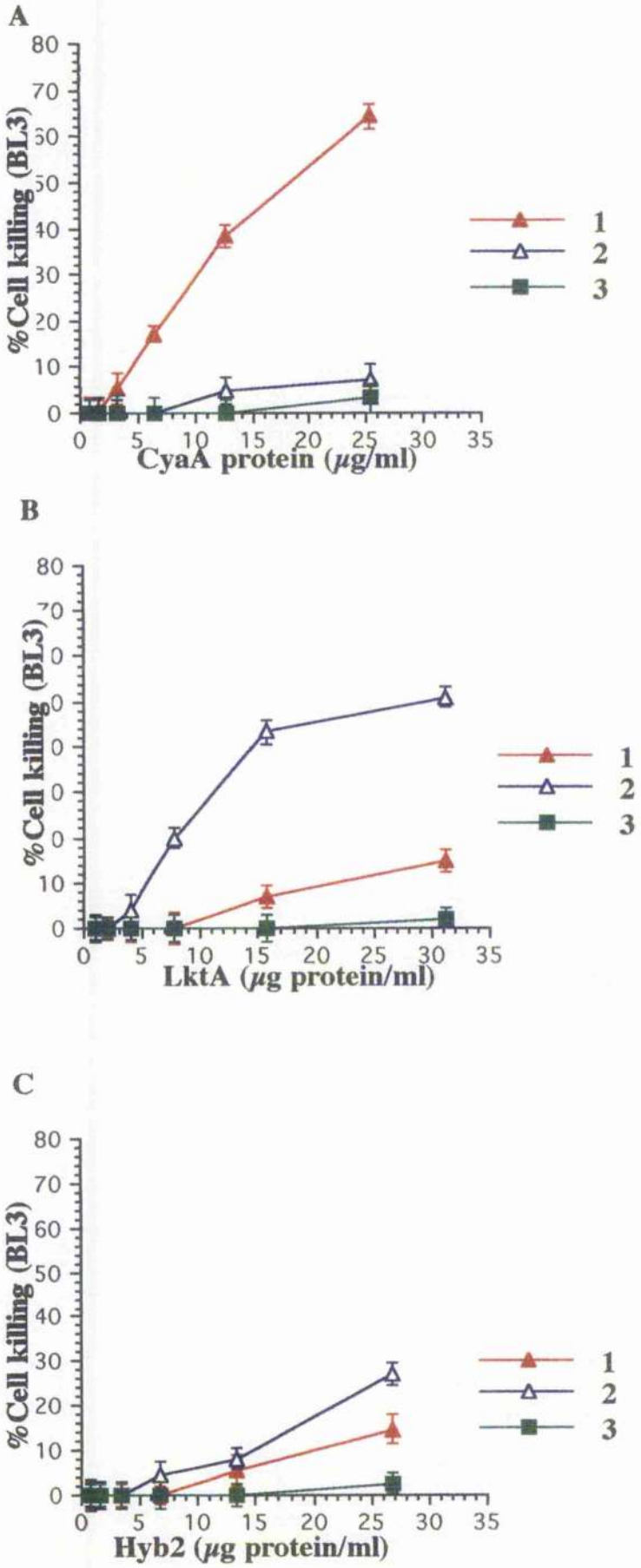

A
%Cell killing (BL3)
CyaA protein (μg/ml)
1
2
3
B
%Cell killing (BL3)
LktA (μg protein/ml)
1
2
3
C
%Cell killing (BL3)
Hyb2 (μg protein/ml)
1
2
3

Fig. 32

Effect of in vitro-activated CyaA or LktA on the chemiluminescence response of rabbit neutrophils to opsonised zymosan.

In vitro activation reactions were carried out as described in the legend to Fig. 28, and then various dilutions of in vitro activated CyaA and LktA were assayed with rabbit neutrophils in the CL inhibition assay (section 2.15). The results represent the mean values of 4 determinations ±SEM (two separate experiments done in duplicate).

Panel A :

1 CyaA+CyaC+CAF

2 CyaA+LktC+CAF

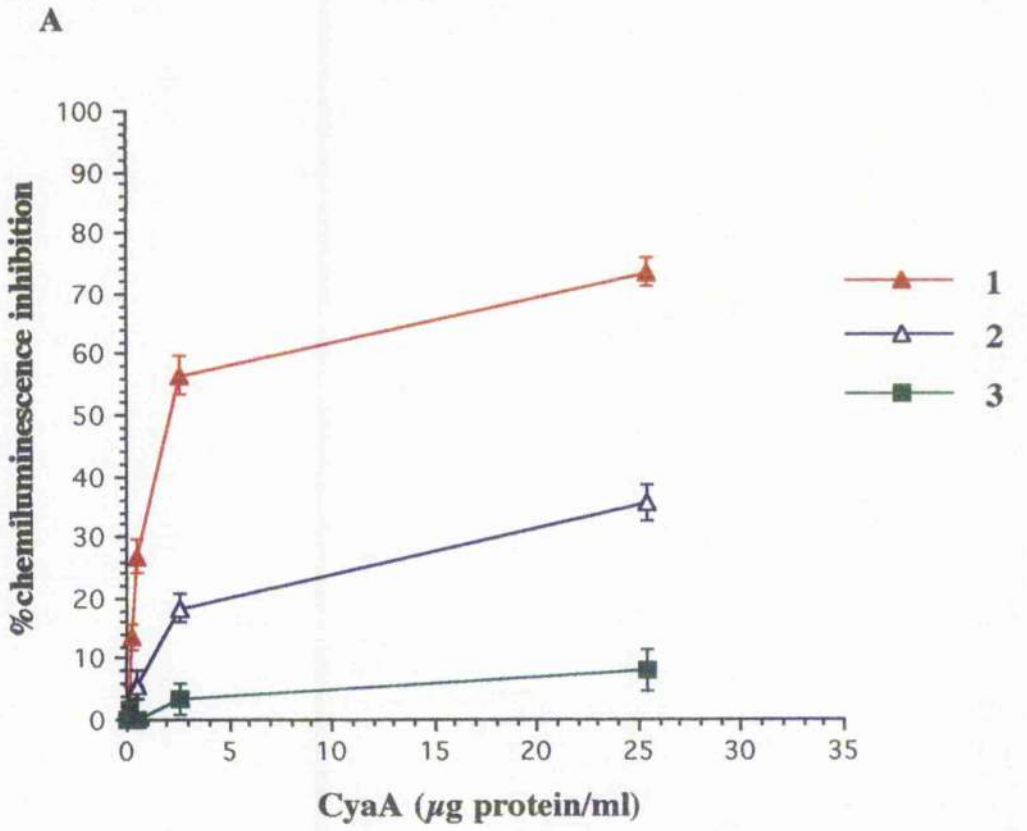
3 CyaA + HEPES buffer pH 7.5

Panel B :

1 LktA+CyaC+CAF

2 LktA+LktC+CAF

3 LktA+HEPES buffer pH 7.5


A
%chemiluminescence inhibition
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
CyaA (μg protein/ml)
1
2
3

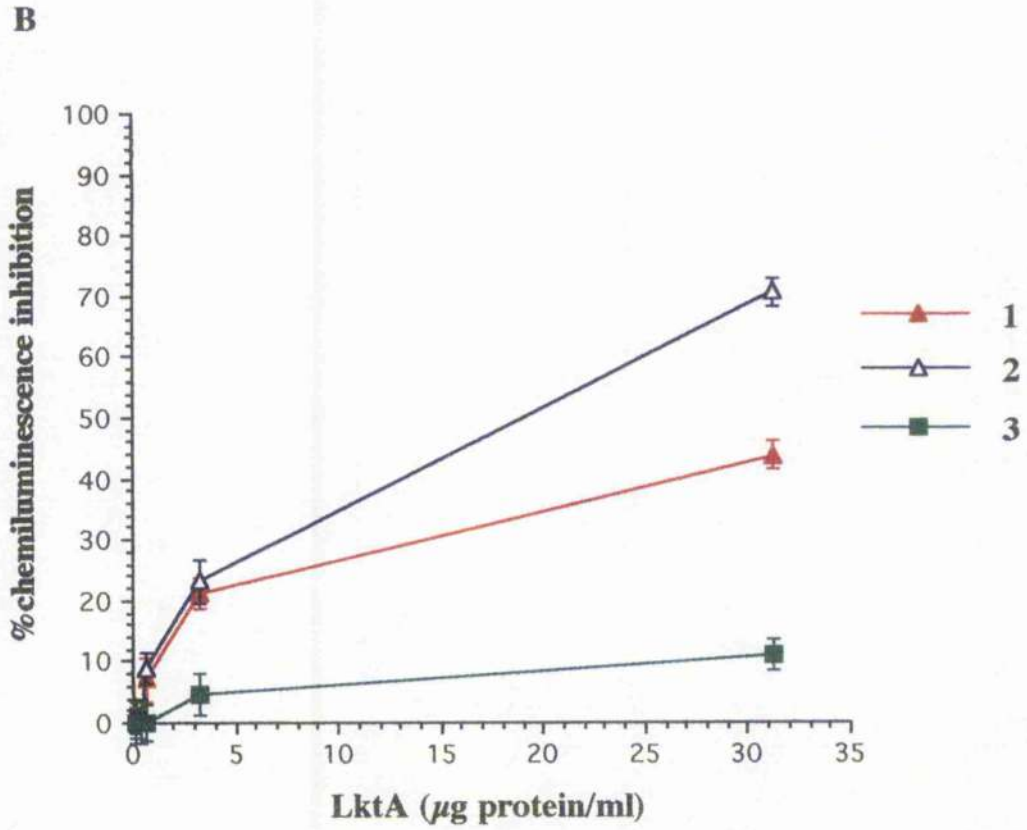

B
%chemiluminescence inhibition
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
LktA (μg protein/ml)
1
2
3

Fig. 33

Effect of in vitro-activated CyaA or LktA on the chemiluminescence response of bovine neutrophils to opsonised zymosan.

In vitro activation reactions were performed as described in the legend to Fig. 28, and then various dilutions of in vitro-activated CyaA and LktA were assayed with bovine neutrophils in the CL inhibition assay (section 2.15). The results are the mean values of 4 determinations ± SEM (two separate experiment performed in duplicate).

Panel A :

1 CyaA + CyaC+CAF

2 CyaA + LktC+CAF

3 CyaA + HEPES buffer pH 7.5

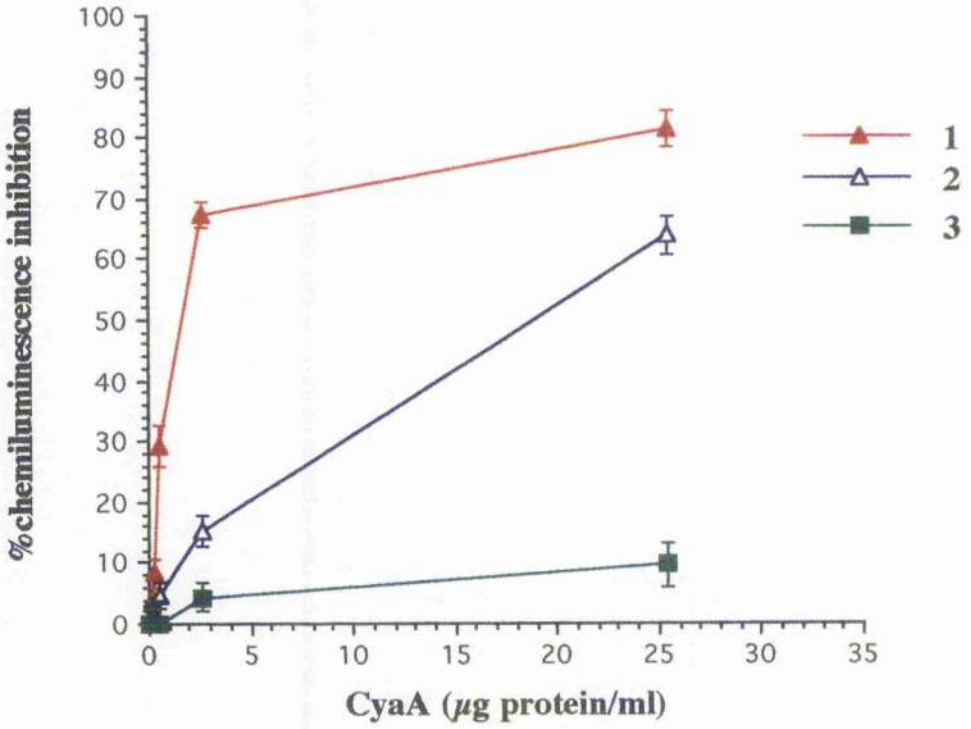
Panel B :

1 LktA + CyaC+CAF

2 LktA + LktC+CAF

3 LktA + HEPES buffer pH 7.5

A


100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
% chemiluminescence inhibition
CyaA (μg protein/ml)
1
2
3

B

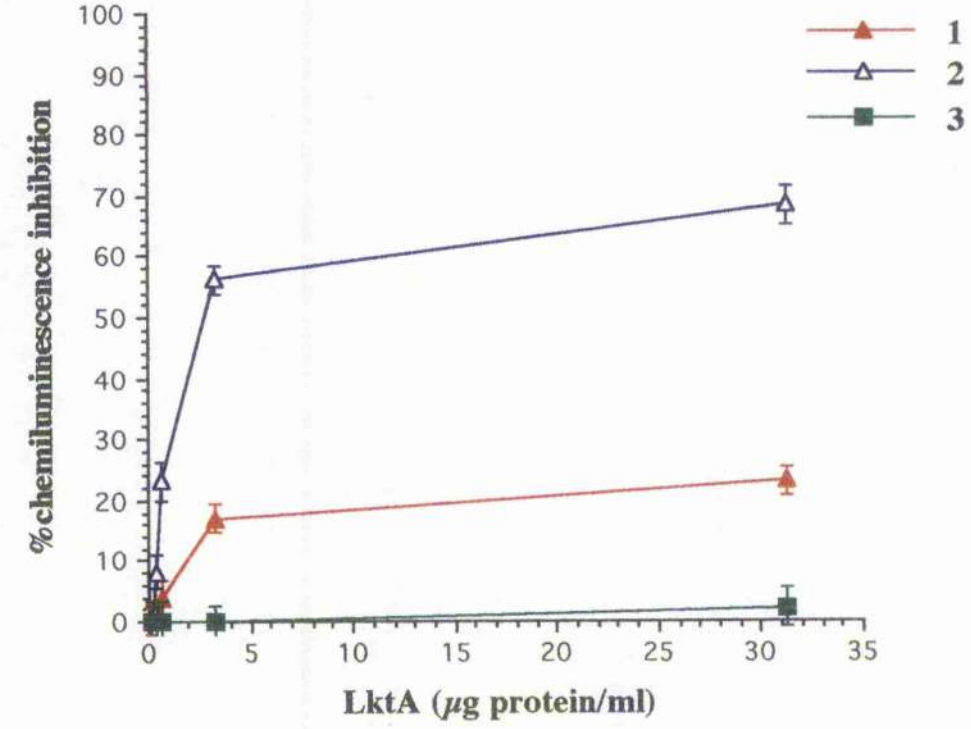

100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
% chemiluminescence inhibition
LktA (μg protein/ml)
1
2
3

Table 8.

In vitro activation of CyaA protoxin

Activation reaction mix	Invasive activity [a] μg protein/ml for 50% stellation of BHK 21 cells	Haemolytic activity [a] μg protein/ml for 20% haemolysis of sheep RBC	Cytotoxic activity [a] μg protein/ml for 50% cell killing of J774.2	BL3
CyaA+CyaC+CAF [b]	27.16±1.86	13.49±2.26	8.15±1.19	17.39±1.64
CyaA+CyaC+dHCAF [c]	>116	>63	>92	>73
CyaA+CyaC+dLCAF [d]	NT	19.34±2.38	14.69±1.63	22.31±2.27
CyaA+CyaC	>257	>81	>154	>164
CyaA+CAF	>155	>58	>149	>152
CAF	>370	>177	>362	>474

a. Results are the mean values ± SEM of 4 determinations.
b. CAF= Cytosolic activating factor
c. dHCAF= represents CAF dialysed with a membrane with a high Mr (>200kDa) cut-off point.
d. dLCAF= represents CAF dialysed with a membrane with a low Mr (<14 kDa) cut off point.

level of chemiluminescence inhibition of bovine and rabbit neutrophils and a background level of cell killing of J774.2 or BL3 cells . CyaA plus CAF alone was also examined and again showed a background level of activity in all assays. Thus in vitro activation of proCyaA required CyaC and a component (s) in CAF.

Dialysis of cytosolic factor (CAF) with a membrane having a high Mr (>200kDa) cut-off point resulted in the loss of CAF and loss of activation ability, but dialysis with a membrane of low Mr (<14kDa) cut-off did not result in the loss of all CAF activity. This showed that CAF was a dialysable cytosolic factor of molecular weight >14 kDa and <200kDa (Table 8).

The in vitro activation of LktA and Hyb2 were also examined under the same conditions. The complete activation mix of LktA, LktC and CAF was active in the haemolysis assay with sheep erythrocytes and cytotoxic assay with BL3 cells. The LktC-activated LktA was not cytotoxic for the mouse macrophage cell line J774.2 in the MTT assay, but it did inhibit the CL response of rabbit neutrophils to OZ stimulation as well as being active against the CL response of bovine neutrophils (Figs 32 and 33, Table 9).

3.12.4 Trans-activation of CyaA, LktA and Hyb2 by CyaC and LktC

Trans-activation of CyaA, LktA or Hyb2 by CyaC or LktC was tested. The CyaC-activated LktA in vitro was haemolytic on sheep erythrocytes and cytotoxic for BL3 cells. LktC could not activate CyaA to an appreciable extent in the cell invasive, haemolytic or cytotoxic assays (Figs 28-31), but there was noticeable activity in the CI assays with both bovine and rabbit neutrophils (Figs 32 and 33). CyaC gave some activation to LktA for all the assays tested

Table 9.

In vitro activation of CyaA and LktA protoxins by complementation with LktC or CyaC

Activation reaction mix	Haemolytic activity [a] μg protein/ml ± SEM* for 20% haemolysis of sheep RBC	Cytotoxic activity [a] μg protein/ml for 50% CL inhibition of neutrophils		Cytotoxic activity [a] μg protein/ml for 50% cell killing of	
		bovine	rabbit	J774.2	BL3
CyaA+CyaC+CAF	15.81±2.31	1.94±0.58	2.26±0.89	10.83±1.73	18.27±1.32
CyaA+LktC+CAF	49.35±4.87	19.81±2.39	33.73±3.74	>186	>213
LktA+CyaC+CAF	32.57±1.38	69.34±4.42	18.92±2.18	>504	>133
LktA+LktC+CAF	11.94±1.42	2.93±0.81	36.14±0.67	>302	30.86±3.26
Hyb2+CyaC+CAF	41.17±3.69	ND	ND	>111	>104
Hyb2+LktC+CAF	21.63±1.65	ND	ND	>98.83	46.43±2.37
CyaA+CyaC	>76	>112	>127	>256	>269
CyaA+LktC	>90	>126	>135	>268	>281
LktA+CyaC	>74	>141	>150	>541	>376
LktA+LktC	>69	>114	>132	>438	>269
Native LktA [b]	ND	1.15	>87	ND	ND

[a] Data are the mean values and ± SEM of 4 determinations.

[b] Leukotoxin extracted from the natural producer *Pasteurella haemolytica*.

(Figs 28-33). LktA showed no cytotoxicity for J774.2 cells irrespective of which C protein was used (Fig. 30). LktA activated by either C protein was active for cytotoxicity on BL3 cells, although LktC produced a more active toxin (Fig. 31. Therefore activation of LktA by CyaC did not alter the specificity of the active LktA for ruminant cells as was found for the in vivo produced toxin (section 3.11). LktC activated Hyb2 to a noticeably higher extent than CyaC for haemolysis of sheep erythrocytes which was similar to that found in vivo (Tables 7 and 9). Hyb2 toxin activated by CyaC or LktC protein showed similar low cytotoxicity for J774.2 cells, but LktC-activated Hyb 2 was more active for BL3 cells. Again this was found in vivo (section 3.11).

3.12.5 In vitro activation with in vitro acylated acyl-ACP

The experiments described in section 3.12.3 showed that the cytosolic activating factor could be lost by dialysis. Previous work on HlyC activation of HlyA had shown that acyl-ACP was a cofactor in the activation reaction (Hardie *et al.*, 1991; Issartel *et al.*, 1991). This was investigated here for CyaC activation of CyaA and LktC activation of LktA (Tables 10-13). Commercial ACP, charged in vitro with palmitic acid, was able to act as a cofactor in place of CAF to activate CyaA or LktA (Tables 10-13). When the activating component of CAF had been removed by dialysis, the dialysed CAF could still act as source of acyl-ACP synthetase if used in the reaction to charge ACP in vitro instead of commercial acyl-ACP synthetase (section 2.18.3) (Table 10). If either palmitic acid or acyl-ACP synthetase were left out of the reaction to

Table 10.

In vitro activation of CyaA protoxin by acyl carrier protein charged with various acyl groups

Activation reaction mix	Cytotoxic activity [a] μg protein/ml for 50% cell killing of J774.2	BL3
CyaA+CyaC+ACP +ACP synthetase+PA	18.43±3.70	23.61±2.19
CyaA+CyaC+ACP +ACP synthetase+MA	26.57±4.83	34.58±3.15
CyaA+CyaC+ACP +ACP synthetase+LA	>32	>48
CyaA+CyaC+ACP +ACP synthetase+SA	>47	>59
CyaA+CyaC+ACP +ACP synthetase+PLA	>29	>38
CyaA+CyaC+ACP +ACP synthetase+OA	>57	>61
CyaA+CyaC+ACP +ACP synthetase	>168	>174
CyaA+CyaC+dHCAF +ACP+PA	33.34±2.83	38.43±4.15
CyaA+CyaC	>175	>184

[a]Results are the mean values ± SEM of 4 determinations.

dHCAF= CAF dialysed with a membrane of high Mr (200 kDa) cut-off ACP= Commercial acyl carrier protein, ACP synthetase= Commercial acyl carrier protein synthetase, PA=Palmitic acid, MA=Myristic acid, LA= Lauric acid, SA= stearic acid, OA= Oleic acid, PLA= Palmitoleic acid.

Table 11.
In vitro activation of LktA protoxin

Activation reaction mix	Cytolytic activity [a] μg protein/ml for 20% haemolysis of sheep RBC	Cytotoxic activity [a] μg protcin/ml for 50% cell killing of J774.2	BL3
LktA+LktC+CAF	12.67±1.35	>302	33.41±2.94
LktA+LktC+ACP +ACP synthetase+PA	16.24±2.73	>364	37.36±2.51
LktA+LktC+ACP +ACP synthetase	>85	>379	>162
LktA+LktC+ACP synthetasc	>91	>484	>175
LktA+LktC+ACP	>120	>461	>270
LktA+LktC	>173	>475	>281
LktA+CAF	>164	>458	>268
CAF	>272	>769	>374

[a]Results are the mean values ± SEM of 4 determinations.
CAF=Cytosolic activating factor, ACP= Commercial acyl carrier protein, ACP synthetase =Commercial acyl carrier protein synthetase, PA=Palmitic acid.

Table 12.

In vitro activation of LktA protoxin by acyl carrier protein charged with various acyl groups

Activation reaction mix	Cytotoxic activity μg protein/ml for 50% cell killing of	
	J774.2	BL3
LktA+LktC+ACP +ACP synthetase+PA	>61	36.43±2.19
LktA+LktC+ACP +ACP synthetase+MA	>74	47.90±2.52
LktA+LktC+ACP +ACP synthetase+LA	>76	59.32±3.79
LktA+CyaC+ACP +ACP synthetase+SA	>81	62.41±4.27
LktA+LktC+ACP +ACP synthetase+PLA	>71	51.26±1.62
LktA+LktC+ACP +ACP synthetase+OA	>78	66.31±3.47
LktA+LktC+ACP +ACP synthetase	>184	>162
LktA+LktC	>286	>175

[a]Results are the mean values ± SEM of 4 determinations.

ACP= Commercial acyl carrier protein, ACP synthetase= Commercial acyl carrier protein synthetase, PA=Palmitic acid, MA=Myristic acid, LA= Lauric acid, SA= stearic acid, OA= Oleic acid, PLA= Palmitoleic acid.

Table 13.

In vitro activation of hybrid 2 protoxin by acylation with various acyl groups

Activation reaction mix	Cytotoxic activity μg protein/ml for 50% Cell killing of J774.2	BL3
Hyb2+CyaC+CAF	>111	>104
Hyb2+CyaC+ACP +ACP synthetase+PA	>132	>124
Hyb2+CyaC+ACP +ACP synthetase+MA	>146	>138
Hyb2+CyaC+ACP +ACP synthetase+LA	>152	>150
Hyb2+CyaC+ACP +ACP synthetase+PLA	>149	>144
Hyb2+LktC+CAF	98.83	46.43
Hyb2+LktC+ACP +ACP synthetase+PA	>115	75
Hyb2+LktC+ACP	>137	>129
Hyb2+LktC+ACP +ACP synthetase+LA	>152	>144
Hyb2+LktC+ACP +ACP synthetase+PLA	>148	>136
Hyb2+LktC+ACP +ACP synthetase	>172	>161

[a]Results are the mean values ± SEM of 4 determinations.

ACP= Commercial acyl carrier protein, ACP synthetase= Commercial acyl carrier protein synthetase, PA=Palmitic acid, MA=Myristic acid, LA= Lauric acid, PLA= Palmitoleic acid.

acylate ACP in vitro, then no activation of either toxin was observed when the reaction mix was added to the C proteins (Tables 10 and 11).

In further experiments, the effect of altering the fatty acid used by acyl-ACP synthetase to charge ACP was investigated (Tables 10, 12 and 13). The principal reason for doing this was to see if changing the fatty acyl group on the active toxin had any effect on target cell specificity of the toxin, particularly for LktA which is normally ruminant cell-specific. The data showed that charging ACP with palmitic acid created the most effective acyl-ACP for the in vitro activation of both CyaA and LktA by their respective C proteins (Tables 10 and 12). Other fatty acids used were less effective. For the fatty acids that were suitable, in all cases the CyaC-activated CyaA was more active against J774.2 cells than against BL 3 cells (Table 10). The fatty acid preference for charging either CyaA or LktA was PA>MA>PLA>LA> OA and SA, indicating that CyaC and LktC exhibited the same fatty acid preference for their action (Tables 10 and 12). LktA charged with either PA, MA, PLA or LA was inactive against J774.2 cells, indicating no alteration in the target cell specificity of the toxin dependent on the activating acyl group (Table 12). The same pattern of fatty acid preference for in vitro activation of Hyb2 with both C proteins was observed (Table 13), although Hyb2 activated by CyaC was barely active. As with the situation in vivo, Hyb2 activated by LktC was more active and again showed greater activity towards BL3 cells than J774.2 cells.

3.12.6 Effect of various CAF preparations on in vitro activation

Cellular extracts were obtained from cultures of *B. pertussis* 348 and *P. haemolytica* (Ph30 isolate) and tested as sources of CAF in the activation

reaction in place of the *E. coli* CAF. None of the extracts was haemolytic on its own. The results are summarised in Table 14 and show that other Gram-negative bacteria could be used as a source of CAF in activation reactions with roughly equal efficiency.

3.12.7 Effect of chloride ions and EGTA on in vitro activation

Previously reports had indicated that Cl^- ions were inhibitory for the in vitro activation of proHlyA by HlyC (Hardie *et al.*, 1991). Results given in Table 14 show that chloride ions had a marked inhibitory effect on activation of proCyaA by CyaC, but the inclusion of EGTA (5 mM) increased activation efficiency by approximately 15%.

3.13 IMMUNOLOGICAL PROPERTIES OF CyaA AND HYBRID TOXINS

3.13.1 Mouse weight gain test for toxicity

The effect of vaccination of mice with CyaA preparations from *B. pertussis* strains and recombinant *E. coli* strains at a dose of 15 μg protein/mouse was studied over 21 days post-vaccination. Male 3-4 week old CD-1 mice were randomised in groups of 10 and injected subcutaneously with two doses of vaccines at a two week interval. The mice were examined and weighed over 21 days, especially during the first 3 days after each vaccination. Any deaths or changes in weight by comparison with control groups were recorded. The experiment was performed twice (Experiments 1 and 2) and the

Table 14.

Effect of chloride ions, EGTA and various CAF preparations on the in vitro activation of CyaA protoxin

Activation reaction mix	Haemolytic activity [a] μg protein/ml for 20% haemolysis of sheep RBC	Invasive activity [a] μg protein/ml for 50% stellation of BHK cells	Cytotoxic activity [a] μg protein/ml for 50% CL inhibition of bovine neutrophils
CyaA+CyaC+Ec CAF[b]	14.51±3.29	25.16±1.43	16.47±1.16
CyaA+CyaC+Bp CAF[c]	12.73±2.73	24.81±1.19	17.86±1.47
CyaA+CyaC+Ph CAF[d]	17.38±3.12	28.13±2.15	15.39±2.03
CyaA+CyaC+ Ec CAF[b] +5mM Na Cl	25.51±1.83	32.57±1.60	28.40±2.19
CyaA+CyaC+ Ec CAF[b] +10mM Na Cl	42.17±3.45	46.82±2.39	39.26±1.32
CyaA+CyaC+ Ec CAF[b] +2mM EGTA	12.62±2.27	22.35±1.09	13.92±0.76
CyaA+CyaC+ Ec CAF[b] +5mM EGTA	12.38±1.83	19.83±0.85	12.66±0.39

a. Data are the mean values ± SEM of 4 determinations

b , c, d= represent CAF preparations from *E. coli* BL21 (Ec), *B. pertussis* BP 348 (Bp) and *P. haemolytica* (Ph) respectively.

data for the two separate experiments are shown in Figs 34 and 35. The results indicated that all mice in each group gained weight over of the 21 day post-vaccination period and no deaths were recorded. There was some indication overall that the active CyaA, whether native or recombinant, had a slightly detrimental effect on mouse weight gain. In a similar experiment (Experiment 3) the effect of vaccination with the hybrid toxin preparations Hyb1 and Hyb 2 was compared with PAR CyaA (Fig. 36, Panel A). Hybrid toxins showed no toxicity in mice over the 21 day post-vaccination period and, in this experiment, neither did the active CyaA.

3.13.2 Mouse protection with CyaA and hybrid preparations

The protective activities of CyaA and hybrid preparations were examined by their abilities to protect mice against intranasal challenge with *B. pertussis* 18323. The mice were vaccinated with two doses of vaccine preparation and were challenged intranasally with a sublethal dose of approximately 10^5cfu/mouse *B. pertussis* 18323 one week after the second vaccination (section 2.21.3). The experiments were performed twice (Experiments 1 and 2). At 7 days post-challenge 5 mice were sacrificed and weighed individually. Their lung pathology, lung weight and lung counts of *B. pertussis* were determined. Mouse weight gain data, the results of lung counts and weight of lungs as percentage body weight for each experiment are given in Tables 15-17 and shown in Figs 36-38.

After challenge, mice vaccinated with active CyaA (CAN CyaA, PAN CyaA, CAR CyaA and PAR CyaA), active Hyb2 (PAR Hyb2/CyaC or PAR Hyb2/LktC) or whole- cell DPT vaccine (Experiments 1, 2 and 3) continued to

Fig. 34

Mouse weight gain after vaccination with crude or purified CyaA preparations, whole cell DPT vaccine or alhydrogel (Experiment No.1).

Male HAM ICR (CD-1) mice, 3-4 weeks old, randomised in groups of 10, were vaccinated subcutaneously twice at a two week interval with 15 μg protein/mouse of crude or purified CyaA preparation from *B. pertussis* or recombinant *E. coli* strains. The CyaA was absorbed on 250 μg alhydrogel. Control mice were vaccinated with 1/5 th human dose/mouse of commercial whole cell DPT vaccine or 250 μg alhydrogel. The mice were weighed at intervals after vaccination.

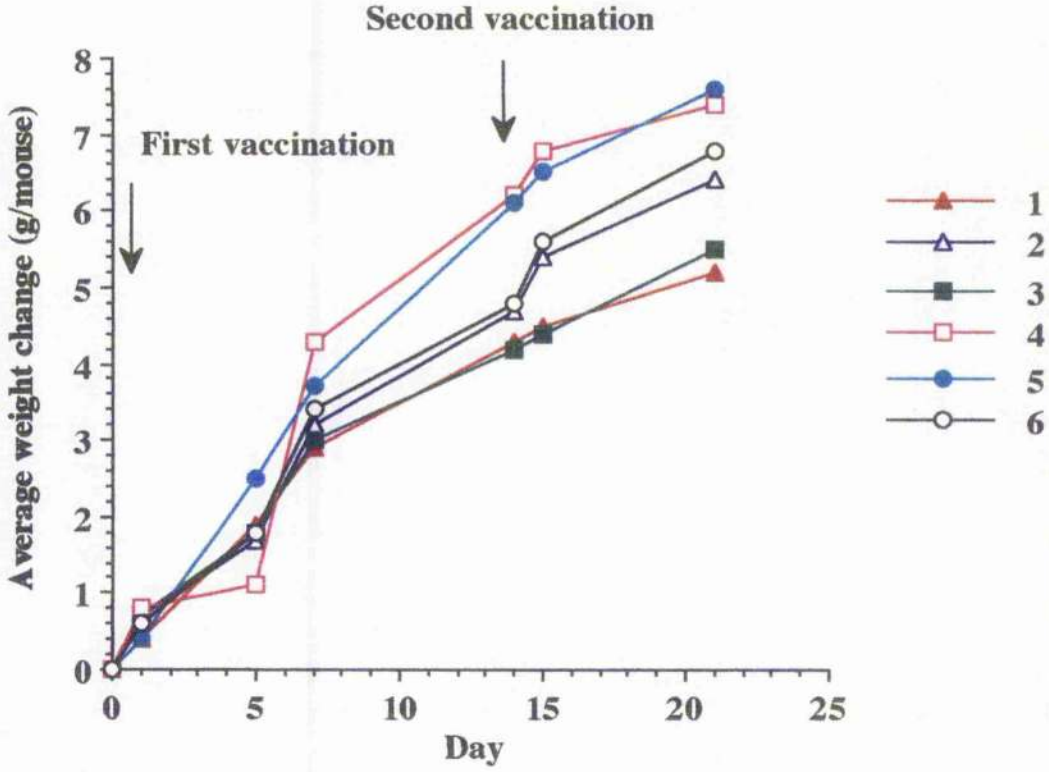
Panel A :

1 CAN CyaA
2 CIN CyaA
3 PAN CyaA
4 PIN CyaA
5 Whole cell DPT vaccine
6 Alhydrogel alone

Panel B :

1 CAR CyaA
2 CIR CyaA
3 PAR CyaA
4 PIR CyaA
5 Control (no vaccination)

A


Second vaccination
First vaccination
Average weight change (g/mouse)
Day
0
1
2
3
4
5
6
7
8
0
5
10
15
20
25
1
2
3
4
5
6

B

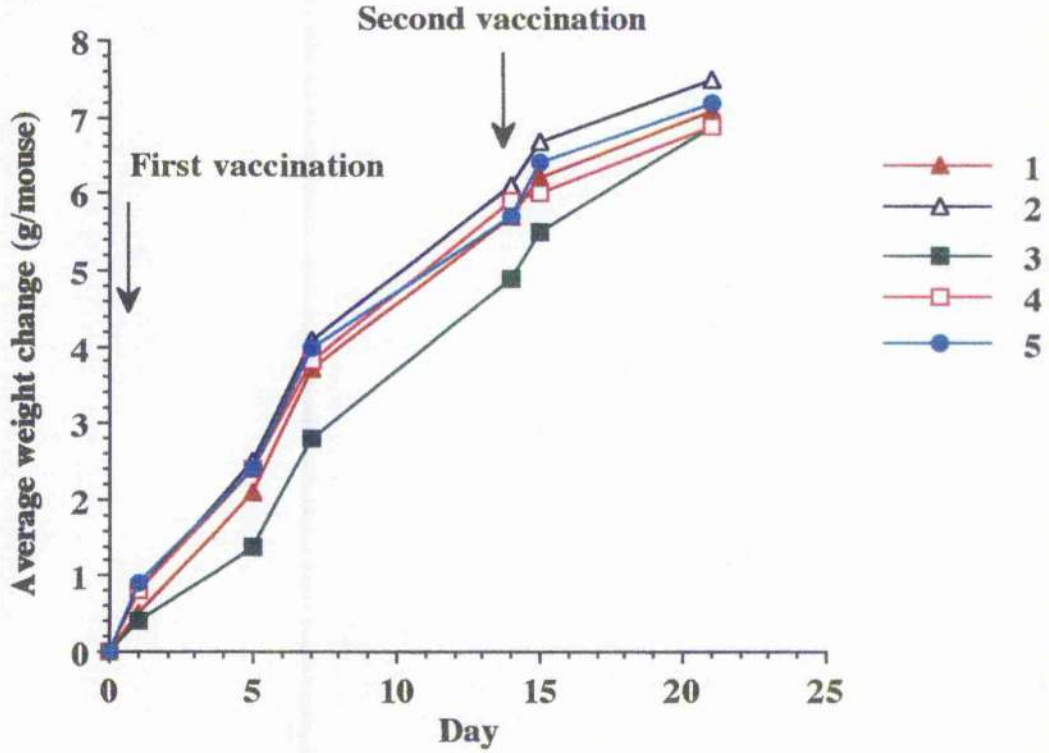

Second vaccination
First vaccination
Average weight change (g/mouse)
Day
0
1
2
3
4
5
6
7
8
0
5
10
15
20
25
1
2
3
4
5

Fig. 35

Mouse weight gain after vaccination with crude or purified CyaA preparations, whole cell DPT vaccine or alhydrogel (Experiment No. 2).

Experiment No. 2 was carried out under the same conditions as Experiment No.1(see legend of Fig.34).

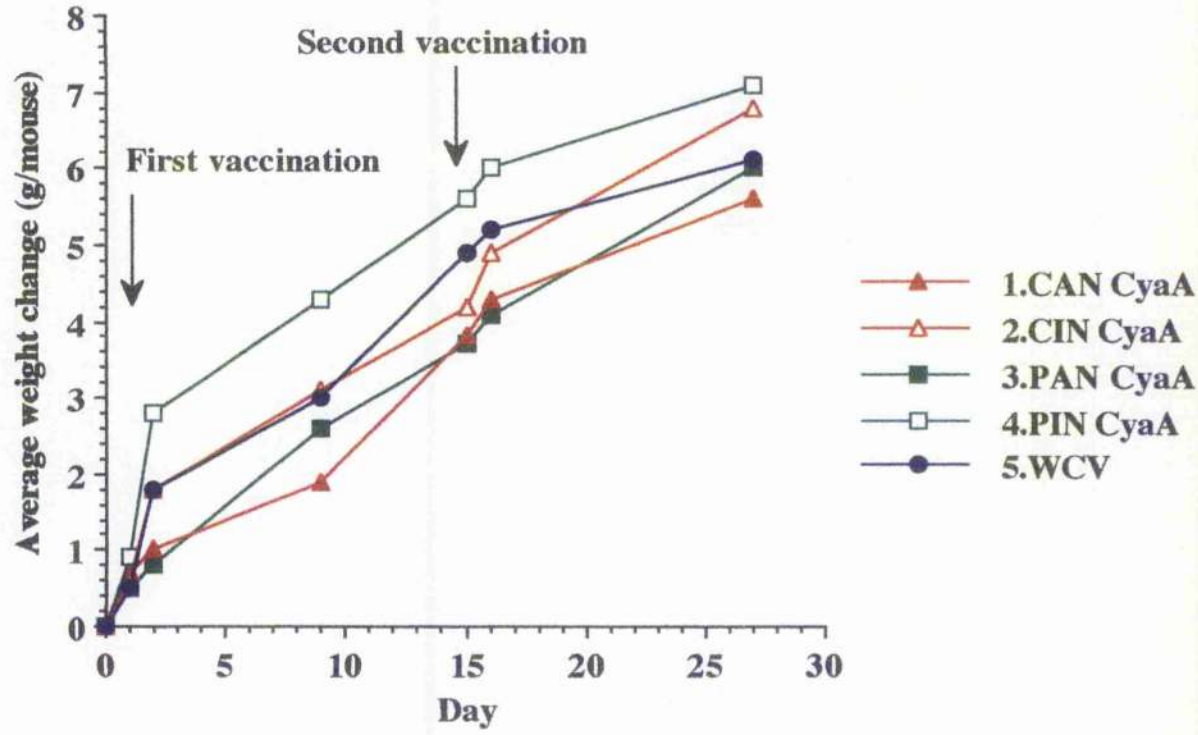
Panel A :

1 CAN CyaA
2 CIN CyaA
3 PAN CyaA
4 PIN CyaA
5 Whole cell DPT vaccine

Panel B :

1 CAR CyaA
2 CIR CyaA
3 PAR CyaA
4 PIR CyaA
5. Alhydrogel alone

A


Second vaccination
First vaccination
Average weight change (g/mouse)
Day
0
1
2
3
4
5
6
7
8
0
5
10
15
20
25
30
1.CAN CyaA
2.CIN CyaA
3.PAN CyaA
4.PIN CyaA
5.WCV

B

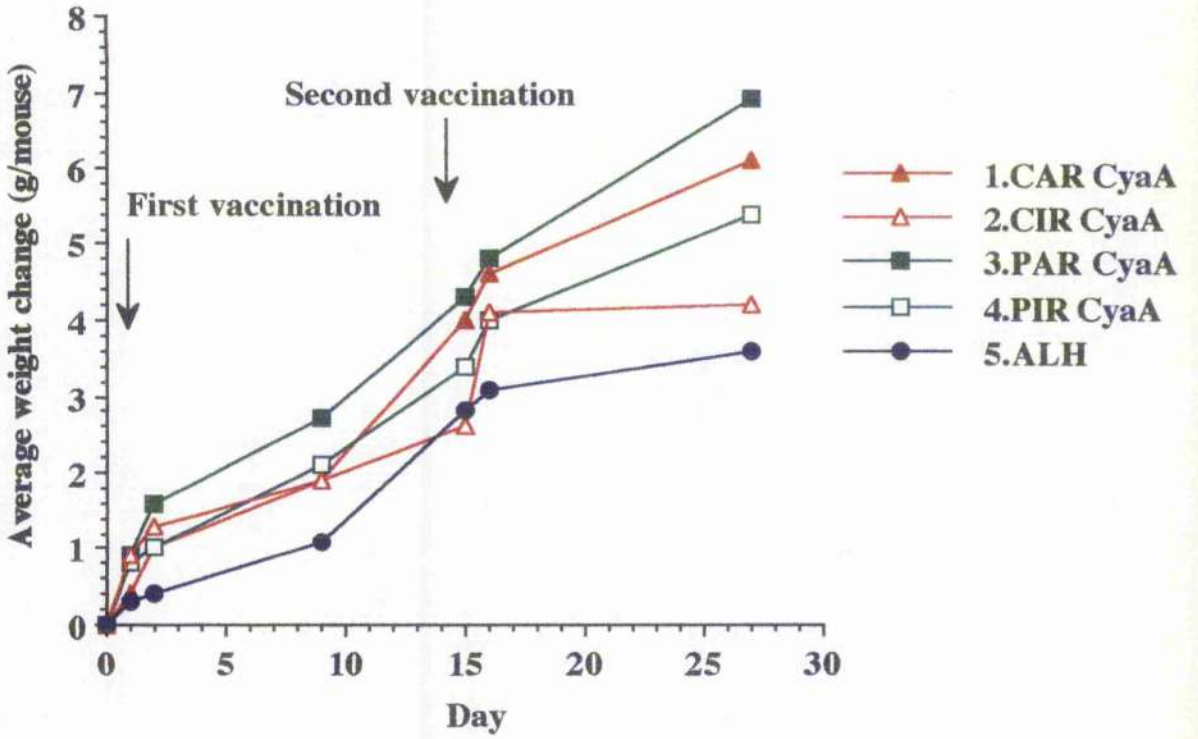

Second vaccination
First vaccination
Average weight change (g/mouse)
Day
0
1
2
3
4
5
6
7
8
0
5
10
15
20
25
30
1.CAR CyaA
2.CIR CyaA
3.PAR CyaA
4.PIR CyaA
5.ALH

Fig. 36

Weight gain of mice vaccinated with purified hybrid1 or hybrid 2 or CyaA preparations , whole cell DPT vaccine or alhydrogel for 3 weeks before challenge and for one week after challenge.

Male HAM ICR (CD-1) mice, 3 weeks old, randomised in groups of 10, were vaccinated subcutaneously twice at a two week interval with 15 μg/mouse of purified hybrid or CyaA preparations from recombinant *E. coli* strains absorbed on 250 μg alhydrogel, 0.2 human dose/mouse of whole cell DPT vaccine or 250 μg alhydrogel.

Panel A,B : Mouse weight gain before challenge (A) or after challenge (B)

1 Pure Hyb 2 from *E. coli* BL21/DE3 (pGW88)

2 Pure Hyb 2/CyaC from *E. coli* BL21/DE3 (pGW88,54)

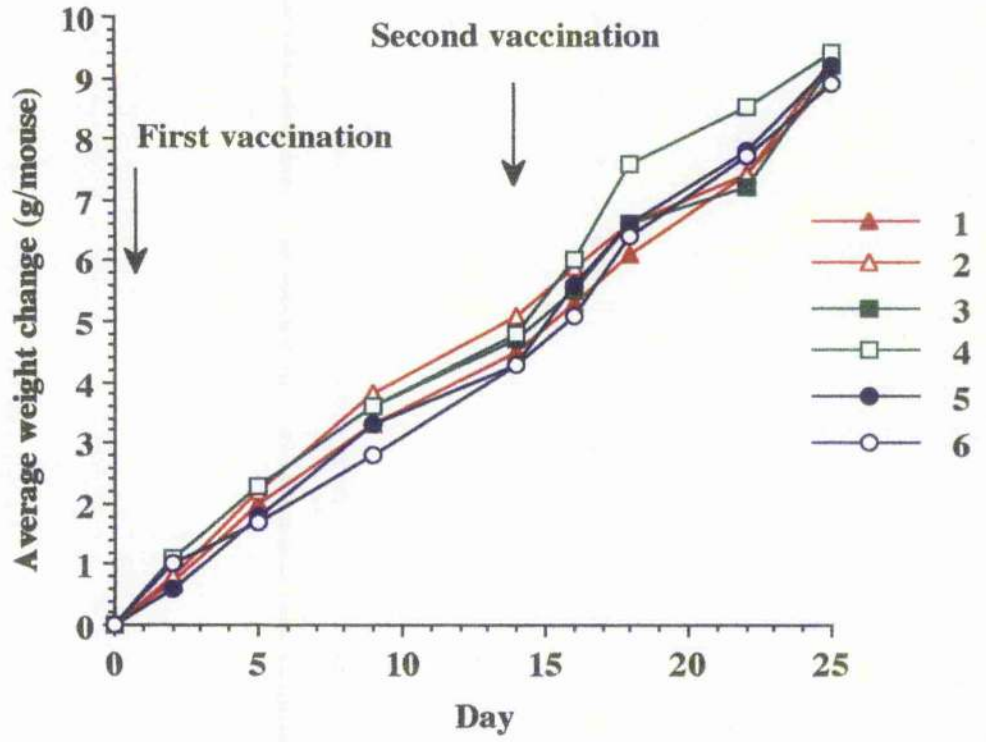
3 Pure Hyb 2/LktC from *E. coli* BL21/DE3 (pGW88,78)

4 Pure Hyb 1 from *E. coli* BL21/DE3 (pGW80,54)

5 Pure CyaA from *E. coli* BL21(pCACT3)

6 Alhydrogel

A


Second vaccination
First vaccination
Average weight change (g/mouse)
Day
0
1
2
3
4
5
6
7
8
9
10
0
5
10
15
20
25
1
2
3
4
5
6

B

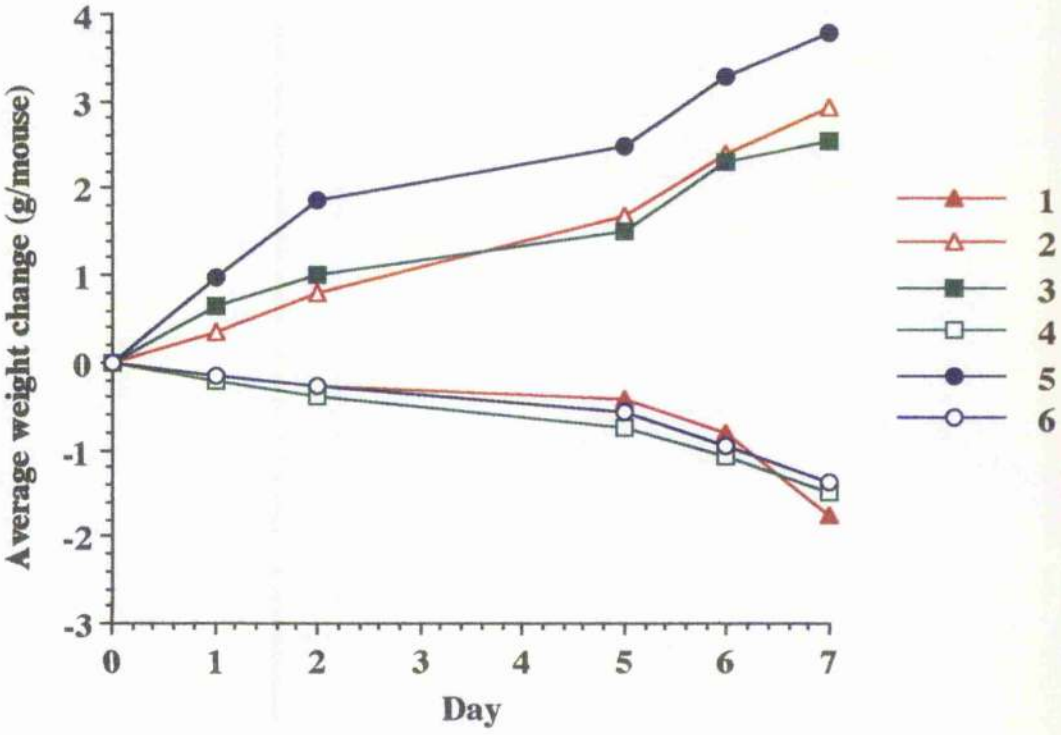

Average weight change (g/mouse)
Day
4
3
2
1
0
-1
-2
-3
0
1
2
3
4
5
6
7
1
2
3
4
5
6

Fig. 37

Weight gain of mice vaccinated with crude or purified CyaA preparations, whole cell DPT vaccine or alhydrogel, after challenge with *B. pertussis* 18323 (Experiment No. 1).

The mice, randomised in groups of 5 were challenged intranasally with *B. pertussis* 18323 (10^5 cfu/mouse), one week after the last vaccination with crude or purified CyaA preparations or whole cell DPT vaccine or alhydrogel. The mice were weighed daily for one week after challenge .

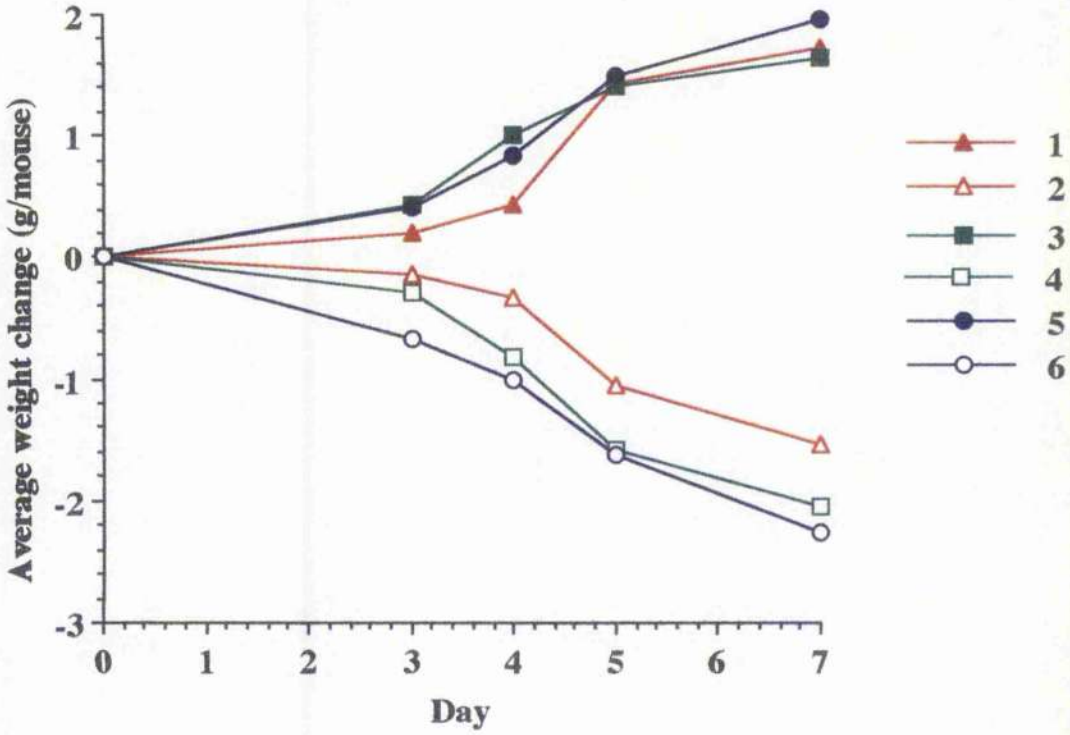
Panel A :

1 CAN CyaA
2 CIN CyaA
3 PAN CyaA
4 PIN CyaA
5 Whole cell DPT vaccine
6 Alhydrogel alone

Panel B :

1 CAR CyaA
2 CIR CyaA
3 PAR CyaA
4 PIR CyaA
5 Control (no vaccination, no challenge)

A


Average weight change (g/mouse)
2
1
0
-1
-2
-3
0
1
2
3
4
5
6
7
Day
1
2
3
4
5
6

B

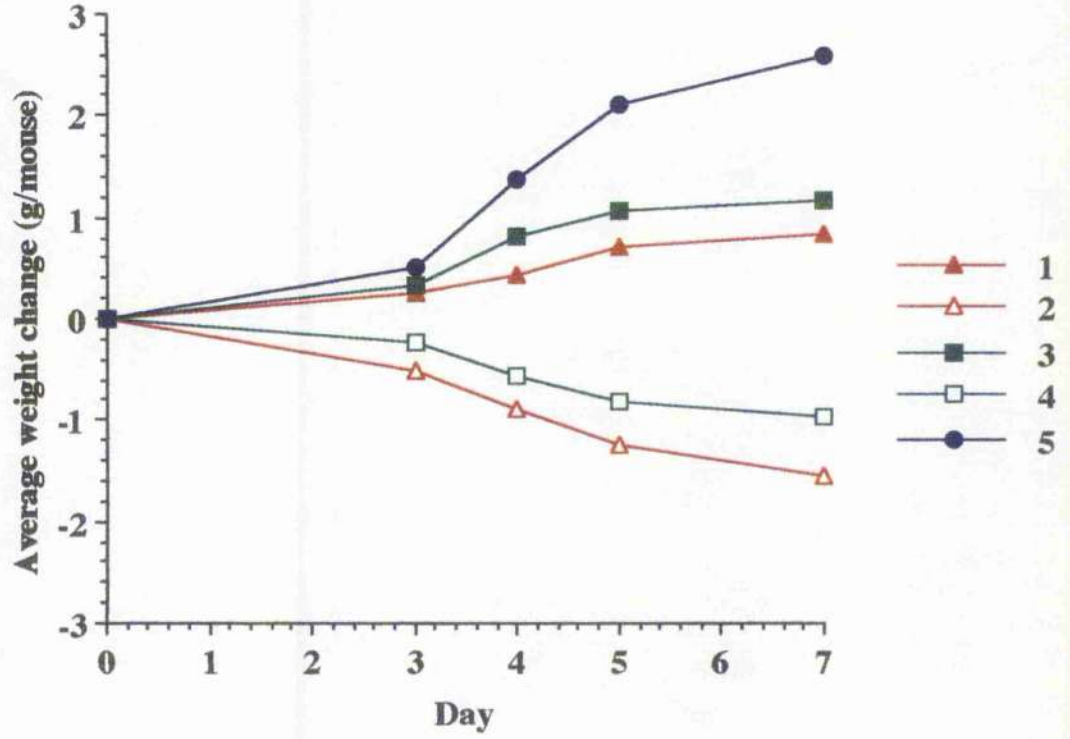

Average weight change (g/mouse)
3
2
1
0
-1
-2
-3
0
1
2
3
4
5
6
7
Day
1
2
3
4
5

Fig. 38

Weight gain of mice vaccinated with crude or purified CyaA preparations, whole cell DPT vaccine or alhydrogel, after challenge with *B. pertussis* 18323 (Experiment No. 2).

Experiment No.2 was done under the same conditions as Experiment No.1 (see legend of Fig. 37)

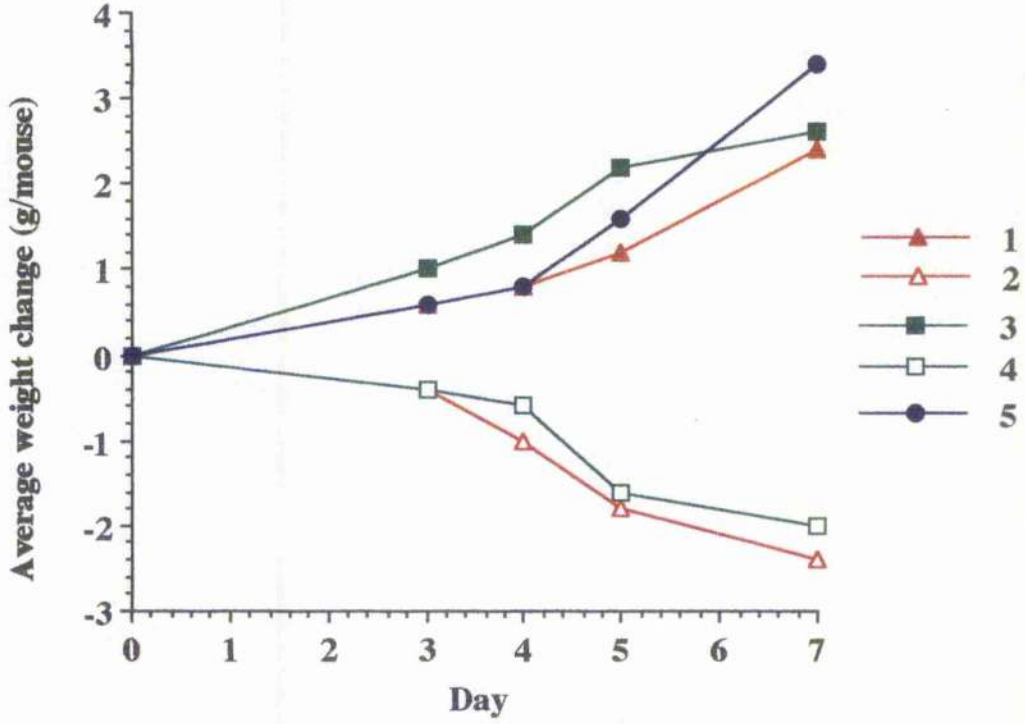
Panel A :

1 CAN CyaA
2 CIN CyaA
3 PAN CyaA
4 PIN CyaA
5 Whole cell DPT vaccine

Panel B :

1 CAR CyaA
2 CIR CyaA
3 PAR CyaA
4 PIR CyaA
5 Alhydrogel

A


Average weight change (g/mouse)
4
3
2
1
0
-1
-2
-3
0
1
2
3
4
5
6
7
Day
1
2
3
4
5

B

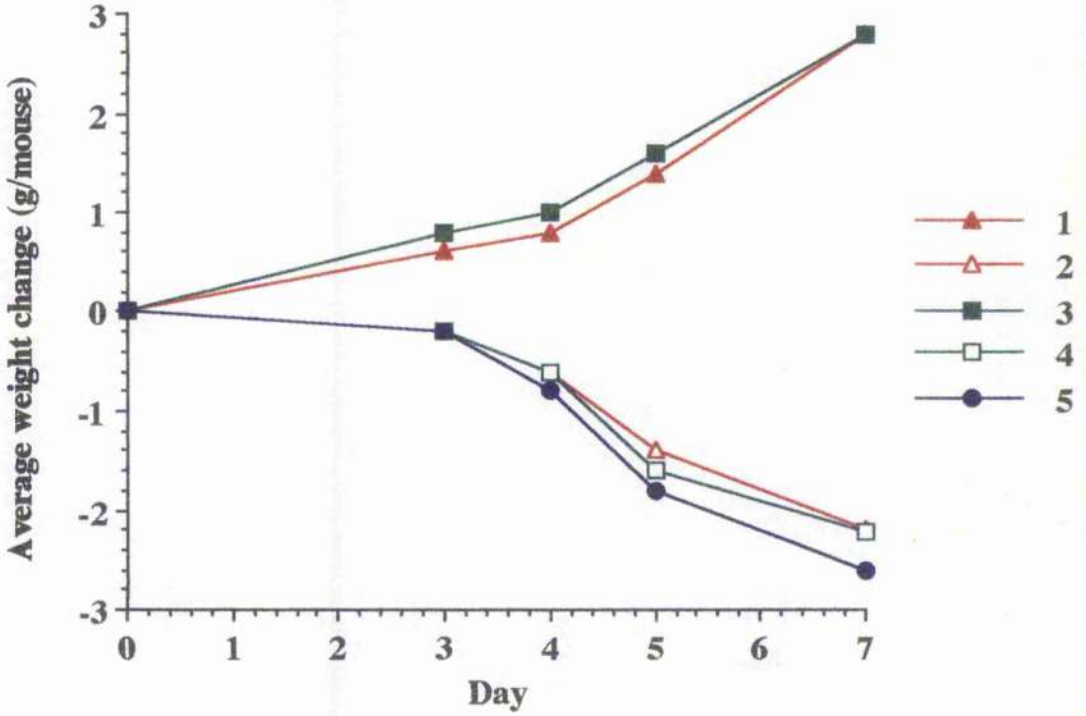

Average weight change (g/mouse)
3
2
1
0
-1
-2
-3
0
1
2
3
4
5
6
7
Day
1
2
3
4
5

Table 15.

Active protection against intranasal challenge with *B. pertussis* 18323 strain by vaccination of mice with various CyaA preparations from *B. pertussis* and recombinant *E. coli* strains (Experiment No. 1).

Vaccine preparation	Mean lung weight (g) as %of body weight [a]	Mean or median *B. pertussis* count (cfu/lung) [a]
PAN CyaA	0.73±0.02	$(2.60\pm 0.11)\times10^3$
CAN CyaA	0.81±0.03	$(5.260\pm 0.82)\times10^3$
PIN CyaA	1.29±0.04	1.70×10^6 (median)
CIN CyaA	1.13±0.02	$(1.64\pm 0.15)\times10^6$
PAR CyaA	0.89±0.02	$(3.52\pm 0.33)\times10^3$
CAR CyaA	1.02±0.04	$(5.50\pm 0.24)\times10^3$
PIR CyaA	1.37±0.03	4.50×10^6 (median)
CIR CyaA	1.33±0.05	3.50×10^6 (median)
Alhydrogel	1.45±0.02	$(4.94\pm 0.64)\times10^6$
WCV	0.93±0.02	$(4.78\pm 1.17)\times10^3$
Control [b]	0.71±0.01	<25

a. Results are the mean values from 5 individual mice ± SEM or median values for lung counts in the cases with counting recorded from confluent culture plates.

b. Control= non-vaccinated and non-challenged

Table 16.

Active protection against intranasal challenge with *B. pertussis* 18323 strain by vaccination of mice with various CyaA preparations from *B. pertussis* and recombinant *E. coli* strains (Experiment No. 2).

Vaccine preparation	Mean lung weight (g) as %of body weight [a]	Mean *B. pertussis* count (cfu/lung) [a]
PAN CyaA	0.82±0.05	$(0.68\pm 0.18)\times10^3$
CAN CyaA	0.97±0.04	$(0.83\pm 0.21)\times10^3$
PIN CyaA	1.42±0.03	$(4.22\pm 0.38)\times10^6$
CIN CyaA	1.25±0.05	$(3.76\pm0.26)\times10^6$
PAR CyaA	1.01±0.04	$(0.91\pm 0.28)\times10^3$
CAR CyaA	1.11±0.06	$(2.76\pm 0.25)\times10^3$
PIR CyaA	1.45±0.06	$>2.5\times10^6$
CIR CyaA	1.31±0.06	$>2.5\times10^6$
Alhydrogel	1.45±0.07	$(5.73\pm0.42)\times10^6$
WCV	0.84±0.01	$<0.5\times10^3$

[a]Results represent the mean values from 5 individual mice ±SEM

Table 17.

Active protection against intranasal challenge with *B. pertussis* 18323 strain by vaccination of mice with purified CyaA and hybrid preparations from recombinant *E. coli* strains (Experiment No. 3).

Vaccine preparation	Mean lung weight (g) as %of body weight [a]	Mean *B. pertussis* count (cfu/lung) [a]
Hyb2	1.47± 0.07	>2.50x10^7
Hyb2/LktC	0.94± 0.01	(1.26± 0.15)x10^4
Hyb2/CyaC	1.00± 0.01	(1.57± 0.13)x10^4
Hyb1/CyaC	1.37± 0.10	>2.50x10^7
PIR CyaA	1.39± 0.06	>2.50x10^7
PAR CyaA	0.86± 0.03	(0.59± 0.16)x10^4
PAR CyaA(pCACT3)	0.91± 0.02	(0.99± 0.12)x10^4
Alhydrogel	1.55± 0.09	>2.50x10^7
WCV	0.87± 0.02	(0.36±0.05)x10^4

[a]Results represent the mean value from 5 individual mice ± SEM .

gain weight in a similar manner to those that were left as non-vaccinated, non-challenged controls. The mice vaccinated with both crude or purified inactive CyaA or alhydrogel alone lost weight quite markedly. Two deaths were recorded in the group vaccinated with alhydrogel two days after challenge, but no deaths were observed in groups vaccinated with CIN, PIN, CIR or PIR preparations.

The lungs of mice vaccinated with any of the inactive toxin preparations as well as those mice vaccinated only with alhydrogel showed extensive consolidation (greyish discoloured areas) in all lobes of the lungs, whereas the mice vaccinated with active toxins as well as the mice vaccinated with whole cell DPT vaccine showed little sign of consolidation (data not shown).

The lung weight data and lung counts of *B. pertussis* for experiments 1 and 2 are presented in Tables 15 and 16. There were clear differences in the lung weight as a percentage of body weight (up to 2 fold) of mice vaccinated with any of the inactive toxins (i.e. CIN, CIR, PIN and PIR CyaA) and alhydrogel compared to mice vaccinated with any of the active toxins (i.e. CAN, CAR, PAN, PAR CyaA) and whole cell DPT vaccine as well as non-vaccinated, non-challenged control mice. The lung counts of *B. pertussis* from mice vaccinated with any of the inactive toxins and alhydrogel showed up to 3 $\log_{10}$ differences compared to mice vaccinated with active toxins or whole cell vaccine. The results from the second experiment were consistent with the first experiment.

The lung counts of mice vaccinated with Hyb2 modified with LktC or CyaC indicated that this hybrid toxin was protective whether modified by CyaC or LktC (Table 17). Interestingly, the CyaC-modified Hyb2 seemed to be slightly less protective than the LktC-modified form, which paralleled the slightly lower toxicity of this hybrid (Table 7). Modified Hyb1 showed no

protective activity. Lack of time did not allow the experiment with the hybrid toxins to be repeated. Taken together, these experiments indicated that only active toxins, as well as whole cell vaccine, were protective, as indicated by the low lung weight, low bacterial numbers in the lung and lack of effect on mouse weight gain compared to non-vaccinated, control mice.

3.14 ANTIGENICITY OF CyaA AND Hyb PREPARATIONS

3.14.1 Immunoblotting of purified CyaA with various mouse sera

Western blot analysis of purified CyaA with 1/1000 dilution of sera of mice vaccinated with purified CyaA preparations are shown in Figs 39 and 40. As is evident from the figures, antisera from mice vaccinated with PAN CyaA or PIN CyaA showed strong cross-reaction with the major 200 kDa CyaA band and bands of lower M_r (presumably degradation products of CyaA). There was a slight indication of cross reaction of PAN and PIN CyaA preparations with antibodies present in mice vaccinated with whole cell vaccine although the bands did not correspond to the CyaA bands (Fig. 39, lanes 9 and 10), and this was also apparent with PAR and PIR samples (Fig. 40 lanes 5 and 11).

3.14.2 Development of ELISA for detection of antibodies to CyaA toxin or ovalbumin

In order to determine the concentration of CyaA or ovalbumin suitable for coating antigen in ELISA, the wells of ELISA Dynatech Immulon-2 plates were coated with different dilutions of PAR CyaA or ovalbumin and the

Fig. 39

Western blot analysis of purified CyaA preparations using sera from mice vaccinated with PAN CyaA, PIN CyaA, DPT and alhydrogel.

Purified CyaA (5 μg protein/lane) preparations from *B. pertussis* or recombinant *E. coli* strains were subjected to electrophoresis on a 10% polyacrylamide gel and transferred to a Hybond C-Super membrane. The nitrocellulose was cut into strips and probed with sera collected from groups of 5 mice in experiment No. 1 on day 7 after the second dose of vaccine i.e. at the time of challenge of identical groups of mice. Mice were vaccinated with various purified CyaA preparations from *B. pertussis* plus alhydrogel, whole cell DPT vaccine or with alhydrogel alone. The numbers on the left of blots indicate the positions of molecular weight standards.

	A: Antigen preparation bound to nitrocellulose	B: Probed with sera from mice vaccinated with:
Lane 1 :	PAN CyaA	PAN CyaA
Lane 2 :	PIN CyaA	PAN CyaA
Lane 3:	PAR CyaA	PAN CyaA
Lane 4 :	PIR CyaA	PAN CyaA
Lane 5 :	PAN CyaA	PIN CyaA
Lane 6 :	PIN CyaA	PIN CyaA
Lane 7:	PAR CyaA	PIN CyaA
Lane 8 :	PIR CyaA	PIN CyaA
Lane 9 :	PAN CyaA	DPT vaccine
Lane 10 :	PIN CyaA	DPT vaccine
Lane 11 :	PAN CyaA	Alhydrogel
Lane 12:	PIN CyaA	Alhydrogel

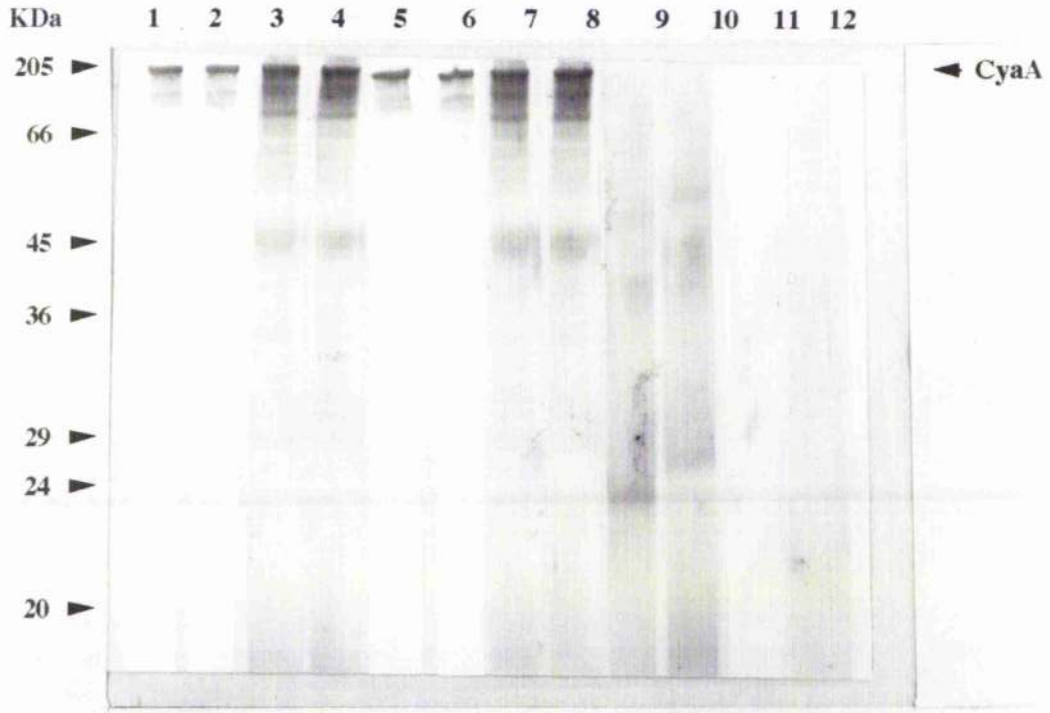
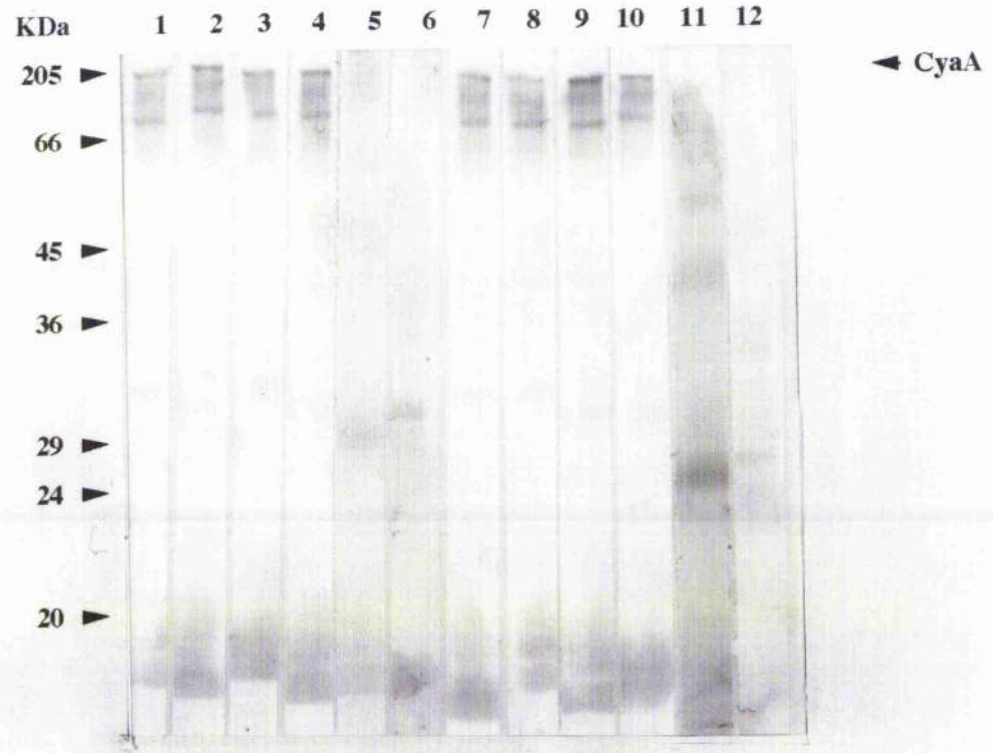

KDa
1
2
3
4
5
6
7
8
9
10
11
12
205
66
45
36
29
24
20
CyaA

Fig. 40

Western blot analysis of purified CyaA preparations using sera from mice vaccinated with PAR CyaA, PIR CyaA, DPT and alhydrogel.

Purified CyaA (5 μg protein/lane) preparations from *B. pertussis* or recombinant *E. coli* strains were subjected to electrophoresis on a 10% polyacrylamide gel and transferred to a Hybond C-Super membrane. The nitrocellulose was cut into strips and probed with sera collected from groups of 5 mice in experiments No. 1 on day 7 after the second dose of vaccine i.e. at the time of challenge of identical groups of mice. Mice were vaccinated with various purified CyaA preparations from recombinant *E.coli* strains plus alhydrogel, whole cell DPT vaccine or with alhydrogel alone. The numbers on the left of blots indicate the positions of molecular weight standards.

	A: Antigen preparation bound to nitrocellulose	B: Probed with sera from mice vaccinated with:
Lane 1 :	PAN CyaA	PAR CyaA
Lane 2 :	PIN CyaA	PAR CyaA
Lane 3:	PAR CyaA	PAR CyaA
Lane 4 :	PIR CyaA	PAR CyaA
Lane 5 :	PAN CyaA	DPT vaccine
Lane 6 :	PIN CyaA	Alhydrogel
Lane 7:	PAR CyaA	PIR CyaA
Lane 8 :	PIR CyaA	PIR CyaA
Lane 9 :	PAR CyaA	PIR CyaA
Lane 10 :	PIR CyaA	PIR CyaA
Lane 11 :	PAR CyaA	DPT vaccine
Lane 12:	PIR CyaA	Alhydrogel


KDa
1
2
3
4
5
6
7
8
9
10
11
12
205
66
45
36
29
24
20
CyaA

routine ELISA procedure using a 1/2000 dilution of anti-CyaA antiserum or a 1/1000 dilution of mouse anti-Oa antiserum was followed (section 2.20). In this experiment, the ELISA dose response curves for titrated antigens indicated that 5 μg of CyaA antigen or 100 μg of ovalbumin was the best concentration for coating antigen in ELISA plates .

3.14.3 Mouse IgG antibody response to CyaA or Hyb antigens

The antigenicity of CyaA and Hyb preparations in mice vaccinated with 15 μg protein/mouse absorbed on 250 μg alhydrogel were determined by ELISA. Sera from individual mice of each group were assayed by ELISA. Examples of the IgG anti-CyaA antibody titrations by ELISA with antisera raised against various antigens are shown in Figs 41 and 42.

The antibody titres were determined in ELISA U/ml of neat mouse sera by assigning an arbitrary unitage of 100 ELISA U/ml to a pooled reference antiserum (section 2.19.2). By interpolating at OD 0.5, the antibody titre in ELISA U/ml was calculated from the ratio of test serum titre to reference serum titre and multiplying by a factor of 100. As is evident from data given in Tables 18 and 19, all CyaA preparations were highly antigenic in stimulating anti-CyaA IgG antibody responses in mice. In addition serum raised to whole cells of *B. pertussis* in the rat (provided by Dr. E. Hall) also showed the presence of a low level IgG antibody which reacted with CyaA. No cross-reaction was evident with antisera raised to PT, FHA or PRN, indicating that the CyaA coating antigen was free of these antigens (Fig. 42). As is also evident from Table 19, the hybrid toxins Hyb1 and Hyb2, also raised anti-

Fig. 41

Examples of ELISA for Mouse IgG antibody responses to crude and purified CyaA antigens from *B. pertussis* and *E. coli* strains at the time of challenge (Experiments No. 1 and 2).

Male HAM ICR (CD-1) mice, 3 weeks old, randomised in groups of 10, were vaccinated subcutaneously twice at a two week interval with 15 μg protein/mouse of crude or purified CyaA preparations from *B. pertussis* or recombinant *E. coli* strains absorbed on 250 μg alhydrogel. Serum IgG antibody responses were measured in individual sera by ELISA 7 days after the second vaccination, using PAR CyaA as coating antigen.

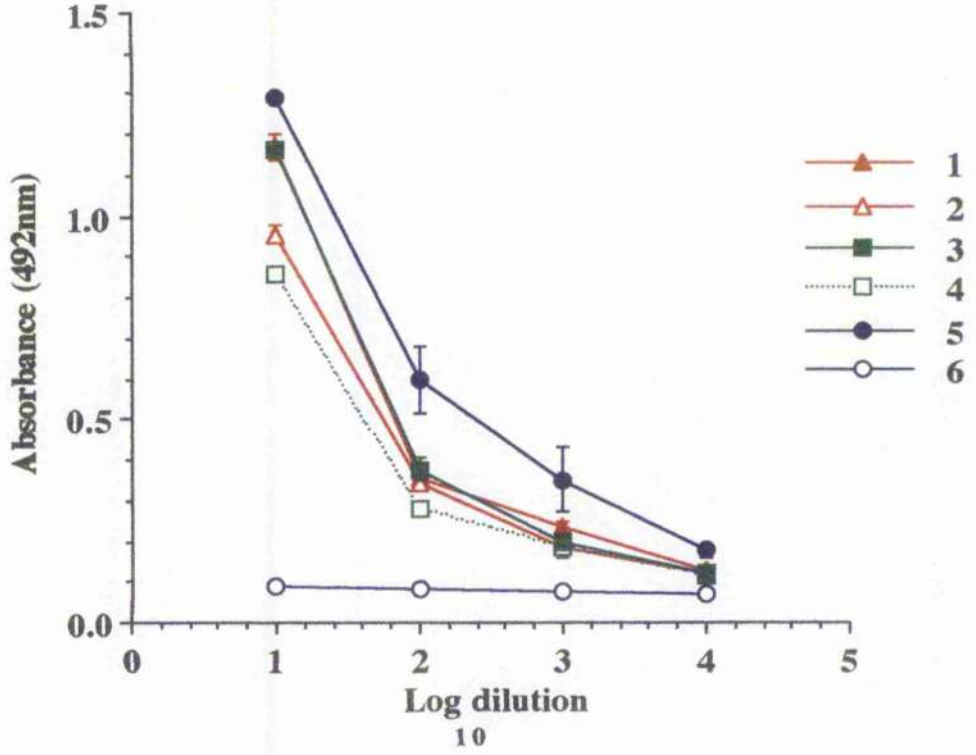
Panel A: Examples of ELISA to measure Serum IgG anti-CyaA antibody in sera of individual mice vaccinated with :

1 PAN CyaA
2 PIN CyaA
3 PAR CyaA
4 PIR CyaA
5 Reference antiserum (anti-CyaA polyclonal antibody)
6 Pooled normal (unvaccinated) mouse serum

Panel B: Examples of ELISA to measure Serum IgG anti-CyaA antibody in sera of individual mice vaccinated with:

1 CAN CyaA
2 CIN CyaA
3 CAR CyaA
4 CIR CyaA
5 Reference antiserum (anti-CyaA polyclonal antibody)
6 Pooled normal (unvaccinated) mouse serum

A


1.5
1.0
0.5
0.0
Absorbance (492nm)
0
1
2
3
4
5
Log dilution
10
1
2
3
4
5
6

B

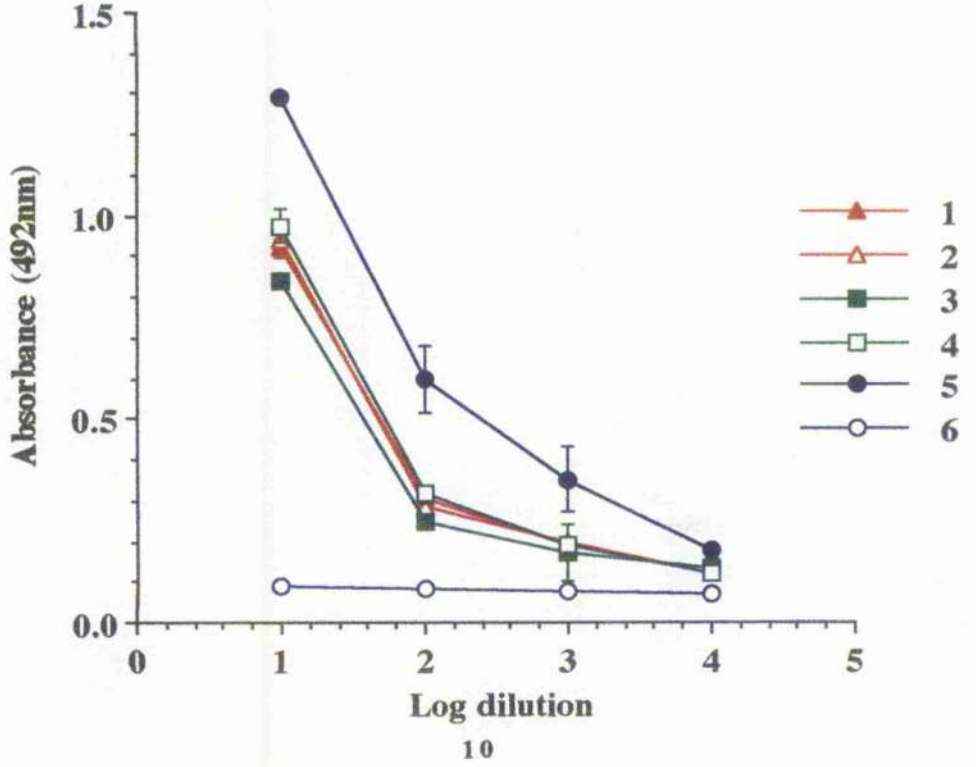

1.5
1.0
0.5
0.0
Absorbance (492nm)
0
1
2
3
4
5
Log dilution
10
1
2
3
4
5
6

Fig. 42

IgG ELISA for antigen content of purified CyaA preparations from *B.pertussis* strain 348 pRMB1.

PAN CyaA was used as a coating antigen in ELISA plates and serum IgG to this antigen was measured in various sera raised against *B. pertussis* components (FHA, PT, PRN) or whole *B. pertussis* 18323 cells (see section 2.19.3 for source of these sera).

Antiserum raised against

1 PAN CyaA from *B.pertussis* 348 pRMB1 in mice
2 Purified filamentous haemagglutinin (FHA) in mice
3 Purified pertussis toxin (PT) in mice
4 Purified pertactin toxin (PRN) in mice
5. Whole *B. pertussis* cells in rats
6 Reference antiserum (anti CyaA polyclonal antibody)
7 Pooled normal (unvaccinated) mouse serum

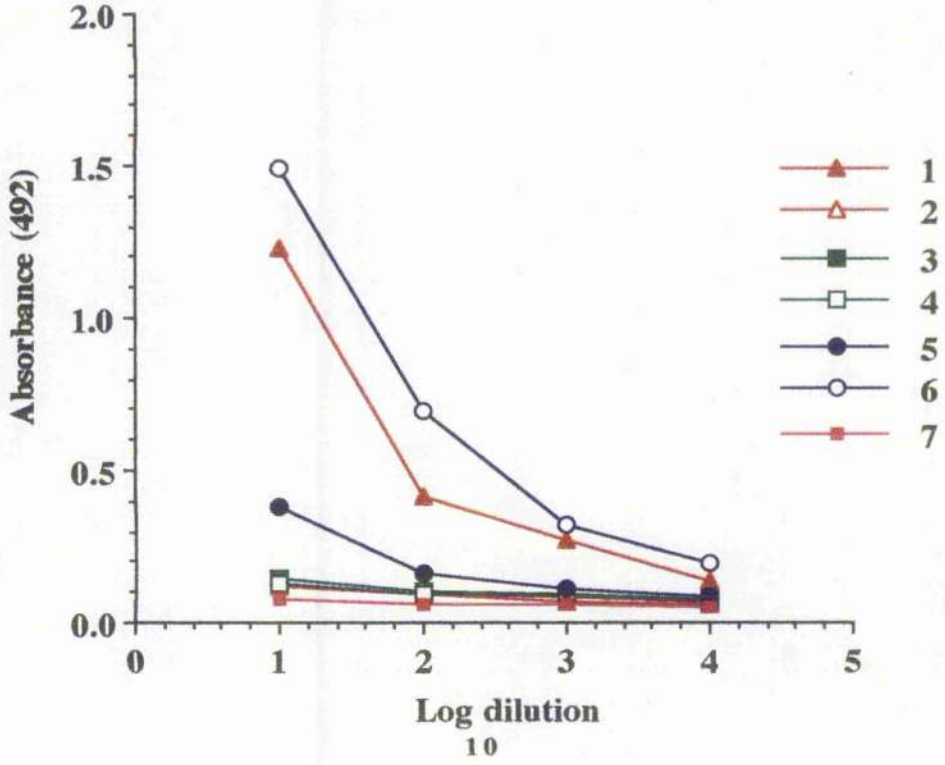

2.0
1.5
1.0
0.5
0.0
Absorbance (492)
0
1
2
3
4
5
Log dilution
10
1
2
3
4
5
6
7

Table 18.

IgG anti-CyaA antibody titres in sera of mice vaccinated with various CyaA antigens were detected in ELISA with PARCyaA as a coating antigen.

Sera	IgG-anti CyaA antibody titres ELISA units/ml [a]	
	Experiment 1	Experiment 2
PAN CyaA	83.07±3.35	78.37±2.19
PIN CyaA	75.89±2.62	72.23±2.54
CAN CyaA	71.36±3.18	73.86±3.73
CIN CyaA	68.80±2.14	66.59±2.21
PAR CyaA	67.34±1.72	71.08±2.49
PIR CyaA	80.36±3.55	66.32±2.31
CAR CyaA	65.81±2.24	62.29±1.68
CIR CyaA	73.08±3.75	64.18±1.42
WCV [b]	18.48±1.73	26.40±1.15
Control [c]	ND [d]	ND

a. Antibody titres (ELISA units/ml) were expressed as the mean value of 5 individual mice sera ± SEM.
b.WCV= Commercial whole cell DPT vaccine (Welcome Trivax-AD)
c. Control= non-vaccinated and non-challenged mice d. ND= not detected.

Table 19.

IgG anti-CyaA antibody titres in sera of mice vaccinated with various CyaA and Hyb antigens were detected in ELISA with PARCyaA as a coating antigen.

Sera	IgG-anti AC antibody titres ELISA units/ml [a]
PAR pCACT3	75.49±2.81
Hyb2	66.59±2.17
Hyb2/LktC	73.31±2.59
Hyb2/ CyaC	69.08±2.42
Hyb1/ CyaC	61.32±1.64
Alhydrogel	ND
WCV	11.83±1.71

a. Antibody titres (ELISA units/ml) were expressed as the mean value of 5 individual mice sera ± SEM.

CyaA antibody levels comparable to those from mice vaccinated with the CyaA antigens.

3.15 ADJUVANTICITY OF CyaA ON ANTIBODY RESPONSES TO OVALBUMIN

In order to investigate a possible adjuvant effect of CyaA on the antibody response, mice were vaccinated twice, with a two week interval, with PAR CyaA or PIR CyaA with or without ovalbumin as the test antigen. Sera were taken over a period of four weeks at one week intervals starting from day 7, in two identical experiments (Experiments 4 and 5) (section 2.22). The sera were then titrated by ELISA and the data for two separate experiments are shown in Figs 43 and 44.

As is evident from the data (Figs 43 and 44, Panel A), the primary IgG anti-Oa antibody response was enhanced 2-3-fold in mice vaccinated with Oa and PAR CyaA compared with the mice vaccinated with Oa alone. The adjuvant effect of PIR CyaA was not as obvious. The secondary IgG anti-Oa antibody response was increased about 4.8 fold in the mice vaccinated with Oa and PAR CyaA compared with the mice receiving Oa alone. In control mice for these experiments i.e. non-vaccinated animals, the anti-Oa and anti-CyaA antibody levels were below the limit of detection (i.e. A492 <0.5).

The IgG subclasses and IgE anti-Oa antibody responses in sera from Experiments 4 and 5 taken at 7 days after the second injection were also determined. Results are shown in Tables 20 and 21. These titres are calculated relative to the IgG subclasses and IgE titres in a pooled reference mouse serum (section 2.19.2) (100 U/ml) and so the antibody level for one immunoglobulin

Fig. 43

Effect of purified CyaA preparations from recombinant *E. coli* strains on primary and secondary anti-Oa or anti-CyaA antibody responses (Experiment No. 4).

Male HAM ICR (CD-1) mice, 4 weeks old, randomised in groups of 20, were vaccinated subcutaneously twice with a two week interval, with 15 μg protein/mouse of PAR CyaA or PIR CyaA preparations from recombinant *E. coli* strains with or without 20 μg ovalbumin/mouse, or with 20 μg ovalbumin/mouse alone. Anti-Oa or anti-CyaA serum IgG antibodies were measured by ELISA (section 2.20). Ovalbumin (Oa) was used as coating antigen in ELISA for results shown in Panel A, whereas PAR CyaA was used as coating antigen for results shown in Panel B.

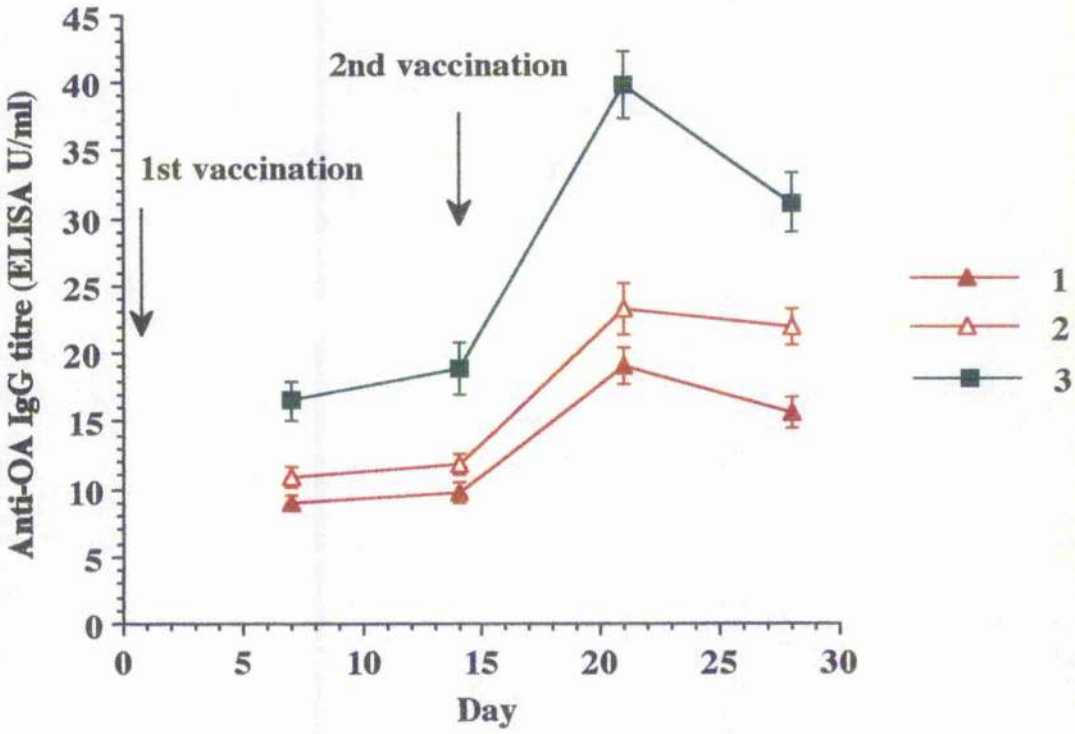
Panel A: Mouse anti-Oa IgG antibody in sera from mice vaccinated with

1 Ovalbumin (Oa)
2 PIR CyaA with Oa
3 PAR CyaA with Oa

Panel B: Mouse anti-CyaA IgG antibody in sera from mice vaccinated with

1 PAR CyaA
2 PIR CyaA
3 PIR CyaA with Oa
4 PAR CyaA with Oa

A


Anti-OA IgG titre (ELISA U/ml)
45
40
35
30
25
20
15
10
5
0
2nd vaccination
1st vaccination
0
5
10
15
20
25
30
Day
1
2
3

B

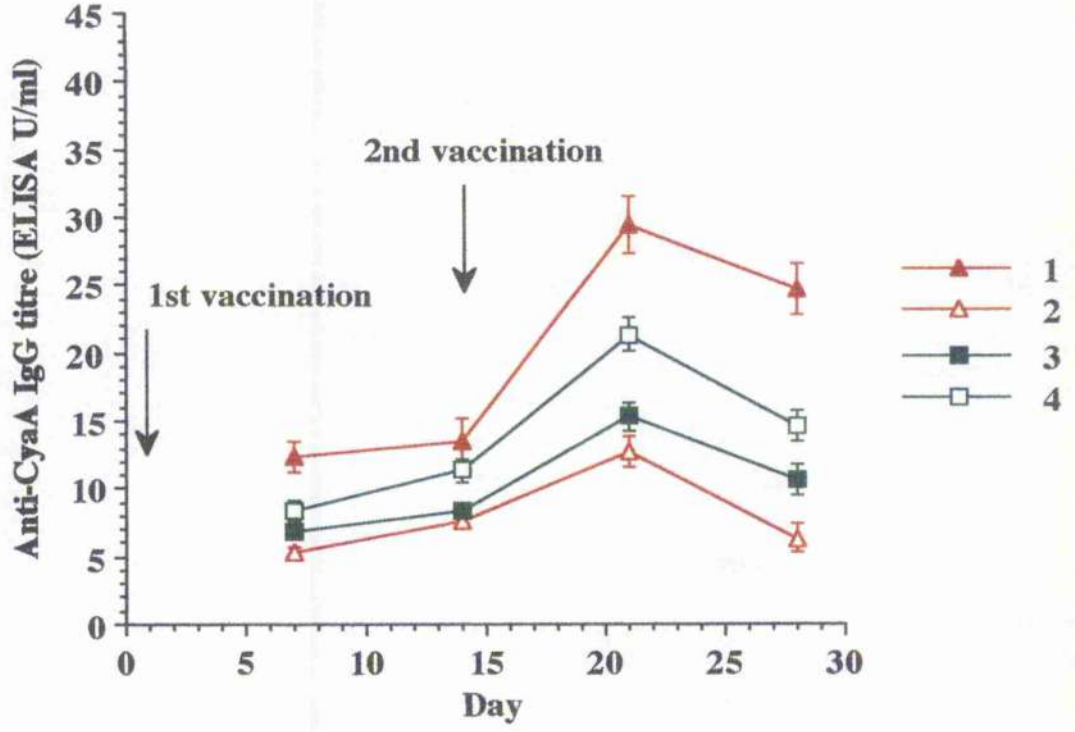

Anti-CyaA IgG titre (ELISA U/ml)
45
40
35
30
25
20
15
10
5
0
2nd vaccination
1st vaccination
0
5
10
15
20
25
30
Day
1
2
3
4

Fig. 44

Effect of purified CyaA preparations from recombinant *E. coli* strains on primary and secondary anti-Oa or anti-CyaA antibody responses (Experiment No. 5).

Experiment No. 5 was carried out under the same conditions as Experiment No. 4 (see legend of Fig. 43).

Panel A: Mouse anti-Oa IgG antibody in sera from mice vaccinated with

1 Ovalbumin (Oa)

2 PIR CyaA with Oa

3 PAR CyaA with Oa

Panel B: Mouse anti-CyaA IgG antibody in sera from mice vaccinated with

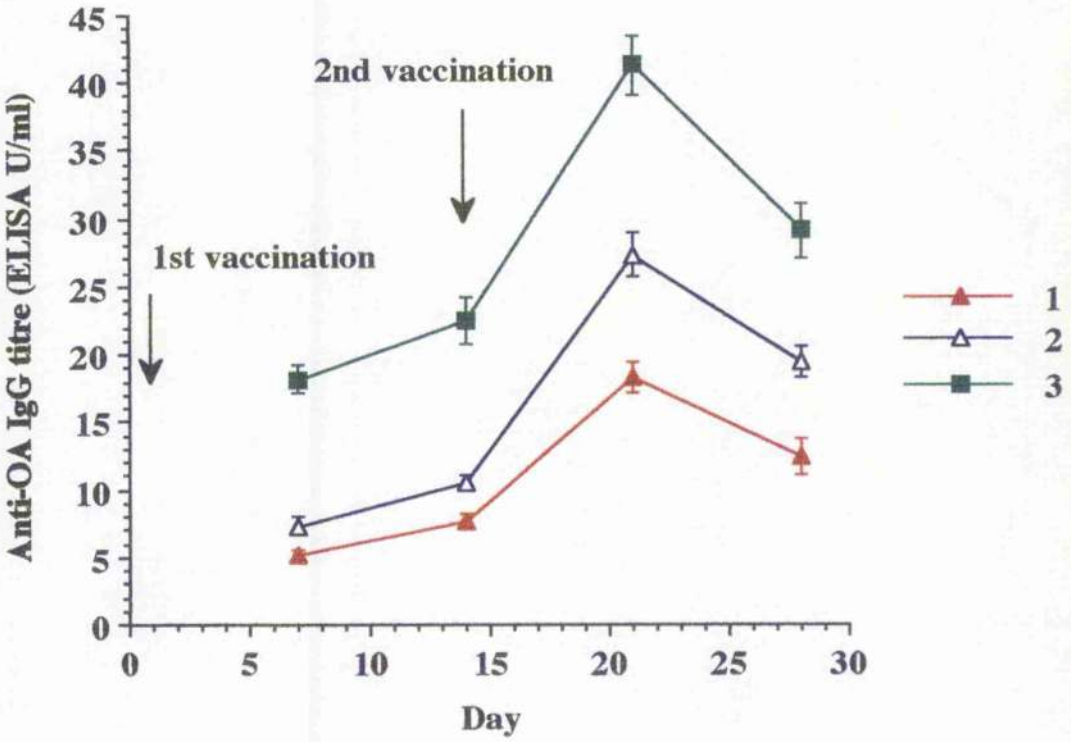
1 PAR CyaA

2 PIR CyaA

3 PIR CyaA with Oa

4 PAR CyaA with Oa

A


Anti-OA IgG titre (ELISA U/ml)
45
40
35
30
25
20
15
10
5
0
2nd vaccination
1st vaccination
0
5
10
15
20
25
30
Day
1
2
3

B

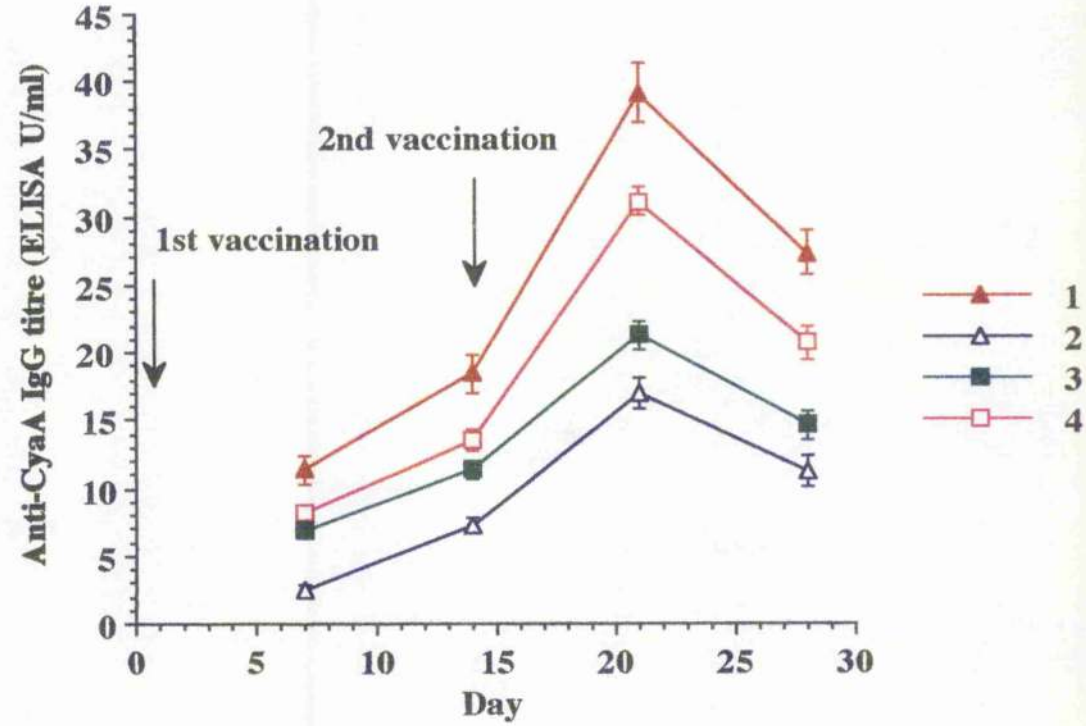

Anti-CyaA IgG titre (ELISA U/ml)
45
40
35
30
25
20
15
10
5
0
2nd vaccination
1st vaccination
0
5
10
15
20
25
30
Day
1
2
3
4

Table 20.

The IgG subclasses and IgE anti-Oa titres in sera of mice vaccinated with Oa, with or without PAR CyaA or PIR CyaA antigens, detected in ELISA with Oa as a coating antigen. Sera were taken at 7 days after the second injection (Experiment No. 4).

Sera	Anti-Oa antibody titres (ELISA units/ml [a])			
	IgE	IgG	IgG1	IgG2a
Oa	2.34±0.48	7.91±1.52	5.11±1.08	3.62±1.15
Oa with PAR CyaA	19.05±1.27	38.41±2.19	26.30±2.24	9.83±1.72
Oa with PIR CyaA	7.63±1.16	16.31±2.73	11.54±1.78	5.24±1.31

[a]Antibody titres (ELISA units/ml) were expressed as the mean value of 5 individual mice sera ± SEM.

Table 21.

The IgG subclasses and IgE anti-Oa titres in sera of mice vaccinated with Oa, with or without PAR CyaA or PIR CyaA antigens, detected in ELISA with Oa as a coating antigen. Sera were taken at 7 days after the second injection (Experiment No. 5).

Sera	Anti-Oa antibody titres (ELISA units/ml [a])			
	IgE	IgG	IgG1	IgG2a
Oa	4.11±1.52	10.91±1.52	8.25±1.38	2.83±1.52
Oa with PAR CyaA	22.48±1.27	36.53±3.49	23.66±2.71	7.26±2.14
Oa with PIR CyaA	8.29±1.16	13.90±2.12	9.53±1.37	3.51±1.63

[a]Antibody titres (ELISA units/ml) were expressed as the mean value of 5 individual mice sera ± SEM.

can not be related to that for other immunoglobulin classes. The IgG and IgG1 anti-Oa antibody responses were markedly enhanced in groups of mice vaccinated with PAR CyaA with Oa compared to control groups vaccinated with Oa alone or Oa with PIR CyaA, The IgG2a response was also increased but to a lower extent and IgG2b antibody was not detectable in any sera. Anti-Oa antibody response was not detectable in control mice (i.e. non-vaccinated mice) or those which had received PAR CyaA alone (data not shown). Similar to the IgG1 antibody response, the IgE anti-Oa antibody response was significantly increased in mice that were primed in the presence of active CyaA compared to a control group who received Oa alone. With Oa and PIR CyaA, there appeared to be some enhancememnt of the anti-Oa IgE response, compared to mice given Oa alone, and this enhancement was also evident in the IgG subclass responses.

The primary and secondary IgG anti-CyaA antibody responses were also evaluated in sera from Experiments 4 and 5 taken over a period of four weeks at one week intervals starting from day 7. The primary IgG anti-CyaA antibody response was enhanced 1.7 fold in mice primed with PAR CyaA compared with the mice vaccinated with PIR CyaA (Figs 43 and 44, Panel B). The secondary IgG anti-CyaA antibody was increased about 2.6 fold in mice vaccinated with PAR CyaA compared to mice given PIR CyaA alone (Figs 43 and 44, Panel B). Ovalbumin had no adjuvant effect on the response to CyaA. The IgG subclasses and IgE anti-CyaA antibody responses in sera from Experiments 4 and 5 taken at 7 days after the second injection were also determined. Interestingly, the IgG and IgG1 anti-CyaA antibody responses in the groups of mice receiving PAR CyaA alone were enhanced significantly compared with mice given PIR CyaA alone (Tables 22 and 23). IgG2a anti-

Table 22.

The IgG subclasses and IgE anti-CyaA titres in sera of vaccinated mice with PAR CyaA with or without Oa, and PIR CyaA with or without Oa antigens, detected in ELISA with PAR CyaA as a coating antigen (Experiment No. 4). Sera were taken 7 days after the second vaccination.

Sera	Anti-CyaA antibody titres (ELISA U/ml [a])			
	IgE	IgG	IgG1	IgG2a
PAR CyaA	14.32±1.48	31.37±1.07	22.52±1.67	8.42±0.71
PAR CyaA with Oa	10.49±1.13	23.19±1.64	14.63±1.41	5.73±0.58
PIR CyaA with Oa	7.36±1.82	15.26±1.19	10.33±1.85	3.16±0.29
PIR CyaA	6.17±0.49	12.73±1.32	7.19±0.54	5.08±0.92

[a]Antibody titres (ELISA units/ml) were expressed as the mean values of 5 individual mice sera ± SEM.

Table 23.

The IgG subclasses and IgE anti-CyaA titres in sera of vaccinated mice with PAR CyaA with or without Oa, and PIR CyaA with or without Oa antigens, were detected in ELISA with PAR CyaA as a coating antigen (Experiment No. 5). Sera were taken 7 days after the second vaccination.

Sera	Anti-CyaA antibody titres (ELISA U/ml [a)]			
	IgE	IgG	IgG1	IgG2a
PAR CyaA	17.29±1.15	39.17±2.11	28.41 ± 1.08	11.74±1.05
PAR CyaA with Oa	11.72±1.36	31.11±1.04	16.90 ± 2.24	7.35±0.65
PIR CyaA with Oa	6.91±0.42	21.27±1.52	12.63±1.19	4.62±0.38
PIR CyaA	5.43±0.66	16.89±1.14	8.36 ± 0.74	6.75±1.21

[a]Antibody titres (ELISA units/ml) were expressed as the mean values of 5 individual mice sera ± SEM.

CyaA responses were enhanced to a lower extent, whereas the IgE anti-CyaA antibody responses in mice who had received PAR CyaA alone were enhanced markedly compared with mice which had received PIR CyaA alone or with Oa (Tables 22 and 23).

The total serum concentration of IgG, IgG1 and IgG2a in sera of mice at 7 days after the second vaccination (Experiments 4 and 5) were also measured by radial immunodiffusion (Figs 45-47). In all cases, the immunoglobulin levels were significantly higher in mice injected with PAR CyaA plus Oa than in mice injected with PIR CyaA plus Oa. The immunoglobulin levels in mice injected with PAR CyaA alone were lower than those in mice given the two antigens together but they were consistently higher than in mice given PIR CyaA alone or control mice (non-vaccinated mice) (Figs 46 and 47). IgG 2b antibodies were not detectable at the lowest dilution tested (1/10) in any of the sera (data not shown).

3.16 CYTOKINE PRODUCTION FROM SPLEEN CELLS

Spleens of 5 mice primed with PAR CyaA or PIN CyaA (Experiments 4 and 5) were removed at 7 days after the second vaccination and homogenised together aseptically. The spleen cells were cultured in the presence of active and inactive CyaA, ConA was also used as a positive control and urea in tissue culture medium as another control (section 2.23). The supernatants were collected from a portion of the culture after 24 h and 48 h and assayed by cytokine ELISA (section 2.24). The cytokine responses at 24 h after in vitro activation of spleen cells was very low in both experiments (data not shown). The cytokine response at 48 h of spleen cells taken from mice vaccinated with

Fig. 45

Measurement of total IgG concentration by radial immunodiffusion in sera of mice vaccinated with recombinant CyaA preparations.

IgG Standards (60% and 10% v/v) and serum samples were loaded on to the wells of agarose gel plates (section 2. 25). After the required diffusion time, antibody in serum or IgG standards had reacted with the anti-IgG antibody in the gel and formed precipitin rings. The ring diameters were measured and the concentration of the IgG in serum samples was read off a standard curve.

1. IgG Standard (60%)
2. IgG Standard (10%)
3. Pooled sera of mice vaccinated with Oa alone
4. Pooled sera of mice vaccinated with Oa and PIR CyaA
5-9. Sera of 5 individual mice vaccinated with Oa and PAR CyaA
10-14. Sera of 5 individual mice vaccinated with PAR CyaA alone

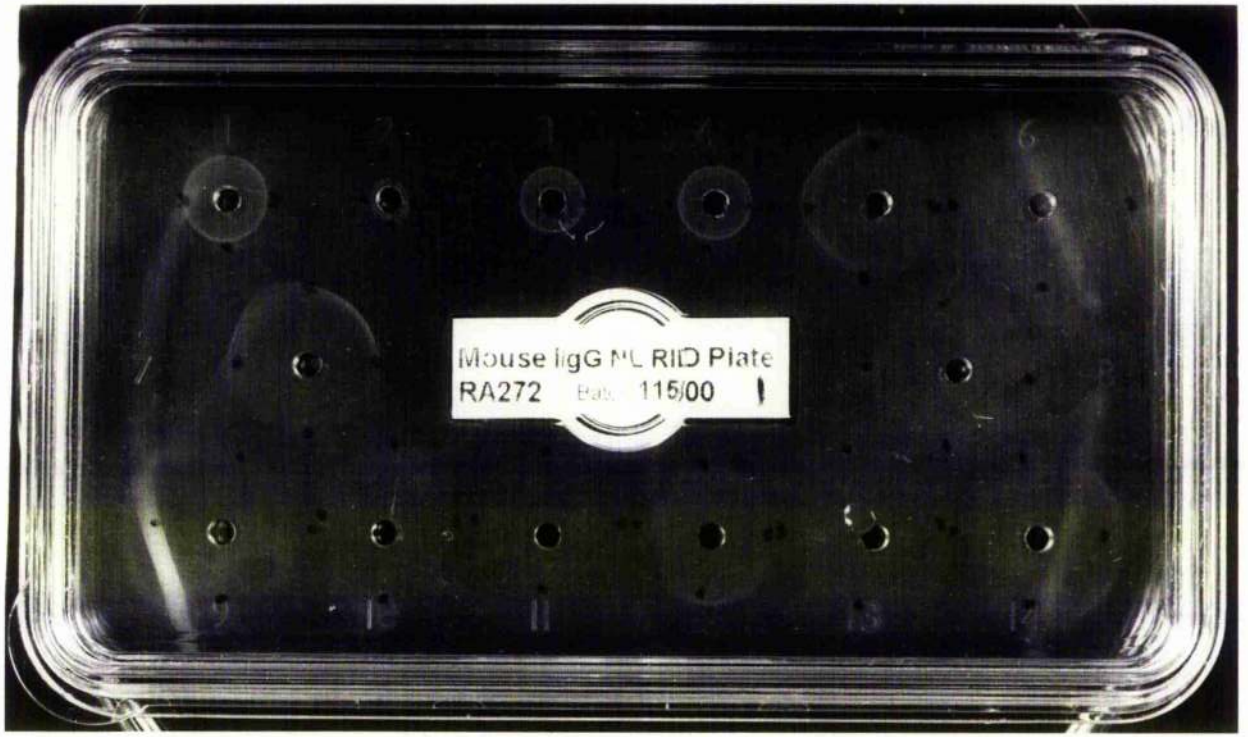

Mouse IgG ML RID Plate
RA272 Batch 115/00

Fig. 46

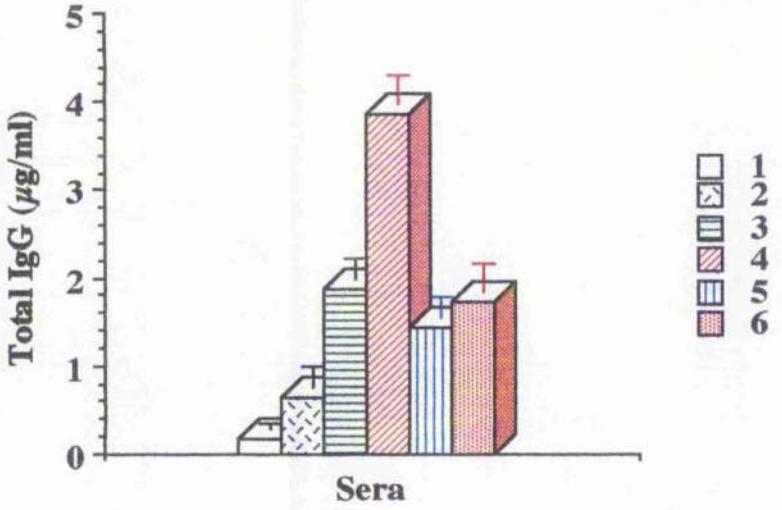
Effect of purified CyaA preparations from recombinant *E. coli* strains on total serum IgG, IgG1, IgG2a (Experiment No. 4).

Male HAM ICR (CD-1) mice, 4 weeks old, randomised in groups of 20, were vaccinated subcutaneously twice at a two week interval with 15 μg protein/mouse of PAR CyaA or PIR CyaA with or without 20 μg ovalbumin/mouse, or with 20 μg ovalbumin/mouse alone. Total serum IgG, IgG1, IgG2a in sera of 5 mice of each group at one week after the second vaccination were measured by radial immunodiffusion (RID) (section 2.25). Data are mean values ± SEM from 5 individual mouse sera.

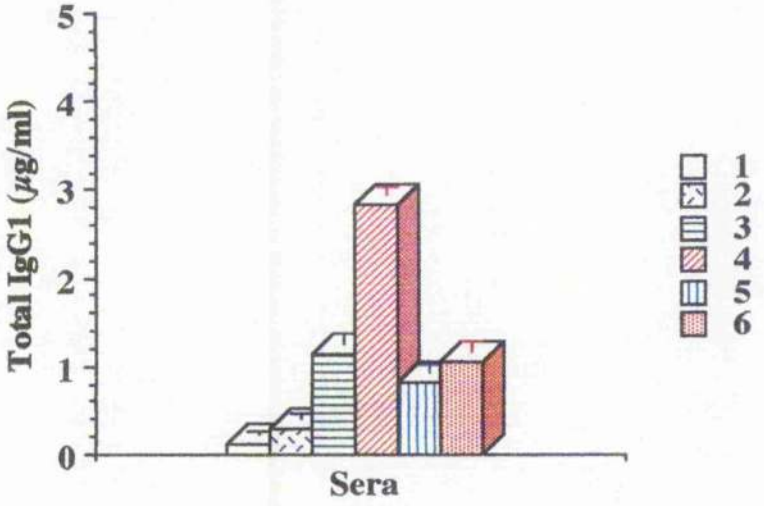
Panel A,B,C : Mouse total serum IgG (Panel A), IgG1 (Panel B), IgG2a (panel C) antibody in sera from mice vaccinated with:

1 Control mice (non-vaccinated and non-challenged)
2 Ovalbumin (Oa) alone
3 Oa with PIR CyaA
4 Oa with PAR CyaA
5 PIR CyaA alone
6 PAR CyaA alone

A


Total IgG (μg/ml)
5
4
3
2
1
0
Sera
1
2
3
4
5
6

B


Total IgG1 (μg/ml)
5
4
3
2
1
0
Sera
1
2
3
4
5
6

C

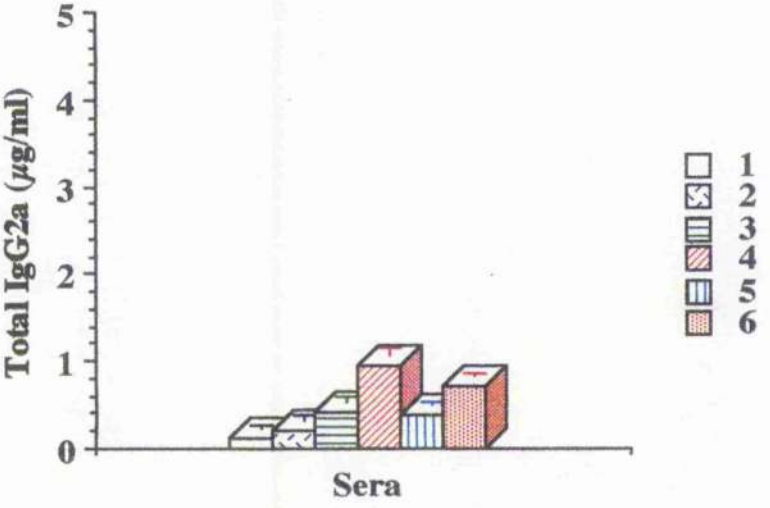

Total IgG2a (μg/ml)
5
4
3
2
1
0
Sera
1
2
3
4
5
6

Fig. 47

Effect of purified CyaA preparations from recombinant *E. coli* strains on total serum IgG, IgG1, IgG2a (Experiment No. 5).

Experiment No. 5 was carried out under the same conditions as Experiment No. 4 (see legend of Fig. 46).

Panel A,B,C : Mouse total serum IgG (Panel A), IgG1 (Panel B), IgG2a (panel C) antibody in sera from mice vaccinated with:

1 Control mice (non-vaccinated and non-challenged)

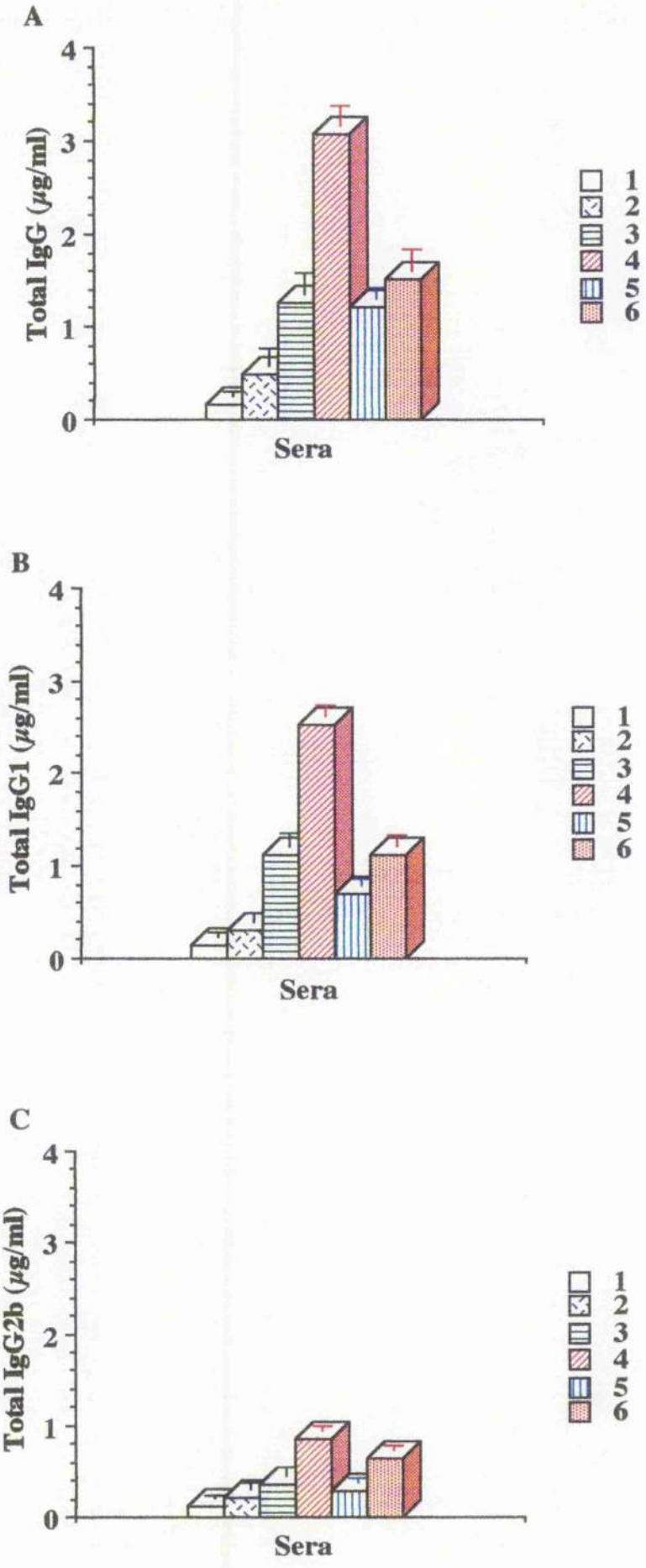
2 Ovalbumin (Oa) alone

3 Oa with PIR CyaA

4 Oa with PAR CyaA

5 PIR CyaA alone

6 PAR CyaA alone


A
Total IgG (μg/ml)
4
3
2
1
0
Sera
1
2
3
4
5
6
B
Total IgG1 (μg/ml)
4
3
2
1
0
Sera
1
2
3
4
5
6
C
Total IgG2b (μg/ml)
4
3
2
1
0
Sera
1
2
3
4
5
6

either active or inactive CyaA, and stimulated in vitro with various stimuli are shown in Table 24. Spleen cells stimulated with Con A generally showed high levels of production of all cytokines except IL 5. Spleen cells from mice vaccinated with PAR CyaA, in nearly every case, gave high cytokine responses. With PIR CyaA as vaccine, responses were lower, but higher than those of cells from non-vaccinated mice. Spleen cells stimulated with either Oa or urea as controls generally showed low or undetectable cytokine responses. The results show that PAR CyaA was more active than PIR CyaA in both priming and stimulating spleen cells for cytokine responses.

Table 24.

Elisa assay of cytokines synthesized by in vitro stimulation for 48 h of spleen cells from mice vaccinated with PAR CyaA or PIR CyaA on day 7 after second vaccination

Pooled spleen cells from 5 mice vaccinated with	In vitro stimulation after 48 h incubation with:	IL 2 conc (U/ml)		IL 4 conc (U/ml)		IL5 conc (U/ml)	IL 10 conc (U/ml)	INF γ conc (U/ml)		TNF conc (U/ml)
		Exp 3	Exp 4	Exp 3	Exp 4	Exp 3	Exp 3	Exp 3	Exp 4	Exp 3
PAR CyaA	PAR CyaA	13.21	17.67	71.26	86.93	10.16	94.96	14.19	11.62	51.94
PAR CyaA	PIR CyaA	3.45	4.72	63.42	76.92	6.52	64.94	11.48	9.53	41.54
PAR CyaA	Ovalbumin	<1.56	<1.56	<1.56	<1.56	<1	17.13	<1	<1	7.32
PAR CyaA	ConcavalinA	51.36	58.78	46.19	53.51	2.82	41.19	25.56	34.21	63.53
PAR CyaA	No stimulation	<1.56	<1.56	<6.13	<6.13	<1	<10.43	<1	<1	<8.29
PAR CyaA	0.2 M urea	<1.56	<1.56	2.15	4.93	<1	<10.43	<1	<1	<8.29
PIR CyaA	PAR CyaA	5.45	4.47	29.73	25.16	1.29	34.18	8.23	6.29	25.17
PIR CyaA	PIR CyaA	2.16	3.81	24.71	22.01	<1	<10.43	4.62	5.87	16.35
PIR CyaA	Ovalbumin	<1.56	<1.56	<6.13	<6.13	<1	<10.43	<1	<1	59.43
PIR CyaA	ConcavalinA	21.83	27.71	32.52	40.10	3.45	26.17	16.29	23.49	13.84
PIR CyaA	No stimulation	<1.56	<1.56	<6.13	<6.13	<1	<10.43	<1	<1	<8.29
PIR CyaA	0.2 M urea	<1.56	<1.56	2.34	1.61	<1	<10.43	<1	<1	<8.29
Non vaccinated	PAR CyaA	<1.56	<1.56	<6.13	<6.13	<1	<10.43	<1	<1	<8.29
Non vaccinated	PIR CyaA	<1.56	<1.56	<6.13	<6.13	<1	<10.43	<1	<1	<8.29
Non vaccinated	Ovalbumin	<1.56	<1.56	<6.13	<6.13	<1	<10.43	<1	<1	<8.29
Non vaccinated	ConcavalinA	2.41	1.70	<6.13	<6.13	<1	<10.43	5.41	7.11	<8.29
Non vaccinated	No stimulation	<1.56	<1.56	<6.13	<6.13	<1	<10.43	<1	<1	<8.29
Non vaccinated	2 M urea	<1.56	<1.56	<6.13	<6.13	<1	<10.43	2.67	5.87	<8.29

[a]Data are the mean values of triplicate determinations in each experiment

DISCUSSION

4.1 PRODUCTION OF CRUDE UREA EXTRACTS OF CyaA, LktA AND HYBRID TOXINS

4.1.1 Production of CyaA from *B. pertussis* strains

The first preparations of CyaA prepared by different investigators from crude extracts of whole *B. pertussis* wild type organisms or concentrated culture supernates were reported to contain multiple forms of toxin with molecular weights ranging from 45 kDa to 206 kDa. In later work, the toxin was extracted with urea which stabilised the 200 kDa form of the enzyme and allowed it to be purified (Hewlett *et al.*, 1989; Rogel *et al.*, 1989). In the present study, *B. pertussis* recombinant strain BP348 (pRMB1) was used (Brownlie *et al.*, 1988). This strain produces large amounts of CyaA encoded by the cosmid pRMB1 and cells were extracted with urea to minimise degradation of the toxin. The 200 kDa toxic form of CyaA was the main product detected in the crude extracts as confirmed by the SDS-PAGE analysis and immunoblotting with monoclonal or polyclonal antibodies (Figs 7, 8 and 14). The large amounts of CyaA in crude extracts enabled purification of sufficient amounts of the 200 kDa form for investigation of its toxic and protective activities.

The production of CyaA was optimised by the addition of MeβCD to the culture medium and by growth of the culture for 48 h (Table 2). The degradation of the toxin was minimised by adding BSA to culture medium during growth and the addition of PMSF during processing stages for purification of the toxin (Tables 5). Under these conditions, the level of the toxic form of CyaA produced by *B. pertussis* strain BP348 (pRMB1) was about

10-fold higher than that from the wild type *B. pertussis* strains. This finding was consistent with the yield of the CyaA from strain BP348 (pRMB1) as previously reported by Brownlie *et al* (1988).

Strain BP DE386, a mutant defective in the activator gene (*cyaC*) produced a good yield of CyaA of 200 kDa in urea extracts (CIN CyaA) which was indistinguishable from the active toxin by SDS-PAGE (Fig. 7). It exhibited a comparable level of the enzymic activity to that of strain BP348 (pRMB1) but had no detectable haemolytic activity on sheep erythrocytes, no toxic activity on J774.2 cells and no invasive activity on BHK21 cells, even at the highest levels tested (Figs. 17, 21 and 22, Table 6). This is in keeping with previous work by Hewlett *et al.* (1993). These investigators showed that inactive CyaA from BPDE386 underwent a calcium dependent conformational change and bound to target cells in a manner comparable to active CyaA, but this was not sufficient for pore forming insertion and translocation of the toxin into target cells, and thus it had no cytolytic activity. Hewlett *et al.* (1993) suggested that the CyaC modification of CyaA is probably critical for conferring an exact conformational change on the CyaA molecule to allow it to participate directly in pore forming events which translocate the CyaA molecule into the target cells.

4.1.2 Production of CyaA and LktA from recombinant *E. coli* strains

Inactive recombinant CyaA of 200 kDa was initially produced in low amounts from the urea cell extracts of a protease-defective recombinant *E. coli* strain H1469 (pRMB3) which expressed the *cyaA* gene on pRMB1 (Brownlie *et al.*, 1988). They reported that the CyaA produced by this strain showed

enzymic activity in the presence of exogenous CaM protein, but no invasive activity was detected.

Sebo *et al.* (1991) reported high level production of active recombinant CyaA toxin of 200 kDa produced from the urea extract of recombinant *E. coli* BL21 (pCACT3) where the *cya*A and *cya*C genes were co-expressed in pCACT3 from the *lac* promoter. It had a comparable level of toxic activity as well as enzymic activity to that of native CyaA toxin from the natural producer *B. pertussis*, but only a weak haemolytic activity (4-10 fold reduction) (Sebo *et al.*, 1991; Betsou *et al.*, 1993; Hackett *et al.*, 1995).

In the present study, a T7 RNA polymerase expression system was used which had been developed in this laboratory for the production of a large amount of the active recombinant CyaA toxin in *E. coli*. A high level of active recombinant CyaA toxin of 200 kDa was produced by co-expression of the *cyaA* and *cyaC* genes from the protease defective *E. coli* strain BL21/DE3 harbouring two separate compatible plasmids, pGW44 and pGW54 (Westrop *et al.*, 1996). This strain produced CyaA and CyaC in comparable amounts to that produced from the strains BL21/DE3 (pGW44) or BL21/DE3 (pGW54) carrying the plasmids separately (Figs 7 and 13). The urea extracts of strain BL21/DE3 (pGW44, pGW54) had similar invasive, toxic and enzymic activities to urea extracts of strain BL21 (pCACT3), but slightly lower haemolytic activity. The inactive recombinant CyaA of 200 kDa produced from the urea extract of *E.coli* BL21/DE3 (pGW44) had a high level of enzymic activity, but no toxic or haemolytic activities (Fig 18, 21 and 22, Table 6) which was comparable with the CyaA produced by *B. pertussis* BP DE386 defective in the *cyaC* gene.

A similar system based on T7 vectors of the translational fusion type was used for production of active recombinant LktA by co-expression of *lktA* and *lktC* genes from separate compatible plasmids by *E. coli* HMS174 /DE3 (pGW64, pGW78). The yield of LktA protein was less than expected when compared to the production of CyaA in the same T7 expression system (Fig. 10) The urea extracts of recombinant active LktA contained a 105 kDa LktA protein and 19 kDa LktC protein as judged by SDS-PAGE analysis and immunoblotting with polyclonal antibody raised against recombinant LktA in rabbits (Figs 10, 13 and 15. B). An inactive LktA protein was also produced from recombinant strain HMS174/DE3 (pGW64) which was indistinguishable from active recombinant LktA by SDS-PAGE but lacked LktC protein in urea extracts (Fig. 10). No detectable toxic activity was found in urea extracts of this recombinant inactive LktA even at high concentrations (Figs 30. B and 31. B, Table 11).

4.1.3 Production of hybrid toxins from recombinant *E. coli* strains

In order to investigate the structure-function relationships of the CyaA toxin, two hybrid toxins (Hyb1 and Hyb2) were produced in *E. coli* by expression of chimeric constructs containing products of both the *cyaA* and *lktA* genes (see section 3.3). Urea extracts of Hyb1 toxin were prepared from cells of strain BL21/DE3 (pGW80, pGW54) by co-expression of *hyb1* and *cya*C or strain BL21/DE3 (pGW80, pGW78) by co-expression of *hyb1* and *lktC* genes. Stable proteins of 150 kDa were produced with comparable AC enzymic activity to that of CyaA, but no detectable haemolytic or toxic activity (Figs 9, 19, 23 and 24, Table 7). Hyb2 toxins were prepared as urea extracts

from strain BL21/DE3 (pGW88, pGW54) by co-expression of *hyb2* and *cyaC* genes or strain BL21/DE3 (pGW88, pGW78) by co-expression of *hyb2* and *lktC* genes. These appeared as stable proteins of 200 kDa as judged by SDS-PAGE and immunoblotting (Figs 9, 10 and 15. A). Again these hybrid toxins had AC enzymic activity comparable to CyaA and they also possessed and partial haemolytic and toxic activity. This is discussed more fully in section 4.3.

4.2 PURIFICATION OF CyaA AND HYBRID PREPARATIONS

4.2.1 Purification of CyaA from *B. pertussis* strains

This study describes the purification of CyaA from strain BP348 (pRMB1) with toxic, haemolytic and enzymic activities, and CyaA from strain BPDE386 with enzymic activity but no detectable toxic or haemolytic activities. In this work, the CaM agarose and DEAE-sepharose methods were chosen for purification of CyaA because these methods were expected to yield a high level of purified toxins with low amounts of degradation products, in keeping with previous reports (Sebo *et al.*, 1991; Sakamoto *et al.*, 1992). The purified CyaA preparations both contained a major polypeptide of 200 kDa, although some minor peptides of lower molecular weight were still present as judged by SDS-PAGE analysis and immunoblotting with monoclonal and polyclonal antibodies. This is consistent with data from previous investigators (Bellalou *et al.*, 1990 a). The presence of other protein components of *B. pertussis* such as FHA, PT and PRN was checked by immunoblotting with anti-FHA, anti-PT and anti-PRN antibodies and no detectable impurities were found (Fig. 14).

4.2.2 Purification of CyaA from recombinant *E. coli* strains

Toxic and non-toxic forms of recombinant CyaA were purified from crude urea extracts of inclusion bodies formed in *E. coli* strains by the convenient procedure of DEAE sepharose chromatography. Large quantities were obtained mainly as a polypeptide of 200 kDa with only a few degradation products as judged by immunoblotting with polyclonal antibody raised against 200 kDa CyaA in rabbits. The purified toxic form of CyaA could not be distinguished from the non-toxic form according to size of the polypeptides on SDS-PAGE gels (Figs 7).

4.2.3 Purification of hybrid toxins from recombinant *E. coli* strains

Hyb1 toxin was purified from inclusion bodies as a major polypeptide of 150 kDa protein, with few degradation products, from crude extracts of recombinant *E. coli* strains harbouring only one plasmid pGW80 (*hyb1*) or two plasmids pGW80 and pGW78 (*lktC*) or pGW54 (*cyaC*) by calmodulin affinity chromatography as judged by SDS-PAGE analysis or immunoblotting. Calmodulin affinity chromatography was used here because DEAE-sepharose was less effective at purification of this hybrid, presumably because it lacked the large number of glycine-aspartate repeat units of the C-terminal end of CyaA which formed the main basis of attraction of CyaA to the DEAE-sepharose. The C-terminal portion of Hyb1 was derived from LktA which has a fewer of these repeat units (Lally *et al.*, 1994). Hyb2 toxin was purified by DEAE sepharose chromatography as this protein possessed the C-terminal

region of CyaA. Purified Hyb2 toxin was detected mainly as a 200 kDa polypeptide according to SDS-PAGE analysis (Fig. 9).

4.2.4 Purification of CyaC, LktC and LktA

These proteins were partially purified from urea extracts of inclusion bodies from recombinant *E. coli* strains by urea washing of the inclusion bodies which removed contaminating soluble protein from the insoluble proteins of the inclusion bodies. The molecular weight of CyaC and LktC were 21 kDa and 19 kDa respectively, according to SDS-PAGE analysis and had very few contaminating polypeptides present (Fig. 13). The molecular weight of both active and inactive recombinant LktA was 105 kDa as judged by SDS-PAGE analysis and immunoblotting (Figs 10 and 15. B), but this preparation still had some contaminating polypeptides.

4.3 PROPERTIES OF CyaA AND HYBRID PREPARATIONS

4.3.1 AC Enzymic activity of CyaA and hybrid toxins

In this study, the AC specific activity of the urea extract of CyaA from *B. pertussis* BP348 (pRMB1) was significantly higher than that reported for either the wild-type *B. pertussis* or strain BPDE386 (Brownlie *et al.*, 1988). This increase in the specific activity was almost certainly due to a gene dosage effect where the presence of several copies of pRMB1 carrying the *cyaA* gene in each cell would increase the amount of CyaA protein overall. The AC

specific activity of purified toxin was enhanced 10-fold due to the removal of impurities from the urea extracts of inclusion bodies (Table 6).

The enzymic specific activity of toxic 200 kDa form of CyaA from the crude urea extracts of recombinant *E. coli* was lower than that of the non-toxic 200 kDa recombinant form of CyaA. This was probably due to the fact that the active form was produced by co-expression of CyaA and CyaC from two separate plasmids whereas the inactive form was produced by expression of *cyaA* alone from one plasmid. In the later case there would undoubtedly be a higher copy number of the CyaA-encoding plasmid. It was almost certainly not due to toxic effects of active CyaA in the cells because the protein was induced by IPTG addition. The enzymic activity of the Hyb1 was higher than that of Hyb2 toxin, which was probably due to the fact that it is a smaller protein (Table 7). Storage of purified CyaA in 8M urea at -70 °C did not reduced the enzyme activity (Table 5).

4.3.2 Haemolytic activity of CyaA, LktA and hybrid toxins

It has been reported previously that purified *B. pertussis* CyaA had comparable cell invasive AC activity and a 4-fold higher haemolytic activity on sheep erythrocytes than that of partially-purified CyaA recombinant toxin from *E. coli*, and thus the ratio of the haemolytic activity to cytotoxic activity of the recombinant active CyaA expressed in *E. coli* was markedly lower than that of CyaA from *B. pertussis* (Sebo *et al* ., 1991; Benz *et al.*, 1994; Hewlett, 1995). Hackett *et al.* (1995) have shown that whereas the CyaA from *B. pertussis* is exclusively palmitoylated at Lys-983, the recombinant CyaA from *E. coli* represented a heterogeneous mixture of CyaA proteins with 13%

myristoylated and 67% palmitoylated at Lys-983 and an additional 67% palmitoylated at Lys-860. Thus the differences in the haemolytic:toxic activity ratios of recombinant CyaA compared to *B. pertussis* CyaA could be due to these differences in the chemical nature of the acylation.

In the present study, the purified recombinant CyaA (PAR CyaA) had significantly weaker haemolytic activity compared to the haemolytic activity of purified CyaA from *B. pertussis* BP348 (pRMB1)(PAN CyaA) (Fig. 18, Table 6), confirming previous work. The recombinant CyaA protein co-expressed with LktC had no haemolytic activity on sheep erythrocytes which is in keeping with previous reports that indicated the strong specificity conferred by LktC. For example, HlyA could not be activated by LktC (Forestier and Welch, 1990). The haemolytic activity of the recombinant LktC-modified LktA on sheep erythrocyte was low, which was also consistent with a previous report (Forestier and Welch, 1990), whereas the CyaC-modified LktA showed markedly higher haemolytic activity for sheep erythrocytes than LktC-modified LktA. This may be due to differences in the way in which these C proteins modify the protoxins, for example, by adding different acyl groups. This was investigated in the in vitro activation experiments and is discussed below (section 4. 4).

The CyaC-modified or LktC-modified Hyb1 showed no haemolytic activity on sheep erythrocytes. Although LktC was unable to activate CyaA, it was more efficient at activating Hyb2 than CyaC. This indicates that a domain between amino acids 379 and 616 of LktA is required for recognition by LktC and that Lys-554 in LktA is a likely activation site, although recent data have indicated that it may not be the only site in the complete LktA toxin (Pellett and Welch, 1996). The CyaC-modified Hyb2 had lower haemolytic activity for

sheep erythrocytes than that of LktC-modified Hyb2 (Fig. 19, Table 7). The fact that LktC- or CyaC-modified Hyb2 had significantly lower haemolytic activity than CyaC-modified CyaA, suggest that the reduction in haemolytic activity could be due to inefficient acylation of the Hyb2 protein molecules or due to efficient acylation but in a manner which either creates a conformational change in the hybrid protein that functions poorly as a haemolysin. Alternatively, creates a variety of acylated derivatives could be produced only some of which are capable of functioning efficiently to form pores in erythrocytes. This is in keeping with dissociation of the haemolytic activity from its invasive activity (Bellalou *et al.*, 1990 b; Iwaki *et al.*, 1995). The haemolytic activity of CyaA, LktA and hybrid toxins was stable for up to 6 months when stored as 8M urea extracts at -70 °C.

4.3.3 Cytotoxic activity of CyaA, LktA and hybrid toxins

The cytotoxic activity of the recombinant toxic form of CyaA against bovine-derived BL3 and mouse-derived J774.2 cells was shown to be comparable to that of *B. pertussis* CyaA (Figs 21 and 22, Table 6). The CyaC-modification was required for cytotoxic activity of CyaA, whereas the LktC-modification did not activate CyaA. Again, CyaC was able to activate LktA protoxin efficiently without affecting the specificity of the modified LktA for ruminant cells. The toxin was still fully active against BL3 cells of ruminant origin, but showed no activity against J774.2 cells of murine origin. As was found for the haemolytic assay, LktC was more efficient at activating Hyb2 than CyaC with regard to activity against BL3 cells and there was some evidence that LktC modification bestowed a degree of target cell specificity

because LktC-modified Hyb2 was more active against BL3 cells of ruminant origin than against J774.2 cells, whereas the CyaC-modified Hyb2 had a similar activity against both cell types. These observations indicated a noticeable change in target cell specifity of the LktC-modified Hyb2 compared to the CyaC-modified hybrid. This may indicate that the mode of activation by LktC may have an influence on the target cell specifity of the native LktA toxin which is only active against ruminant cells. However, the LktC-modified Hyb2 was still not ruminant specific which shows that other features of the LktA protein, not contained in Hyb2, are necessary for this phenotype.

4.4 IN VITRO ACTIVATION OF CyaA, LktA AND HYB2

Barry *et al.* (1991) reported that *B. pertussis* adenylate cyclase protoxin required post-translational activation by the product of the *cyaC* gene (CyaC protein) in order to acquire haemolytic and toxic activities,. Similarly, the other members of the RTX toxins such as the HlyA of *E. coli* or LktA of *P. haemolytica* require activation by C-proteins. Issartel *et al.* (1991) reported that activation of proHly by HlyC was achieved by the transfer of a fatty acyl group from acyl carrier protein to proHlyA. By analogy to the HlyA activation system, *B. pertussis* CyaA protoxin activation also could involve fatty acylation. Hackett *et al.*, (1994) have demonstrated that CyaA was modified in vivo by amide linked paltimoylation of the ε-amino group of Lys-983. Because of the limitations in the in vivo studies of activation for examining the mechanism of protoxin activation, the in vitro approach to understanding the activation mechanism which converts inactive protoxins to the cell invasive toxins was investigated.

In the present study, an in vitro system was developed in order to examine the mechanism of CyaC-dependent acylation of proCyaA and LktC-dependent activation of LktA. The in vitro activation of HlyA by HlyC was the only in vitro system described when this work was undertaken, and although the in vivo acylation of CyaA had been confirmed by Hackett *et al.* (1994), it was only assumed that LktA was modified in a similar manner. It was hoped to relate biological activity to the manner in which the toxin is activated and finally to investigate the effect of variation of the modifying fatty-acyl group or C-proteins on target cell specificity and toxic activity of CyaA and LktA.

Previous work on in vitro activation of proHlyA by HlyC had shown a requirement for overproduction and purification of HlyA and HlyC from recombinant *E. coli* strains (Hardie *et al.*, 1991). In the present study, proCyaA and CyaC were overproduced by using the *E. coli* T7 expression system for the *cyaA* and *cyaC* genes and purified from inclusion bodies. The inclusion bodies were washed with 1M urea in HEPES buffer to remove any contaminating soluble proteins and then the aggregated insoluble proteins were solubilized with 8M urea in HEPES buffer to obtain partially purified preparation of CyaA and CyaC. They were prepared in urea and HEPES, because Cl^- ions and Tris were found to be inhibitory for the activation reaction of HlyA by HlyC. In fact, in our system, EGTA was found to be beneficial for activation (Table 14), indicating that the binding of Ca^{2+} by proCyaA is not necessary for activation by CyaC. This would suggest that the two post-translational requirements of proCyaA activation, Ca^{2+} binding and CyaC-dependent modification are completely independent of each other.

The purified CyaA preparations were not used in the assays because it was found in early experiments that the partially purified CyaA, prepared by

washing of inclusion bodies, did not contain any contaminating CAF; CyaA and CyaC mixed in the absence of CAF produced no active toxin (Table 8). The purified CyaA was kept for immunological studies as large amounts were needed for this work. When only the partially purified CyaA and CyaC were incubated together, no active CyaA protein was generated, but active CyaA toxin was generated when 20-fold dilutions of the 200 kDa proCyaA and 21 kDa CyaC proteins were mixed with a 20 fold dilution of a cytosolic (S100) extract (CAF) of *E. coli* (Tables 8). The fact that proCyaA, produced from intracellular inclusion bodies, could be activated indicated that the CyaB/CyaD/CyaE-dependent secretion process was not involved in the activation of CyaA. Dilution to remove excess urea was favoured instead of dialysis of the urea extracts before use in the activation reaction because dialysis resulted in protein aggregation, particularly of CyaC, and the extent of activation was markedly reduced. The cellular factor (CAF) required for activation was lost if the *E. coli* S100 extract was dialysed with a membrane of high M_r (200 kDa) cut-off before addition to the activation mix and only a low level of cytotoxic activity of CyaA was generated. Dialysis of the cellular factor (CAF) with a membrane having a low M_r cut off (<14 kDa) allowed retention of most of the activating factor, and thus approximately the same level of cytotoxic activity was achieved as with undialysed CAF (Table 8). S100 extracts of *B. pertussis* strain BP348, which is defective in CyaA production, and of *P. haemolytica* (Ph30) lacking LktA were also used in the activation reaction as a source of cellular factor and supported the activation (Table 14). These findings indicated that the cellular factor had a low molecular mass which was consistent with a requirement for acyl -ACP in the activation reaction (Hardie *et al.*, 1991; Issartel *et al.*, 1991). This was

supported by additional experiments, where commercial acyl-ACP, charged in vitro with various fatty acids, was able to act as a co-factor to activate CyaA (Table 10). The results demonstrated that acylation conferred cytotoxic and haemolytic activities upon the protoxin, and the level of the activation depended upon the fatty acid tested, with palmitoylation showing highest activity and myristoylation showing less activity with the other fatty acids producing very low activity. Fatty acid preference for the acylation reaction with CyaA was palmitic acid (PA, C_{16})> myristic acid (MA, C_{14})> palmitoleic acid (PLA, $C_{16.1}$)> lauric acid (LA, C_{12})>oleic acid (OA, $C_{18.1}$)>stearic acid (SA, $C_{18.1}$) (Tables 10). These fatty acids were chosen because they were commercially available and were convenient to solubilise for use in the in vitro activation reactions. These observations indicated that the chain length or the rigidity of the fatty acids could effect the interaction of the toxin with target cells. The results are similar to those reported by Issartel *et al.* (1991) for acylation of HlyA by HlyC. In their studies, however, after acylation of ACP in vitro with a range of fatty acids, myristoylated ACP was the most active in the conversion of proHlyA to a haemolytic toxin with palmitoleic acid next, closely followed by palmitic acid with lauric acid and cis-octadecenoic acid showing much less activity. In both cases, however, the C_{14} and C_{16} fatty acids proved most efficient at activating the CyaA and HlyA protoxins. Whichever fatty acid addition was used in the in vitro activation reaction of CyaA by CyaC, the in vitro-modified CyaA showed greater cytotoxicity for J774.2 cells than BL3 cells. This was true also for CyaA activated in vivo by CyaC (Table 6). The in vitro activation of CyaA by LktC revealed no activation which was also true of the in vivo situation.

The partial purification of LktA and LktC was also achieved by washing inclusion bodies. The in vitro activation of LktA as another member of RTX family of toxins was investigated to compare the in vitro activation of LktA with that of CyaA and to examine the effect on the biological activity of heterologous activation of the protoxins CyaA and LktA by LktC and CyaC respectively. CyaA was not activated by LktC, but LktA was activated by CyaC as well as by LktC, again showing the high specificity of LktC compared with CyaC, which was apparent from the in vivo activation. It was interesting to see that the specificity of LktC for ruminant cells was preserved in this in vitro reconstituted system.

It was found that LktA in vitro-activated by LktC or CyaC showed marked inhibition of rabbit neutrophil chemiluminescence, whereas the native LktA had very little effect on rabbit neutrophils, even at the highest concentration. Moreover the in vitro activated LktA inhibited the CL response of rabbit neutrophils to a 10-fold greater extent than the in vivo activated form. However in vitro activated LktA retained specificity for BL3 cells with regard to cytotoxicity as measured by the MTT assay. These data showed apparently that active LktA produced by in vitro activation or partially purified from inclusion bodies from *E. coli* had an effect on the zymosan-induced chemiluminescence response of rabbit neutrophils which was not exhibited by the native LktA toxin found in the culture medium after growth of *P. haemolytica*. The reason for this loss of target cell specificity by recombinant LktA is not clear as it was only observed in the chemiluminescence inhibition (CLI) assay. No activity of LktA toxins from whatever source was observed in the MTT cytotoxicity assay against J774.2 cells. The result implies that the toxins are triggering different types of responses in the target cells which are detected in the respective

assays. The MTT cytotoxicity assay is measuring the killing capacity of the toxin whereas the CLI assay is measuring inhibition of the respiratory burst. It is possible that the recombinant LktA toxins can bind to rabbit neutrophils and inhibit their response to opsonised zymosan but can not penetrate the cells in order to kill them. The native LktA, on the other hand, can not interact with rabbit neutrophils in such a way as to inhibit the respiratory burst. All the RTX toxins in their host strains are exported from the cell and it is possible that this process is crucial in some way for determining the final properties of the toxins. For example, there is evidence to suggest that LPS may become closely associated with the toxins, perhaps as they are exported from the cell (Czuprynski and Welch, 1995). The synthesis, export and activity of HlyA are closely linked to LPS biosynthesis. RTCH is a transcriptional activator of the *rfa* operon which encodes enzymes for the synthesis of the LPS core, but it also activates the *hly* operon (Bailey *et al.*, 1992). Inactivation of *rfa*P, one of the genes of the *rfa* operon, causes a loss of extracellular HlyA activity associated with aggregation of HlyA (Stanley *et al.*, 1996). Interestingly, a similar phenomenon to that seen here was reported by Tu *et al.* (1994) working with the ApxIIA toxin of *A. pleuropneumoniae*. The ApxIIA toxin, when expressed in *E. coli* existed in two forms: the intracellular form had both haemolytic and cytolytic properties, but the secreted form was cytolytic, but non-haemolytic. This indicated that during the secretion process, the conformation of the toxin was altered so that it lost haemolytic activity.

The order of fatty acid preference for the in vitro activation of LktA was the same as that of CyaA by in vitro activation. Thus the C-proteins do not differ in fatty acylation preference and the precise nature of the acyl group added was not important for cell specificity (Tables 10-12). The in vitro

activation of Hyb2 with regard to fatty acid preference, whether activated by CyaC or LktC was the same as LktA or CyaA (Table 13). In fact, the biological activity of in vitro-activated Hyb2 followed the same pattern as Hyb2 activated in vivo. Hyb2 activated in vitro by LktC had greater activity against BL3 cells than J774.2 cells, whatever fatty acid was used, but Hyb2 activated in vitro by CyaC was less active, and had similar cytotoxicity against both cell types which was the same as the in vivo situation (Table 7).

Kinetic studies revealed that the haemolytic activity of the in vitro-activated CyaA toxin was highly labile (Fig. 27). The initial rates of activation appeared to increase with increasing temperature from 4 °C to 37 °C, indicating that the interaction between the activation components is more ionic than hydrophobic. Maximal rates were obtained after 10 min incubation at 37 °C and after 30 min at 24 °C and 4 °C. However, the haemolytic activity was rapidly lost after a long period of incubation of the activation components and little or no haemolytic activity was detected after incubation for 60 min . The reason for this decline or loss of activity is not clear, but it appears to be temperature dependent and may be due to loss of the modifying acyl group or it may be due to the aggregation of the toxin.

In vitro activated CyaA was assayed by using various assays for invasiveness, cytotoxicity and haemolytic activity (Table 9). When the ratio of protein concentrations required for 50% haemolysis and 50% stellation or cytotoxicity for different cell types were compared to that of in vivo activated CyaA, the level of haemolytic activity observed for the in vitro-activated CyaA was less than 10% of that expected from its invasive CyaA activity. This may be due to lack of specificity inherent in an in vitro sytem using a crude S100 extract as a source of acyl-ACP and fatty acids. Because the CyaA toxin

contains 47 lysine residues, it is reasonable to suppose that several residues are randomly acylated in vitro and the modification results in a mixture of differently modified populations, which may have different toxic and haemolytic activities.

Heveker *et al.* (1994) observed similar differences in invasive and haemolytic activities for CyaA activated in vitro by chemical acylation of proCyaA toxin using water soluble acylpyrophosphates. This system leads to random acylation of multiple lysine residues and the active toxin probably represents a sub-population that is able to assume a physiologically active conformation. The chemically myristoylated toxin showed 10-50 fold lower haemolytic activity than toxin modified in vivo by co-expression of *cyaA* and *cyaC* in *E. coli*.

4.4.1 Overall conclusions

The capacity to express the *A* and *C* genes for CyaA and LktA separately in *E. coli* mean that each protoxin could be co-expressed with a heterologous C protein. It was found that LktC could not interact with CyaA to produce an active toxin, indicating complete specificity of LktC for LktA. On the other hand, CyaC was able to activate LktA to produce an active toxin which showed that CyaC was less specific than LktC in its activity. CyaC-modified LktA however was still completely ruminant-cell specific indicating that the manner of acylation of the protoxin had no effect on target cell specificity. This was borne out also by the in vitro activation studies where, regardless of the fatty acyl group used to charge the LktA protoxin, no change in target cell specificity was detected.

Incorporation of a 238 amino acid sequence, spanning part of the activation region of LktA, into CyaA allowed LktC to interact with the Hyb2 molecule to produce a partially active toxin. Thus this sequence must contain the modification site for LktA and presumably has a structural conformation different to that of equivalent region in CyaA. This region presumably defines part of the domain determining ruminant target cell specificity to LktA. Hyb2 constitutes a good starting point for creation of a completely ruminant-specific adenylate cyclase toxin. The ability to define in general terms the region specifying cell tropism in RTX toxins may have wider implications because it may allow the creation of cell specific toxins for selective destruction of cells or for delivery of specific antigenic determinants to specific cell types.

4.5 PROTECTION PROPERTIES OF CyaA, Hyb1 AND Hyb2 IN MICE

4.5.1 Extent of toxicity of toxin preparations in mice

The data presented in this study (Figs 34 and 35) from the weight-gain test (toxicity test) following immunization with active and inactive *B. pertussis* CyaA preparations, shows that there was a small effect of each toxin on mouse weight gain. This may have been due to impurities of preparations with other *B. pertussis* antigens such as LPS, even in small amounts in the purified preparations, because the non-toxic form of *B. pertussis* CyaA exhibited the same pattern of toxic effects. The crude extract preparations were more toxic than purified CyaA, and the crude extract from *B. pertussis* showed more toxicity than crude extract from *E. coli*, again indicating the effect of impurities

in crude preparations. There was, however, no marked differences between mice vaccinated with the active invasive toxin and those given the inactive non-invasive toxin. This indicated that, at least at the dosage used here, active CyaA was not creating a toxic physiological response.

4.5.2 Protective activities of CyaA from *B. pertussis* strains

Guiso *et al.* (1989) demonstrated that passive immunization with monoclonal and polyclonal anti-CyaA antibodies raised against the purified 45 kDa catalytic fragment of CyaA in mice, protected mice passively against lethal intranasal challenge with *B. pertussis* and *B. parapertussis*. This indicated that protection might occur either by antibody inhibiting penetration of the CyaA into the host cell lymphocytes or by inhibiting bacterial colonization, but not by inhibiting the catalytic activity of the enzyme in the host cells because the monoclonal anti-CyaA had no inhibitory effect on the catalytic activity of the enzyme. They also reported that active immunization with 45 kDa CyaA purified from culture supernate and with catalytic activity but no cytotoxic activity in vitro protected mice against intranasal or intracerebral challenge with *B. pertussis* or *B. parapertussis* as efficiently as whole cell vaccine (Guiso *et al.*, 1991). This suggested that the major protective epitopes of CyaA were located on the N-terminal region of the enzyme. In subsequent reports, active immunization with purified *B. pertussis* CyaA of 200 kDa, but which still contained several lower molecular weight degraded proteins, protected mice as efficiently as whole cell vaccine against *B. pertussis* and *B. parapertussis* colonization in the murine intranasal model, although only the activated toxin was effective and inactive toxin made in

E. coli was not protective (Guiso *et al.*, 1991; Betsou *et al.*, 1993). This indicated that CyaC-mediated modification of CyaA was important not only for invasive activity of the protein but also for its protective activity. Betsou *et al* (1995), by analysis of the protective activity of different purified truncated proteins derived from recombinant CyaA, showed that the important protective epitopes were located at the C-terminal end of CyaA in the last 800 residues which contradicted the earlier report by the same group (Guiso *et al.*,1991) that they were N-terminally located. They explained this apparent anomaly by suggesting that the 45 kDa preparation used in the previous work was contaminated with C-terminal fragments with no AC enzymic activity. These fragments spanned the modification and repeat region of the CyaA and were cleaved from the rest of the CyaC-modified CyaA molecule during secretion of the toxin.

In the present study, the crude extract of active *B. pertussis* CyaA, as well as purified active *B. pertussis* CyaA, both with M_r of 200 kDa, exhibited protective activity in mice against intranasal challenge with *B. pertussis* strain 18323 similar to the protective efficacy of the whole-cell DPT vaccine. This was shown by the low lung; body weight ratio, low bacterial numbers in the lungs and the minor effect on mouse weight gain (Figs 37 and 38, Tables 15 and 16), compared to mice vaccinated with either crude or purified inactive CyaA of 200 kDa expressed in strain *B. pertussis* DE386. These had no protective activity against *B. pertussis* colonization in mice confirming, with native CyaA, that CyaC-mediated modification of CyaA toxin is critical for its protective activity. Presumably the CyaC modification creates an immunodominant epitope (s) required for protective activity probably by

changing the conformation of the CyaA protein molecule in such a way that the protective epitope (s) is now presented to the immune system.

The protective effect of active CyaA could be due to antibodies preventing CyaA acting on alveolar macrophages which would normally allow multiplication of *B. pertussis* on the surface of the respiratory epithelium early in the infection. This study, as well as previous work strongly suggests that CyaA should be considered as a useful component of an acellular vaccine, because of the presumed important role of immunity to CyaA in preventing colonization. The purified CyaA showed little evidence of toxicity but any possible effects would be minimised by site-specific mutation that either abolishes AC enzymic activity or prevents pore formation. However, the means by which CyaA promotes a protective immune response is not clear. It is a toxin that penetrates target cells and it is possible that it may be presented via the MHC class I antigen presentation pathway which is important for cell mediated immunity. This point was investigated to some extent in this work (see section 4.8).

4.5.3 Protective activities of recombinant CyaA and hybrid toxins from *E. coli* strains

Betsou *et al.* (1993) showed that a purified preparation of active CyaA derived from recombinant *E. coli* containing a major polypeptide of 200 kDa and several proteolytic fragments exhibited protective activity, but to a lesser extent than that of native CyaA, against *B. pertussis* colonization in the intranasal mouse model. They suggested that this may have been related to its

lower haemolytic activity. They also found that purified inactive recombinant CyaA of 200 kDa had no protective activity.

In the present investigation, the protective activity of crude extracts and purified preparations of recombinant active CyaA of 200 kDa synthesised in *E. coli* showed a similar pattern of protective activity to that of the native CyaA or the whole-cell DPT vaccine against intranasal challenge with *B. pertussis* in mice. As was found with inactive native CyaA, inactive recombinant CyaA was not protective.

The hybrid toxins Hyb1 and Hyb2 were also evaluated for their protective activity against *B. pertussis* challenge. Preliminary experiments with Cya C- or LktC-mediated Hyb1 toxins showed that it had no protective activity and exhibited a similar pattern of response in mice to inactive CyaA. The Cya C- or LktC-modified Hyb2 toxins were, however somewhat protective against *B. pertussis* challenge, and with the LktC-modified Hyb2 showing the better protective properties. Thus this hybrid toxin, which showed only 5% of the cell invasive activity of active CyaA, acted nevertheless as a protective antigen. This indicated that much of the protective epitope(s) structure determined by the C-terminal portion of CyaA had been retained even though part of the C-modification region spanning 238 amino acids had been replaced by the equivalent region from LktA. The CyaC- or LktC-modified Hyb2 toxin is suggested as a candidate for inclusion in future recombinant pertussis vaccines, since it exhibited no significant cytoxicity and may represent a safe vaccine component with efficient protective activity.

4.6 ANTIBODY RESPONSES TO CyaA AND HYBRID TOXINS IN MICE

4.6.1 Anti-CyaA antibody response

Betsou *et al.* (1995) showed that anti-*B. pertussis* CyaA polyclonal antibody used in Western blots recognized recombinant CyaA polypeptide of 200 kDa as well as the purified *B. pertussis* CyaA polypeptide. Antiserum from infants, children, or adults infected with *B. pertussis*, and obtained between 4 and 8 weeks from the beginning of the cough, was shown to recognize *B. pertussis* CyaA or *E. coli* CyaA (Betsou *et al.*, 1993). Similarly, sera from mice infected with strain *B. pertussis* 18323 recognized *B. pertussis* CyaA or *E. coli* CyaA, and thus both types of toxin were immunologically similar in Western blotting. In addition, the presence of antibodies neutralizing CyaA toxin activity is not a reliable measure of the induced protection against *B. pertussis* infection because a truncated-recombinant CyaA protein, which was not protective at all, induced a strong neutralizing antibody response (Betsou *et al.*, 1995). No inhibition of the CaM-dependent AC activity by anti-CyaA antibodies has been observed (Rogel *et al.*, 1988). Thus antibodies directed mainly against C-terminal hemolysin portion of CyaA are able to neutralize its haemolytic activity but not the enzymic AC activity of its N-terminal domain (Betsou *et al.*, 1995). However, antibodies against a truncated protein consisting of the N-terminal domain and the last 217 amino acids of the CyaA molecule were able to neutralize both enzymic and cytotoxic activity of the recombinant active CyaA (Betsou *et al.*, 1995). Farfel *et al.* (1990) reported that anti-CyaA antibodies were produced in man during

pertussis infection in titres higher than after vaccination, but these antibodies did not neutralise the enzyme activity or penetration activity of CyaA. This does not rule out the possibility that locally produced antibodies neutralise the enzymic and invasive activities and may be important for defence against the CyaA toxin in the respiratory tract.

In the present study, mice developed strong specific IgG antibody responses against the active or inactive form of CyaA toxin 21 days post-vaccination with CyaA preparations, and the dominant subclass was IgG1. Low titres of IgE specific for CyaA were also induced in mice after immunization with active and inactive CyaA toxins (Tables 20-23). In these mice the higher ratio of IgG1 to IgG2a specific for CyaA and the lack of an IgG2b specific antibody response indicated a Th2 cell response to CyaA toxin which enhances the secretion of IgG1 by induced B-lymphocytes. These findings indicated that a strong Th2-enhanced antibody response following immunization with CyaA toxin may be responsible for the reduction in the level of *B. pertussis* colonization in respiratory tracts of mice, perhaps by interaction of CyaA-specific antibody with the CyaA toxin which stops its entry into immune effector cells.

4.6.2 Immunoblot analysis of anti-CyaA immune sera

The immunoblot study of sera from the mouse protection experiments 1 and 2 raised against crude and purified preparations of CyA either from *B. pertussis* or recombinant *E. coli* strains, and probed against purified CyaA preparations demonstrated the presence of antibodies to the 200 kDa toxin in all sera taken 21 days after first immunization. The sera also reacted with

proteins of lower molecular weights (Figs 39 and 40) that were presumably breakdown products of the CyaA molecule in the preparations. A monoclonal antibody specific to the enzymic domain of the CyaA molecule also recognized these proteolytic fragments indicating that they contained the N-terminal domain of CyaA.

The sera raised against either the purified non-toxic or the toxic form of recombinant CyaA of 200 kDa recognised both toxic or non-toxic forms of recombinant CyaA as well as toxic or non-toxic forms of CyaA purified from *B. pertussis*, indicating that all recombinant CyaA preparations and *B. pertussis* CyaA preparations were immunologically similar in Western blots. This indicates that certain epitopes are the same in all CyaA preparations. In addition, immunoblotting of antisera raised against purified *B. pertussis* CyaA preparations did not recognize FHA or PT or PRN, indicating the absence of these factors in the purified preparations of CyaA toxins.

The CyaA-specific polyclonal antibody raised against purified recombinant CyaA in mice recognized Hyb2 in Western blots as well as CyaA, but failed to recognize Hyb1. This indicated that the anti-CyaA antibodies were directed predominantly against epitopes present in the C-terminal domain of CyaA or conformational epitope(s) formed when the C-terminal region, the modification regions and N-terminal domains are intact. This observation was in keeping with previous findings by Betsou *et al.* (1995), who reported that truncated recombinant CyaA proteins were recognized by the polyclonal sera of *B. pertussis* infected humans or mice as long as they contained the C-terminal 800 amino acids. This indicated that the intact modification and repeat region in the last 800 amino acid residues of the CyaA molecules are essential for a protective antibody response. The sera raised in the rabbit against LktA

recognized LktA and Hyb1, but failed to recognize the Hyb2 protein. This indicated again that the immunodominant region of LktA was in C-terminal region, within the last 575 amino acids. The data as a whole showed the immunological relatedness between the two proteins of the RTX family of cytolytic toxins and addressed the importance of the C-terminal repeat region as the immunodominant region of both toxins.

4.7 ADJUVANTICITY OF CyaA

Bottomly (1988) showed that the use of Alum as an adjuvant may induce a Th2 response and production of IL-4 and IL-10 which in turn could down-regulate the Th1 response and enhance the antibody response. The present work also showed that Alum had an adjuvant effect on the antibody response to CyaA, because a higher level of antibody response to CyaA was detected in sera of mice when both active and inactive CyaA were co-administrated with Alum (Experiments 1 and 2) compared with adminstration of CyaA alone (Experiments 4 and 5). Interestingly, in the mouse protection experiments (1 and 2) the administration of active CyaA even with alum in mice showed a higher anti-CyaA antibody response compared with adminstration of inacive CyaA with alum. For this reason, the present study examined the adjuvant effect of active CyaA alone on the antibody response to itself and to another protein antigen (Oa) administrated at the same time.

4.7.1 Effects of CyaA on primary and secondary anti-Oa responses

The present study has shown that the co-adminstration of invasive CyaA with a protein antigen such as ovalbumin in mice enhanced several-fold the specific IgG, IgG1 and IgE antibody responses to Oa compared with adminstration of Oa alone. The Ig2a anti-Oa response was enhanced 2-3 fold, whereas IgG2b was not detectable in any sera (Figs 43 and 44, Table 20 and 21). Interestingly, such an enhancement in antibody response to active CyaA was not observed with the inactive form of CyaA, indicating that invasive activity of CyaA toxin on the T helper lymphocytes may be responsible for the enhancement effect on the antibody responses to Oa. The findings also suggest that the effect of CyaA on antibody responses may not be specific to Oa, but similar experiments with other immunizing antigens will need to be done. The more interesting test will be to study any enhancing effect of CyaA on the antibody response to PT, FHA and PRN in future acellular vaccines. It has been reported previously that PT can enhance antibody responses to another antigen (Samore and Siber, 1996). The adjuvant effect of both CyaA and PT could be due to the fact that they both increase the level of intracellular cAMP in the target cells or immune effector cells. It has been reported that cAMP upregulates IL4 and IL5 production by activated $CD4^+$ T cells (Lacour et al, 1994).

4.7.2 Effects of CyaA on primary and secondary anti-CyaA responses

The primary and secondary IgG anti-CyaA responses were enhanced in mice vaccinated with PAR CyaA compared with mice vaccinated with PIR

CyaA or PIR CyaA with Oa, although the antibody response to PAR CyaA alone was lower compared to antibody response to PAR CyaA with alum. These findings indicate that activity of active form of CyaA may stimulate a greater Th2 response than the inactive form of CyaA.

4.8 EFFECTS OF CyaA ON CYTOKINE PRODUCTION

It was reported by Redhead *et al.* (1993) that infection in the mouse or vaccination with whole cell vaccine induced a strong activation of the type1 CD4+ T helper cells (Th1 response) associated with the induction of cell mediated immunity. High levels of IL-2 and INF-γ and low levels of IL-4 were produced and there was little antibody production. This work suggested that phagocytic cells such as macrophages and neutrophils were being activated under these conditions. Immunization with acellular vaccines containing mainly PT induced a strong antibody response (humoral immunity) and production of high levels cytokines such as Il-4 and IL-5 associated with the activation of type 2 $CD4^+$ T cells (Redhead *et al.*, 1993).

In this preliminary study, the effect of CyaA toxin on cytokine release from spleen cells of mice immunized with CyaA, was investigated and provided indirect evidence for the induction of a predominantly Th2 lymphocyte response by CyaA although there was low level production of cytokines associated with a Th1 response as well. The spleen cells from mice immunized with active toxin produced high levels of IL-4 and IL-5 and IL-10 after in vitro stimulation with CyaA toxin for 48 h and low levels of INF-γ and IL-2, indicating a mixed, but predominantly Th2 response to the toxic form of CyaA. The cytokine response of spleen cells of mice immunised with inactive

CyaA was similar, but the cytokine levels were lower (Table 24). These findings, suggest that the invasive CyaA toxin may stimulate or activate Th2 lymphocytes directly, or indirectly via other cytokines, to secrete IL-4 and IL-5 which then induce B lymphocytes to produce IgG1. The possibility that, because of its cell invasive nature, the CyaA toxin also stimulates a Th1 response requires further investigation.

4.9 OVERALL CONCLUSIONS

The work on the immunogenicity of the CyaA toxin showed clearly that only activated invasive CyaA was protective and that the recombinant CyaA was as effective as CyaA from *B. pertussis*. The capability now exists to overproduce the CyaA toxin for possible inclusion in an acellular vaccine. Development of acellular vaccines for pertussis has emphasised the inclusion of PT, FHA and other adhesins such as PRN. CyaA has been overlooked, mainly because of difficulty in obtaining sufficient purified material. The ability to produce the toxin in *E. coli* should overcome this problem. It will be of interest to investigate fully the type of protective response promoted by active CyaA. As an invasive protein which is degraded intracellularly it may be processed via the MHC class I-restricted pathway to activate the cell mediated arm of the immune system. Some evidence for this was provided by the synthesis of small amounts of IL-2 and INFγ, both of which promote this response. It will be important to try and separate Th1 and Th2 specific $CD4^+$ T cell clones from the spleens of vaccinated mice and to compare the cytokine profiles created by other protective antigens (such as PT and FHA) and WCV with that of CyaA.

CyaA was shown to act as an adjuvant and increase the antibody response to itself and to the co-adminstrated antigen Oa. It will be important to assess if CyaA can act as an adjuvant for other protective antigens such as FHA and PRN and perhaps even PT itself. Further work will also need to investigate whether or not the AC enzymic activity of CyaA is required for the protective and adjuvant effect of the toxin.

APPENDICES

APPENDIX I: MEDIA

A. Bordet-Gengou Agar (BG) (pH 7.4)

BG-Agar base (Gibco-BRL)	7.2	g
Glycerol	1%	(v/v)
Distilled water	160	ml

Dissolved and autoclaved at 121°C for 15 min. 20%(v/v) sterile defibrinated horse blood and 10μg/ml tetracycline was added to the autoclaved BG agar cooled previously to 45 °C.

B. Cyclodextrin liquid medium (CDL) (pH 7.4)

L-Proline	0.24	g
NaCl	2.5	g
KH_2PO_4	0.5	g
KCl	0.2	g
$MgCl_2, 6H_2O$	0.1	g
$CaCl_2$	0.02	g
Tris	6.1	g
Casamino acids	10	g
Methyl β cyclodextrin	1	g
Distilled water	990	ml

Dissolved and autoclaved at 121 °C for 15 min

C. Supplement for 1 litre of CDL medium

L-cysteine	0.04	g
$FeSO_4, 7H_2O$	0.01	g
Nicotinic acid (Niacin)	0.004	g
Glutathione	0.15	g

Ascorbic acid	0.4	g
Distilled water	10	ml

Dissolved and sterilized by syringe filter (0.45 µm pore size, Gelman)

D. 2 xYT broth

Tryptone	16	g
Yeast extract	10	g
NaCl	5	g
Distilled water	1	l

Dissolved and autoclaved at 121°C for 15 min.

E. 2 xYT agar

Tryptone	16	g
Yeast extract	10	g
NaCl	5	g
Agar technical	12	g
Distilled water	1	l

Dissolved and autoclaved at 121°C for 15 min.

F. Brain heart infusion broth (BHIB) (pH 7.4)

Brain heart infusion dehydrated (Oxoid)	37	g
Distilled water	1	l

Dissolved and autoclaved at 121°C for 15 min.

G. Brain heart infusion agar (BHIA) (pH 7.4)

Brain heart infusion dehydrated (Oxoid)	37	g
Agar technical	12	g
Distilled water	1	l

Dissolved and autoclaved at 121°C for 15 min.

H. Tissue culture medium for BHK21 cells

MEM (Gibco-BRL)	100 ml
HEPES pH 7.4	22 mM
Fetal calf serum	2%(v/v)

I. Tissue culture medium for J774.2 cells

RPMI 1640 (Gibco-BRL)	100 ml
L-Glutamine	200 mM
Penicillin/Streptomycin	10 mg/ml
Amphotericin B (Fungizone)	250 µg/ml
Fetal calf serum	10%(v/v)
HEPES (pH 7.4)	20 mM

J. Tissue culture medium for BL3 cells

RPMI 1640 (Gibco-BRL)	100 ml
L-Glutamine	200 mM
Fetal calf serum	10%(v/v)
HEPES (pH 7.4)	20 mM

APPENDIX II Buffers and reagents

A. SDS-PAGE

a. Acrylamide/Bisacrylamide

Acrylamide	29.2 g
N, N-methylene bis acrylamide	0.8 g
Distilled water	100 ml

b. Lower gel buffer (pH 8.8)

Tris (1.5M)	18.2	g
SDS	0.4	g
Distilled water	100	ml

The pH was adjusted to 8.8 with 2N HCl and the final volume made up to 100 ml with distilled water and the buffer filtered through Whatman No. 1 filter paper and stored at 4°C.

c. Upper gel buffer (pH 6.8)

Tris	6.2	g
SDS	0.4	g
Distilled water	100	ml

The pH was adjusted to 6.8 with 2N HCl and the final volume made up to 100 ml with distilled water and the buffer filtered through the Whatman No. 1 filter paper and stored at 4°C.

d. TEMED (Sigma)

Undiluted stock, stored in dark.

e. Ammonium persulphate (APS)

A 10% solution (100 mg/ml in distilled water) made up freshly before use

f. Solubilising buffer

Glycerol	10	ml
2-mercaptoethanol	5	ml
SDS	4	g
Bromophenol blue	0.01	g

Upper gel buffer (pH 6.8) (1/4 dilution)	100	ml

g. Running buffer (8.3)

Tris	3.03	g
Glycine	14.4	g
SDS	1	g
Distilled water	1	l

h. Staining solution

Coomasie Blue R250	1.525	g
50% (v/v) methanol	454	ml
Glacial acetic acid	46	ml

i. Destaining solution

Methanol	50	ml
Glacial acetic acid	75	ml
Distilled water	875	ml

j. Slab gel preparation

1. Lower (separating) gel

	Percent acrylamide			
	7.7%	10%	12%	
Acrylamide/Bis (30%)	5	10	15	ml
Lower gel buffer (pH 8.8)	10	10	10	ml
Distilled water	25	20	15	ml
APS (10%)	200	200	200	µl
TEMED	20	20	20	µl

2. Upper (stacking) gel

	4% acrylamide	
Acrylamide/Bis (30%)	2.6	ml
upper gel buffer (pH 6.8)	5	ml
Distilled water	9	ml
APS (10%)	30	µl
TEMED	20	µl

B. Phosphate-buffered saline (PBS, pH 7.38)

a. Solution A

K_2HPO_4	2.7	g
Deionized water	100	ml

b. Solution B

Na_2HPO_4	2.8	g
Deionized water	100	ml

c. Solution C

KCl	0.199	g
NaCl	7.99	g
Deionized water	100	ml

38 ml of solution B was added to solution C and pH was adjusted with solution A

C. Hanks HEPES (pH 7.4)

Sodium chloride	150	mM
Potassium chloride	5	mM
Calcium chloride	3	mM

Magnesium chloride	1	mM
D-Glucose	5	mM
HEPES	10	mM

adjusted to pH 7.4 with 5 M NaOH.

D. Western blotting/Immunoblotting

a. Electroeluting buffer

Tris	3.03	g
Glycine	14.4	g
Methanol 20% (v/v) in distilled water	1	l

b. Blot developing substrate solution

3,3'-diaminobenzidine (DAB)*	0.05	g
Cobalt chloride 1% (w/v) in distilled water	2.00	ml
PBS (pH 3.38)	98.0	ml
Hydrogen peroxide (30%)	0.10	ml

*DAB is a carcinogen, so was handled with care.

c. Ponceau-S solution

Ponceau-S	0.5	g
Glacial acetic acid	1	ml
Distilled water	100	ml

d. Blocking buffer

Skim milk (3%)	3	g
Tween 20 (0.2%)	0.2	ml
Distilled water	100	ml

e. Horseradish peroxidase-anti-Ig conjugate

A variety of species-specific anti-Ig-conjugates were obtained from Scottish Antibody Production Unit (SAPU) and Serotec (Oxford). These were diluted as indicated by the manufacturers and stored frozen in 20 µl aliquots.

E. Protein reagent for protein estimation

Brilliant Blue G-250	100	mg
ethanol 95% (v/v)	50	ml
phosphoric acid 85% (v/v)	100	ml

F. Purification

a. Equilibrating buffer (DEQ buffer)

Tris-HCl (pH 8.0)	50	mM
$CaCl_2$	0.2	mM
Urea	8	M
NaCl	150	mM

b. Eluting buffer (DEL buffer)

Tris-HCl (pH 8.0)	50	mM
$CaCl_2$	0.2	mM
Urea	8	M
NaCl	2	mM

c. Calmodulin agarose affinity chromatography

1. Buffer A

Tris-HCl (pH 8.0)	10	mM

EGTA	2	mM
EDTA	0.5	mM
NaCl	1	M

2. Buffer B

Tris-HCl (pH 8.0)	10	mM
EDTA	2	mM
$Mg\ Cl_2, 6\ H_2O$	3	mM

3. Buffer C

Tris-HCl (pH 8.0)	10	mM
$CaCl_2, 6\ H_2O$	1	mM
NaCl	0.5	M

4. UE buffer (Urea extract buffer)

Buffer A (pH 8.0) for 1 g wet weight cell pellets	8	ml
Urea	8	M

d. Equilibrating buffer for gel filtration

Tris-HCl (pH 7.5)	50	mM

e. Eluting buffer for Gel filtration

Tris-HCl (pH 7.5)	50	mM
NaCl	100	mM

f. Electroeluting buffer for electroelution

Tris-HCl (pH 7.4)	200	mM
SDS	1%	(w/v)
Dithiothreitol	100	mM

g. Running buffer for electroelution

Tris-HCl (pH 7.4)	50	mM
SDS	0.1%	(w/v)
Sodium thioglycolate	0.5	mM

G. Reagent for Salomon assay

a. Reaction mix (RM)

1. Solution A

Tris-HCl	1	M
$MgCl_2$	100	mM
$CaCl_2$	10	mM
BSA	100	mg/ml
Calmodulin (CaM)	0.1	mM
cAMP	10	mM
H_2O	4	ml

2. Solution B

^3H cAMP (1 mCi/ml)	2	µl
H_2O	4	ml

Mix 4 ml solution A with 1 ml solution B. store at -20 °C.

b. Substrate mix (SM)

ATP (100 mM)	200	µl
α-^{32}P ATP (10 mCi/ml)	2	µl
H_2O	798	µl

H. ELISA

a. Coating buffer (carbonate buffer, pH 9.8)

Sodium carbonate	1.59	g
Sodium bicarbonate	2.93	g
Distilled water	1	l

b. Washing buffer

PBS	l	l
Tween-20	50	µl

c. Blocking buffer

PBS	100	ml
Tween-20	50	µl
BSA	2	g

d. Citrate-phosphate buffer (pH 5.0)

1. Solution A: 0.1 M citric acid	21.01	g
2. Solution B: 2 M Na_2HPO_4	28.31	g

To prepare 0.15 M citrate-phosphate(pH 5.0), solution A (49 ml) was mixed with solution B (51 ml) just before use.

e. Developing solution

OPD (O-phenylenediamine, Sigma)	17	mg
Citrate phosphate buffer (pH 5.0)	50	ml
Hydrogen peroxide	10	µl

f. Stop solution

12.5% (v/v) sulphuric acid in distilled water.

I. TEN buffer

Tris-HCl (pH 8.0)	50	mM
NaCl	150	mM
EDTA	100	mM

J. TC buffer

Tris-HCl (pH 8.0)	50	mM
$CaCl_2$	0.2	mM

K. TSB (Tranformation and storage buffer)

TSB is 2xYT broth suplemented with:

PEG	10%	v/v
DMSO	5%	v/v
$MgCl_2$	10	mM
$MgSO_4$	10	mM

L. GTE buffer (Glucose, Tris and EDTA)

Glucose	50	mM
Tris-HCl (pH 8.0)	25	mM
EDTA (pH 8.0)	10	mM

Solution autoclaved for 15 min at 10 lb/sq inch and stored at 4 °C.

M. NSD buffer (NaOH, SDS)

Sodium hydroxide (0.2 N)	10	ml
Sodium dodecyl sulphate (SDS)	1%	(w/v)

Freshly prepared solution inverted five times and stored on ice for 5 min.

N. KAC buffer

Glacial acetic acid	60	ml
Potassium acetate (5M)	11.5	ml
Distilled water	28.5	ml

Freshly prepared solution vortexed gently and stored on ice for 5 min.

O. Dialysis buffer

Tris-HCl (pH 7.5)	10	mM
NaCl	100	mM
$CaCl_2$	1	mM

P. Acylation reaction mix for enzymatic synthesis of acyl-ACP

Adenosine triphosphate (ATP)	5	mM
Dithiothreitol (DTT)	2	mM
Triton X-100	2%	v/v
Tris-HCl (pH 8.0)	100	mM
LiCl	400	mM
$MgCl_2$	10	mM
fatty acid (sodium salt)	160	μM
Commercial ACP-SH	155	μM
Commercial ACP synthetase	3	units

Freshly prepared reaction mix incubated at 37 °C for 3 h and then incubated for another 16 h at 30 °C.

REFERENCES

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

ALTMEIER, W.A. and AYOUB, E.M. (1977). Erythromycin prophylaxis for pertussis. *Pediatrics*, **59** : 623-625.

ARAI, H. and SATO, Y. (1976). Separation and characterization of two distinct haemagglutinins contained in purified leukocytosis-promoting factor from *Bordetella pertussis*. *Biochimica et Biophysica Acta*, **444** : 765-782.

ARCINIEGA, J.L., HEWLETT, E.L., JONSON, F.D., DEFOREST, A., WASSILAK, S., ONORATO, I.M., MANCLARK, C.R. and BURNS, D.L. (1991). Human serological responses to envelope associated proteins and adenylate cyclase toxin of *Bordetella pertussis*. *Journal of Infectious Diseases*, **163** : 135-142.

ARICO, B., MILLER. J.F., ROY, C., STIBITZ, S., MONACK, D.M., FALKOW, S., GROSS, R. and RAPPUOLI, R. (1989). Sequences required for expression of *Bordetella pertussis* virulence factors share homology with prokaryotic signal transduction proteins. *Proceedings of the National Academy of Sciences, USA.*, **86** : 6671-6675.

ASHWORTH, A.E., IRONS, L.A. and DOWSETT, A.B. (1982). Antigenic relationship between serotype specific agglutinogen and fimbriae of *Bordetella pertussis* endotoxin. *Infection and Immunity*, **37** : 1287-1281.

AYME, G., CAROFF, M., CHABY, R., HAEFFNER-CAVAILLON, N., LEDUR, A., MOREAU, M., MUSET, M., MYNARD, M.C., ROUMIANTZEFF, M., SCHULZ, D. and ZSABO, L. (1980). Biological activities of fragments derived from *Bordetella pertussis* endotoxin : Isolation of a nontoxic, Shwartzman-negative lipid A possessing high adjuvant properties. *Infection and Immunity*, **27** : 739-745.

BAILEY, M.J.A., KORONAKIS, V., SCHMOLL, T. and HUGHES, C. (1992).Escherichia coli HlyT protein, a transcriptional activator of haemolysin synthesis and secretion, is encoded by the *rfaH* (*sfrB*) locus required for expression of sex factor and lipopolysaccharide genes. *Molecular Microbiology*, **6** : 1003-1012

BARAFF, L.J., WILKINS, J. and WEHRLE, P.F. (1978). The role of antibiotics, immunizations and adenovirus in pertussis. *The Lancet*, ii : 830-831.

BARNARD, A., MAHON, B.P., WATKINS, J., REDHEAD.K. and MILLS, K. H.G. (1996). Th1/Th2 cell dicotomy in aquired immunity to *Bordetella pertussis*: variables

in the in vivo priming and in vitro cytokine detection techniques affect the classification of T-cell subsets as Th1, Th2 of Th0. *Immunology*, **87** : 372-380.

BARRY, E.M., WEISS, A.A., EHRMANN, I.E., GRAY, M.C., HEWLETT, E.L. and GOODWIN, M.M. (1991). *Bordetella pertussis* adenylate cyclase toxin and hemolytic activities require a second gene, *cya C*, for activation. *Journal of Bacteriology*, **173** : 720-726.

BASS, J.W. (1985). Pertussis : Current status of prevention and treatment. *Pediatric. Infectious Disease Journal*, **4** : 614-619.

BEATTIE, D.T., SHAHIN, R. and MEKALANOS, J.J. (1992) A vir-supressed gene of *Bordetella pertussis* is required for virulence. *Infection and Immunity*, **60** : 571-577.

BELLALOU, J., LADANT, D. and SAKAMOTO, H. (1990 a). Synthesis and secretion of *Bordetella pertussis* adenylate cyclase as a 200-Kilodalton protein. *Infection and Immunity*, **58** : 1195-1200.

BELLALOU, J., SAKAMOTO, H., LADANT, D., GEOFFROY, C. and ULLMANN , A. (1990 b). Deletions affecting hemolytic and toxic activities of *Bordetella pertussis* adenylate cyclase. *Infection and Immunity*, **58** : 3242-3247.

BEMIS, D.A. (1992). *Bordetella* and mycoplasma respiratory-infections in dogs and cats. *Veterinary Clinics of North America Small Animal Practice*, **22** : 1173-1186.

BENZ, R., MAIER, E., LADANT, D., ULMANN, A. and SEBO, P. (1994). adenylate cyclase toxin (CyaA) of *Bordetella pertussis* : evidence for the formation of small ion-permeable channels and comparison with HlyA of *Escherichia coli*.. *Journal of Biological Chemistry*, 27231-27239.

BERKOWITZ, S.A., GOLDHAMMER, A.R., HEWLETT, E.L. and WOLF, J. (1980). Activation of prokaryotic adenylate cyclase by calmodulin. *Annals of the New York Academy of Sciences*, 356, 360.

BETSOU, F., SEBO, P. and GUISO, N. (1993). Cya C mediated activation is important not only for toxic but also for protective activities of *Bordetella pertussis* adenylate cyclase -haemolysin. *Infection and Immunity*, **61** : 3583-3589.

BETSOU, F., SEBO, P. and GUISO, N. (1995). The C-terminal domain is essential for protective activity of the *Bordetella pertussis* adenylate cycalase-haemolysin. *Infection and Immunity*, **63** : 3309-3315.

BORDET, J. and GENGOU, O.(1906). Le microbe de la coqueluche. *Annales de Institut Pasteur* , **20**, 731-741.

BOTTOMLY, K. (1988). A functional dichotomy in $CD4^+$ T lymphocytes. *Immunology Today*, **9** : 268-274.

BRADFORD. M. (1976). A rapid and sensitive method for the quantitation of microgram of protein, utilizing the principle of protein binding. *Analytical Biochemistry*, **72** : 248-257.

BRENNAN, M.J., LI, Z.M., COWELL, J.L., BISHER, M.E., STEVEN, A.C. and MANCLARK, C.R. (1988). Comparison of agglutinogens on serotype 3 and serotype 6 strains of *Bordetella pertussis*. pp. 183-193. In : *Proceedings of the Federation of European Microbiological Societies, Symposium on Pertussis* (Eds. Mebels. et al.), Berlin. GDR.

BREZIN, G., GUISO, N., LADANT, D., OHANIANCE, D.L., MARGARET, F., ONYEOCHA, I. and ALONSO, J.M. (1987). Protective effects of anti-*Bordetella pertussis* adenylate cyclase antibodies against lethal respiratory infection of the mouse. *FEMS Microbiology Letters*, **42** : 75-80.

BRODEUR, B.R., MARTIN. D., MARTIN, D., HAMEL, J., SHAHIN, R.D. and LAFERRIER, C. (1993). Antigenic analysis of the saccharide moiety of the lipooligosaccharide of *Bordetella pertussis. Springer Seminars in Immunopathology*, **15** : 205-215.

BROMBERG, K. (1989). Whooping cough (pertussis). pp. 346-350. In : *Infectious diseases*. (Eds. Hoeprich, P.D. and Jordon, M.C). Fourth Ed. Lippincott, Philadelphia.

BROMBERG, K., TANNIS, T. and STEINER, P. (1991). Detection of *Bordetella pertussis* asssociated with the alveolar macrophages of children with human immunodeficiency virus infection. *Infection and Immunity*, **59** : 4715-4719.

BROWNLIE, R.M., COOTE, J.G., PARTON, R., SCHULTZ, J.E., ROGEL, A. and HANSKI, E. (1988). Cloning of the adenylate cyclase genetic determinant of *Bordetella pertussis* and its expression in *Escherichia coli* and *Bordetella pertussis. Microbial Pathogenesis*, **4** : 335-344.

CHABY, R. and CAROFF, M. (1988). Lipopolysaccharides of *Bordetella pertussis* endotoxin. pp. 247-271. In: *Pathogenicity and Immunity in Pertusssis*, (Eds Wardlaw, A.C.and Parton, R.). John Wiley and Sons, Chichester.

CHANG, A.C.Y. and COHEN, S.N. (1978). Construction and characterisation of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *Journal of Bacteriology*, **134** : 1141-1156.

CHANG, Y.F., RENSHAW, H.W., MARTENS, R.J. and LIVINGSTON, J.R. (1986). *Pasteurella haemolytica* leukotoxin : Chemiluminescence response of peripheral blood leukocytes from several different mammalian species to leukotoxin and opsonin treated living and killed *Pasteurella haemolytica* and *Staphylococcus aureus*. *American Journal of Veterinary Research*, **47** : 67-74.

CHARLES, I., FAIRWEATHER, N., PICKARD, D., BEESLEY, J., ANDERSON, R., DOUGAN, G. and ROBERTS, M. (1994). Expression of the *Bordetella pertussis* P.69 pertactin adhesin in *Escherichia coli* : Fate of the carboxy-terminal domain. *Microbiology* , **140**: 3301-3308.

CHERRY, J.D. (1993). Acellular pertussis vaccines : A solution to the pertussis problem. *Journal of infectious Diseases*, **168** : 21-24.

CHERRY, J.D., BARAFF, L.J. and HEWLETT, E. (1989). The past, present, and future of pertussis. The role of adults in epidemiology and future control. Western Journal of Medicine, 150 : 319-328.

CHERRY, J.D., BRUNELL, P.A., GOLDEN, G.S. and KARZON, D.T. (1988). Report of the task force on pertussis and pertussis immunization. *Paediatrics*, 81 : 939-984.

CLINKENBEARD, K.D. and UPTON, M.L. (1991). Lysis of bovine platelets by *Pasteurella haemolytica* leukotoxin. *American Journal of Veterinary Research*, **52** : 453-457.

CONFER, D.L. and EATON, J.W. (1982). Phagocyte impotence caused by the invasive bacterial adenylate cyclase. *Science*, **217** : 948-950.

CONFER, D.L. and EATON, J.W. (1985). *Bordetella* adenylate cyclase : Host toxicity and diagnostic utility. *Developments in Biological Standardization*, **61** : 3-10

CONFER, D.L., ZACKRISSON, G., LAGERGAGD, T. and EATON, J.W. (1990). Rapid diagnosis of pertussis. *Scandanavian Journal of infectious Diseases*, **22** : 175-177.

COOKSON, B.T., VANDAMME, P., CARLSON, L.C., LARSON, A.M., SHEFFIELD, JVL., KERSTERS, K. and SPACH, D.H. (1994). Bacteremia caused by a novel *Bordetella* species, *B. hinzii*. *Journal of Clinical Microbiology*, **32** : 2569-2571.

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

COOTE, J.G. (1996). The RTX toxins of Gram-negative bacterial pathogens : Modulators of the host immune system. *Reviews in Medical Microbiology*, **7**: 53-62.

COWELL, J.L., HEWLETT, E.L. and MANCLARK, C.R. (1979). Intracellular localization of the dermonecrotic toxin of *Bordetella pertussis*. *Infection and Immunity*, **25** : 896-901.

CZUPRYNSKI, C. and WELCH, R. (1995). Biological effects of RTX toxins : The possible role of lipopolysaccharide. *Trends in Microbiology*, **3** : 480-483.

DE MAGISTRIS, M.T., DI TOMMASO, A., MARSILI, I., BUGNOLI, M. and RAPPUOLI, R. (1993). Formaldehyde-treatment of proteins constrains recognition by T cells in vitro but not in vivo. *Biologicals*, **1** : 23.

DE MAGISTRIS, M.T., ROMANO, M., NUTI, S., RAPPUOLI, R. and TAGLIABUE, A. (1988). Dissecting human T cell responses against *Bordetella* species. *J.Exp. Med*, **168** :1351-1362.

DOMENIGHINI, M., RELMAN, D., CAPIAU, C., FALKOW, S., PRUGNOLA, A., SCARLATO, V. and RAPPUOLI, R. (1990). Genetic characterization of *Bordetella pertussis* filamentous haemagglutinin : A protein processed from an unusually large precursor. *Molecular Microbiology*, **4** : 787-800.

ELDERING, G., HORNBECK, C. and BAKER J. (1957). Serological study of *Bordetella pertussis* and related species. *Journal of Bacteriology*, **74** : 133-136.

EMSLEY, P., CHARLES, I.G., FAIRWEATHER, N.F. and ISACCS, N.W. (1996). Structure of *Bordetella pertussis* virulence factor P.69 pertactin. *Letters to Nature*, **381** : 90-92.

ENDOH, M., AMITANI, M. and NAKASE, Y. (1986). Purification and characterization of heat labile toxin from *Bordetella bronchiseptica*. *Microbiology and Immunology*, **30** : 659-673.

ENDOH, M., NAGAI, M., BURNS, D.L., MANCLARK, C.R. and NAKASE, Y. (1990). Effects of exogenous agents on the action of *Bordetella parapertussis* heat-labile toxin on guinea pig skin. *Infection and Immunity*, **58** : 1456-1460.

EVEREST, P., LI, J., DOUCE, G., CHARLES, I., DE AZAVEDO, J., CHATFIELD, S., DOUGAN, G. and ROBERTS, M. (1996). Role of the *Bordetella pertussis*

P.69/pertactin protein and the 69/pertactin RGD motif in the adherence to and invasion of mammalian cells. *Microbiology*, **142** : 3261-3268.

FAHEY, J.L. and McKELVEY, E.M. (1965). Quantitative determination of serum immunoglobulins in antibody-agar plates. *Journal of Immunology*, **94** : 84-90.

FARFEL, Z., KONEN, S., WIERTZ, E., KLAPMUTS, R., ADDY, A. and HANSKI, E. (1990). Antibodies to *Bordetella pertussis* adenylate cyclase are produced in man during pertussis infection and after vaccination. *Journal of Medical Microbiology*, **32** : 173-177.

FERNANDEZ, R.C. and WEISS, A.A. (1994). Cloning and sequencing of a *Bordetella pertussis* serum resistance locus. *Infection and Immunity* , **62** : 4727-4738.

FIELD, L.H. and PARKER, C.D. (1979). Differences observed between fresh isolates of Bordetella pertussis and their laboratory derivatives. pp. 124-132. In : *International symposium on pertussis* (Ed. Manclark, C.R), US, DHEW, Washington D.C.

FIELD, L.H. and PARKER, C.D. (1982). Effects of fatty acids on the growth of *Bordetella pertussis* in chemically defined medium. *Journal of Clinical Microbiology*, **9** : 651-653.

FINE, P.E. AND CLARKSON, J.A. (1982). The re-occurrrance of whooping cough : Possible implications for vaccine efficacy. *Lancet*, **i** : 666-668

FINN, T.M. and STEVENS, L.A. (1995). Tracheal colonization factor : *Bordetella pertussis* secreted virulence determinant. *Molecular Microbiology*, **16** : 625-643.

FORESTIER, C. and WELCH, R.A. (1990). Nonreciprocal complementation of the *hlyC* and *lktC* genes of the *Escherichia coli* haemolysin and *Pasteurella haemolytica* leukotoxin determinants. *Infection and Immunity*, **58** : 828-832

FRIEDMAN, E., FARFEL, Z. & HANSKI, E. (1987). The invasive adenylate cyclase of *Bordetella pertussis* : Properties and penetration kinetics. *Biochemical Journal*, **243** : 145-151.

FRIEDMAN, R.L. (1987a). *Bordetella pertussis* adenylate cyclase : Isolation and purification by calmodulin-sepharose 4 B chromatography. *Infection and Immunity* , **55** : 129-134.

FRIEDMAN, R.L. (1988). Pertussis : The disease and new diagnostic methods. *Clinical Microbiology Reviews*, **1** : 365-376.

GEARING, A.J.H., BIRD, C.R., REDHEAD, K. and Thomas, M. (1989). Human cellular immune responses to *Bordetella pertussis* infection. *FEMS Microbiol. Immunol.*, **47** : 205-212.

GLASER, P. EMAOGLOU-LAZARIDOU, A., KRIN, E., LADANT, D., BARZU, O. and DANCHIN. (1989). Identification of residues essential for catalysis and binding of calmodulin in *Bordetella pertussis* cyclase by site directed mutagenesis. *EMBO JOURNAL*, **8** : 967-962.

GLASER, P., LADANT, D., SEZER, O., PICHOT, F., ULLMANN, A. and DANCHIN, A. (1988 a). The calmodulin sensitive adenylate cyclase of *Bordetella peretussis* : Cloning and expression in *Escherichia coli. Molecular Microbiology*, **2** : 19-30

GLASER, P., SAKAMOTO, H., BELLALOU, J., ULLMANN, A. and DANCHIN, A. (1988 b). Secretion of cyclolysin : the calmodulin sensitive adenylate cyclase-haemolysin bifunctional protein of *Bordetella pertussis*. *EMBO. Journal*, **7** : 3997-4004.

GOLDHAMMER. A. and WOLFF, J. (1982). Assay of calmodulin with *Bordetella pertussis* adenylate cyclase. *Analytical Biochemistry*, **124** : 45-52.

GOLDMAN, W.E. (1988). Tracheal cytotoxin of *Bordetella pertussis*. pp. 231-243. In : *Pathogenesis and Immunity in Pertussis* (Eds. A.C Wardlaw and R. Parton.). JohnWiley and Sons Ltd. Chester.

GOLDMAN, W.E. and BASEMAN, J.B. (1980). In vitro effect of *Bordetella pertussis* culture supernatant on hamster trachea epithelial cells. *Abstract, Annual Meeting of American Society of Microbiology*, **80** : 49.

GOODNOW, R.A. (1980). Biology of *Bordetella bronchiseptica*. *Microbiological Reviews*, **44** : 722-738.

GOODWIN, M.S. and WEISS, A.A. (1990) Adenylate cyclase toxin is critical for colonization and pertussis toxin is critical for lethal infection by *Bordetella pertussis* in infant mice. *Infection and Immunity*, **58** : 3445-3447.

GORDON, V.M., YUONG, W.W., LECHLER, S.M., GRAY, M.C., LEPPLA, S.H. and HEWLETT, E.L. (1989). Adenylate cyclase toxins from *Bacillus anthracis* and *Bordetella pertussis*. Different processes for interaction with and entry into target cells. *Journal of Biological Chemistry*, **264** : 14792-14796.

GORRINGE, A.E., ASHWORTH, L.A.E., IRONS, L.I. and ROBINSON, A. (1985). Effects of monoclonal antibodies on the adherence of *Bordetella pertussis* to Vero cells. *FEMS Microbiology Letters*, **26** : 5-9.

GOYARD, S. AND ULLMANN, A. (1991). Analysis of Bordetella pertussis *cya* operon regulation by use of *cya-lac* fusions. *FEMS Microbiology Letters*, **77** : 251-256.

GREENLEE, D.V., ANDREASEN, T.J. and STORM, D.R. (1982). Calcium independent stimulation of *Bordetella pertussis* adenylate cyclase by calmodulin. *Biochemistry*, **21** : 2759-2764.

GROSS, M.K., AU, D.C., SMITH, A.L. and STORM, D.R. (1992). Targeted mutations that ablate either the adenylate cyclase or hemolysin function of the bifunctional CyaA toxin of *Bordetella pertussis* abolish virulence. *Proceedings of the National Acacdemy of Sciences* , USA, **89** : 4898-4902

GUISO, N., ROCANCOURT, M., SZATANIK, M. and ALOSO, J.M. (1989). *Bordetella* adenylate cyclase is a virulence associated factor and an immunoprotective antigen. *Microbial Pathogenesis*, **7** : 373-380.

GUISO, N., SZATANIK, M. and ROCANCOURT, M. (1991). Protective activity of *Bordetella* adenylate cyclase and haemolysin against bacterial colonization. *Microbial Pathogenesis*, **11** : 423-431.

HACKETT, M., GUO, L., SHABANOWITZ, J., HUNT, D.F. and HEWLETT, E.L. (1994). Internal lysine palmitoylation in adenylate cyclase toxin from *Bordetella pertussis*. *Science* , **266**: 433-435.

HACKETT, M., WALKER, C.B., GUO, L., GRAY, M.C., VANCUYK, S., ULLMANN, A., SHABANOWITZ, J., HUNT, D.F., HEWLETT, E.L. and SEBO, P. (1995). Haemolytic but not cell-invasive activity of adenylate cyclase is selectively affected by differential fatty-acylation in *Escherichia coli*. *Journal of Biological Chemistry*, **270** : 20250-20253.

HAEFFNER-CAVAILLON, N., CAVAILLON, J.M., MOREAU, M., and SZABO, L. (1984). Interleukin-1 secretion by human monocytes stimulated by the isolated polysaccharide region of *Bordetella pertussis* endotoxin. *Molecular Immunology*, **31** : 389-395.

HANSKI, E. (1989). Invasive adenylate cyclase toxin of *Bordetella pertussis*. *Trends in Biochemical Sciences*, **14** : 459-463.

HANSKI, E. (1994). Mechanism of penetration of *Bordetella pertussis* adenylate cyclase toxin into target cells. *Journal of Toxicology Toxin Reviews*, **13** : 111-113.

HANSKI, E. and FARFEL (1985). *Bordetella pertussis* adenylate cyclase : partial resolution and properties of cellular penetration. *Journal of Biological Chemistry* , **260** : 5526-5532.

HARDIE, K.R., ISAARTEL, J.-P., KORONAKIS, E., HUGHES, C. and KORONAKIS, V. (1991). In vitro activation of *Escherichia coli* prohaemolysin to the mature membrane-targetted toxin requires Hly C and a low molecular-weight cytosolic polypeptide. *Molecular Microbiology* , **5** : 1669-1679

HEININGER, U., STEHR K., SCHMITT GROHE, S., LORENZ, C., ROST, R., CHRISTENSON, P.D., UBERALL, M. and CHERRY, J.D. (1994). Clinical characteristics of illness caused by *Bordetella parapertussis* compared with illness caused by *Bordetella pertussis*. *Pediatric. Infectious Disease. Journal*, **13** : 306-309.

HEISS, L.N.and LANCHASTER, J.R., CORBETT, J.A. and GOLDMAN, W.E. (1994). Epithelial autotoxicity of nitric oxide : Role in the respiratory cytopathology of pertussis. *Proceedings of the National Academy of Sciences,USA*, **91** : 267-270.

HEVEKER, N., BONAFFE, D. and ULLMANN, A. (1994). Chemical fatty acid acylation confers hemolytic and toxic activities to adenylate cyclase protoxin of *Bordetella pertussis* . *Journal of Biological Chemistry*, **269** : 32844-32847.

HEWLETT, E. and WOLFF, J. (1976). Soluble adenylate cyclase from the culture medium of *Bordetella pertussis* : Purification and characterisation. *Journal of Bacteriology*, **127** : 890-898.

HEWLETT, E.L. (1995). *Bordetella* species. pp. 2078-2084. In : *Principles and practice of infectious diseases, 3rd edition*, (Eds. Mandel, G.L., Douglas, G.R. and Bennett, J.E.). New York : Churchill Livingstone.

HEWLETT, E.L. and MALONEY, N.J. (1994). Microbial Toxins. pp. 425-439. In : *Hand book of Natural Toxins, volume 8*, (Eds. Iglewski, B., Moss, J., Tu, A.T., Vaughan, M.). Marcel Dekker, New York.

HEWLETT, E.L., GORDON, V. M., McCAFFERY, J.D.., SUTHERLAND, W.M. and GRAY, M.C. (1989). Adenylate cyclase toxin from *Bordetella pertussis* : Identification and purification of the holotoxin molecule. *Journal of Biological Chemistry*, **264** : 19379-19384.

HEWLETT, E.L., GRAY, L., ALLIETTA, M., EHRMAN, V., GORDON, M. and GRAY, M. (1991). Adenylate cyclase from *Bordetella pertussis* : conformational change associated with toxin activity. *Journal of Biological Chemistry*, **266** : 17503-17508.

HEWLETT, E.L., GRAY, M.C., and PEARSON, R.D. (1987). Delivery of *Bordetella pertussis* adenylate cyclase toxin to target cells by intact bacteria. *Clin ical Res earch*, **35** : 477A.

HEWLETT, E.L., GRAY, M.C., EHRMAN, I.E., MALONEY, N.J., OTERO, A.S., GRAY, L., MARGARETTA, A., SZABO, G., WEISS, A.A. and BARRY, E.M. (1993). Characterization of adenylate cyclase toxin from a mutant of *Bordetella pertussis* defective in the activator gene, *cyaC*. *J. Biol. Chem.*, **268** : 7842-7848.

HEWLETT, E.L., UNDERHILL, L.H., COOK, G.H., MANCLARK, C.R. and WOLFF, J. (1979a). A protein activator for adenylate cyclase of *Bordetella pertussis*. *Journal of Biological Chemistry*, **254** : 5602-5605.

HEWLETT, E.L.and GORDON, V.M. (1988). Adenylate cyclase toxin of *Bordetella pertussis*. pp. 193-209. In : *Pathogenicity and Immunity in Pertusssis*, (Eds Wardlaw, A.C. and Parton, R.). John Wiley and Sons, Chichester.

HIGHLANDER, S.K., CHIDAMBARAM, M., ENGLER, M.J. nad WEINSTOCK, G.M. (1989). DNA sequence of the *Pasteurella haemolytica* leukotoxin gene cluster. *DNA* **8** : 15-28.

HILDEBRANDT, J.D., SEKURA, R.D., CODINA, J., IYENGAR, R., MANCLARK, C.R., BIRNBAUMER, L. (1983). Stimulation and inhibition of adenylyl cyclases mediated by distinct regulatory proteins. *Nature*, **302** : 706-709

HORNIBROOK, J.W. (1940). Nicotinic acid as a growth factor for *H. pertussis*. *Proceedings of the Society for Experimental Biology and Medicine*, **45** : 598-599.

IMAIZUMI, A., SUZIKI, Y., ONO, S., SATO, H. and SATO, Y. (1983). Heptakis (2,6-O-Dimethyl) β-Cyclodextrin : A novel growth stimulant for *Bordetella pertussis* phase 1. *Journal of Clinical Microbiology*, **17** : 781-786.

IRONS, L.I., ASHWORTH, L.A.E., ROBINSON, A. (1985). Release and purification of fimbriae from *Bordetella pertussis*. *Developments in Biological Standardization*, **61** : 153-163.

ISH-HOROWICZ, D, and BURKE, J.F. (1981). Rapid and efficient cosmid cloning. *Nucleic Acids Research*, **9** : 2989-2998.

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

IWAKI, M., ULLMANN, A. and SEBO, P. (1995). Identification by in vitro complementation of regions required for cell-invasive avtivity of *Bordetella pertussis* adenylate cyclase toxin. *Molecular Microbiology*, **17** : 1015-1024.

KARIMOVA, G., BELLALOU, J. and ULLMANN, A. (1996). Phosphorylation-dependent binding of BvgA to the upstream region of the *cyaA* gene of *Bordetella pertussis*. *Molecular Microbiology*, **20** : 489-496.

KATADA, T., TAMURA, M., UI, M. (1983). The A-protomer of islet-activating protein, pertussis toxin, as an active peptide catalysing ADP-rybosylation of a membrane protein. *Archives of Biochemistry and Biophysics*, **224** : 290-298.

KEOGH, E.V. NORTH, E.A. and WARBURTON, M.F. (1947). Haemagglutinin of *Haemophilus* group. *Nature*, **160** : 63-69.

KERSTERS, K., HINTZ, K.H., HERTLE, A., SEGERS, P., LIEVENS, A., SIEGMANN, O. AND DILEY, J. (1984). *Bordetella avium* sp. nov., isolated from the respiratory tracts of turkeys and other birds. *International Journal of Systematic Biology*, 34 : 56-70.

KHELEF, N., BACHELET, C.M., VARGAFTIG, B.B. and GUISO, N. (1994). Characterization of murine lung inflammation after infection with parental *Bordetella pertussis* and mutants deficient in adhesins or toxins. *Infection and Immunity*, **62** : 2893-2900.

KIMURA, A. and HIKINO, N. (1985). Results with a new DTP vaccine in Japan. *Developments in Biological Standardizations*, **61** : 545-561.

KIMURA, A., MOUNTZOUROS, K.T., RELMAN, D.A., FALKOW, S. and COWELL, J.L. (1990). *Bordetella pertussis* filamentous haemagglutinin : Evaluation as a protective antigen and colonization factor in a mouse respiratory infection model. *Infection and Immunity*, **58** : 7-16.

KOBISCH, M. and NOVOTNY, P. (1990). Identification of a 68-kilodalton outer membrane protein as the major protective antigen of *Bordetella bronchiseptica* by using specific pathogen-free piglets. *Infection and immunity*, **58** : 352-357.

KORONAKIS, V., KORONAKIS, E. and HUGHES, C. (1989). Isolation and analysis of the C-terminal signal directing export of *Escherichia coli* hemolysin protein across both bacterial membranes. *EMBO Journal*, **8** : 595-605.

KRAIG, E., DAILEY, T. and KOLODRUBETZ, D. (1990). Nucleotide sequence of the leukotoxin gene from *Actinobacillus actinomycetemcomitans* : Homology to the alpha hemolysin / leukotoxin gene family. *Infection and Immunity*, **58** : 920-929.

LACEY, B.W. (1960). Antigenic modulation of *Bordetella pertussis*. *Journal of Hygeine*, **58**: 57-03.

LACOUR, M., ARRIGHI, J.F., MULLER, K.M., CARLBERG, C., SAURAT, J.H. AND HAUSER, C. (1994). cAMP up-regulates IL-4 and IL-5 production from activated CD4+ T cells while decreasing IL-2 release and NF-AT induction. *Journal of International Immunology*, **6** :1333-1341.

LADANT, D. (1988). Interaction of *Bordetella pertussis* adenylate cyclase with calmodulin. *Journal of Biological Chemistry*, **263** : 2612-2618.

LADANT, D., MICHELSON, S., SARFATI, R., GILLES, A.M, PREDELEANU, R. and BARZU, O. (1989). Characterisation of the calmodulin binding and catalytic domains of *Bordetella pertussis* adenylate cyclase. *Journal of Biological Chemistry*, **264** : 4015-4120.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature*, London, **227** : 680-685.

LALLY, E.T., GOLAB, E.E. and KIEBA, I.R. (1994). Identification and immunological characterisation of the domain of *Actinobacillus actinomycetem-comitans* leukotoxin that determines its specificity for human target cells. *Journal of Biological Chemistry* , **269** : 31289-31295.

LALLY, E.T., GOLAB, E.E., KIEBA, I.R., TAICHMAN, N.S., ROSENBLOOM, J., ROSENBLOOM, J.C., GIBSON, C.W. and DEMUTH , D.R. (1989). Analysis of the *Actinobacillus actinomycetemcomitans* leukotoxin gene: delineation of unique features and comparison to homologous toxins. *Journal of Biological Chemistry*, **264** : 15451-15456.

LAOIDE, B.M. and ULLMANN, A. (1990). Virulence dependent and independent regulation of the *Bordetella pertussis* cya operon. *EMBO Journal*, **9** : 999-1005.

LAPIN, J.H. (1943). Whooping cough. Charles C. Thomas (Publisher). Spring Field.

LEININGER, E., KENIMER, J.G and BRENNAN, M.J. (1990). *Surface proteins of Bordetella pertussis* : *Role in adherence*. pp. 100-105. In : Sixth international symposium on pertussis (Ed.Manclark, C.). US DHEW publication. Washington DC.

LEININGER, E., ROBERTS, M., KENIMER, J.G., CHARLES, I.G., FAIRWEATHER, N.F., NOVOTNY, P. and BRENNAN, M.J. (1991). Pertactin, an Arg-Gly-Asp-containing *Bordetella pertussis* surface protein that promotes adherence to mammalian cells. *Proceedings of the National Academy of Sciences*, *USA*, **88**: 345-349.

LEPPARD, K., TOTTY, N., WATERFIELD, M., HARLOW, E., JENKINS, J. and CRAWFORD, L. (1983). Purification and partial amino acid sequence analysis of the cell ular tumour antigen, from mouse SV40-transformed cells. *EMBO. Journal* **2** : 1993-1999.

LEPPLA, S.H. (1982). Anthrax toxin edema factor : A bacterial adenylate cyclase that increases cyclic AMP concentrations in eukaryotic cells. *Proceedings of National Academy of Sciences*, USA, **79** : 3162-3166.

LEVINE, L.and PIERONI, R.E. (1966). A Unitarian hypothesis of altered reactivity to stress mediated by *Bordetella pertussis*. *Experentia*, **22** : 797-782.

LIVEY, I. and WARDLAW, A.C. (1984). Production and properties of *Bordetella pertussis* heat labile toxin. *Journal of Medical Microbiology*, **17**: 91-103.

LIVEY, I., PARTON, R. and WARDLAW, A.C. (1978). Loss of heat-labile toxin activity from *B. pertussis* grown in modified Hornibrook medium. *FEMS Microbiology letters*, **3** : 203-205.

LOCHT, C., BERTIN, P., MENOZZI, F.D.and RENAULD, G. (1993). The filamentous haemagglutinin, multifaceted adhesin produced by virulent *Bordetella* spp. *Molecular Microbiology* , **9**: 653-660.

LONG, S.S., WELKON, C.J, and CLARK, J.L. (1990). Widespread silent transmission of pertussis in families: antibody correlation of infection and immunity. *Journal of Infectious Diseases*, **16**: 480-486.

LUDWIG, A., GARCIA, F., BAUER, S., JARCHAU, T., BENZ, R., HOPPE, J. and GOEBEL, W. (1996). Analysis of the in vivo activation of hemolysin (Hly A) from *Escherichia coli*. *Journal of Bacteriology*, **178** : 5422-5430.

MACKMAN, N., NICAUD, J.M., GRAY, L. and HOLLAND, I.B. (1988). Genetical and functional organisation of the *Escherichia coli* haemolysin determinant. *Molecular and General Genetics*, **201** : 282-288.

MANCLARK, C.R. and COWELL, J.L. (1984). Pertussis, pp. 60-106. In : *Bacterial vaccines*. (Ed. R.Germanier). Academic Press. New York.

MARCHITTO, K.S., SMITH, S.G., LOCHT, C. and KEITH, J.M. (1987). Nucleotide sequence homology to pertussis toxin gene in *Bordetella bronchiseptica, Bordetella parapertussis*. *Infection and Immunity*, **55** : 497-501.

MASURE, H.R., AU, D.C., GROSS, M.K., DONOVAN, M.G. and STORM, D.R. (1990). Secretion of *Bordetella pertussis* adenylate cyclase from *Escherichia coli* containing the hemolysin operon. *Biochemistry*, **29** : 140-145.

MASURE, H.R., SHATTUCK, R.L. and STORM, D.R. (1987). Mechanisms of bacterial pathogenicity that involve production of calmodulin-sensitive adenylate cyclases. *Microbiological Reviews*, **51** : 60-65.

MASURE, H.R.and STORM, D.R. (1989). Characterization of the bacterial cell associated calmodulin-sensitive adenylate cyclase from *Bordetella pertussis* *Biochemistry*, **28** : 438-442.

MEADE, B.D. and BOLLEN, A. (1994). Recommendations for use of polymerase chain reaction in the diagnosis of *Bordetella pertussis* infections. *Journal of Medical Microbiology*, **41** : 51-55.

MOCK, M. and ULLMANN, A. (1993). Calmodulin-activated bacterial adenylate cyclases as virulence factors. *Trends in Microbiology*, **1** : 187-192.

MONNERON, A., LADANT, D., ALAYER, J., BELLALOU, J., BARZU, O. and ULLMANN, A. (1988). Immunological relatedness between *Bordetella pertussis* and rat brain adenylyl cyclases. *Biochemistry*, **27** : 536-539.

MOOI, F.R. (1994). Genes for the filamentous haemagglutinin and fimbriae of *Bordetella pertussis* : Colocation, coregulation and cooperation. pp. 144-155. In : *Molecular Genetics of Bacterial Pathogenesis*, Eds. MILLER, V.L., RAPER, J.B. *et al* American Society for Microbiology, Washington DC.

MORENO-LOPEZ, M. (1952). El genero *Bordetella* . *Microbiology Espagnol* , **5**: 117-181.

MOSMANN, T. (1983). Rapid colorimetric assay for cellular growth and survival : application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, **65** : 55.

MULLER, A.S., LEEUWENBURG, J. and PRATT, D.S (1986). Pertussis: epidemiology and control. *Bulletin of the World Health Organization*, **64**: 321-331.

MUNIER, H., GILLES, A.M., GLASER, P., KRIN, E., DANCHIN, A., SARFATI.R. and BARZU, A. (1991). Isolation and characterization of catalytic and calmodulin-binding domains of *Bordetella pertussis* adenylate cyclase. *European Journal of Biochemistry*, **196** : 469-474.

MUNOZ, J.J. and BERGMAN, R.K. (1979). Biological activities of *Bordetella pertussis*. pp. 143-150. In : *International symposium on pertussis* (Eds Manclark, C.R. and Hill, J.C). US DHEW publication. No. (NIH) 79-1830, Washington DC.

MUNOZ, J.J. and PEACOCK, M.G. (1990). Action of pertussigen (pertussis toxin) on serum IgE and on Fc-epsilon receptors on lymphocytes. *Cellular Immunology*, **127** : 327-336.

NAKASE, Y. and ENDOH, M. (1988). Heat-labile toxin of *Bordetella pertussis*. pp. 211-229. In: *Pathogenicity and Immunity in Pertusssis* , (Eds Wardlaw, A.C.and Parton, R.). John Wiley and Sons, Chichester, :

NICAUD, J.M., MACKMAN, N., GRAY, L. and HOLLAND, I.B. (1985). Characterisation of HlyC and mechanism of activation and secretion of hemolysin from *Escherichiacoli* . *FEBS Letters*, **187** : 339-344.

OLSON, L.C. (1975). Pertussis. *Medicine*, **54**: 427-469.

ONORATO, I.M. and WASSILAK, S.G.F. (1987). Laboratory diagnosis of pertussis: the state of the art. *Paediatric Infectious Diseases* , **6**: 145-151.

PARTON, R. (1991). Changing perspectives on pertussis and pertussis vaccination. *Reviews in Medical Microbiology*, **2** : 121-128.

PARTON, R. (1997). *Bordetella*. .In: *Topley and Wilson's Microbiology and Microbial Infections* , Vol 2, (Ed Collier, L.). Edward Arnold, London (in press).

PEARSON, R.D., SYMES, P., CONBOY, M., WEISS, A.A. and HEWLETT, E.L. (1987). Inhibition of monocyte oxidative responses by *Bordetella pertussis* adenylate cyclase toxin. *Journal of Immunology*, **139** : 2749-2754

PELLETT, S. and WELCH, A. (1996). *Escherichia coli* hemolysin mutant with altered target cell specificity. *Infection and Immunity*, **64** : 3081-3087.

PEPPOLONI, S., NENCIONI, L.and DI TOMMASO. (1991). Lymphokine secretion and cytotoxic activity of human $CD4^+$ T-cell clones against *Bordetella pertussis*. *Infection and Immunity*, **59** : 3768-3773.

PERKINS, F.T., SHEFFIELD, F.W., OUTSCHOORN, A.S. and HEMSLEY, D.A. (1973). An international collaborative study on the measurement of the opacity of bacterial suspensions. *Journal of Biological standardization*, **1** : 1-10.

PITTMAN, M. (1970). *Bordetella pertussis* : Bacterial and host factors in the pathogenesis and prevention of whooping cough. pp. 239-270. In : Infectious agents and host reactions, (Ed. Mudd, S.). W.B. Saunders. Co. Philadelphia.

PITTMAN, M. (1974). Genus *Bordetella* Moreno-Lopez. pp. 401-412. In : *Bergey's Manual of Determinative Bacteriology, 8th*. (Eds. Buchanan, R.E. and Gibbons, N.E.) Williams and Wilkins. Baltimore.

PITTMAN, M. (1979). Pertussis toxin : the cause of the harmful effects and prolonged immunity of whooping cough. A hypothesis. *Reviews of Infectious Diseases*, **1** : 401-412.

PITTMAN, M. (1984). The concept of pertussis as a toxin-mediated disease. *Pediatric Infectious Disease Journal*, **3** : 467-486.

PITTMAN, M. (1986). Neurotoxicity of *Bordetella pertussis*. *Neurotoxicology*, **7** : 53-68.

PITTMAN, M. and WARDLAW, A.C. (1981). The Genus *Bordetella*. pp. 1075-1085. In : *The prokaryotes. A handbook on habitats, isolation and identification of bacteria*, (Eds. Starr, M.P, Stolp, H., Truper, H.G., Balows, A.and Schlegel, H.G. Springer-Verlag.). Berlin, Heidelberg, New York.

PITTMAN, M., FURMAN, B.L. and WARDLAW, A.C. (1980). *Bordetella pertussis* respiratory tract infection in the mouse : Pathophysiological responses. *Journal of Infectious Diseases*, **142** : 56-66.

PODDA, A., DELUCA, E.C., TITONE, L., CASADEI, A.M., CASCIO, A., BARTALINI, M., VOLPINI, G., PEPPOLONI, S., MARSILI, I., NENCIONI, L. and

RAPPUOLI, R. (1993). Immunogenicity of an acellular pertussis vaccine composed of genetically inactivated pertussis toxin combined with filamentous hemagglutinin and pertactin in infants and children. *Journal of Pediatric*, **123** : 81-84.

PRESTON, N.W. (1987). Eradication of pertussis by vaccination. *Lancet*, **1**: 1312.

PRESTON, N.W. (1988). Pertussis today. pp. 1-8. In : *Pathogenesis and Immunity in Pertussis*, (Eds. Wardlaw, A.C.and Parton, R.). John Wiley and Sons, Chichester.

PRESTON, N.W., TIMEWELL, R.M. and CARTER, J. (1982). A reappraisal of serotype factors 4, 5 and 6 of *Bordetella pertussis*. *Journal of Hygiene* (Cambridge), **88** : 39-46.

PRUGNOLA, A., ARICO, B., MANETTI, R., RAPPUOLI, R. and SCARLATO, V. (1995). Response of the *bvg* regulon of *Bordetella pertussis* to different temperatures and short-term temperature shifts. *Microbiology*, **141** : 2529-2534.

RAPPUOLI, R. (1993). Development and clinical results with an acellular vaccine containing genetically detoxified pertussis toxin. *Biologicals*, **21** : 2-3

RAPPUOLI, R. (1996). Acellular pertussis vaccines : A turning point in infant and adolescent vaccination. *Infectious Agents and Disease- Reviews Issues and Commentary* . **5:** 21-28.

RAPPUOLI, R., PIZZA, M., PODDA, A., DE MAGISTRIS, D.M. and NENCIONI, L. (1991). Towards third-generation of whooping cough vaccines. *Trends in Biotechnology*, **91** : 232-238.

REDHEAD, K., WATKINS, J., BARNARD, A. and MILLS, K.H.G. (1993). Effective immunization against *Bordetella pertussis* respiratory infection in mice is dependent on the induction of cell-mediated immunity. *Infection and Immununity Journal*, **61** : 3190-3198.

ROBINSON, A., GORRINGE, A.R., FUNNELL, S.G.P. and FERNANDEZ, M. (1989). Serotype protection of mice against intranasal infection with *Bordetella pertussis*. *Vaccine*, 7 : 321-324.

ROCK, C.O., GARWIN, J.L. and CRONAN, J.E. (1981). Preparative enzymatic synthesis of acyl-acyl carrier protein. *Methods in Enzymology*, **72** : 397-403

ROGEL, A. and HANSKI, E. (1992 a). Distinct steps in the penetration of adenylate cyclase toxin of *Bordetella pertussis* into sheep erythrocytes. Translocation of the toxin across the membrane. *Journal of Biological Chemistry*, **267** : 22588-23605.

ROGEL, A., FARFEL, Z., GOLDSCHMIDT, S. , SHILOACH, J. and HANSKI, E. (1988). *Bordetella pertussis* adenylate cyclase : Identification of multiple forms of the enzyme by antibodies. *Journal of Biological Chemistry* , **263** : 13310-13316.

ROGEL, A., MELLER, R. and HANSKI, E. (1991). Adenylate cyclase toxin from *Bordetella pertussis* : The relationship between induction of cAMP and hemolysis. *Journal of Biological Chemistry,* **266** : 3154-3161.

ROGEL, A., SCHULTZ, J.E., BROWNLIE, R.M., COOTE, J.G., PARTON, R. and HANSKI, E. (1989). *Bordetella pertussis* adenylate cyclase : Purification and characterisation of the toxic form of the enzyme. *EMBO Journal* , **8** : 2755-2760.

RUTTER, J.M. (1985). Atrophic rhinitis in swine. *Advances in Veterinary Science and Comparative Medicine*, **29** : 239-279.

SAADATI, M., GIBBS, H.A., COOTE, J.G. and PARTON, R. (1997). Characterisation of the leukotoxin produced by different strains of *Pasteurella haemolytica.* microbiology. *Journal of Medical Microbiology*, **46** : 276-284.

SAKAMOTO, H., BELLALOU, J., SEBO, P. and LADANT, D. (1992). *Bordetella pertussis* adenylate cyclase toxin. Structural and functional independence of the catalytic and hemolytic activities. *Journal of Biological Chemistry*, **267** : 13598-13602

SALOMON,Y., LONDOS, C. and RODBELL, M.(1974). A highly sensitive adenylate cyclase assay. *Analytical Biochemistry*, **56**:341-548.

SAMBROOK, J., FRITSCH, E.F. and MANIATIS, T. (1989). *Molecular cloning, a laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SAMORE, M.H. and SIBER, G.R. (1996). Pertussis toxin enhanced IgG1 and IgE responses to primary tetanus immunization are mediated by interleukin-4 and persist during secondary responses to tetanus alone. *Vaccine*, **14** : 291-297.

SANDROS, J. and TUOMANEN, E. (1993). Attachment factors of *Bordetella pertussis* : Mimicry of eukaryotic cell recognition molecules. *Trends Microbiology*, **1** : 192-196.

SATO, Y., IZUMIYA, K., SATO, H., COWELL, J.L. and MANCLARK, C.R. (1981). Role of antibody to leukocytosis-promoting factor hemagglutinin and to filamentous hemagglutinin in immunity to pertussis. *Infect.ion and Immunity*, **31** : 1223-1231.

SATO, Y., KIMURA, M. and FUKUMI, H. (1984). Development of a pertussis component vaccine in Japan. *Lancet*, **1**: 122-126.

SCARLATO, V., ARICO, B., PRUGNOLA, A. and RAPPUOLI, R. (1991). Sequential activation and environmental regulation of the virulence genes in *Bordetella pertussis*. *EMBO. Journal*, **10** : 3971-3975.

SCARLATO, V., PRUGNOLA, A., ARICO, B. and RAPPUOLI, R. (1991). Positive transcriptional feedback at the *bvg* locus controls expression of virulence factors in *Bordetella pertussis*. *Proceedings of the National Academy of Sciences, USA*, **87** : 6753-6757.

SCHLESINGER, M.J., VEIT, M. and SCHMIDT, M.F.G. (1993). pp. 1-19. In : *Lipid modifications of proteins*, Schlesinger,M.J, Ed., C.R.C. Press, Boca Raton, FL.

SEBO, P., GLASER, P., SAKAMOTO, H. and ULLMANN, A. (1991). High-level synthesis of active adenylate cyclase of *Bordetella pertussis* in a reconstituted *Escherichia coli* system. *Gene*, 104 : 19-24.

SHEWEN, P.E. and WILKIE, B.N. (1982). Cytotoxin of *Pasteurella haemolytica* acting on bovine leukocytes. *Infection and Immunity*, **35** : 91-94.

STAINER, D.W. and SCHOLTE, M.J.(1971). A simple chemically defined medium for the production of phase 1 *Bordetella pertussis*. *Journal of General Microbiology* , **63**: 211-220.

STANDFAST, A.F.B. (1958). The comparison between field trials and mouse protection tests against intracerebral challenge with *Bordetella pertussis*. *Immunology*, : 135-143

STANLEY, P., KORONAKIS, V., HARDIE, K. and HUGHES, C. (1996). Independent interaction of the acyltransferase HlyC with two maturation domains of the *Escherichia coli* toxin HlyA. *Molecular Microbiology*, **20** : 813-822.

STANLEY, P., PACKMAN, L., KORONAKIS, V. and HUGHES, C. (1994). Fatty acylation of two internal lysine residues required for the toxic activity of *Escherichia coli* hemolysin. *Science*, **286** : 1992-1966

STEFFEN, P., GOYARD, S. and ULLMANN, A. (1996). Phosphorylated BvgA is sufficient for transcriptional activation of virulence regulated genes in *Bordetella pertussis*. *EMBO. Journal*, **15** : 102-109.

STIBITZ , S., WEISS, A.A. and FALKOW, S. (1988). Genetic analysis of a region of the *Bordetella pertussis* chromosome encoding filamentous haemagglutinin and the pleiotropic regulatory locus *vir*. *Journal of Bacteriology*, **170** : 2904-2913.

STIBITZ, E. and MILLER, J.F. (1994). Co-ordinate regulation of virulence in *Bordetella pertussis* mediated by the *vir* (*bvg*) locus. pp. 407-422. In : *Molecular Genetics of Bacterial Pathogenesis* , Eds. Miller, V.L., Raper, J.B. *et al* . American Society for Microbiology, Wa shington DC.

STIBITZ, S. and GALETTS, T.L. (1992). Derivation of a physical map of the chromosome of *Bordetella pertussis* Tohama I. *Journal of Bacteriology*, **174** : 7770-7777

STIBITZ, S. and YANG, M.S. (1991). Subcellular localization and immunological detection of proteins encoded by the *vir* locus of *Bordetella pertussis*. *Journal of Bacteriology*, **173** : 4288-4296.

STORSAETER, J., HALLANDER, H., FARRINGTON, C., OLIN, P., MOLLBY, R. and MILLER, E. (1990). Secondary analysis of the efficacy of two acellular pertussis vaccines evaluated in a Swedish phase III trial. *Vaccine*, **8** : 457-461.

STRATHDEE, C.A. and LO, R.Y.C. (1987). Extensive homology between the leukotoxin of *Pasteurella haemolytica* A1 and the alpha hemolysin of *Escherichia coli*. *Infection and Immunity*, **55** : 3233-3236.

STRATHDEE, C.A. and LO, R.Y.C. (1989). Regulation of expression of the *Pasteurella haemolytica* leukotoxin determinant. *Journal of Bacteriology*, **171** : 5955-5962.

SZABO, G., GRAY, M.C. and HEWLETT, E.L. (1994). Adenylate cyclase toxin from *Bordetella pertussis* produces ion conductance across artificial lipid bilayers in a calcium- and polarity-dependent manner. *Journal of biological chemistry*, 269 : 22496-22499.

TAICHMAN, N.S., SIMPSON, D.L., SAKURADA, S., CRANFIELD, M., DIRENZO, J.and SLOTS, J. (1987). Comparative studies of the biology of *Actinobacillus actinomycetemcomitans* leukotoxin in primates. *Oral Microbiology and Immunology*, **2** : 97-104.

TAMURA, M., NOGIMORI, K., MURAI, S., YAJIMA, M., ITO, K., KATADA, T., UI, M. and ISHII, S. (1982). Subunit structure of the islet activating protein, pertussis toxin, in conformity with the A-B model. *Biochemistry* , **21**: 5516-5522.

TEJADA, G.M., MILLER, J.F. and COTTER, P.A. (1996). Comparative analysis of the virulence control systems of *Bordetella pertussis* and *Bordetella bronchiseptica*. *Molecular Microbiology*, **22** : 895-908.

TOWBIN, H., STAEHELIN, T. and GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets : Procedure and some applications. *Proceedings of the National Academy of Sciences*, USA, **76**: 4350-4354.

TU, A.H.T., HAUSLER, C., YOUNG, R. and STRUCK, D.K. (1994). Differential expression of the cytotoxic and hemolytic activities of the ApxIIA toxin from *Actinobacillus pleuropneumoniae*. *Infection and Immunity*, **62** : 2119-2122.

TUOMANEN, E. (1988). *Bordetella* adhesins. pp. 75-94. In: *Pathogenicity and Immunity in Pertusssis* , (Eds Wardlaw, A,C., & Parton, R.). John Wiley and Sons, Chichester.

TUOMANEN, E. and WEISS, A. (1985). Characterisation of two adhesins of *Bordetella pertussis* for human respiratory epithelial cells. *Journal of Infectious Diseases*, **152**: 118-125.

TUOMANEN, E.I., PRASAD, S.M., GEORGE, J.S, HOEPELMAN, A.I., IBSEN, P., HERON, I. and STARZYK, R.M. (1993). Reversible opening of the blood-brain barrier by anti-bacterial antibodies. *Proceedings of the National Academy of Sciences. USA*, **90** : 7824-7828.

UI, M. (1988). The multiple biological effects of pertussis toxin. pp. 273-282.In : *Pathogenesis and immunity in pertussis*, (Eds Wardlaw, A.C. and Parton, R.). John Wiley and Sons, Chichester.

UTSUMI, S., SONODA, S., IMAGAVA, T. and KANOH, M. (1978). Polymorphonuclear leukocyte-inhibitory factor of *Bordetella pertussis* : Extraction and partial purification of phagocytosis- and chemotaxis-inhibitory activities. *Biochemistry Journal*, **21** : 121-135.

VANDAMME, P., HOMMEZ, J., VANCANNEYZ, M., MONSIEURS, M., HOSTE, B., COOKSON, B., VONKONIG, C.H.W., KERSTERS, K. and BLACKALL, P.J. (1995).*Bordetella hinzii* Sp.nov., isolated from poultry and humans. *International Journal of Systematic Bacteriology* , **45**: 37-45.

WALKER, E. (1988). Clinical aspects of pertussis. pp. 273-282. In: *Pathogenicity and Immunity in Pertusssis* , (Eds Wardlaw, A.C. and Parton, R.). John Wiley and Sons, Chichester.

WALKER, K.E. and WEISS, A.A. (1994). Characterization of the dermonecrotic toxin in members of the Genus *Bordetella*. *Infection and Immunity*, **62** : 3817-3828.

WARDLAW, A.C. and PARTON, R.(1988). The host parasite relationship in pertussis. pp. 327-347. In: *Pathogenicity and Immunity in Pertusssis* , (Eds Wardlaw, A.C. and Parton, R.). John Wiley and Sons, Chichester.

WARDLAW, A.C. and STEVENSON, L.Q. (1984). Antitoxic immunity in protection against pertussis. pp. 203-209. In : *Bacterial protein toxin*. Academic Press. London.

WEDEGAERTNER, P.B., CHU, D.H., WILSON, P.T., LEWIS, M.J. and BOURNE, H.R. (1993). Palmitoylation is required for signaling functions and membrane attachment of G (Q) alpha and G (S) alpha. *Journal of Biological Chemistry*, **268** : 25001-25008.

WEISS, A.A. (1992). The genus *Bordetella*. pp. 2530-2543. In : *The prokaryotes* (2nd edition), Eds. Balows A, Truper HG et al..Springer-Verlag, Berlin.

WEISS, A.A. and GOODWIN, M.S.M. (1989). Lethal infection of *Bordetella pertussis* mutants in the infant mouse model. *Infection and Immunity* , **57**: 3757-3764.

WEISS, A.A. and HEWLETT, E.L. (1986). Virulence factors of *Bordetella pertussis*. *Annual Review of Microbiology* , **40**: 661-686.

WEISS, A.A., HEWLETT, E.L., MYERS, G.A. and FALKOW, S. (1984). Pertussis toxin and extracytoplasmic adenylate cyclase as virulence factors of *Bordetella pertussis*. *Journal of Infectious Diseases*, **150** : 219-222.

WEISS, A.A., MELTON, K.E., SELIM, A.C. and MEIDL, J.J. (1989). Use of the promoter fusion transposon Tn*5* *lac* to identify mutations in *Bordetella pertussis* vir-regulated genes. *Infection and Immunity*, **57** : 2674-2682.

WELCH, R.A. (1991). Pore forming cytolysins of Gram-negative bacteria. *Molecular Microbiology*, **5** : 521-528.

WESTROP, G.D., CAMPBELL, G., KAZI,Y., BILLCLIFFE, B., COOTE, J.G., PARTON, R., FREER, J.H. and EDWARDS, J.G. (1994). A new assay for the invasive adenylate-cyclase toxin of *Bordetella-pertussis* based on its morphological

effects on the fibronectin-stimulated spreading of BHK21 cells. *Microbiology*, **140**: 245-253.

WESTROP, G.D., HORMOZI, E.K., DA COSTA, N.A., PARTON, R. and COOTE, J.G. (1996). *Bordetella pertussis* adenylate cyclase toxin proCyaA and CyaC proteins synthesised separately in *Escherichia coli* produce active toxin *in vitro*. *Gene*, **180**: 91-99.

WESTROP, G.D., HORMOZI, E.K., DA COSTA, N.A., PARTON, R. and COOTE, J.G. (1997). Structure-function studies of the adenylate cyclase toxin of *Bordetella pertussis* and the leukotoxin of *Pasteurella haemolytica* by heterologous C protein activation and construction of hybrid proteins. *Journal of Bacteriology*, **179** : 871-879.

WEYANT, R.S., HOLLIS, D.G., WEAVER, R.E., AMIN, M.F.M., STEIGERWALT, A.G., O'CONNOR, S.P., WHITNEY, A.M., DANESHVAR, M.I., MOSS, C.W., BRENNER, D.J.(1995). *Bordetella holmseii* , sp. nov., a new gram-negative species associated with septicaemia. *Journal of Clinical Micribiology*, **33**: 1-7.

WILSON, R.R., THOMAS, M., RUTMAN, A., HARRISON, K., LUND, V., COOKSON, B., GOLDMAN, W., LAMBERT, H. and COLE, P. (1991). Effect of *Bordetella pertussis* infection on human respiratory epithelium in vivo and in vitro. *Infection and Immunity*, **59** : 337-345.

WINTERS, A.L., BAGGET, D.W., LEE, J.D., SLOAN, G.L., LEMMON, R.D. and stinson, r.s. (1985). Immunomodulation by *Bordetella pertussis* antiviral effects. *Developments in Biological Standardisation*, **61** : 233-240.

WIRSING VON KONIG, C.D. and FINGER.H. (1994). Role of pertussis toxin in causing symptoms of *Bordetella pertussis* infection. *European Journal of Microbiology and Infectious Diseases*, **4** : 243-255

WOLFF, J. and COOK, G.H. (1973). Activation of thyroid membrane adenylate cyclase by purine nucleotide. *Journal of Biological Chemistry*, **248** : 350-355.

WOLFF, J. and COOK, G.H. (1982). Amphiphile-mediated activation of soluble adenylate cyclase of *Bordetella pertussis*. *Archives of Biochemistry and Biophysics*, **215** : 524-531.

WOOLFREY, B.F. & MOODY, J.A. (1991). Human infections associated with *Bordetella bronchiseptica* . *Cinical Microbiology Reviews*, **4:** 243-55.

YAJIMA, M., HOSODA, K., KANBAYASHI, Y., NAKAMURA, T., TAKAHASHI, I. and UI,M. (1978). Biological properties of islets activating protein purified from the culture medium of *Bordetella pertussis*. *Journal of Biochemistry* Tokyo, **80** : 305-312.

ZU, T., MANETTI, R., RAPPUOLI, R. and SCARLATO, V. (1996). Differential binding of BvgA to two classes of virulence genes of *Bordetella pertussis* directs promoter selectivity by RNA polymerase. *Molecular Microbiology*, **21 :** 557-565.