Isolation and Characterization of Different Forms of *Bordetella pertussis* Adenylate Cyclase

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DEDICATION

I should like to dedicate this thesis to my late respected father, Faiz Mohammad A. Kazi for his enthusiasm and interest in our education.

BACTERIA .....AND WE!

Behave as friends, foes and non-aligned ,
Opportunists, thou, but limited and needed,
Criminals, thieves, bandits, yet un-defeated!
You the mini.....

Dr. Badar Memon.

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DECLARATION

This thesis is the original work of the author: Yasmeen F. Kazi.

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Abbreviations

AC	Adenylate cyclase
ACT	Adenylate cyclase toxin
ADP	Adenosine diphosphate
AGG	Agglutinogen
Ap	Ampicillin
ATP	Adenosine triphosphate
BG	Bordet-Gengou agar
BHK	Brain Heart Infusion Broth Baby hamster kidney
bvg	*Bordetella* virulence regulatory gene
CAA	Casamino acids
CaM	Calmodulin
cAMP	Cyclic-adenosine monophosphate
cfu	Colony forming units
CHO	Chinese hamster Ovary
CL	Chemiluminescence
Cm	Chloramphenicol
cm	Centimetre
cya	Adenylate cyclase gene
Cya	Adenylate cyclase phenotype
CyaA	Adenylate cyclase protein
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNDH	Dimethylamino-naphthalene-1,2-dicarbonic acid hydrazide
EDTA	Ethylenediamine tetra-acetic acid
EGTA	Ethyleneglycol-bis N,N'-tetraacetic acid.
ELISA	Enzyme linked immunosorbent assay
FHA	Filamentous haemagglutinin
fha	Filamentous haemagglutinin gene
fim	Fimbrial gene
fMLP	Formyl methionine-leucine-phenyl alanine
g	Gramme
HLT	Heat labile toxin
HLY	Haemolysin
Hly	Haemolytic phenotype

HSF	Histamine sensitizing factor
in	Intranasal
IPTG	Isopropyl ß -D-thiogalactopyranoside
kDa	Kilo Dalton
Km	Kanamycin
lac	Lactose operon gene
LPS	Lipopolysaccharide
M	Molar
mA	Milliamps
MeßCD	Heptakis (2, 6-0-methyl) ß-cyclodextrin
mg	Milligram
ml	Millilitre
mM	Millimolar
mm	Milimetre
NA	Nutrient agar
nm	Nanometre
OD	Optical density
OMP	Outer membrane protein
ou	Opacity units
PBS	Phosphate buffered saline
PMA	Phorbol myristate acetate
pmol	Picomole
PT	Pertussis toxin
ptx	Pertussis toxin gene
P-69	Protein -69
RNA	Ribonucleic acid
rpm	Revolutions per minute
SEM	Standared error of the mean
SDS	Sodium dodecyl sulphate
Tc	Tetracycline
TCT	Tracheal cytotoxin
Tn	Transposon
WHO	World Health Organization
X-gal	5-Bromo-4-chloro-3-indoyl-ß-D-galactopyranoside
µg	Microgram
ml	Millilitre
mm	Millimetre

$^{\circ}C$	Degrees Celsius
^{32}P	Radioisotope phosphorus
^{3}H	Radioisotope hydrogen

SUMMARY

This thesis describes the different forms of AC produced by *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and those expressed in *E. coli* . The work was done with both crude and purified preparations of AC and also with whole cells of *B. pertussis*. Immunoblot analysis with various antisera, including anti-AC monoclonal, monospecific, and polyclonal antibodies, revealed the production of two different forms of AC with different electrophoretic mobilities i.e., 210 and 200 kDa as the major cell- associated forms in *B. pertussis* and *B. bronchiseptica*. In *B. parapertussis* and *E. coli* , the predominant form was 210 kDa. These forms may represent the toxic and non-toxic forms observed by Rogel *et al.* (1989).

Production of the holotoxic AC was not observed in culture supernates under the growth conditions used. Only low MW forms of 45-50 kDa were observed and these forms also appeared to be produced by proteolysis of AC during storage and other experimental manipulations.The breakdown was partially prevented by using a protease inhibitor. The enzymic activity of AC was found to be unstable in purified and crude forms of AC in the absence of urea. Thus,urea seemed to be beneficial in protecting loss of activity of AC during storage.

A noteworthy observation of the present study was that the different Bordetellae, including *B. pertussis* Tn*5*-insertion mutants deficient in individual virulence factors, and a recombinant *B. pertussis* strain, produced different amounts of AC enzymic and toxic activities under the same set of condition which indicate that the production varied from strain to strain . The highest yields of enzymically-active and toxic AC were obtained from recombinant strain *B. pertussis* BP348 (pRMB1) and from *B. pertussis* Tn*5*- insertion mutant BP357 (deficient in a functional PT gene). On the other hand, *B. pertussis* Tohama and Tn*5* mutant BP353 showed the lowest AC enzymic and toxic activities of the *B. pertussis* strains examined. *B. bronchiseptica* produced an intermediate level of AC enzymic and toxic activities compared to those of the *B. pertussis* strains.

Enzymically and biologically- active AC was obtained from the *E. coli* constructs. However, the toxic activity was approximately half than that of native AC. *E. coli* strains harbouring the cloned *B. pertussis cyaA, B, D* genes together with *C* genes either from *P. vulgaris, E. coli,* or *B. pertussis* on separate plasmids, showed production of CyaA (AC), as assessed by immunoblotting and the Salomon assay,and its activation by *C* genes, as assessed by inhibition of chemiluminescence.

Various attempts were made to develop more convenient, economical , and sensitive methods for detection of AC and determination of its enzymic and toxic

activities. An alternative method for detection of AC by calmodulin- affinity probing of a Western blot was found to be reproducible and suitable for use when specific anti-AC antibodies were not available. This is the first report of such a use for calmodulin. A microplate assay was developed for detection of AC enzymic activity and this proved economical, reproducible, and convenient for preliminary screening of AC-producing strains and as a semi-quantitative assay. The method has some advantages over conventional assays, such as the Salomon assay and the Binding protein assay , in that it does not require radioisotope and it is rapid. Further development of this assay as a diagnostic test for pertussis may be possible.

A comparative study was made of different assays for determination of toxic effects of AC on a variety of target cells . These included : haemolysis of sheep and rabbit RBCs, CHO cell elongation, arborization of BHK cells, inhibition of the nitroblue tetrazolium reduction capacity of rabbit neutrophils, and inhibition of the chemiluminescence response of neutrophils to various stimuli. The study indicated that CL inhibition by AC toxin was a sensitive assay and could detect ACT in crude preparations at the level of 1μg protein /10^6 cells and purified ACT at 5 nmol cAMP/min/ml AC enzymic activity applied to 10^6 cells. The much lower concentration of 0.05 pmol cAMP /min/ml AC enzymic activity caused a stimulation rather than an inhibition of CL response which could provide an extremely sensitive assay for sub-toxic concentrations of ACT.

This stimulatory effect also provides further evidence that ACT is a member of the RTX family of toxins since a similar effect at sub-lytic concentration has been observed very recently with *E. coli* alpha haemolysin (Bhakdi and Martin, 1991). An immunological relationship of AC to other RTX toxins i.e., *Pasteurella haemolytica* leukotoxin (LktA) , *Actinobacillus actinomycetemcomitans* leukotoxin (AktA), and *E. coli* alpha haemolysin (HlyA) was also supported by an immunoblot study of these toxins.These toxins reacted with anti- AC monoclonal 9D4 at their appropriate MW. This is the first report providing the demonstration of an immunological relatedness among these toxins. and provides a novel method for detection and identification of the newly recognized leukotoxin from *Actinobacillus actinomycetemcomitans* , an important human pathogen.

A time-dependent suppression of CL response was observed when rabbit neutrophils were exposed to live, AC-producing *B. pertussis* . This impairment of host immune effector cell functions indicates that ACT may account for the survival and proliferation of *B. pertussis* during the disease.

AC toxin appears to be an important antigen of *B. pertussis* since antibodies against it were detected by immunoblotting of human pertussis convalescent sera. These findings substantiate the recently published studies by Farfel *et al.* (1990).

However, in the present study, the antisera were found to be capable of neutralization of AC enzymic and toxic activities which does not agree with the findings of Farfel *et al.* (1990). Another anti-AC antiserum (Ms1), raised during this study, was also capable of neutralizing AC enzymic and toxic activities. This antiserum was raised against nitrocellulose -bound AC using a novel method for production of highly specific anti-AC antibodies. This antiserum was used in passive protection tests in mice against intranasal challenge with *B. pertussis* 18323 and a partial protection was observed.

INTRODUCTION

THE BORDETELLAE

The genus *Bordetella* includes four species: *B. pertussis* , *B. parapertussis* , *B. bronchiseptica* , and *B. avium.* All these species are obligate pathogens and cause upper respiratory tract infections in mammalian or avian species.

Bordetella pertussis

B. pertussis was first discovered as the causative agent of the severe respiratory disease ' whooping cough ' by Bordet and Gengou in 1906. Since the isolation was carried out on a medium containing glycerated potato extract and 50% (v/v) blood, the organism was placed in the genus *Haemophilus* because of its presumed requirement for blood in the culture medium and termed *Haemophilus pertussis*. Later , it was revealed that blood was not obligatory for growth and the organism was able to grow in relatively simple media containing only a few amino acids, growth factors and salts, with either charcoal, soluble starch or anionic exchange resins to neutralize the growth inhibitors (Hornibrook,1939; Kawajima *et al.*, 1957). Subsequently, the genus *Bordetella* was proposed and the 'whooping cough bacillus' was placed in this genus (Moreno-Lopez,1952,cited by Wardlaw, 1988) and the species is now known as *Bordetella pertussis.*

Peptone, colloidal sulphur, peroxides, manganese, fatty acids and some structural analogues of vitamin B-complex are inhibitory to the growth of *B.pertussis* (Field and Parker 1979; McPheat and Wardlaw,1982). The addition of charcoal, ion exchange resins or blood may overcome this effect . *B. pertussis* can now be grown relatively easily on a large scale in chemically- defined media such as Stainer and Scholte medium (Stainer and Scholte,1971).This medium was modified by Imaizumi *et al.* (1983), in particular by the addition of heptakis (2, 6-O-dimethyl) ß-cyclodextrin, which rendered the medium suitable for the growth, even from small inocula and, as a solid medium, gave rise to the same number of colonies and growth rate as Bordet -Gengou medium (BG). However, with various modifications, BG is still widely used for primary isolation and maintenance of *B. pertussis* .

B. pertussis is a Gram-negative, strictly aerobic, minute cocco-bacillus (0.3-0.5 x 0.5-1mm) . It is chemoorganotrophic, and requires organic nitrogen in the form of amino acids e.g., glutamate, and organic sulphur in the form of cysteine, plus nicotinamide or nicotinic acid. Growth on BG gives smooth, raised, pinpointed

colonies with a slight metallic lustre and a narrow diffuse zone of haemolysis. Incubation for 72 h is usually required for the appearance and development of colonies and upon prolonged incubation the colonies acquire opaque and brownish centres.

In liquid media under constant agitation , *B. pertussis* grows as a uniform cloudy suspension but in two to three days, the culture develops a ropy, mucoid mass . The cells from liquid cultures are usually longer than those from solid media. L-forms have also been observed and spheroplasts may be produced in special media (Mason,1966). *In vivo* , growth of *B.pertussis* appears to be highly selective. Growth occurs on and among the cilia of the human upper respiratory tract and does not invade the parenchyma (Bromberg, 1989). However, an intracellular state has recently been reported in which *B. pertussis* cells have been found within pulmonary macrophages of infected animals or inside tissue culture cells (Bromberg *et al.*, 1991).

Other Bordetellae

The other three species of the genus *Bordetella* are also respiratory tract pathogens. *B. parapertussis* was isolated and identified as the causative agent of a mild type of pertussis (parapertussis) in humans (Lautrop, 1971).

B. bronchiseptica causes respiratory diseases in various mammals e.g., kennel cough in dogs and atrophic rhinitis in swine (Goodnow, 1980).

B. avium is a more recent addition to the genus and was first proposed as a species by Kersters *et al.* (1984) for the agent that causes turkey coryza (turkey rhinotracheitis or bordetellosis) and other respiratory diseases in birds (Kersters *et al.*, 1984). All bordetellae were formerly classified under different names, before the introduction of the genus *Bordetella* by Moreno-Lopez (1952,cited by Wardlaw,1988). The four species have common features: all are obligate parasites of the respiratory tract where they localize on ciliated epithelial cells.

The genetic relatedness of the species within the genus *Bordetella* has been explored by various investigators. According to a study of DNA - DNA hybridization , a high degree of homology between the chromosomal DNA of *B.pertussis* , *B. parapertussis* and *B. bronchiseptica* has been observed (Kloos *et al.*, 1981) and the G+C content (67-69 mole%) of their DNA is very similar (Table 1) whereas *B. avium* is more distantly related (61-62 mole%). A repeat sequence with at least 20 copies/genome has been identified in *B. pertussis*, but shown to be present in low

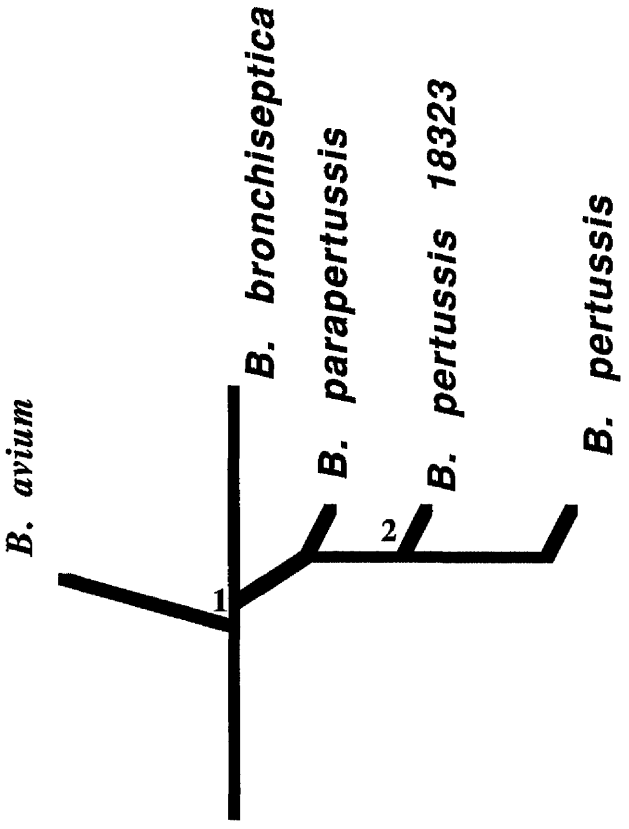
copy number (1 copy/genome) in *B. parapertussis* and *B. bronchiseptica*, and absent from *B. avium* (McPheat and MacNally, 1987).

In a survey, by multilocus enzyme electrophoresis, of 60 strains of the mammalian bordetellae obtained from world-wide sources, Musser *et al.* (1986) reported that only a limited degree of diversity occurs between these species.However,*B. pertussis* 18323, a strain used for vaccine potency testing and vaccine production, appeared to be more closely related to *B. bronchiseptica* and *B. parapertussis* than to *B. pertussis* . Arico *et al.* (1987) and Gross *et al.* (1989) have proposed a phylogenetic tree (Fig.1) of the genus *Bordetella*, based on the nucleotide sequence of pertussis toxin (PT) operon. Both *B. parapertussis* and *B. bronchiseptica* possess the mutated (silent) PT toxin genes, but *B. pertussis* strains alone express and produce pertussis toxin. Sequencing of the PT operon of *B. pertussis, B. parapertussis*, and *B. bronchiseptica* revealed a > 95% DNA homology between these species (Arico and Rappuoli, 1987).Other characterstic features of the species are given in Table 1.

Fig.1

Phylogenetic Tree of the Genus *Bordetella*

The phylogenetic tree proposed by Gross *et al.* (1989), based on the sequencing of the pertussis toxin operon. The figure indicates the acquisition of the pertussis toxin operon by *B. parapertussis* and *B. bronchiseptica* after being dissociated from *B. avium* (1).The gain of pertussis toxin expression by *B. pertussis* and *B. pertussis* 18323 (2) suggests an evolutionary link between *B. parapertussis* and *B. bronchiseptica* which possess silent genes for pertussis toxin.



Phylogenetic tree of Bordetellae

Table.1 Characterstics of *Bordetella* Species

Phenotype	Bp	Bpp	Bb	Ba
Flagella	_	_	+	+
Nitrate reduction	_	_	+	?
Urease activity	_	+	+	_
Oxidase activity	V	_	+	?
Catalase utilization	_	V	+	V
Growth on BG (days)	3	1-2	1	1
Growth on MA	_	+	+	+
Browning pigment	_	+	_	_
LPS	+	+	+	+
HLT	+	+	+	+
TCT	+	+	+	+
ACT	+	+	+	_
PT	+	_	_	_
FHA	+	+	+	?
AGGs	+	+	+	+
Species specific				
1	+	_	_	?
14	_	+	_	?
12	_	_	+	?
Strain specific				
2,3,4,5,6,13	+	_	_	?
8,9,10	_	+	_	?
8,9,10,11,13	_	_	+	?
G+C (mole%)	67.7-68	68.1-69	68.2-69.5	61.6

Data from: Eldering *et al.* (1957); Pittman (1984); Kersters *et al.* (1984); Goldman (1988); and Wardlaw (1988).

Bp=*B. pertussis*, Bpp=*B. parapertussis*, Bb=*B. bronchiseptica*,Ba=*B. avium.*

BG=Bordet-Gengou medium, MA=MacConkeys agar, LPS= lipopolysaccharide, HLT=heat labile toxin, ACT= adenylate cyclase toxin, PT= pertussis toxin, FHA= filamentous haemagglutinin, AGGs = agglutinogens.

+= present, - = absent, V= variable, ?=not known.

ANTIGENIC VARIATION IN *B. PERTUSSIS*

B. pertussis produces a variety of biologically active components which directly or indirectly contribute to its pathogenicity and thus may be termed 'virulence factors'. The established virulence factors are:
Heat labile toxin (HLT), Tracheal cytotoxin (TCT), Endotoxin or lipopolysaccharide (LPS), Pertussis toxin (PT), Filamentous haemagglutinin (FHA), Agglutinogens (AGGs), Pertactin (P-69) outer membrane protein, and Adenylate cyclase toxin (ACT). Detailed description of these virulence factors is given on page 15.

A significant phenomenon in *B. pertussis* is its capacity to undergo antigenic variation in which expression of these virulence factors is altered. Three types of variation have been described; 1) Antigenic modulation, 2) Phase variation, 3) Serotype variation. These phenomena in *B. pertussis* are reviewed by Coote and Brownlie (1988) and Coote (1991).Similar variation processes probably occur in the other *Bordetella* species but these have been less well characterized.

1) Antigenic Modulation

Lacey (1960) observed a change of antigenic expression when *B. pertussis* cells were grown under different conditions. He designated the resulting cells 'C'-mode from the bluish (cyanic) appearance of the confluent growth on media containing $MgSO_4$ instead of NaCl. The opposite of this was X-mode for the yellowish (Xanthic) colour of the confluent growth of cells on normal media at 37°C where cells possessed all the features of freshly isolated virulent strains. The variation process also involved a change in the surface of *B. pertussis* from hydrophobic to hydrophilic (Robinson *et al.*, 1986) and altered adherence to mouse lung tissue (Burns and Freer ,1982) and unciliated human tissue culture cells (Gorringe *et al.*, 1985). In addition, C-mode cells are unable to bind the dye Congo red (Parton, 1988) and have lost virulence factors such as agglutinogens (McPheat *et al.*, 1983), haemolysin and haemagglutinin (Lacey, 1960), pertussis toxin (Wardlaw *et al.*, 1976), heat-labile toxin (Livey *et al.*, 1978), adenylate cyclase toxin (Parton and Durham, 1978) and certain outer membrane proteins (Parton and Wardlaw, 1975).Other workers have confirmed and extended the findings of Lacey (1960) and have shown that growth in the presence of other factors e.g., nicotinic acid (Pusztai and Joo, 1967), quinaldic acid and other pyridines (Schneider and Parker, 1982), sodium sulphate, sodium acetate and sodium tartarate (Brownlie *et al.*, 1985) can also induce modulation.

2) Phase Variation

Leslie and Gardner (1931) first observed the loss of virulence in *B. pertussis* after repeated subculturing. They noted four antigenically distinct phases i.e., I and II being virulent and III and IV being relatively avirulent, and termed this process 'Phase Variation'. Phase IV strains have an increased resistance to certain antibiotics (Bannatyne and Cheung, 1984), and fatty acids (Peppler and Schrumpf, 1984) and are capable of growing on media not supplemented with blood, charcoal or cyclodextrin (Peppler, 1982). Phase IV variants can also be distinguished from Phase I strains by their inability to take up Congo Red from media containing the dye (Parton, 1988). An alternative classification has been proposed by Weiss and Falkow (1984) for phase variants i.e., virulent (Vir^+) for the isolates of *B. pertussis* which possess all the virulence factors and avirulent (Vir^-) for those which have lost these factors including PT, ACT, FHA, HLT and the fimbrial AGGs (page 15). Genes encoding these virulence factors were found to be regulated by a trans-acting factor produced by a single locus which Weiss & Falkow (1984) designated *vir* . A single Tn*5* insertion into this region of the *B. pertussis* chromosome was able to create the avirulent phenotype as in BP347. This strain, however, was not equivalent to a Phase IV strain since it was not able to grow on nutrient agar.

Relatedness between phase variation and antigenic modulation.

Mutations in the *B. pertussis* genetic locus previously designated as a *vir* , now more commonly known as *bvg* (bordetella virulence genes) (Miller *et al.*,1990), cause loss of expression of AC, FHA, PT, and AGGs. The same locus is involved in the down regulation of virulence factor expression by environmental factors such as low temperature (28°C) or 40 mM $MgSO_4$ (Knapp and Mekalanos.,1988).

The nucleotide sequence of *bvg* DNA revealed two open reading frames : *bvgA* which codes for a 23 kDa protein BvgA and *bvgS* which codes for a 140 kDa protein, BvgS (Scarlato *et al.*, 1990). BvgA is located in the cytoplasm and BvgS in the membrane fraction. Regions of nucleotide homology with other two component sensor/regulator systems (Arico *et.al.*, 1989) suggests BvgS to be the signal sensor and transmitter molecule and BvgA as a signal receiver and DNA binding protein. The *bvg* locus thus encodes a transmembrane transcriptional control system (Braun and Focareta, 1991) . BvgS may act as a transmembrane protein with a periplasmic N-terminal receiver domain which senses external signals and transmits them to the second component,

BvgA via phosphorylation which allows BvgA to regulate specific loci (Coote, 1991).It has been suggested that excess phosphate may interfere with the phosphorylation mechanisms whereby BvgS transmits external signals to BvgA (Melton and Weiss, 1989). Recent results suggest that BvgA, upon modification by BvgS, binds to specific promoter sequences to activate or repress transcription (Roy and Falkow,1991).A direct involvement of the Bvg locus in modulation has become evident from the report of Miller *et al.* (1992) who showed that a single amino acid substitution in BvgS resulted in an insensitivity to the environmental signals such as $MgSO_4$, nicotinic acid, and 28 °C temp for growth, thus confirming the role of the *bvg* locus in signal transmission. It seems that the two processes (antigenic modulation and phase variation) have a common switch mechanism that regulates the simultaneous expression of several virulence factors of *B. pertussis*, and it is revealed from these data that the phase I and X-mode organisms may be essentially the same, as are the phase IV and C-mode cells. The difference is that the X-mode-C-mode changes are environmentally regulated, affect all the cells in the culture, and are freely reversible, whereas the phase I - phase IV changes are fairly rare mutational events which require subsequent clonal selection for recognition and are usually permanent. However, in some instances, phase variation has been shown to be reversible and caused by insertion or deletion of a cytosine residue in a run of six cytosine residues within the *bvgS* gene (Stibitz *et al.*, 1989). In other cases, stable variants have been found to posses small deletions or additions in the *bvg* locus (Monack *et al.*, 1989).

The possible role of these phenomena during the disease was summarized by Wardlaw and Parton (1988). Colonization, by avirulent *B. pertussis* of children and adults may provide carriers in a 'pertussis cycle' and antigenic modulation may aid in the process of transmission of the disease. Recent studies suggest a possibility of modulation *in vivo* . A mutant strain of *B. pertussis* expressing ACT independent of the control of *bvg* continued to produce AC enzymic activity, whereas the parent strain showed a reduction of the AC enzymic activity when the bacteria were located in macrophages (Masure and Barrett,1991), suggesting the involvement of modulation during this phenomenon. As well as virulence activated- genes (*vag*) such as those encoding PT, AC, FHA, AGG, and P-69, other genes have been identified, by Tn*5phoA* insertions, as *vrg* (*vir* -repressed genes) (Knapp and Mekalanos, 1988).

3) Serotype Variation

Serotype variation in *B. pertussis* was first described by Cameron (1967) who

observed that pure cultures of *B. pertussis* can display serotype heterogeneity. Variants of a single clone of *B. pertussis* can independently gain or lose AGGs 2 and 3 with a frequency between 10^{-3} to 10^{-4} (Stanbridge and Preston, 1974), a range of frequencies similar to those reported by Stocker (1949) for flagella variation in *Salmonella typhimurium*.. Serotype variation can occur in experimental animals (Preston *et al.*, 1980) and in the infected child (Preston and Stanbridge, 1972).

The contribution of serotype variation to the disease is not clear ; however, it is thought that changes in AGGs may allow the bacteria to evade the host immune system. Robinson *et al.* (1989) reported that in mice immunized with AGG3 , a *B. pertussis* challenge strain of serotype 1,2,3 underwent a change first to 1,2,0 than to 1,0,0 suggesting that selective pressure occurs against organisms expressing the homologous AGGs.Willems *et al.* (1990) have produced evidence that serotype variation may occur due to insertion/deletion events within the cytosine-rich region located 70 bp upstream of each of the *fim* genes.

PERTUSSIS,THE DISEASE

Whooping cough or pertussis is a non-invasive, acute infection of ciliated cells of the upper respiratory tract which mainly affects infants and young children. Muller *et al.* (1986) estimated that the vast majority of the world's unvaccinated children acquire the infection by the age of 5 years and life threatening cases occur in very young children (under 6 months). However, persons of all ages are susceptible, and although outbreaks in adults are uncommom (Linnemann and Nasenbury,1977) there is increasing evidence that adults serve as an important reservoir of infection (Muller *et al.*, 1986). Mortality from pertussis in developing countries can be as high as 1 in 100 . In developed countries, mortality is much lower (from 1 in 10,000 to 1 in 100,000) but the disease still persists (Rappuoli *et al.*, 1991) . Each year there are 60 million cases and over 1 million deaths world-wide, due to pertussis (Muller *et. al.*, 1986).

In most countries the disease is endemic with epidemics erupting every 3 to 4 years, depending on the number of susceptibles in the population (Cherry *et al.*, 1988). Transmission is usually due to droplet infection from infected carriers and the attack rate may be high even in immunized individuals, when they are exposed within a closed environment (Long *et al.*, 1990). There is no evidence for the existence of true (i.e., long-term, asymptomatic) carriers (Bass and Weiss, 1990) and no natural, non-human host, reservoir or vector has been reported.

Clinical Aspects of Pertussis

The severity, duration and likelihood of complications in pertussis vary with age, general health, and immune status of the individual (Parton, 1991). Pertussis can be a very severe disease in infants but in grown ups, the disease may be a mild, sometimes asymptomatic, atypical type of pertussis. Typical pertussis can be divided into three symptomatic stages, 1) catarrhal, 2) paroxysmal and 3) convalescent (Cherry *et al.*, 1988; Walker,1988).

Catarrhal stage . After an incubation period of 6 to 20 days (Lapin, 1943),the symptoms begin with a mild cough which resembles any ordinary respiratory infection. The chances of recovery of the organism are high at this stage (Weiss and Hewlett,1986). The catarrhal stage lasts 1-2 weeks and then the disease enters into its second , paroxysmal stage (Olson,1975).

Paroxysmal stage . In this stage the cough is initially more frequent at night, and progresses with time to become more frequent by day. In severe cases, 15-20 episodes of coughing may occur in a day. Each forceful cough eventually ends when air is rapidly inspired into the lungs and often causes the characteristic 'whoop'.As the paroxysm ends, the patient often vomits (Pittman,1970).

Sometimes, due to prolonged episodes of coughing, the patients may suffer anoxia, and cyanosis. Weight loss, dehydration and malnutrition are quite common in young children of substandard nutritional status. Most complications and deaths occur in this stage. Respiratory complications and central nervous system damage are relatively common and are mainly due to hypoxia and apnoea (Cherry *et al.*, 1988).Other long-term complications attributed to pertussis, such as impaired lung function and predisposition towards other respiratory complications, have not been proved in recent studies (Krantz *et al.*, 1990). The paroxysmal stage lasts 1-4 weeks and, in severe cases, up to 20 weeks (Cherry *et al.*; 1988, Walker, 1988).

Convalescent stage . The convalescent stage approaches with a reduction in the number and frequency of paroxysms, but these can occur sporadically for as long as 6 months after the infection is over (Lawrence, 1965) and may recur with subsequent respiratory infections (Fine and Clarkson, 1982).

Diagnosis.

The catarrhal phase of the disease represents the period of maximal proliferation and transmission of *B. pertussis* (Olson, 1975). Different diagnostic methods for

pertussis have been reviewed by Friedman (1988). The 'gold standard' for diagnosis of *B. pertussis* infection is recovery of the organism from nasopharyngeal swabs by culturing (Onorato *et al.*, 1987). The organism is however, difficult to recover from previously immunized persons and patients treated with antibiotics (Baraff *et al.*, 1978). An alternative method for diagnosis, a fluorescent antibody test has been used and was claimed to be more sensitive than culture (Olson, 1975) while other studies found culture more sensitive (Field and Parker, 1977). However false positive results may occur due to nonspecific binding to background material in respiratory specimens or due to cross-reactivity with other organisms sharing common antigenic determinants (Onorato *et al.*, 1987). Other serological methods such as agglutination (Manclark *et al.*, 1986), immunofluorescence and complement fixation (Bradstreet *et al.*, 1973) have been used. However, none of these methods shows high diagnostic sensitivity (Onorato *et al.*, 1987). The ELISA technique for diagnosis of pertussis (Finger and Koenig, 1985) has been shown to be a useful aid to the original culture method. Recently, Mertsola *et al.* (1990) have reported diagnosis of pertussis by measuring anti-pertussis toxin IgA responses in patients sera and have proposed this technique to be used as an additional tool, with culturing, for diagnosis and confirmation of pertussis.

Another method for diagnosis of pertussis is the exploitation of the components of *B. pertussis* produced during the course of disease. A method for detection of *B. pertussis* AC *in vitro* was accomplished by incubating alginate swabs coated with *B. pertussis* in a medium containing ATP and calmodulin and cAMP production was measured (Confer and Eaton, 1985). Recently this method has been applied to clinical specimens (Confer *et al.*, 1990).

A diagnostic method for pertussis based on DNA recombinant technology, using DNA probes has been proposed by Friedman (1988). This method has been used for screening of enterogenic *E. coli* (Moseley *et al.*, 1982).

Treatment

Treatment of pertussis with antibiotics has a negligible effect for preventing the progression, clinical course, or the establishment of disease (Bass *et al.*, 1969). However antibiotic treatment is useful as a prophylactic measure for the prevention of secondary infections (Altmeier and Ayoub, 1977). Erythromycin may reduce infectivity (Bass, 1983) and has been used along with glucocorticoids (Zoumbovlakis *et al.*, 1973) which have been reported to ease the coughing and shorten the duration of paroxysm

Barrie, 1982). Hyperimmune human gamma globulin is still used occasionally but there are no reliable data on its efficacy.

PERTUSSIS VACCINES

Whole-Cell Vaccine

An effective vaccine against whooping cough was developed in the 1940s (cited by Rappuoli *et al.*, 1991). This vaccine was composed of whole *B. pertussis* cells, killed by formalin and treated at 56°C for 30 min.With few modifications, this conventional pertussis vaccine is still in general use except in Japan.In the United Kingdom, this cellular vaccine is usually administered in combination with diphtheria and tetanus toxoids (DTP), adsorbed to a carrier like aluminium hydroxide and with thiomersal as a preservative (Health Department, 1990).

The development and efficacy of whole-cell vaccines for pertussis has been reviewed by Griffiths (1988). The methods to standardize the vaccine potency include :1) the use of standard opacity units in the cell suspensions and 2) the intracerebral mouse protection test (Kendrick *et al.*,1947). The whole-cell pertussis vaccine has been shown to protect against whooping cough. However, a high frequency of adverse reactions and a temporal relationship with serious neurological events have been observed following administration of this vaccine (Miller *et al.*,1981). Adverse effects ranging from transient local reactions to permanent brain damage have been reported (Cherry *et al.*, 1988; Griffith, 1988 ; Golden, 1990).

The concern about these reactions, in many Western countries, led to a sharp decrease in vaccine acceptance (from 90% to 30% in some areas) and a reappearance of pertussis epidemics (Cherry *et al.*, 1988). However, in a recent review it was concluded that if pertussis vaccine does cause brain damage, it must be a very rare event and it may never be demonstrated conclusively (Marcuse and Wentz, 1989). Therefore, although whole-cell pertussis vaccines may cause some transient local and systemic effects, there is no convincing evidence that they cause permanent neurological disorders. It now seems to be generally accepted that the benefits of the whole-cell vaccine are greater than the risks, and the uptake rate of whole-cell pertussis vaccine in the U.K has returned to its former level of 80% (Parton, 1991). The World Health Organization recommended continuing vaccination with whole-cell vaccine, while focussing research on the development of new vaccines devoid of side effects.

Acellular Vaccines Containing Chemically Toxoided *B. pertussis* Components

Whole-cell pertussis vaccines are undoubtedly reactogenic and different preparations may differ in their reactogenicity. Mild common reactions like swelling and redness at the site of injection, high fever or uncontrollable crying may occur after the administration of whole-cell vaccine (Cherry *et al.*, 1988). In an effort to reduce these reactions and to increase the vaccine acceptance and efficacy, acellular pertussis vaccines containing purified specific bacterial components are being developed. The first of these vaccines essentially contained filamentous haemagglutinin (FHA) and pertussis toxoid (PTd), and have been shown to confer protection in animal models (Sato *et al.*, 1981) and in man (Biritwum *et al.*, 1985; Isomura *et al.*, 1985).

Acellular pertussis vaccines have been used to immunize children in Japan since 1981 (Kimura *et al.*, 1990). They are composed mainly of toxoided PT and FHA in equal amounts (Biken type) or FHA and toxoided PT (9:1) along with small amounts of AGGs and 69kDa protein (P-69) (Takeda type). These vaccines are reported to be protective in children (Biritwum *et al.*, 1985; Isomura *et al.*, 1985) and less reactogenic than whole-cell vaccine (Kimura and Hikino, 1985).

In Sweden, a large scale, double blind placebo- controlled clinical trial was started in 1986. Two acellular vaccines; the two-component Biken type and toxoided PT alone were field tested. According to the reports of the Ad Hoc group for the study of pertussis vaccine (1988), the acellular vaccines had somewhat lower efficacy in protecting children against pertussis than would have been expected for a whole-cell vaccine. Both vaccines however, gave good protection (80%) against severe pertussis

The mono-component vaccine containing PT toxoid alone did not prevent infection, whereas the Tadeka-type vaccine gave some protection against infection, suggesting that inclusion of FHA was helpful (Storsaeter *et al.*, 1990).

The results of the Swedish vaccine trials of pertussis toxoid alone or pertussis toxoid in combination with FHA suggests that other antigens may be needed to confer full protection.

A new British vaccine containing individually-purified PT, FHA and AGGs has been developed at the Centre for Applied Microbiology and Research, Porton Down and is currently under consideration for future Phase-III clinical trials .

Acellular Vaccine Containing Genetically-Detoxified *B.pertussis* Components

During the clinical trials of acellular vaccines in Sweden, PT toxoid prepared with formaldehyde showed some reversion to toxicity on storage at 37 ^{o}C (Kimura and Kuno, 1990). In order to avoid the possible risks of such reversion, other methods of toxoiding have been investigated. With the application of modern molecular genetic and immunological techniques, a genetically inactivated PT is currently being evaluated as a candidate vaccine antigen (Nencioni *et al.*, 1990). A Phase-II clinical trial to determine the safety and immunogenicity of this material has recently been performed on a group of 250 infants.Preliminary results show that the vaccine is safe and highly immunogenic (Rappuoli *et al.*, 1991). Furthermore, in a phase I clinical trial on adult volunteers, acellular pertussis vaccine composed of genetically detoxified PT , FHA and P-69 outer membrane protein was able to produce a marked humoral response against these antigens, and a vaccine-acquired cellular immunity was also observed after the second dose (Podda *et al.*, 1991).

Several of the virulence-associated genes of *B. pertussis* have now been cloned and sequenced. DNA sequencing has allowed the deduction of amino-acid sequences and the identification of potential epitopes. For the production of new acellular vaccines, the known epitopes of cloned genes could be manipulated to provide protective but non-toxic components. Increased expression of cloned genes could also prove advantageous for vaccine production. Expression of the virulence genes in *E. coli* has proved difficult so far because of the requirements for the trans-activating virulence regulatory genes (Weiss and Falkow, 1984). However in *B. pertussis*, overproduction of genetically-inactivated PT by strains containing multiple copies of *tox* Lys ^{9} -Gly^{129} has been reported very recently by Zealey *et al.* (1992).

VIRULENCE FACTORS

A virulence factor can be defined as 'any bacterial component that promotes pathogenicity by enabling the organism to infect the host and cause disease' (Wardlaw, 1988). The virulence factors of *B. pertussis* are:

Heat Labile Toxin (HLT)
Tracheal Cytotoxin (TCT)
Endotoxin or Lipopolysaccharide (LPS)
Pertussis Toxin (PT)
Filamentous Haemagglutinin (FHA)
Agglutinogens (AGGs)
P-69 outer-membrane protein
Adenylate Cyclase Toxin (ACT)

Heat Labile Toxin (HLT)

HLT is a dermonecrotic toxin present in all bordetellae. The levels of HLT do not vary much between different phase I strains, but phase IV and C-mode strains have reduced HLT (Livey *et al.*, 1978). This toxin has proved very difficult to purify on account of its instability and no purification method has yet had independent confirmation (Nakase and Endoh, 1988). Estimations of molecular weight have also varied : 89kDa (Livey and Wardlaw, 1984), 102 kDa (Endoh *et al.*, 1986) and 130 kDa (Zhang *et al.*, 1985). HLT is mainly cytoplasmic but may be partially secreted on the cell surface (Livey and Wardlaw, 1984). Exogenous agents incorporated during the preparation of crude HLT have been shown to have an impact on the dermonecrotic reaction of the toxin and therefore the reliablity of this assay involving the measurement of skin lesions seems questionable (Endoh *et al.*, 1990) .

The toxin is found to be lethal and causes vasoconstriction in experimental animals. This results in ischaemia, haemorrhage, leukocytic influx and spleen atrophy (Cowell *et al.*, 1979; Nakase and Endoh, 1985). A Tn*5* mutant deficient in HLT production, BPM1809 (Weiss *et al.*, 1989) has been found to be unimpaired in its ability to cause lethal infection suggesting that this toxin does not contribute significantly to the disease process at least in mice.

Since HLT is inactivated upon heating at 56°C for 10 min (Manclark and Cowell, 1984), it is destroyed during the conventional method of pertussis vaccine preparation.

This suggests a non-essential role for HLT in protection.

Tracheal Cytotoxin (TCT)

TCT is produced by all four species of *Bordetella* (Goldman, 1988). Chemically, TCT is a disaccharide tetrapeptide derived from the peptidoglycan of the bacterial cell wall and thus has a low MW , estimated to be only 921 Da(Goldman, 1988).

The role of TCT in pathogenicity involves destruction of ciliated cells followed by extrusion of these cells from the epithelium.This activity has been noted in hamster tracheal organ-cultures. In non-ciliated cells, a reduction of DNA synthesis has been observed (Goldman and Baseman, 1980). These effects may reflect a hidden toxicity of this virulence factor and may explain the persistence of respiratory tract abnormality in pertussis long after bacteria have left the host. The destruction of ciliated cells may also contribute to the secondary bacterial infections which may account for complications and predisposition to secondary infections (Wardlaw, 1988) . However, TCT appears to be non-immunogenic (Goldman, 1988).

Endotoxin (Lipopolysaccharide)

Endotoxin or lipopolysaccharide (LPS) is produced by all the four species of *Bordetella* and it comprises around 2% of the dry weight of the bacterial cells. *Bordetella* LPS, besides sharing the common features of adjuvant action, pyrogenicity and the Shwartzman reactivity with other Gram-negative LPS, has a unique property of generating non-specific antiviral activity (Winters *et al.*, 1985). Purified *B. pertussis* LPS is also reported to activate mononuclear phagocytes (Haeffner-Cavaillon *et al.*, 1984).

B. pertussis strains contain two immunologically and chemically distinct molecular components, LPS I and LPS II ((Aprile and Wardlaw, 1973; Chaby and Caroff, 1988). In *B. pertussis*, both LPS I and II lack the long chain polysaccharide O-antigens, characteristic of LPS of enteric bacteria, and they have been termed lipo oligosaccharide (Ayme *et al.*, 1980). Recent comparison of LPS from phase variants of different strains of *B. pertussis* has shown a difference in their composition, antigenicity and reactogenicity. Phase I strains contained LPS I whereas phase IV had LPS II (Ray *et al.*, 1991). *B. pertussis* LPS produces a typical lipooligosaccharide electrophoretic pattern whereas *B. parapertussis* and *B. bronchiseptica* produce a smooth LPS. *B. bronchiseptica* LPS is antigenically different from LPS I and LPS II of

B. pertussis (Ray *et al.*, 1991).

Regarding the role of LPS in pathogenicity, a comparative study of *B. pertussis, B. parapertussis* and *B. bronchiseptica* LPS has recently been reported by Byrd *et al.*, (1991).In this report, survival of the *Bordetella* strains (other than *B. pertussis)* against complement-mediated killing was observed which, it was suggested, was due to the presence of high MW O-side chains and described that this structure of LPS was responsible for the inactivation of the complement complex (C5 - C9) and blocking its access to outer cell membrane, thereby preventing cell lysis.

Despite the presence of LPS in the whole-cell vaccine, it is considered not to be a protective antigen (Pittman, 1984). However, Standfast (1958) reported the protective role of boiled pertussis vaccine against lethal intranasal challenge with *B. pertussis* in a mouse model. Moreover, high titres of anti-LPS antibody have been reported in tracheobronchial washings from mice but not in serum (Wardlaw and Stevenson,1894)

On the other hand, the reactogenicity of the commercial whole-cell vaccine may be largely due to the presence of LPS and the reactogenicity is one of the major reasons for developing an acellular vaccine. In recent trials in the U.S.A., whole-cell vaccine was compared with acellular vaccine having 95% less LPS. The reactogenic symptoms like fever, local reactions, and other common reactions were significantly less with the acellular vaccine (Cherry *et al.*, 1988).

Pertussis Toxin (PT)

Unlike other *Bordetella* virulence factors, the production of pertussis toxin (PT) is restricted to *B. pertussis* . This product is considered to play a major role in the pathogenesis of whooping cough (Pittman, 1979; Irons and Gorringe, 1988). Before the term 'Pertussis toxin' was proposed by Pittman (1979), the toxin was known as histamine-sensitizing factor (HSF), leukocytosis and lymphocytosis promoting factor (LPF), islet activating protein (IAP) (Yajima *et al.*, 1978) or pertussigen (Munoz and Bergman, 1977).

PT has been the subject of several reviews including Wardlaw and Parton (1983), Sekura *et al.* (1985), Ui (1988), Furman *et al.* (1988), Munoz (1988) and Irons and Gorringe (1988).

PT is a protein complex of MW ~ 105 kDa which is made of six protein sub-units, two of which are identical. The toxin resembles cholera toxin in that it is an A-B subunit toxin where the A-subunit protomer consists of a single polypeptide chain containing a disulphide bond which must be reduced for activity. The B- subunit is an oligomer

consisting of two dimers (S2-S4 and S3-S4) connected by S5.

It has been demonstrated that the A-subunit, S1 is an enzyme active as an ADP-ribosyl transferase and also as an NAD-glycohydrolase. The B-subunit is responsible for binding the intact toxin to susceptible cells.

PT causes an acute increase in cellular cAMP levels by ADP-ribosylation of components of the host cell's adenylate cyclase enzyme complex. However, unlike cholera toxin where adenylate cyclase is stimulated by modification of the GTP-dependent regulatory component responsible for stimulation of the adenylate cyclase (the alpha-subunit of N_S), pertussis toxin acts by ADP-ribosylation of the 41 kDa GTP-dependent regulatory component responsible for inhibition of the adenylate cyclase system (N_i). When the alpha $_i$ component binds to GTP, it is activated and the adenylate cyclase enzyme is inactivated. It seems that ADP-ribosylation of the alpha i subunit of N_i by PT leads to a permanent inactivation of the component by preventing GDP from being replaced at the nucleotide binding site by GTP. Thus PT causes an intracellular increase in cAMP by inhibiting hormonally-controlled deactivation of AC with a consequent disturbance to normal cell function. Studies of the biological activities of A and B components of PT revealed that although many diverse effects of PT depend on A-subunit activity, others such as T-cell mitogen activity are mediated by the cross-linking effect of B-subunit on mammalian membrane proteins. In addition to its toxic activities, pertussis toxin functions as an adhesin (Tuomanen, 1988).

Transposon-insertion mutagenesis has shown that PT is an essential virulence determinant in a mouse model of infection.For example, *B. pertussis* strain BP357 (PT deficient) was found to be severely impaired in the ability to cause lethal infection in the infant mouse model (Weiss *et al.*, 1984; Weiss and Goodwin, 1989; Goodwin and Weiss, 1990). The ability of PT to act as a protective antigen has been referred to on page 13 & 14. Molecular cloning of PT genes (Locht and Keith, 1986; Nicosia *et al.*, 1986) has revealed that similar genes are also carried by *B. parapertussis* and *B. bronchiseptica*, but not by *B. avium*. However, these genes are not expressed in these species mainly because of defective promoter regions (Marchitto *et al.*, 1987).

Filamentous Haemagglutinins (FHA).

FHA is produced by *B. pertussis, B. parapertussis* and *B. bronchiseptica* , but has not been reported in *B. avium.*

B. pertussis produces 3 distinct haemagglutinins namely filamentous haemagglutinin (FHA), pertussis toxin and an ornithine containing lipid (Kawai *et al.,* 1982). Among these, FHA is the most potent (Sato *et al.,* 1983).

FHA has a highly hydrophobic fine filamentous structure, measuring 2 nm in diameter, and 40-100 nm in length (Arai and Sato, 1976). It appears to be a non-fimbrial surface protein (Ashworth *et al.*, 1982). SDS-PAGE and Western blot analysis of purified FHA has revealed a major component of MW 220kDa (Domenighini *et al.*, 1990).

The role of FHA in pathogenesis of pertussis seems to be as an adhesin that anchors the bacteria against the mucociliary stream in the respiratory tract. However, the strong adhesion of *B. pertussis* to ciliary tufts of human respiratory tract cells *in vitro* required both FHA and PT (Tuomanen, 1988). Both molecules are thought to act in concert as adhesins. PT and FHA, in the soluble form, have been reported to adhere either to *B. pertussis* itself or to unrelated bacteria e.g. ,*Streptococcus pneumoniae*, or *Haemophilus influenzae* which thereby become sticky and attach to cilia. This phenomenon termed 'piracy' of adhesins has been suggested as an explanation for the prevalence of secondary infections in childhood pertussis (Tuomanen, 1988). Transposon insertion mutants of *B. pertussis* lacking either FHA or PT (BP353 and BP357 respectively) showed decreased adherence to rabbit and human ciliated cells (Tuomanen *et al.*, 1983). However,Weiss and Goodwin (1989) have shown that a Tn*5* mutant, unable to produce FHA, was as virulent as the wild-type strains in a mouse model. Moreover, the recovery rates were also similar to those obtained with wild-type strains (Goodwin and Weiss, 1990). Studies in the rabbit with another FHA Tn*5* mutant showed that there was lack of efficient localization to cilia which resulted in more severe lesions in the lungs than with the wild- type strain (Tuomanen *et al.*, 1985). This suggests that attachment to the human respiratory tract could halt entry of organism into the lungs and the subsequent development of lethal pneumonia.

FHA generates humoral and cellular immune responses in humans during natural disease. In an adult mouse model, FHA proved to be a protective antigen and the animals showed reduced lung colonization after immunization with FHA (Kimura *et al.*, 1990), followed by intranasal aerosol challenge with *B. pertussis*. The use of FHA in acellular pertussis vaccines is mentioned on pages 13 &14 .

The gene for FHA has been cloned into *E. coli* (Domenighini *et al.*, 1990). Stibitz *et al.* (1988) cloned into *E. coli* a *B. pertussis* DNA fragment carrying the *bvg* locus together with the *fha* locus. Immunologically detectable FHA was expressed by *E. coli*. There was no FHA expressed however when a plasmid carryhing the *fha* locus alone was used, confirming the importance of trans-acting *bvg* locus gene products.

Agglutinogens (AGGs)

Agglutinogens (AGGs) are surface antigens capable of eliciting cell agglutinating antibodies (agglutinins) and which determine the serotypes of isolates (Preston *et al.*, 1982). There are 14 agglutinogens reported in the genus *Bordetella* (Eldering *et al.*, 1957). AGGs 8-12 and 14 are not produced by *B . pertussis* . *B. pertussis* strains produce 8 AGGs, of which 2-6 and 13 are strain specific, 1 is species specific , AGG 7 is shared with *B. parapertussis* and AGG 13 with *B. bronchiseptica* AGGs 1-3 are major antigens and AGG 4-6 are minor (Preston *et al.*, 1982).The four widely recognized serotypes of *B. pertussis* are : 1.2.3, 1.2, 1.3, and 1.0, of which only the first three infect man (Preston and Stanbridge, 1972; Preston, 1988). AGG 2 and AGG 3 are fimbrial proteins (Irons *et. al.*, 1985).

Mooi *et al.* (1987) studied the amino acid sequences of the fimbrial subunits from *B. pertussis* and found that AGGs 2 and 3 are closely related. The amino terminal amino acid sequence shared 50% homology with that of *E. coli* K99 fimbrial subunits. A gene *fim* 2 encoding AGG 2 has been cloned and sequenced by Livey *et al.* (1987) and the *fim* 3 gene has been isolated (Mooi *et al.*, 1987) and sequenced (Mooi *et al.*, 1990). Another gene, distinct from the above, *fim* X, has been characterized by Pedroni *et al.* (1988). The three genes do not appear to be linked on the chromosome (Pedroni *et al.*, 1988). Expression of AGG2 and AGG3 are controlled by the *bvg* locus (McGregor *et al.*, 1991).

The role of AGGs in pathogenicity is still unclear. However, a role in the initial attachment of bacteria to ciliated epithelial cells of the respiratory tract for establishment of infection has been suggested by Gorringe *et al.* (1985). Monoclonal antibodies to type 2 or type 3 fimbriae blocked the adhesion of *B. pertussis* to Vero cells (Gorringe *et al.*, 1985). However, *B. pertussis* cells without fimbriae adhered well to human ciliated cells (Tuomanen and Weiss, 1985).Thus Tuomanen (1988) has argued that there is little evidence for a role of AGGs as adhesins to ciliated cells of the human respiratory tract.

Following the epidemiological studies of Preston (1963) and his recommendations that pertussis vaccine should contain AGGs 1, 2 and 3, a mixture of strains of

representative serotypes is generally used to prepare vaccines (World Health Organization Committee on Biological Standards, 1979). There is some evidence that whole-cell vaccines should contain these components in order to protect adequately against all strains of *B. pertussis* (Preston, 1988).

Protein - 69 Outer-Membrane Protein

A recently identified AGG (Brennan *et al.*, 1988) is a non-fimbrial outer membrane protein P-69, the gene for which has been cloned and sequenced (Charles *et al.*, 1989). An antigenically related protein is also produced by *B. parapertussis* and *B. bronchiseptica* (Novotny *et al.*, 1985; Kobisch and Novotny, 1990).

P-69 is a *bvg* -regulated protein and is synthesized as a polypeptide of 93 kDa, which is then processed to a 69 kDa form in the outer-membrane (Charles *et al.*, 1989). Purified P-69 has been shown to enhance binding of cultured eukaryotic cells to plastic surfaces.The name'pertactin' has been proposed for P-69 to reflect its adhesive properties (Leininger *et al.*, 1990).

Roberts *et al.* (1991) have reported construction of P-69 mutants which produced normal levels of other *bvg* -regulated factors except P-69. The ability of these mutants to adhere and invade HEp2 cells was not significantly different from that of the parent strain. However, a greater reduction of adhesion and invasion was observed in a P-69, FHA^- double mutant. Arginine-glycine-aspartic acid (RGD) sequences in eukaryotic extracellular matrix proteins are involved in binding to integrin receptors of mammalian cells. The RGD sequence has also been found in both FHA and P-69 (Leininger *et al.*, 1990) thus supporting the view that these component have a role in binding of *B. pertussis* to mammalian cells.

ADENYLATE CYCLASE TOXIN (ACT)

Adenylate cyclase toxin is produced by three species i.e., *B. pertussis, B. parapertussis* and *B. bronchiseptica* but not by *B. avium* (Gentry-Weeks *et al.*, 1987; Hewlett and Gordon, 1988).*B. pertussis* AC activity was first observed in the supernate of a whole-cell pertussis vaccine (Wolff and Cook, 1973). It was later purified from *B. pertussis* culture supernates and whole-cell extracts by Hewlett and Wolff (1976).

The enzymic activity of AC is increased several hundred-fold in the presence of the eukaryotic calcium-binding protein calmodulin (CaM) which is absent in *B. pertussis* (Wolff *et al.*, 1980). Calmodulin is a small heat- stable protein (MW 16,700), which

activates a large number of enzymes in a Ca^{++} dependent, 2-step mechanism (Klee *et al* ., 1980).

Calmodulin (CaM) + Calcium (Ca^{++}) ----------> CaM - Ca^{++} Complex (1)

Inactive enzyme + CaM-Ca^{++} Complex----------> CaM-Ca^{++}- active enzyme (2)

However, Greenlee *et al.* (1982) found CaM-mediated activation of *B. pertussis* AC to be unique in that there was no requirement for Ca^{++} for the majority of the activation observed.

B. pertussis ACT has been identified in two forms. 1) The AC-enzymic form (AC) and 2) AC-toxic form (ACT). The enzymic AC is able to generate cAMP from ATP *in vitro*, whereas the AC toxic form has the ability to penetrate target eukaryotic cells, where it interacts with host-cell CaM and generates supra-physiological levels of cAMP from endogenous ATP (Confer and Eaton,1982; Hanski and Farfel,1985).These uncontrolled, high levels of cAMP impair the ability of polymorphonuclear leukocytes and macrophages to undergo chemotaxis, phagocytosis, superoxide generation and microbial killing (Confer and Eaton, 1982; Weiss and Hewlett, 1986; Friedman *et al.*, 1987 b; Hanski, 1989).

Bacillus anthracis, a Gram-positive bacillus that causes anthrax also produces extracytoplasmic CaM-dependent AC which upon entry into target cells generates cAMP from host cell ATP (Leppla, 1982). However, this protein is unrelated to ACT (Gordon *et al.*, 1989). Several other bacterial toxins also elicit their effect by alteration of intracellular concentrations of cAMP in animal cells and act by means of ADP-ribosylation of GTP-binding proteins that regulate the host membrane- bound AC system (Gilman,1984).These include, cholera toxin, pertussis toxin and *E. coli* heat-labile enterotoxin. All these toxins are A-B type toxins and enter the target cell via endocytic vesicles. However, AC from *B. pertussis* is different from these toxins in its structure, mechanism of penetration into target cells and duration of its toxic signal within the host (Gordon *et al.*, 1988; 1989; Gilboa *et al.*, 1989; Hanski, 1989).

The structural - functional relationships of ACT

AC has been isolated and purified by many workers, either from *B. pertussis* culture supernates as soluble AC or from bacterial cell extracts (Table 2).
Many different forms of AC have been associated with *B. pertussis*.. These include polypeptides of various molecular masses ranging from 43 kDa to 660 kDa (Hanski, 1989). Because of this multiplicity of forms , it was not clear whether the larger forms of the enzyme represented aggregates of the catalytic form, either with itself or with other polypeptides, **or** multiple molecular mass forms of the enzyme.The structural-functional relationship became clearer when Ladant *et al.* (1986) isolated and purified three species of AC enzyme of molecular mass 43, 45 and 50 kDa and found them to be structurally related. Furthermore, purified AC from concentrated bacterial extracts revealed two forms of AC, of 200 kDa and 47 kDa molecular mass (Rogel *et al.*, 1989). These forms were shown to have immunologically related domains because polyclonal antiserum raised against the smaller (47 kDa) form of AC cross-reacted with the 200 kDa form (Rogel *et al.*, 1989). It was concluded that the low MW form which was non-toxic, was probably formed by proteolytic cleavage of the high MW 200 kDa toxic form of AC. Bellalou *et al.* (1990 a) have produced evidence that production of the 40-50 kDa form (s) may not occur under physiological conditions. They showed that under certain conditions, the 200 kDa polypeptide could be secreted into the external medium of virulent strains of *B. pertussis* without any proteolytic processing.

The AC locus has been cloned and expressed in *E. coli* (Glaser *et. al.*, 1988; Brownlie *et.al.*, 1988).These studies revealed that the CyaA protein (AC) is synthesized as a large precursor of 1706 amino acid. The calmodulin-stimulated catalytic activity resides in the amino- terminal 400 amino acids whereas the 1300 amino acid carboxy -terminal part of the precursor possesses haemolytic activity. Biochemical analysis indicated that the ATP- and CaM-binding sites lay within the first 450 aa* N-terminal region .Tryptic fragments of this region show that amino acids 1-235/237 form the ATP binding site whereas residues 235/237-399 constitute a CaM binding site (Glaser *et al.*, 1989).

*amino acid

Table 2 Isolation and Characterization of AC by Different Investigators

Investigator & Year	Source	M_r	Enzymic activity	Toxic activity
Hewlett & Wolff (1976)	CS,C	70k	142*	_
Confer & Eaton (1982)	UE,C	_	1000*	1800 pmol/min/mgT
Wolff *et al.* (1984)	BE,C	39k	_	2000 pmol/10^7Tc
Hewlett *et al.* (1985)	UE,C	>350&70k	_	1000 pmol/min/mgT
Hanski &Farfel (1985)	UE,C	>600,190&50k	_	4500 pmol/10^7Tc
Shattuck & Storm (1985)	CS,C	_	_	300-800 pmol/10^8Tc
Shattuck *et al* (1985)	CS,C	43.4k	608000*	_
Kessin & Franke (1986)	CS	700, 60-70k	100,000*	_
Ladant *et al* (1986)	CS	45,43k	1600,000*	_
Weiss *et al.* (1986)	UE	2000,200,60k	3034*	1278 pmol/min/mgT
Friedman *et al.* (1987 a)	UE,P	200,60k	52.6*	47 pmol/10^7Tc
Pearson *et al.* (1987)	UE	_	360,000*	9400 pmol/min/mgT
Glaser *et al.* (1988 b)	CS	45k	100nmol/min/ml	_
Rogel *et al* .(1988)	UE,P	200,70k	460,1685 µmol•	
Glaser *et al.* (1989)	CS,P	_	2000 µmol•	_
Masure &Storm (1989)	UE,P	215k &45	2.8 µm/10^7 cpm	_
Rogel *et al.* (1989)	UE,P	200 k	2-4 µmol•	206µmol/mgT
Hewlett *et al.* (1989)	UE,P	216 k	589 µmol•	_
Gentile *et al.*(1990)	UE,P	220 k	0.4 mm^{o}	0.5µmol/mgT
Bellalou *et al.* (1990 a)	UE,P	200 k	100-150 nmol*	_
Bellalou *et al.* (1990 b)	CS,P	200 k	?	?
Leusch *et al.*(1990)	UE,P	200 k	17000 µmol•	6,250nmol/2x10^6Tc
Rogel *et al.* (1991)	UE,P	200 k	10-30 µmol•	?

CS=culture supernate,UE=urea extracts from bacterial cells, C=crude , P=purified, k= kilodalton,T = target cell protein,Tc= target cells, • = µmol/min/mg protein, or otherwise stated, * = nmol/min/mg protein, ^{o} mmol/min/mg protein .The data taken from Hewlett and Gordon (1988) and updated.

Generation of two fragments : 28 kDa and 19 kDa by further cleavage of the 400 - residue domain has been reported recently by Munier *et al.* (1991) . The 28 kDa fragment corresponding to the N-terminal has been shown to have ATP-binding capacity even after dissociation whereas the 19 kDa fragment was shown to possess the CaM binding site. Both fragments were found susceptible to denaturation in dissociated form and regained their activity when associated.

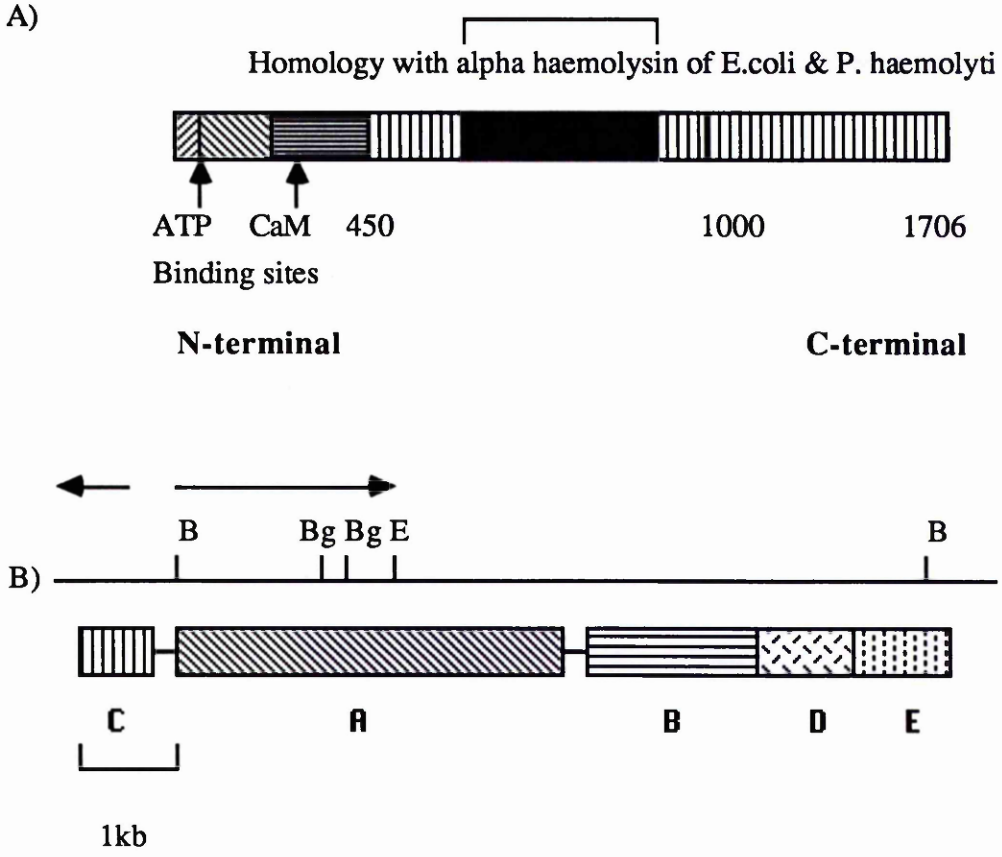
The C-terminal amino acids are essential for invasiveness and haemolytic activity (Rogel *et. al.*, 1989; Hewlett *et.al.*, 1990; Bellalou *et al.*, 1990 b), and share 25% sequence homology with alpha -haemolysin (HlyA) of *E. coli* (Glaser *et al.*, 1988 b). This homology serves to explain the capacity of ACT to act as a weak haemolysin. (Bellalou *et al.*, 1990 b ; Rogel *et al.*, 1991; Ehrmann *et al.*, 1991).It was suggested therefore, that the 200 kDa form of AC represented the full length transcript, whereas the 45-50 kDa form represented the 450 N-terminal aa product .

Three open reading frames have been identified downstream from the *cyaA* gene, *cyaB, cyaD* and *cyaE*, encoding polypeptides of 712, 440 and 474 aa residues, respectively. The gene products of *cyaB* and *cyaD* are highly similar to HlyB and HlyD of *E. coli*, known to be essential for the transport of HlyA across the cell envelope and for its release into the external medium. Complementation and functional studies indicate that the *B. pertussis* AC is secreted by a mechanism similar to that described for *E. coli* alpha -haemolysin, but requiring in addition to *cyaB* and *cyaD* gene products, the presence of a third gene product specified by *cyaE* gene (Glaser *et al.*, 1988 b). Schematic representation of the *cyaA* gene structure and the *cya* operon is given in Fig.2 . CyaE, the third secretory component of *B. pertussis* AC has obvious sequence similarity with Prtf (one of several products involved in the secretion of proteases B and C by *Erwinia chrysanthemi*) which has shown homology with TolC protein (an outer membrane protein of *E. coli*) (Wandersman and Delepelaire , 1990). Within the *prtf* operon, the transport genes *prtD* and *prtE* have significant homology with *hlyB* and *hlyD* of *E. coli* (Letoffe *et al.*, 1990). Since *tolC* mutations have a pleotropic effect on the expression of outer membrane proteins,this protein (TolC) may interact in the outer membrane with the HlyB and HlyD complex to aid translocation of HlyA to the cell surface.This suggests that CyaE may function in *B. pertussis* as a TolC protein analogue. This is supported by the fact that CyaA can be transported from *E. coli* (constitutive for TolC) when *hlyB* and *hlyD* are provided in *trans* (Masure *et al.*, 1990) and not secreted/exported by *B. pertussis* in the absence of CyaE (Glaser *et al.*, 1988 b).

Fig.2
Schematic Representation of the *cyaA* Gene Structure and the *cya* Operon.

A) The *cyaA* gene structure showing the catalytic N-terminal moiety (first 450 amino acids) with the ATP and CaM binding sites and the C-terminal portion responsible for binding to the target cell and haemolytic activities of AC. The figure is based on the data of Glaser *et al.* (1988 a).

B) The *cya* operon showing the organization of different genes responsible for the production of active AC . C represents the *cyaC* gene ; A represents the structural gene for AC : B, D, E represent the secretion and transport genes *cyaB, cyaD,* and *cyaE* respectively. Symbols for the endonucleases are : B, Bam H1; Bg, BgIII; E, EcoR1. The arrow shows the direction of transcription. The figure is based on the data from Glaser *et al.* (1988 b) and Barry *et al.* (1991).


A)
Homology with alpha haemolysin of E.coli & P. haemolyti
ATP
CaM
450
1000
1706
Binding sites
N-terminal
C-terminal
B)
B
Bg Bg E
B
C
A
B
D
E
1kb

Normally in Gram-negative bacteria, the secretion of proteins occurs by an N-terminal leader peptide-dependent two-step process involving translocation of protein to the periplasm and loss of the leader peptide and then transfer of protein across the outer membrane by a specific export mechanism (Hirst and Welch, 1988). AC however, is assumed to be secreted without the formation of a periplasmic interaction in a similar manner to HlyA of *E. coli.* (Coote,1992).

Transcription of the *cya* determinants starts 115 bp upstream of *cyaA* and transcribes a 4.8 kb *cyaA* transcript along with a low level production of a 10 kb *cyaB, D, E* transcript (Laoide and Ullmann,1990).An active *bvg* locus is essential for transcription from this promoter.However, another much weaker initiation site for transcription has been identified in the intragenic *cyaA-cyaB* region.This is located 30 bp upstream of *cyaB* and its activity is constitutive,and not dependent on the *bvg* locus.

ACT production is lost along with other virulence factors during the phenomena of phase-variation and antigenic modulation (Parton and Durham, 1978) .Activation of the AC precursor is a pre-requisite for its interaction with target cells.Recently a *cyaC* gene, located upstream of *cyaA* and in the opposite orientation has been shown to be necessary for haemolytic and invasive activities of the *cyaA* gene product (Barry *et al.*, 1991).This gene is homologous to the *hlyC* gene of *E. coli* required for the activation of the *hlyA* gene product. Electrophoresis under denaturing conditions did not reveal any difference in size between active and non-active ACT, but they have shown differences in mobility under partially-denatured conditions, and different aggregation properties after gel filtration (Rogel *et al.*, 1989). However, the active form of CyaA has been found to be unstable (Rogel *et al.*, 1991).

Mode of Action of ACT

ACT has little target cell specificity (Hanski and Farfel, 1985; Hewlett and Gordon, 1988).The fact that ACT penetration of target cells raises the internal cAMP concentration provides a sensitive assay for ACT with a wide range of eukaryotic cells. The exact mechanism involved in cell invasion by ACT is not known. Agents that interfere with endocytosis have no effect on the penetration of the toxin (Farfel *et al.*,1987;Gordon *et.al.*,1988;Gentile *et. al.*, 1988).

Penetration is presumably determined by folding of the toxin which facilitates its interaction with cell receptors and/or its ability to create pores in cell membranes.For CyaA,there is some evidence that a glycoconjugate receptor may be involved(Gordon *et*

al., 1989). An indication that the target cell receptor for CyaA might be a ganglioside had come from earlier reports that toxin (ACT) penetration into human neutrophils was blocked by bovine brain gangliosides or by treatment of neutrophils with neuraminidase to remove sialic acid residues (Gable *et al.*, 1985; Farfel *et al.*, 1987).

Two types of receptors are involved in bacterial toxin entry, a) glycoprotein receptors which are responsible for receptor-mediated endocytosis, and b) glycolipids which allow toxin entry by an alternative mechanism.Cholera toxin, for example, reacts/binds with the oligosaccharide moiety of ganglioside GM_1, via its B-subunit and creates a pore through which the A-subunit enters (Ribi *et al.*, 1988). A similar mechanism may be active in ACT entry. Analysis of the kinetics of *B. pertussis* ACT entry into target cells showed almost instant accumulation of cAMP which then proceeds to a constant level within 10-40 min (Friedman *et.al.*, 1987; Farfel *at.al.*, 1987) . This level rapidly declined, presumably due to intracellular inactivation of ACT (Gilboa *et al.*, 1989), when target cells were transferred to an enzyme-free medium (Farfel *et al.*, 1987; Gilboa *et.al.*, 1989). The CyaA protein possesses haemolytic activity which is closely associated with the capacity of the protein to penetrate target cells (Bellalou *et al.*, 1990 b; Rogel *et al.*, 1991; Ehrman *et al.*, 1991). Studies on haemolytic activity of ACT have suggested that the lytic process has features which distinguish it from the penetrative activity which results in elevated cAMP levels. Unlike immediate accumulation of cAMP in target cells, the onset of haemolytic activity takes at least 60 min (Rogel *et al.*, 1991; Ehrman *et al* ., 1991). The half maximal activity of toxin required for haemolysis was at least 10-fold higher than that required for invasive activity (Bellalou *et al.*, 1990 b; Rogel *et al.*, 1991).

CaM has been shown to inhibit the entry of ACT into erythrocytes (Shattuck and Storm, 1985; Gentile *et al.*, 1988). Haemolysis however, either remained unaffected or was increased by CaM (Rogel *et al.*, 1991; Ehrman *et al.*, 1991). Thus with bound CaM, the configuration of the N-terminal portion of ACT may prevent access to the cell interior, but pore formation still takes place and causes cell lysis (Rogel *et al.*, 1991).

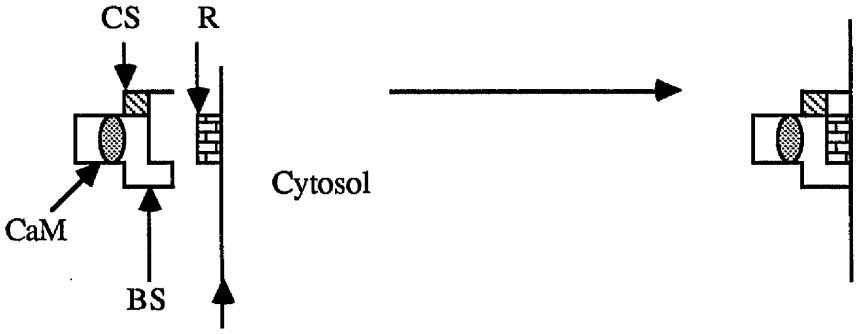
Truncated AC, produced by in-phase deletion of a hydrophobic membrane- spanning region of CyaA, did not raise cAMP levels in target cells , but retained 12-50% of haemolytic activity (Bellalou *et al.*, 1990 b). This suggests a possible mechanism of entry of ACT where the C-terminal portion of CyaA would create a pore through which the catalytic portion (N-terminus) could enter the cytosol. A simplified view of possible events involved in invasion of target cells is given in Fig.3 .

Fig.3

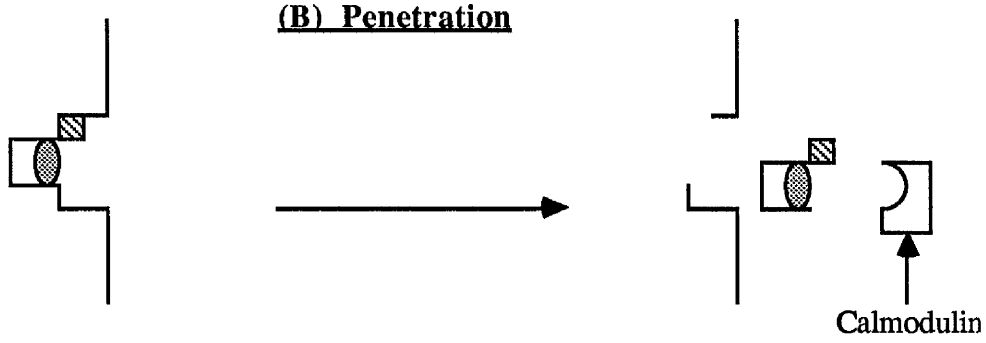
Possible Mode of Action of ACT.

A proposed view of the mode of action of ACT . (A) Binding of ACT to its receptor via its C- terminal moiety , (B) pore formation and penetration of the catalytic and CaM - binding sites (N-terminal moieties) into the cell interior through the pore , (C) activation via interaction with CaM and uncontrolled generation of cAMP. Abbreviations : BS = binding site for cell surface receptor(R), CS = catalytic site, CaM = calmodulin binding site , R = cell surface receptor .

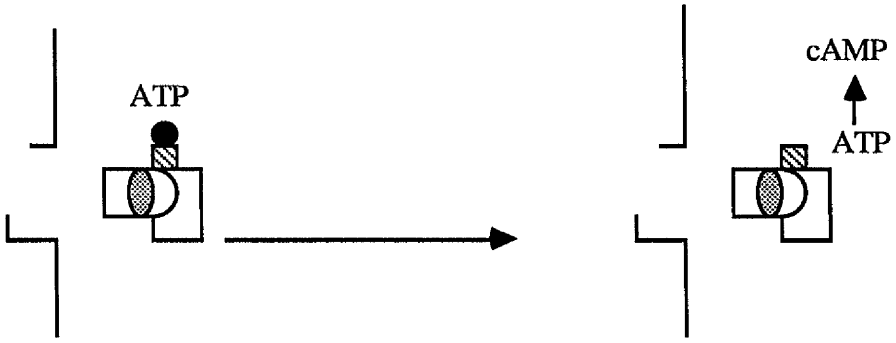
(A) Binding



(B) Penetration



(C) Activation and cAMP generation



Influence of calcium on ACT

The role of calcium in the catalytic and invasive activities of ACT has been investigated by several workers. Masure *et al.* (1988) reported that calcium influenced several properties of ACT, including its chromatographic behaviour, the Stokes radius of the enzyme, the mobility of the protein on SDS-PAGE and the effectiveness of ACT to elevate cAMP in target cells.A greater M_r in the presence of calcium was noted. It was suggested that AC interacts directly with calcium which affects its physical and enzymatic properties. Hanski and Farfel (1985) observed that on fractionation of AC toxin by ultrogel the peak with toxin activity had an apparent size of 190 kDa in the presence of calcium and 340 kDa in the absence of calcium .The presence of calcium is not an essential feature for the enzymic activity of AC since the activity was shown both in the presence and absence of calcium (Greenlee *et al.*, 1988) . Gentile *et al.* (1990) have substantiated this finding and reported that calcium is not an absolute requirement for catalytic activity but an indispensible cofactor for the invasive activity of ACT.

There is some evidence that calcium might be involved in attachment of ACT to target cells. *E. coli* alpha haemolysin requires calcium for its action (Boehm *et al.*, 1990). The presence of 11 tandemly- repeated sequences each consisting of 9 amino acids have been found necessary for the binding of *E. coli* haemolysin to the erythrocyte membrane and these sequences appear to bind calcium (Boehm *et al.*, 1990). Similar sequences have been identified on *B. pertussis* ACT (Glaser *et al.*, 1988 b). Very recently, Hewlett *et al.* (1991) have also shown a role for calcium in ACT structure and function in that calcium binding caused a conformational change in ACT. The exposure of purified ACT to free calcium at concentrations between 1-10 μM resulted in a conformational alteration as viewed by negative staining and electron microscopy.Calcium induced conformational changes in the ACT molecule from folded to linear, which was found necessary for the insertion of ACT into target cells and for delivery of its catalytic domain to the cell interior .

The effect of calcium on the haemolytic activity of ACT has been reported recently. Bellalou *et al.* (1990 b) have shown that the haemolytic activity of ACT was calcium-dependent. However, in another report, exclusion of calcium and addition of a calcium chelating agent (EDTA) did not prevent the binding and haemolytic activity of AC (Rogel *et al.*, 1991). This group has shown a distinct dissociation of haemolytic and cAMP accumulation activities of ACT by manipulating the calcium concentration in the incubation medium for sheep RBCs. Recently, it has been shown that haemolytic activity of *E. coli* grown in the absence of calcium was calcium- dependent and grown in the presence of

calcium was calcium - independent (Boehm *et al.*, 1990).
A similar possibility of calcium- dependent haemolytic activity could be anticipated for *B. pertussis* ACT.

Influence of calmodulin on ACT

Wolff *et al.* (1980) discovered that *B. pertussis* AC was stimulated by calmodulin (CaM) and the stimulation of cell bound AC was 100- fold higher than the AC recovered from culture supernates. The half-maximal stimulation of AC associated with intact cells occurred at a concentration of 45 nM CaM (Masure *et al.*, 1987). CaM regulation of *B. pertussis* AC differs from CaM stimulation of mammalian AC in several respects. Calcium is an obligatory requirement for CaM-stimulation of brain AC but not the bacterial AC and the addition of EGTA to the CaM - stimulated *B. pertussis* AC did not readily reverse the stimulation of the enzyme (Wolff *et al.*, 1980). In fact *B. pertussis* AC is stimulated by CaM both in the presence and absence of calcium (Greenlee *et al.*, 1982 ; Kilhoffer *et al.*, 1983).

In contrast to the stimulation of AC enzymic activity by CaM, the toxic activity of AC may be inhibited by exogenous calmodulin.It has been demonstrated that addition of μM CaM prevented the formation of cAMP in target cells catalyzed by partially purified AC (Shattuck and Storm, 1985). Rogel *et al.* (1989) reported the CaM- mediated inhibition of purified ACT activity and showed that the inhibitory effect of CaM was dose dependent.

Exogenous CaM has recently been shown to potentiate the haemolytic activity of ACT. The lag time for haemolysis was shortened and the extent of lysis was considerably increased in the presence of exogenous CaM. Under similar conditions, CaM almost completely blocked intracellular cAMP generation in target cells (Rogel *et al.*, 1991; Ehrman *et al.*, 1991). Thus with bound CaM, the configuration of the N-terminus of ACT prevents access of the toxin to the cell interior, but pore formation still takes place and causes cell lysis (Rogel *et al.*, 1991). However, the blocking effect seems to be target cell specific, depending upon the type and nature of the cells, since in chinese hamster ovary (CHO) cells, accumulation of cAMP was unaffected when CaM was added (Gentile *et al.*, 1990).

ACT as a virulence factor.

ACT is believed to be a major virulence factor of *B. pertussis* and presumably of *B. parapertussis* and *B. bronchiseptica* on the basis of the following arguments:

(1) ACT, after being activated by a host cell protein, calmodulin, can raise internal cAMP levels of many eukaryotic cells. Neutrophils and macrophages attain cAMP levels high enough to paralyse those functions that are effective in combating infection (Confer and Eaton, 1982; Hanski and Farfel, 1985; Friedman *et al.*, 1987 b; Pearson *et al.*, 1987).

(2) *B. pertussis* Tn*5*- insertion mutants lacking ACT are avirulent in a mouse model (Weiss *et al.*, 1984).

(3) The cloned ACT gene was able to restore virulence to an ACT-deficient Tn*5* insertion mutant of *B. pertussis* when introduced on a plasmid (Brownlie *et al.*, 1988).

(4) Although PT is widely considered to be a major virulence factor of *B. pertussis*, *Bordetella* species in which the pertussis toxin gene is not expressed i.e., *B. parapertussis* and *B. bronchiseptica* (Arico and Rappuoli, 1987) retain virulence and secrete an active, CaM-sensitive ACT (Pearson *et al.*, 1987).

(5) ACT production is a feature of virulent *B. pertussis* and the production is lost together with several other virulence factors, in phase variant, avirulent strains (Parton and Durham, 1978).

(6) ACT and antibodies to AC appear to be protective in animal models (Brezin *et al.*, 1987; Guiso *et al.*, 1989; Guiso *et al.*, 1991).

(7) High titres of anti-AC antibodies are produced in man during pertussis infection (Farfel *et al.*, 1990., Arciniega *et al.*, 1991).

Homology of ACT with RTX toxins.

AC has been included in the family of pore forming toxins, designated as RTX (Repeat in Toxin). The family comprises broadly disseminated cytolytic toxins including *E. coli* alpha- haemolysin (HlyA), *Pasteurella haemolytica* leukotoxin (Lkt A) and *Bordetella pertussis* adenylate cyclase toxin (CyaA). Other toxins related to this group are found in *Actinobacillus actinomycetemcomitans* (AktA), *Proteus vulgaris*, and *Morganella morganii* (Welch,1991; Coote,1992). The common structural feature among these toxins is the presence of a tandem array of a nine amino acid repeats (L-X-G-G-X-G(N/D)-D-X) in each of the toxin proteins (Welch,1991; Coote, 1992 a).The amino acid sequence of the C-terminal 1250 aa of AC exhibits 25% similarity with *E. coli* haemolysin and 22% similarity with *P. haemolytica* leukotoxin (Glaser *et al.*, 1988 b).

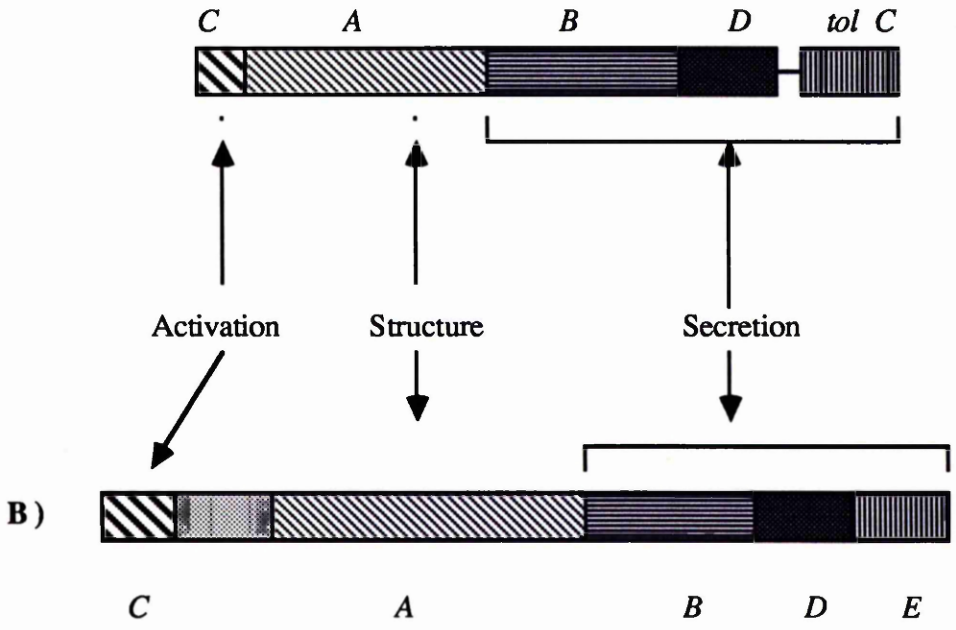
Analysis of the sequence homology between these toxins shows that starting from aa 50 (350 in *cyaA*),all toxins reveal obvious similarity up to and including the glycine -rich repeats. These are followed by a C- terminal end of 134 residues in HlyA 128 in LktA, 77 in CyaA, and 209 in AktA, Within the homologous region, all the proteins share two important domains; several hydrophobic, potentially membrane spanning regions in the N-terminal half and the glycine- rich repeats towards the C- terminal end. A comparison of the genetic organization of the RTX determinants is given in Fig. 4

Fig. 4

Genetic organization of RTX toxins.

Comparison of the genetic organization of the RTX toxin determinants. A) The genes encoding the HlyA, LktA, AktA and associated proteins are identically organized. *tolC* is unlinked to the *hly* operon. The *tolC* equivalent on the *cya* operon, B), is *cyaE .. cyaC* is encoded in the opposite orientation on *cya* operon. The figure is based on the data from Glaser *et al.* (1988 a,b); Barry *et al.* (1991) and Welch (1991).

A)



***Cloning and expression of the cya locus in* E.coli**

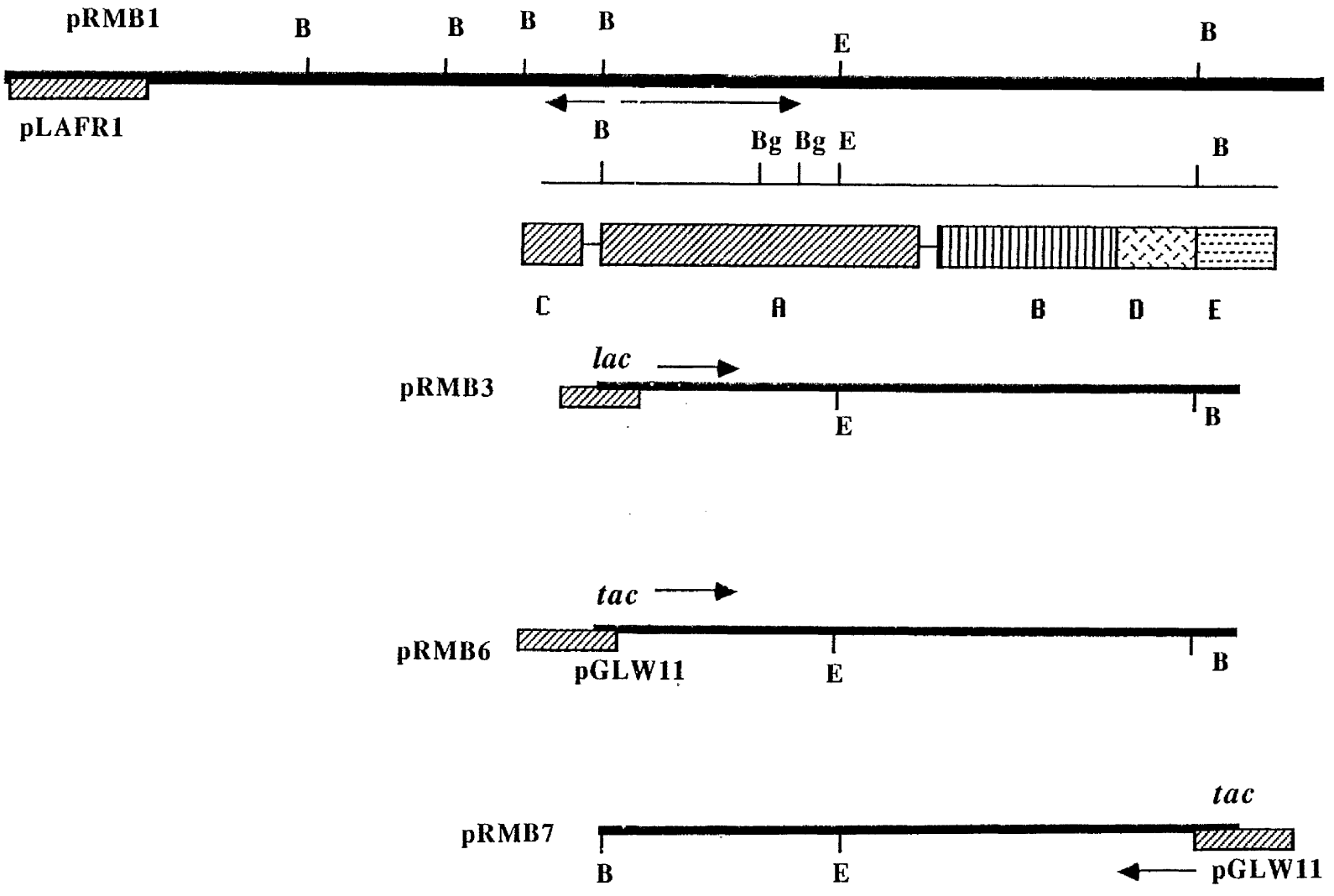
Using the interaction between ACT and CaM as a probe, Glaser *et al.* (1988 a,b) determined its complete nucleotide sequence after cloning the corresponding *cya* gene from *B. pertussis* to a *cya* -defective *E. coli* harbouring a plasmid which expressed high levels of calmodulin. According to their findings, ACT is synthesized as a large precursor of 1706 amino acid residues, endowed with catalytic and haemolytic activity. The protein has been given the name of cycolysin to indicate its activities (Glaser *et al.*, 1989). The secretion of the CyaA protein was found to require the products of three genes : *cya B, D*, and *E* coordinately expressed with the *cyaA* (the structural gene)

Brownlie *et al.* (1988), with the help of appropriate expression vectors, cloned the *B. pertussis* adenylate cyclase structural gene into *E. coli* and demonstrated AC activity in *E. coli* . Moreover, this enzyme activity was enhanced by calmodulin, although not to the levels found in parent strain extracts of *B. pertussis*.. They cloned the AC genetic determinant on a broad- host -range plasmid pLAFR1. The recombinant plasmid, designated as pRMB1 was identified by its ability to restore the AC enzymic and haemolytic activities of a transposon insertion mutant *B. pertussis* strain BP348, deficient in both these activities. A 10 kb *Bam* H1 fragment contained in pRMB1 was subcloned into the vector pIC20H to give the plasmid pRMB3. Expression of AC in *E. coli* was found to be dependent on the orientation of the insert, and was thus expressed from the *lacZ* promoter present in the vector. A 3kb *Bam* H1/ *Eco* R1 fragment was subcloned into pIC20H to give pRMB9. AC enzymic activity was reported from both the constructs which was shown to be increased 250- fold in the presence of exogenous CaM. However,these constructs were unable to show haemolysis on BG medium and extracts from them were found non-toxic in a S49 mouse lymphoma cell assay. The 10 kb *Bam* H1 fragment was subcloned in another vector, pGLW11, in two orientations to give plasmids pRMB6 and pRMB7 and the expression of AC was determined in these constructs . Production of AC was found in pRMB6 and not in pRMB7 indicating again that the insert in the former depended on the *tac* promoter in the vector and needed an exogenous promoter for expression . Comparison of the open reading frame (ORF) for *B. pertussis* AC (Glaser *et al.*, 1988 b) with pRMB3 and pRMB9 showed that pRMB3 contained the entire ORF whereas pRMB9 contained only 5' end (2.6 kb) of the ORF and produced a 95 kDa protein. (Brownlie *et al.*, 1988) which includes the CaM-binding and catalytic domains of the polypeptide. The restriction maps of plasmids containing cloned *B. pertussis* DNA and which were used in the present work are given in Fig.5

Fig.5

Restriction Maps of Plasmids Containing *B. pertussis cya* DNA.

Restriction sites are shown for *Bam* H1, B., *Eco* R1, E. The position of promoters and direction of transcription are indicated as arrows over each plasmid . The genetic organization of the *cya* operon is also shown.The Fig. is based on the data from Brownlie *et al.* (1988); Glaser *et al.* (1988 a,b); and Barry *et al.* (1991).


pRMB1
B
B
B
B
E
B
pLAFR1
B
Bg Bg E
B
C
A
B
D
E
pRMB3
lac
E
B
tac
pRMB6
pGLW11
E
B
tac
pRMB7
B
E
pGLW11

Rogel *et al.* (1989) reported the production of 200 kDa CyaA protein by expression of pRMB3 in a Lon- protease defective *E. coli* strain. This protein was recognized by anti-AC monoclonal antibody but had no haemolytic activity and was non - toxic in S49 lymphoma cells. .

The export of CyaA from *E. coli* using the transport system of *E. coli* alpha -haemolysin (*hlyB*, *hlyD* and *tolC*) has been reported recently by Masure *et al.*. (1990). This group suggested that the gene products of the *hly t*operon may have recognized the signal sequence of CyaA and facilitated the secretion of 215 kDa AC. However, only 20% of the total AC activity was released into the culture medium (cf > 70% for *B. pertussis* Tohama). This form exhibited the enzymic activity but was found non-toxic in mouse neuroblastoma cells.

Expression of toxic AC from cloned *E. coli* has very recently been reported (Sebo *et al.*, 1991) after the identification of the fifth gene, *cyaC*, of the *cya* operon responsible for the activation of *cyaA* product. Reconstruction of the *cya* locus by cloning different genes on appropriate vectors, and under the control of *E. coli* promoters and tran`slational signals enabled the production of a 200 kDa CyaA in *E. coli* with full invasive and partial haemolytic activity (Sebo *et al.*, 1991).

POLYMORPHONUCLEAR (PMNs) LEUKOCYTES: ROLE IN PHAGOCYTOSIS.

Phagocytes were the first eukaryotic cells to be used by Confer and Eaton (1982), as target cells to detect the toxic activity of ACT.In order to understand the effects that AC toxin may have on phagocytes, their activities and functions should be explained.

The role of PMNs in health and disease is well-established. Bacterial pathogenicity is heavily dependent on the capacities of the microbial cells to avoid or to resist the antimicrobial activities of poplymorphonuclear leukocytes, the first line of phagocytic defence against infection (Spitznagel, 1983).

Neutrophils are the most active and common (60-70%) leukocytes in the blood (Van Furth and Willemze, 1979) and exist in blood as circulating cells but in pathological conditions they adhere to the post- capillary venules, leave blood stream and migrate towards damaged tissue (Beesley *et al.*, 1978).Neutrophils are attracted to the area of infection or damage by microbial or host-derived chemotactic factors, including complement components C5a and C3b, f-Met-tri peptides, collagen peptides, lymphokines, interleukin 1, leukotriene B4, and platelet activating factors. In immune complexes, neutrophils are attracted by formation of activated complement components

following Ab-Ag reactions (Romeo,1982).

Phagocytosis occurs with sequential receptor-ligand binding between the PMN and the microorganism. The motive force of phagocytosis resides in actin, myosin and actin-binding proteins. It has been proposed that the cytoplasm near the site of particle-PMN contact undergoes gelation and cross linking of F actin. This causes puckering of the plasma membrane at the site of contact because of attachment of microfilaments to the membrane. Bulging of the membrane around the particle occurs, and new particle-membrane contacts results. The net result is the formation of pseudopodia and a phagocytic vacuole (Yin and Stossel, 1982). Observations suggest that the concentration of free cytoplasmic Ca^{++} is involved in either activation of NADPH oxidase (enzyme responsible for superoxide production) or degranulation of primary granules into the phagosome (Sawyer *et al.*, 1989) or in both processes (Clark ,1990). Calcium and magnesium ions are also required for optimal phagocytosis (Wilkins and Bangham 1964 ; Janah *et al.*, 1990; Baggiolini and Wymann 1990).

In general, bacteria and other particles must be coated or opsonized for attachment and ingestion to occur. The major opsonins are IgG (the only class of immunoglobin that promotes phagocytosis in PMNs) and complement. IgG binds to bacteria with the Fab end, allowing interaction between the Fc portion and Fc receptors on the phagocytic cell. Interaction of IgG and Fc receptors opens calcium-dependent channels that may be important in regulation of phagocytosis and post-phagocytic events (Young *et al.*, 1985). Cell wall components of Gram-positive bacteria (Wilkinson *et al.*,1978) and outer-membrane components of Gram-negative bacteria (LPS) can combine with serum factors to form C3 esterase and to fix C3b to microbial surfaces (Morrison and Kine 1977). Capsules of bacteria may prevent exposure of these outer-wall or -membrane components to the serum, and in such cases specific antibody to the capsule is required before complement activation can occur (Horwitz and Silverstein, 1980).

Microbiocidal Activty

A consequence of phagocytosis is the fusion of the phagosomal vacuole with the lysosomal granules which are involved in killing and digestion of the engulfed bacteria. Neutrophils contain two types of granules 1) primary (azurophilic), containing myeloperoxidase, lysozyme, acid hydrolases, cationic proteins and proteases.
2) Secondary (specific granules) containing the lysozyme, cytochrome b, collagenase and the high affinity iron - binding protein lactoferrin which limits the availability of iron to bacteria (Sawyer *et al.*, 1989).

There are two main arms of the microbiocidal activity of the PMNs: oxygen-dependent mechanisms and oxygen-independent mechanisms. Oxygen-dependent mechanisms rely on toxic molecules produced as a result of the oxidative burst. Resting PMNs consume little oxygen, since most of their energy comes from glycolysis. Oxygen-independent mechanisms are responsible for microbial killing in anaerobic environments such as the gastrointestinal tract, and vaginal mucosa. These mechanisms utilize lysozyme, lactoferrin, and cationic proteins called defensins with MW between 3 kDa and 3.9 kDa , substances which are localized in the azurophilic granules and possess antimicrobial activity against bacteria, fungi, and certain enveloped viruses (Ganz *et al.*, 1985). Another cationic protein with microbicidal activity in PMN azurophilic granules is the bactericidal/ permeability-increasing protein (BPI). BPI is membrane-associated, and the intracellular presence of bacteria is required for its bactericidal activity (Weiss *et al.*, 1978) .

Oxygen-dependent mechanisms of phagocytes involve the production of the respiratory burst which consists of a marked increase in oxygen consumption that occurs during and subsequent to ingestion of microbes by PMNs. This increased oxygen consumption results from activation of a NADPH oxidase, which accepts two electrons from NADPH and transfers them to an FAD-flavoprotein associated with cytchrome b245 and from there to oxygen (Sawyer *et al.*, 1989).

$NADPH^{-e}$---->FAD-$Protein^{-e}$-----> Cytochrome $b245^{-e}$-----> O_2 ---> O_2^-(superoxide)

NADPH oxidase is not expressed in the resting PMNs. Part of the cytochrome b245 is translocated from the cytoplasm (perhaps from the secondary granules) to the plasma membrane following stimulation. In an acidic milieu, superoxide anion is converted to H_2O_2 by superoxide dismutase. Hydrogen peroxide with myeloperoxidase (released from primary granules) and a chloride ion produces hypochlorous acid, a potent microbicidal agent. Other halides, such as iodide, can be used in the myeloperoxidase-hydrogen peroxide reaction. Bacterial halogenation is closely correlated with bacterial death.

The hexose monophosphate shunt is activated during the respiratory burst and provides reduced pyridine nucleotide and reduced glutathione, which protects the PMN from oxidative damage by removing excess H_2O_2 (Babior, 1984).

CHEMILUMINESCENCE AS AN ASSAY.

Generation of chemiluminescence (CL) by human neutrophils during phagocytosis was originally described by Allen *et al.* (1972) . PMNs in their resting stage consume little oxygen . Perturbation or stimulation via the plasma membrane initiates a burst of metabolic activity with an accompanying dramatic increase in oxygen consumption, superoxide generation, formation of hydrogen peroxide, singlet oxygen, and activation of the hexose monophosphate shunt (HMP-shunt). All contribute to phagocytosis and the phenomenon is collectively referred to as the respiratory burst (Babior, 1978). The generated oxidative species postulated to be involved in chemiluminescent reactions and their decay result in light production but the reactions of these species with endogenous substrate yields ultra-weak chemiluminescence (< 1 photon / cell / sec) which needs to be amplified by addition of a synthetic substrate e.g., luminol or DNDH, (see Materials and Methods, page 59) .This increases the light emission 10^3 - 10^4 - fold (Anthony, 1986) by acting as a bystander substrate for these oxidative species and the oxidation results in the production of an excited aminophthalate anion which when relaxed to the ground state, emits light. The mechanism of light emission is totally myeloperoxidase - hydrogen peroxide dependent (Dahlgren and Stendahl, 1983).

In addition to resulting from phagocytosis of particulate matter, the respiratory burst may be elicited by certain soluble stimuli e.g., fMLP, concanavalin A and PMA (Dechatelet and Shirley, 1976;1982) . Any membrane - associated event may alter the ablity of PMNs to generate chemiluminescence when induced with stimulant , e.g., PMA in a luminol or DNDH amplified response, and this is the basis for assesment of toxin activity. Light emitted, with the return of high energy state to ground state levels , during the activities of phagocytosis can be quantitated as chemiluminescence (CL) as originally described by Allen *et al.* (1972). It can be regarded as a cumulative measure of more than one function of the cellular response to stimulants.

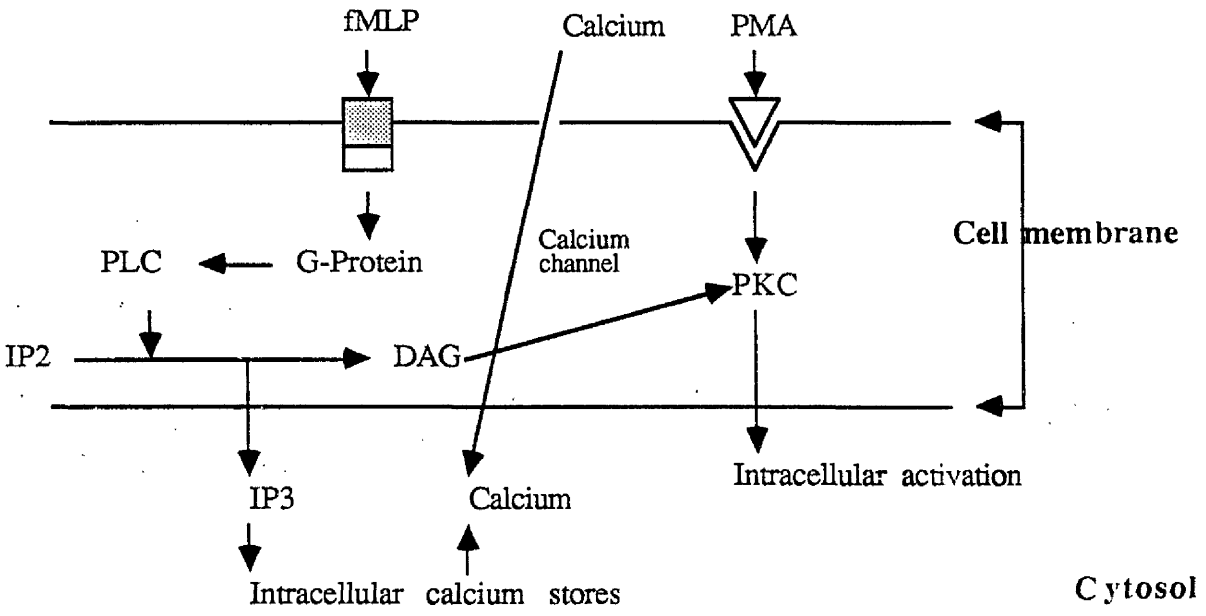
CL has also been used to evaluate the effect of serum opsonins (Robinson *et al.*, 1984). Lillius *et al.* (1985) showed that CL can be used to detect the presence of opsonins against *B. pertussis* ; higher levels of opsonins against *B. pertussis* were found in pertussis patients than in vaccinated individuals .

Mechanism of Oxidative Burst.

The oxidative burst begins with the binding of stimulant to cell surface receptors activating a guanine nucleotide -binding protein (G protein), which in turn activates plasma membrane phospholipase.The latter hydrolyses phosphatidyl biphosphate, generating two intracellular secondary messengers, 1) 1,4,5- inositol triphosphate (IP3) and 2) diacylglycerol (DAG).IP3 releases Ca^{++} from intracellular stores,including the endoplasmic reticulum and microsomal organelles, calciosomes (Sawyer *et al* ., 1989), increases the affinity of DAG for cytosolic protein kinase C (PKC), which is activated and translocated to the cell membrane (Janah *et. al.,* 1990).The PKC phosphorylates the cellular proteins (actins) which brings about the cellular function in response to chemotattactants,e.g.,formyl-methionine-leucine-phenylalanine(fMLP) (Berridge,1984). Another stimulant, phorbol myristate acetate (PMA), on the other hand, circumvents the initial steps by acting directly on PKC, after which the course of events is similar to that evoked by fMLP (Niedel, 1983). A schematic representation of the events involved in the process of phagocytic cell response to the fMLP and PMA is shown in Fig. 6

Fig. 6
A Simplified View of the Events Involved After Stimulation of PMNs with fMLP and PMA.

Coupling of fMLP to its cell surface receptor leads to G- protein activation of phospholipase C(PLC), PLC hydrolyse phosphatidylinisitol 1,4,5- biphosphate (IP2) to produce two secondary messenger molecules : Inositol 1,4,5-trihosphate (IP3) and 1,2-diacylglycerol (DAG). IP3 mobilizes calcium from intracellular stores, thereby increasing the concentration of cytosolic calcium, which may also be elevated by influx of calcium from the extracellular space via calcium channels. DAG is a direct stimulus of protein kinase C (PKC). PMA directly effects PKC. These processes lead to activation of a variety of intracellular processes collectively referred to as the respiratory burst. The figure is based on the data from Sawyer *et al.* (1989).


fMLP
Calcium
PMA
Cell membrane
PLC
G-Protein
Calcium channel
PKC
IP2
DAG
IP3
Calcium
Intracellular activation
Intracellular calcium stores
Cytosol

OBJECT OF RESEARCH

Among the many biologically active factors produced by *B. pertussis*, adenylate cyclase (AC) is one which is presumed to contribute to the pathogenesis of whooping cough in a unique manner, by inhibiting the various activities of host phagocytes by elevating their cAMP levels.This AC has been reported to exist in various forms . The aim of the present investigation was to identify and characterize these different forms of AC in *B. pertussis, B. parapertussis , B. bronchiseptica,* and also in recombinant *E. coli* . A further aim was the development of more convenient, rapid and sensitive procedures for detection of AC and for the determination of its enzymic and toxic activities.

AC should be considered as a potential immunogen for inclusion in future acellular pertussis vaccines, but as yet, there is a little evidence that AC may act as protective antigen . This study also involved the screening of human sera from pertussis patients for the presence of anti-AC antibodies in order to determine whether AC is one of the major antigens of *B. pertussis* recognized during pertussis infection. Intranasal passive protection tests in a murine model were planned to determine the protective activity of *B. pertussis* AC.

Another important aspect of AC is its genetic homology with a family of pore forming toxins (RTX toxins). This study also aimed at revealing the immunological relatedness of AC to the other RTX toxins which may help in understanding the structure- function relationship of AC.

MATERIALS AND METHODS

BACTERIAL CULTURES

Bordetellae

The strains used in this study and their relevant characteristics are listed in Table 3. These strains were obtained from the culture collection of the Department of Microbiology, University of Glasgow. Plate cultures of *B. pertussis* , *B. parapertusis* *B. bronchiseptica* and *B. avium* were grown on Bordet-Gengou (BG,Gibco-BRL) medium with 1% glycerol and 15% defibrinated sheep blood (Becton-Dickinson) incubated at 37 °C for 72 h in a humidified box. Broth cultures were grown in cyclodextrin liquid medium (CDL, Imaizumi *et al.*,1983, App.I) at 37°C for 48 h on an orbital shaker with 80 rpm for aeration.

E. coli .

The strains of *E. coli* used in this study are listed in Table 4 and the plasmids used in Table 5. Cultures were grown on nutrient agar (Oxoid) with their respective antibiotics (see Table 4). Liquid cultures were grown either in nutrient broth or brain heart infusion broth (Oxoid) at 37 °C for 24 hr on an orbital shaker at 80 rpm.

EXTRACTION OF ADENYLATE CYCLASE (AC) FROM *B. PERTUSSIS* AND *E. COLI* STRAINS WITH UREA.

Crude preparations of adenylate cyclase (AC) were made by an adaptation of the method of Brownlie *et al.* (1988) and Confer and Eaton (1982). *B. pertussis* cells were grown as lawns on BG plates with their respective antibiotics (Table 3) for 72 h at 37 °C in a moist atmosphere. The resulting growth was used as inoculum in a ratio of 1 plate / 1 litre of CDL medium. *E. coli* cultures containing pRMB3 were grown at 37 °C for 24 h on nutrient agar with their respective antibiotics, and inoculated, as above, into 1litre volumes of BHI broth. Cultures were incubated on a orbital shaker at 80 rpm for 48 h in the case of *B. pertussis* and for 24 h in the case of *E. coli* or until the cultures reached OD_{650}=2, monitored on a Shimadzu recording spectrophotometer. The cells were harvested at 6000 g for 10 min at 4 °C (Sorvall RC5B). The pellet (approx. 1.3 g wet weight of the cells) was resuspended in 10 ml of distilled water containing 0.5mM of the protease inhibitor, phenyl methyl sulphonyl fluoride (PMSF, Sigma) and kept frozen overnight at - 20 °C. On the following day, the suspensions

were thawed at room temp and crystalline urea (BDH) was added to the final concentration of 4 M. Suspensions were stirred at 4 ^{o}C for 10 min . In order to solublize the intracellular AC from *E. coli* , cells were sonicated 3 times with a MSE sonifier at maximal output for 30 s on an ice bath. Low speed centrifugation of the *B. pertussis* and *E. coli* suspensions was carried out at 8000 g for 15 min at 4 ^{o}C to remove the insoluble material. The supernate was ultracentrifuged at 160,000 g for 1h at 4 ^{o}C in an OTD-Combi / A 148561 machine. The resultant supernate was collected and stored at - 20 ^{o}C as ' urea extracts '. It is important to mention here that the samples prepared for toxicity assays did not receive PMSF to avoid the possiblility of interference of the protease inhibitor in the assays.

E. coli cells containing cloned AC under the control of the inducible *tac* promoter (Table 4 & 5) were grown in NB until OD $_{650}$= 0.4 - 0.5, after which, IPTG (Sigma) was added to 0.5mM final concentration and cells were harvested at OD_{650}=1. The rest of the steps were performed as above.

DIALYSIS

Urea extracts were dialyzed against dialysis buffer (100 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$) before any toxicity assay to remove urea, according to the method of Brownlie *et al.* (1988). Conventional dialysis was carried out in dialysis membrane (size 1 - 8 / 32 ") against 2000 volumes of dialysis buffer at 4 ^{o}C for 48 h with continous stirring. The buffer was changed twice per day. After completion of dialysis, the samples were centrifuged at 6000g for 5 min to remove insoluble precipitates, and stored at -20 ^{o}C, usually for not more than one month if the samples were to be used for toxicity assays. An alternative method to the conventional dialysis, a microdialyzer (Pierce. USA) was used according to the manufacturer's instructions. The molecular mass- cut off of the membrane was 50,000. This method of dialysis was used with the small volumes of purified AC.

Table 3 **Strains of Bordetellae Used in this Study.**

Strains	Plasmid/mutation/ antibiotic marker	Phenotype
B. pertussis 348pRMB1•	Recombinant, Tc^R pRMB1, *vir* +	AC^{++}, HLY^+
B. pertussis Tohama	*vir* +	Wild type
B.pertussis 165	*vir* +	Wild type
B. pertussis 18323	*vir* +	Wild type
B. pertussis 77/18319	*vir* +	Wild type
B. pertussis BP347*	*vir* -1:: Tn5 Km^R	vir-
B. pertussis BP349*	*hly* -2:: Tn5 Km^R	$AC^{\pm}$, HLY^-
B. pertussis BP348*	*hly* -1:: Tn5 Km^R	AC^-, HLY^-
B. pertussis BP353*	*fha* -1:: Tn5 Km^R	FHA^-, AC^+
B. pertussis BP357*	*ptx* -2:: Tn5 Km^R	PT^-, AC^+
B. parapertussis 5952	Wild type	AC^+
B. avium 4148	Wild type	$A C^-$
B. bronchiseptica 214	Wild type	AC^+

* = Tn5 insertion mutants (Weiss *et al.*, 1983).

• = Recombinant strain (BP348 [Tn5 mutant, HLY^- AC^-]harbouring plasmid pRMB1 Brownlie *et al.*, (1988).

Table 4 ***E.coli*** **Strains Used in This Study**

Strain ***E. coli***	**Plasmid**	**Genotype & antibiotic marker**
H1469		*lon*-::Tn10-Tc^R
H1469 (pRMB3)	pRMB3	*cya A,B,D*, Amp^R,Tc^R
H1469 (pRMB9)	pRMB9	*cyaA*, Amp^R,Tc^R
H1469 (pRMB6)	pRMB6	*cya A,B,D*, Amp^R,Tc^R
H1469 (pRMB6,pANN202)	pRMB6, pANN202	*cya A,B,D hly C* (*E. coli* Amp^R,Tc^R,Cm^R.
H1469 (pANN202)	pANN202	*hly C* (*E. coli*) Tc^R,Cm^R.
H1469 (pRMB6,pKIM1)	pRMB6, pKIM1	*cyaA,B,D.hlyC* (*P.vulgaris*) Amp^R,Tc^R,Cm^R
H1469 (pKIM1)	pKIM1	*hly C* (*P.vulgaris*) Amp^R,Tc^R
H1469 (pRMB6,pANGE3)	pRMB6, pANGE 3	*cya A,B,D, C* Amp^R,Tc^R,Cm^R
H1469 (pANGE3)	pANGE3	*cyaC*
E. coli 582	pSF4000	Cm^R
E. coli G802		Δ *cya*

pRMB3, pRMB9, pRMB6 (Brownlie *et al.*,1988), pANN202, pKIM1 obtained from Kim Hardie (University of Cambridge), pANGE3 (G.Westrop,Department of Microbiology,University of Glasgow).

Table 5

Plasmids of Recombinant *E.coli* Strains Used in This Study

Plasmid	Vector	Promoter
pRMB3	pIC20H	*l a c*
pRMB9	pIC20H	*lac*
pRMB6	pGLW11	*tac*
pANN202	pACYC184	*hlyC* (*E. coli*)
pKIM1	pACYC184	*hlyC* (*P. vulgaris*)
pANGE3	pACYC184	*tet* ^{r}

pRMB3, pRMB9, pRMB6. Brownlie *et al.* (1988)

pANN202, pKIM1. Obtained from Kim Hardie (University of Cambridge)

pANGE3 G.Westrop (Department of Microbiology, University of Glasgow)

CHROMATOGRAPHY TECHNIQUES.

Phenyl-Sepharose Chromatography.

The method was essentially that of Hewlett *et al.* (1989) . 4 M Urea extracts (2ml, 2 mg protein / ml) of *B. pertussis* BP348 (pRMB1) were diluted 1:1 with buffer A1 (10 mM Tricine pH 8, 0.5 mM ethylenediamine - tetraacetic acid, di sodium salt [EDTA, BDH], 0.5mM ethylene glycol bis N N-N'-N'tetraacetic acid [EGTA, Sigma]) to reduce the urea concentration to 2 M. This material was loaded onto 3 ml phenyl-sepharose CL4 B (Sigma) previously equilibrated with buffer A1. The column was washed with 3 bed volumes of buffer A1 containing 2 M urea. Bound protein was eluted with 8M urea in buffer A1, and the eluates were collected in 0.5ml volumes . Protein concentration was determined, and fractions were stored at -20^{o}C .

Calmodulin - Agarose Chromatography (Affinity chromatography)

Purification of AC from urea extracts of *B. pertussis* BP348 (pRMB1) was carried out as described by Bellalou *et al.* (1990 a). 4M urea extracts (2 ml) were diluted 1 in 4 in buffer A2 (50 mM Tris-HCl pH 8, 0.22 mM $CaCl_2$ and 0.1% Nonidet-P40, [Sigma]) and left to adsorb onto 3 ml Calmodulin-agarose (Sigma) in polystyrene tubes on a rotating mixer with a speed of 15 rpm for 12 h at 4 ^{o}C. The gel was loaded into a column and washed with 50 ml of buffer A2 containing 0.5 M NaCl. Bound proteins were eluted with 8 M urea in Buffer A2. The eluates were collected sequentially in 0.5 ml aliquots by gravity flow. The purified AC was not dialysed as described in the original method but was stored frozen immediately at -20 ^{o}C.

PROTEIN ESTIMATION.

Protein estimation was carried out by the method of Bradford (1976), using bovine serum albumin (Sigma) as standard. The protein was measured at A_{595} using a Shimadzu recording spectrophotometer.

SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE was performed acording to the method of Laemmli (1970) . 7.5% resolving gels were used throught this study unless otherwise stated. Stacking gels contained 4.5% acrylamide. Samples were solubilised in an equal vol of sample buffer (App.II) and boiled in a water bath at 100 ^{o}C for 3 min . The same procedure was applied to the molecular weight standards (SDS-6H), obtained from Sigma. Loading volume of samples was adjusted according to the capacity of the respective gel i.e.,50 µl (1mg protein/ml) for 1.5 mm thick gels and 25-30 µl (1mg protein/ml) for 0.8 mm thick gels . Samples were electrophoresed into the stacking gel at 20 mA and at 25 mA in the separating gel for 0.8 mm thick gels. For 1.5 mm thick gel, the current for the stacking gel was increased to 30 mA and for the separating gel to 45 mA . Gels were run until the tracking dye reached the bottom of the gel, after which gels were stained either by Coomassie blue or by the silver staining method adapted from the procedure of Oakley *et al* .(1980).

IMMUNOBLOTTING / WESTERN BLOTTING

Immunoblotting was performed according to the method of Towbin *et al.* (1984) in a Bio-rad 'Transblot ' transfer apparatus. A sandwich of nitrocellulose membrane (Schleicher and Schuell) or Hybond-C (Amersham) and gel was prepared between two sheets of Whatman paper (3mm) , with one scotch bright pad on each side. This sandwich was loaded into a sandwich assembly and placed in a tank of transfer buffer (App.III) with the nitrocellulose facing the anode. Protein was transferred at 30V across the sandwich for 18 h followed by 50V for 1 h in order to transfer the higher molecular weight proteins . Then , the nitrocellulose membrane was stained with 0.5% Ponceau S in 0.1% acetic acid for 5 min and then destained with distilled water with three to four changes. Transferred protein bands were visualized and bands of MW standard were marked. A complete destaining was then performed with multiple rinses with PBS (pH7.3). Non-specific binding sites were blocked with Blotto/ Tween (3% skimmed milk + 0.2% Tween 20 in PBS,DulbeccoA, pH 7.3, Oxoid). by incubation at room temp for 1h on a shaker. Blots were probed with the appropriate antibody, diluted in blotto/ Tween, at room temperature for 1h under shaking condition. After probing, nitrocellulose membrane was washed two times for 15 min each, with PBS (DulbeccoA,pH 7.3,Oxoid) and then incubated with anti-immunoglobulin serum conjugated to horseradish peroxidase (Scottish Antibody Production Unit, SAPU),

diluted in Blotto/ Tween . Subsequent washing was carried out two times for 15 min each in PBS (Dulbecco A, pH 7.3, Oxoid).Colour development was performed with diaminobenzidine (DAB,Aldrich) substrate solution (App.III).The reaction was stopped after approximately 3 min with distilled water and the blots were stored in the dark .

ISO-ELECTRIC FOCUSING.

A method from the instruction manual 1-2117-E01 (LKB -Pharmacia) was applied for fractionation of urea extracts and purified AC in isoelectric focusing gels. Stock solutions of acrylamide (29.1%),N,N-bis-methylene acrylamide (bis)0.9% and ammonium persulphate (1%) were prepared in deionized water and filtered through Whatman paper 1, stored at 4 ^{o}C in dark and used within two weeks of preparation. Ampholines of pH range 3.5-9.5 (product no 1804-101) were obtained from LKB Pharmacia .Electrodes solutions used were : cathode=1M NaOH, anode=1M H_3PO_4.
A total volume of 60 ml was prepared by mixing 10 ml of bis acrylamide, 7 ml of 87% glycerol, and 3.5 ml of ampholines. The volume was adjusted to 58.5 ml with deionized water, 1.5 ml of ammonium persulphate was added and the contents were mixed by gentle swirling and immediately poured into the casting mould. Gels containing 6M urea were also used , in which case , 20 ml of 6 M urea was prepared first and rest of solutions were added step by step to this solution . After polymerisation (20-30 min), the gel was removed from the thick glass plate and excess water from the surroundings was removed with filter paper by capillary action . The gel was then placed on the cooling plate of LKB model 2117 Multiphor system . Electrode wicks (LKB 1850-911) were dipped in electrode buffers, and laid along the cathode and anode sides of the plate. Samples (30 μl) were applied to the sample applicators provided in the kit (LKB1850-901). Three different positions for the application of each sample were selected i.e., near the cathode , in the middle of the gel, and near the anode. The p*I* standard marker (Pharmacia) was also applied near the cathode at position 1 .Gels were run at 8 ^{o}C , initially at 25 V /cm at a current of 30 mA. The voltage was increased in steps after every 10 min to 100 V/cm after 70 min and sample applicators were removed. The run was terminated after 2.5 h or until the tracking dye of standard reached the anode .Gels were placed in fixing solution (App.III) for 30 min and in staining solution (App.III) for 10 min preheated at 60^{o}C.Destaining was carried out with several changes of destaining solution (App.III) .

Table 6 **Antibodies / antisera used in this study**

Antibodies/antisera	Description
R164	Polyclonal anti-AC antibody, raised in mice against purified 50 and 200 kDa AC obtained from E.Hanski,Israel.Reacts with 50 kDa,95 kDa and 210 and 200 kDa AC on immunoblots at 1:500 dilution.
McU	Monoclonal anti-AC antibody, obtained from A.Ullmann, Pasteur Institute, Paris. Reacts with 210, 200 kDa AC on imunoblots at 1:4000 dilution
PcU	Polyclonal anti-AC antibody, raised in rabbit against 45 kDa AC, obtained from A. Ullmann, Pasteur Institute, Paris. Reacts with 200 and 210 kDa and other forms of AC on immunoblots at 1:1000 dilution.
Mc9D4	Monoclonal anti-AC antibody, ascites $IgG2_a$, obtained from E.Hewlett, University of Virginia, USA., reacts with 200 and 210 kDa AC on immunoblots at 1:1000 - 1:1500 dilution
Mc1H6	Monoclonal anti-AC antibody, ascitesIgG_1, obtained from E. Hewlett, University of Virginia, USA.Reacts with 200 and 210 kDa AC on immunoblots at 1:1000 dilution.
Ms1	Monospecific anti-AC serum, raised in rabbits against CaM - affinity purified AC (this study). Reacts with 200 and 210 kDa AC on imunoblots at 1:1000 dilution.
McFHA5	Monoclonal anti-FHA antibody, obtained from Dr.A.E Ashworth, PHLS,CAMR,UK.Reacts on immunoblots at 1:1000 dilution.
HS	Convalescent sera of culture-confirmed pertussis patients. Provided by Prof. A.C Wardlaw, Dept. of Micribiology, University of Glasgow.

PREPARATION OF RTX TOXINS.

The range of RTX toxins used in the investigation included the leukotoxin of *P. haemolytica* strain S/C 82/ 1, the leukotoxin of *A. actinomycetemcomitans* strains JP 2 and 29523 , the α-haemolysin of *E. coli* strain 582, and the ACT of *B. pertussis* . Leukotoxin of *P. haemolytica* S/C 82/1 was kindly provided by Mr Qurban Ali, Department of Microbiology, University of Glasgow, as a lyophilized culture supernate, which was reconstituted in sterile distilled water to the final protein concentration of 2 mg / ml. Exotoxin of *A. actinomycetemcomitans* was prepared from whole cells grown on blood agar for 24 h in the presence of 5% CO_2 . The growth was directly solubilized in sample buffer (App.II) .*E. coli* alpha -haemolysin was prepared by growing the cells in BHI broth with Cm (20 μg/ml) for 24 h at 37 ° C on an orbital shaker at 80 rpm. The culture supernate was filtered through a 0.45 μm filter (Acrodisc, Gelman) and concentrated by Aquacide (Sodium salt of carboxymethyl-cellulose, Calbiochem.). 10 ml culture supernate was concentrated down to 1 ml (protein = 0.4 mg/ml). All these toxin preparations were solubilized (except *A. actinomycetemcomitans* which was already prepared in sample buffer) in sample buffer and boiled for 3 min prior to SDS-PAGE.

PRODUCTION OF ANTIBODIES (Ms1) AGAINST NITROCELLULOSE -BOUND ACT.

The method was adapted and modified from the procedure of Diano *et al.* (1987). CaM-affinity purified AC obtained from the urea extracts of *B. pertussis* BP348 pRMB1(AC^{++}) was resolved by SDS-PAGE using a 7.5% acrylamide gel and transferred to nitrocellulose membrane (Schlercher &Schuell) overnight at 30V and 15 ^{o}C. The transferred protein was stained with Ponceau S (0.5% Ponceau S in 0.1% acetic acid) for five min. The membrane was rinsed in sterile deionized distilled water for 3-4 times after which, the bands at 210-200 kDa were carefully cut out . Precautions were taken to handle the material carefully with gloved hands and alcohol - sterilized scalpel and forceps to avoid contamination. Bands were destained in sterile PBS (Dulbecco A , pH 7.3, Oxoid) and stored frozen at - 20 ^{o}C.

When material from 20 bands was collected, (representing 50 - 60 μg protein) the frozen bands were further chopped into 2-3 mm^3 pieces.The material was suspended in

sterile PBS (500 µl / band) and sonicated at maximal output, with 20 -seconds cooling intervals in an ice bath until the nitrocellulose was reduced to a fine suspension, and the particle size was small enough to pass through an 18-gauge hypodermic needle.

The suspension of AC-bearing nitrocellulose in PBS was emulsified in Freund's incomplete adjuvant (Sigma) in 1:1 ratio. A female New Zealand Albino rabbit was injected with 1.5 ml subcutaneously at two sites. Before injection, the rabbit was bled for pre-immune serum. The first injections were followed by two boosters at 15 day intervals.The rabbit was bled on day 40 and serum was collected.

CONCENTRATION OF MS1 BY SALTING-OUT TECHNIQUE.

Ice-cold (10 ml) anti-AC antiserum raised against nitrocellulose bound AC, was stirred in a beaker. While stirring , 7 ml saturated ammonium sulphate was added. The mixture was stirrred for 1 h at room temperature and then centrifuged at 6000 g for 10 min at 4 ^{o}C. The pellet was collected and resuspended in saturated ammonium sulphate as before, The suspension was stirred for another 30 min and then centrifuged at 6000 g for 10 min at 4 ^{o}C. The pellet was resuspended in 5 ml of saline (0.15 M NaCl), and dialyzed against 2000 volumes of saline overnight at 4 °C to remove the salt. The concentrated immunoglobulin was stored at -20 ^{o}C for future use.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

The specificity and titre of the antiserum raised against nitrocellulose bound AC was determined by ELISA. CaM-affinity purified AC was diluted to give a protein conc of 4 µg/ml in 50 mM carbonate /coating buffer (App. III). 100 µl of this diluted antigen was dispensed in each well of the microplate (Nunc ImmunoplateF). The plate was incubated at 4 ^{o}C after which the wells were emptied and washed three times by carefully flooding with washing buffer (App. III) . Washing buffer containing 1% bovine serum albumin was dispensed into the wells as blocking agent . The plate was incubated for 1h at 37 °C. Wells were again washed three times with washing buffer. Antiserum was diluted 10-fold up to 1in 20,000 in washing buffer and 100 µl/well was added . The plate was incubated for 1h at 37 °C , then was washed three times with washing buffer. HRP-anti rabbit IgG conjugate (1in 5000 in washing buffer) was added (100 µl/ well), incubated at 37 °C for 1h and washed 3 times with washing buffer. Finally, 100 µl /well chromogenic substrate O- phenylene diamine (OPD, Sigma , App.III) was added to the wells and the plate was incubated at room temp for 30 min

in dark for colour development . The reaction was stopped by the addition of 50 µl 12.5 % H_2SO_4. Absorbance (A_{620}) in each well was measured on an ELISA reader (Anthos reader, 2001). Net absorbance was determined by substracting the A_{620} of controls from the values obtained from antiserum- containing wells.

INTRANASAL PASSIVE PROTECTION TESTS IN MURINE MODEL.

Test 1. *B.pertussis* 18323 was grown on Bordet-Gengou agar supplemented with 15% defibrinated sheep blood, at 37 °C for 48 h . The growth was suspended in 1% casamino acids (CAA, in PBS Dulbecco A, pH 7.3, Oxoid) was made up to 10 opacity units (ou) (2 x 10^9 cfu / ml) by comparison with the 5th International Reference of Opacity (WHO, 1975) . The suspension was diluted 1 in 5 (~4 x 10^8 cfu/ ml=Dilution 1) and 1 in 10 (~ 2 x 10^8 cfu / ml = Dilution 2).

Monospecifc antiserum Ms1 (Table 6) , was raised against nitrocellulose bound, CaM-affinity purified AC, as described on page 53. The antiserum reacted with urea extracts at 1:1000 dilution on immunoblots.

High dilution and low dilution challenge suspensions (0.5 ml each) were mixed with 0.5 ml 1% casamino acids (in PBS Dulbecco A, pH7.3, Oxiod) . These served as positive controls of the high challenge dose = 2x 10^8 cfu / ml and low challenge dose = 1x10^8 cfu / ml respectively .

Ms1 (0.5 ml) , heated at 56 °C for 20 minutes to inactivate the complement activity was incubated with 0.5 ml each of the original suspensions of the challenge at room temperature for 1 h. .

Groups of 10, 3 - 4 week old, CD-1 strain mice were anaesthetised with ether, and infected by intranasal administration of 25 µl bacterial suspension in casamino acids with or without antiserum . One group of mice was given CAA only and one group of mice was left uninoculated as control. Mice were weighed every two days and any fatalities noted over a period of 3 weeks.

Bacterial counts from the inocula were performed by sampling 10-fold serial dilutions on BG. Three weeks post infection, mice were individually weighed and sacrificed and leukocyte count (by Coulter counter) and lung pathology were recorded. Lungs were removed and weighed, and pieces of lungs were spread aseptically over BG plates. Plates were incubated at 37 °C in a moist atmosphere for 72 h and examined.

Test 2 . The immunoglobulins in Ms1 antiserum were concentrated by salting out (see

page 54), and dialyzed against saline. The experiment as described in test 1was repeated with concentrated Ms1 and halothane as an anaesthetizing agent.

ASSAYS FOR ADENYLATE CYCLASE ENZYMIC ACTIVITY.

Salomon Assay

Adenylate cyclase activity was determined according to the method of Salomon *et al.* (1974). The reaction involved the conversion of [alpha ^{32}P] ATP to [alpha ^{32}P] cAMP, which was then separated from substrate [alpha ^{32}P] ATP by two sequential chromatography steps using Dowex 50 x4 (200-400 mesh) and Neutral alumina columns. The assay was performed in a final volume of 100 µl at 30 ^{o}C containing 25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 µM CaM (bovine brain calmodulin, Sigma), 1mM ATP, and 10 - 20 cpm / pmol [alpha 32P] ATP. The reagents were incubated for 10 min and the reaction was terminated by addition of 100 µl of 2% SDS (Sigma), and ATP (1.4 mM), after which 100 µl of cAMP recovery standard ([^3H] cAMP) was added. The samples were loaded onto the first column which bound cAMP (by a non-specific interaction with the resin) allowing other labelled compounds to be washed away . The cAMP was then washed with distilled water directly onto the second column (alumina) to which it absorbed together with any remaining ATP. Bound cAMP was recovered by washing the column with imidazole buffer (0.3 mM, pH 7.4) and quantified by liquid scintillation counting.

Amersham Assay (Binding Protein Assay)

This involved using a cyclic AMP assay kit (Amersham TRK428) as per manufacturer's instructions. The assay is based on the competition between unlabelled cAMP of the test sample and a fixed quantity of the tritium - labelled compound for binding to a protein. The detection limits for this assay are 0.2 - 16 pmol. The standard amounts of 1,2,4,8, and 16 pmol cAMP were used and a blank count was also performed. Since the assay has a narrow range for detection of cAMP, the urea extracts of *B. pertussis* and cloned *E .coli* had to be tested at a range of dilutions i.e., neat and 10 - fold diluted (10^{-1}, 10^{-2}, 10^{-4}) in a total volume of 40 µl . The samples were mixed with 120 µl of 0.002 M ATP in 0.02M $MgCl_2$, 80 µl 0.18 M Tricine pH 8, and an optional 250 units bovine brain calmodulin (Sigma). Controls with no ATP were also

included. After incubation for 15 min at 30 °C , the reaction was terminated by boiling for 20 min. Test samples at this stage could be stored at - 20 °C. Subsequent steps involved the measurment of cAMP in these test samples by use of the cAMP assay kit (Amersham TRK428) according to the protocol provided by the manufacturers.

Microplate Assay

A simple qualitative assay for cAMP was developed for the detection of enzymatic activity of adenylate cyclase . *E.coli* G802 (Δ *cya*) indicator strain (see Table 4) was streaked onto NA+ 0.025% IPTG+ 0.025% X-gal and incubated at 37 °C for 12 h. A single white colony was used to inoculate a 10 ml test suspension (BHIB+ 0.025% IPTG+ 0.025% X-gal). For qualitative analysis, 25 µl of reaction mixture (App.III) was dispensed in the wells of a microtitre plate followed by 25 µl of dialysed urea extracts./ culture supernate / CaM-affinity purified AC. The contents were mixed and the plate was incubated at 37 °C for 1h , then 150 µl of test culture (*E.coli* G802, grown at 37 °C until OD_{650} 0.4 in test suspension) was added. The contents were mixed and the plate was incubated at 37°C for 12h . Adenylate cyclase transforms ATP into cAMP which permeates into the indicator strain where it stimulates the production of ß-galactosidase from the *lac* operon. This enzyme utilizes the X-gal, a substrate analogue, and hydrolysis produces a blue colour.

For the semi-quantitative analysis of the AC enzymic activity in the cyclase preparations, the assay was further developed.A standard curve of exogenously added cAMP (Sigma) in known concentrations (micromol) was obtained against OD_{620} using an ELISA reader (Anthos reader, 2001). cAMP was diluted in a range of 64 micromol to 0.5 micro mol / 50 µl . The dilutions were added to the wells in triplicate. Samples of unknown were prepared . A 50 µl sample was incubated with equal volume of reaction mixture at 37°C for 15 min , after which the sample was boiled at 100 °C for 15 min to destroy any residual AC activity. A 2-fold dilution of the samples was made in distilled water. Test culture (150 µl) was added to the wells containing cAMP and unknown, plus to two extra rows as a control . The volume was adjusted with reaction mixture. The contents were mixed and the plate was incubated for 12 h at 37°C. OD_{620} was measured on ELISA reader (Anthos reader, 2001) after 12 h incubation .The standard curve of OD_{620} was plotted against the concentration of cAMP added.

ASSAYS FOR ADENYLATE CYCLASE TOXIC ACTIVITY.

Neutrophil Preparation

Glycogen-induced rabbit peritoneal neutrophils were obtained from female New Zealand white rabbits according to the method of Lackie (1977). Normal sterile saline (500 ml) containing (0.1%) oyster glycogen (Sigma) was injected intraperitoneally via a 18 gauge needle. The peritoneal exudate was collected after 4 h stored at 4 ^{o}C and used within 2 days of isolation . PMNs were recovered from the fluid by centrifugation at 500 g for 10 min. Contaminating erythrocytes were removed from the resulting neutrophil pellet by hypotonic lysis (5 ml distilled water for 5 sec) and cells washed once in divalent -cation -free HEPES buffer (App. III) .Washed cells were resuspended in HEPES buffer and were used within 1 day of preparation. A monodisperse population of cells was obtained by passing the cells through a 10 μ m Nitex filter (Plastok Associates, Birkenhead) . This technique produced a PMN suspension of > 95% purity and viability as determined by the Trypan blue dye exclusion test . Cell numbers were standardized after counting in an improved Neubauer counting chamber (Weber Scientific International Ltd.England.).

Nitroblue Tetrazolium Reduction (NBTR) Assay.

An assay based on the method reported for pertussis toxin by Craig and Parton (1988) and Craig *et al.* (1990) was used for the detection of toxic activity of AC using nitroblue tetrazolium reduction (NBTR) activity of rabbit peritoneal neutrophils as a probe. Rabbit neutrophils from peritoneal exudate were purified as described above. Rabbit neutrophils (50 μl of 10^6 cells/ml) were dispensed into the wells of a microtitre plate. Test sample (50 μl of dialysed urea extracts) and its 2-fold dilutions was added to the neutrophils , the contents were mixed and the plate was incubated at 37^{o}C for 1h in a humidified box.After incubation, 50 μl of NBT (2.3 mM in HEPES) and 20 μl of a soluble stimulant, phorbol myristate acetate (PMA, 0.1μg/ml in HEPES) was added to the wells. The contents were mixed and the plate was incubated at 37 ^{o}C in a humidified box. The results were recorded between 30 min to 1h after incubation. The toxin activity of ACT was judged by failure of neutrophils to reduce nitroblue tetrazolium to blue formazan. Unaffected neutrophils showed a purplish blue colour , an indication of actively metabolizing neutrophils.

Chemiluminesence Assay (CL Assay)

The CL assay was performed using an automated luminometer (Wallac LKB 1251 luminometer) connected to an Acorn BBC 'B microcomputer . Chemiluminesence emission was measured in millivolts (mV) at 37 ^{o}C. The number of neutrophils per assay tube was 10^{6} and the final volume of sample per tube was 1000 µl. Dimethylamino-naphthalene-1,2-dicarbonic acid hydrazide (DNDH,Boehringer Mannheim GmbH , W.Germany) was used instead of luminol because of its 2 -3 times larger quantum yield than luminol in CL emitting oxidation - reduction reactions (Allen, 1982). DNDH was first prepared as a 10^{-2} M stock solution in dimethyl sulphoxide, diluted 100-fold in HEPES buffer (App II), then stored at -20 ^{o}C until used. After adding neutrophils plus buffer , DNDH was added to a final concentration of 10^{-5} M. The stimulus for CL was either the soluble chemotactic factor,PMA (Sigma) or as otherwise stated. PMA was first prepared as a 1 mg/ml stock solution in dimethyl sulphoxide and diluted to the working dilution of 1 µg/ml. The final concentration of PMA per assay tube was 0.1µg/ml. Neutrophils were always pre-warmed at 37 ^{o}C for 5 min before use. Samples were usually assayed in duplicate and the CL results shown are the mean values. The CL curves shown in the results are derived from a number of replicate observations. The peak values and the total CL is also presented in tabulated form for the ease of comparison of the data. The percentage inhibition was calculated according to the method of Al-Tuwaijri *et al.* (1990).

Opsonisation of *B. pertussis* Cells for CL Assay

The method followed an adaptation of the procedures of Chang *et al.* (1986) and Friedman *et al.* (1987 b). *B. pertussis* strains : BP348 (pRMB1) (AC^{++}) and BP348 (AC^{-}) were grown on BG medium for 72 h at 37 ^{o}C in a humidified box. Bacterial growth was collected and washed with HEPES buffer (App.II) at 6,000 g for 6 min at 4 ^{o}C. The pellet was resuspended in HEPES buffer and the opacity was adjusted to 10 ou. by comparison with the 5^{th} International Reference Preparation of Opacity, WHO (1975). This suspension (= ~$2x10^{9}$ cells / ml) was diluted 200 times in HEPES buffer to 10^{7} cells / ml. To one volume of bacterial suspension, 0.5 volume of fresh normal rabbit serum (heated at 56°C for 30 min for inactivation of complement), and 0.5 volume HEPES buffer was added. The suspension was tumbled end to end in polystyrene tubes for 30 min at 37^{o}C. After incubation with serum, the suspension was centrifuged at 2000 g for 3min and then washed 2 times with ice cold HEPES buffer .The pellet was

resuspended to the original volume of the bacterial suspension . 500 µl of this suspension was added per assay tube. The final density of rabbit neutrophils was adjusted in HEPES buffer to $5x10^5$ / ml and 250 µl of this cell suspension was added per assay tube. After adding 200 µl DNDH and 100 µl HEPES, the ratio of bacterial cells to PMNs was ~ 40:1.

Effect of Calcium on AC Toxic Activity in CL.

Rabbit peritoneal neutrophils were obtained as described above. The neutrophils were washed in Ca^{++} - and Mg^{++}- free HEPES containing 0.8 mM EDTA and 0.8 mM EGTA and resuspended in the same buffer. A stock solution of $CaCl_2$ (50 mM) was prepared in distilled water .The calcium requirements of rabbit neutrophils for CL response were measured on 10^6 cells / assay tube by adding the calcium from the stock to the vials giving the final concentrations of 0.1mM, 0.3 mM , 1mM , 3 mM , and 10 mM. The effect of ACT (dialyzed urea extracts) for each calcium concentration was investigated.The neutrophils were incubated with 20 µg protein / assay tube in the presence of different concentration of calcium as described above. After 15 min incubation at 37 ^oC, the stimulus (PMA) was added.The sum of CL measured in mV and the % inhibition of CL compared with the controls was calculated for each concentration of calcium.

Haemolytic Assay for AC.

Haemolytic activity of CaM-affinity purified AC from the urea extracts of *B. pertussis* BP348 (pRMB1) was determined by the method of Bellalou *et al.* (1990 b) except that 2% RBCs were used instead of 1%. RBCs from rabbit and sheep were compared for their sensitivity and were prepared as follows:
The blood was centrifuged at 5000 rpm at room temp for 3 min . The pellet was washed three times in haemolysis buffer (buffer H, 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1mM $CaCl_2$) and resuspended in buffer H to give final concentration of 2%. Neat and 2 fold dilutions of CaM-affinity purified ACT (representing 100 nmol cAMP / min / ml enzymic activity in a total volume of 50 µl of H buffer and its 2-fold dilutions) were used. Dilutions were prepared in duplicate in a round bottom microplate. Equal volumes of 2% sheep or rabbit RBCs were added to the wells. The plate was incubated at 37 ^oC for 12-15 h in a humidified box.The haemolytic activity after incubation was

measured by transferring with great care, 30 µl of the supernates into another empty microtitre plate. This plate was read on ELISA reader (Anthos 2001) at A_{540}. The assay was performed in duplicate and the mean was calculated.

In order to observe any blocking effect of anti- AC antisera on the haemolytic activity, the same assay was performed in the presence of monospecific anti-AC antiserum (Ms1, Table 6). Neat and 2-fold dilutions of purified ACT (representing 60 nmol cAMP/ min/ml AC enzymic activity in total volume of 50 µl of buffer H before dilution) were prepared in a round - bottom microtitre plate. A duplicate set of the same samples was prepared initially with 60 nmol cAMP / min / ml enzymic activity and its 2-fold dilution in a total volume of 25 µl of haemolysis buffer and incubated with 25 µl of anti-AC monospecific antiserum Ms1 for 1h at room temp. Controls of buffer and Ms1+ buffer were also included in this assay. After incubation, equal volumes of 2% sheep RBCs were added to all the wells.The plate was incubated at 37 ^{o}C for 12-15 h in a humidified box.The haemolytic activity after incubation was measured by transferring with a great care , 30 µl of the supernates into another empty microtitre plate. The plate was read on an ELISA reader at A_{540}. The assay was performed in duplicate and the mean was calculated.

Chinese Hamster Ovary (CHO) Cell Assay for AC Toxic Activity.

The CHO cell assay was performed as described by Hewlett *et al.* (1983).Cultures of CHO cells showing confluent growth were trypsinized for 5 min and diluted in 10 % foetal calf serum (GIBCO) to a concentration of approximately 2 x 10^4 cells / ml. 250 µl of this cell suspension was dispensed into each of the wells of a sterile flat bottom microtitre plate and incubated at 37 °C in the presence of 5 % CO_2 . After 16 h of incubation , the viability and adherence was checked by microscopy. When the cells showed a monolayer growth, the filter sterilized, CaM- affinity purified AC (200 nmol cAMP/ min / ml enzymic activity and its 2-fold dilutions in 100 mM phosphate buffer containing 0.5 M NaCl) was added under sterile conditions under the laminar flow cabinet. The plate was incubated in the same conditions and the morphological changes were recorded after 16-24 h growth. Medium was aspirated carefully from the wells and cells were air dried and fixed in 4 % formalin in PBS (Dulbecco A, pH 7.3 Oxoid, 200 µl / well) for 15-20 min at room temp. After fixing, the formalin solution was poured off and cells were stained for 15 min with 50 µl / well of Coomassie blue staining solution (App. II). The wells were rinsed with distilled water and the plate was air dried.

results were recorded by observing AC - treated cells for elongation in comparison with control cells under the microscope.The assay was performed in duplicate and the controls of buffer without toxin were also included.

BHK Cell Assay

Baby hamster kidney (BHK) cells were trypsinized with trypsin -versene solution (TV= 0.5 ml of 2.5% trypsin in 20 ml of versene [1 mM EDTA in PBS pH 7.4]) and the cell sheet was rinsed 2 times with 5 ml HEPES buffer (App III). The cells were treated again with TV , swirled and TV was drained. After five min the cells were checked under microscope and found to be detached and round. 5 ml HEPES containing 10% calf serum (GIBCO) was added to the cells and with a pasteur pipette, cells were mixed by aspirating and expelling the cell suspension 20 times. Cells were centrifuged at 1000 rpm and the pellet was resuspended in HEPES. The cell density was estimated in a counting chamber. For assaying each sample, 5 ml of cell suspension containing 10^6 BHK cells was needed.

Preparation of cover slips Detergent -washed and dried cover slips ($1mm^2$) were immersed in 2 ml of coating solution (20 µg fibronectin /ml in HEPES) in a petri dish and left for 15 min and then coating solution was drained and replaced by 2 ml HEPES containing 0.5 mg/ml haemoglobin for another 15 min, after which this solution was drained and replaced with HEPES.

Assay The samples to be tested and positive control (0.1 mM dibutyryl cAMP) were prepared in a final volume of 5 ml of the cell suspension containing 10^6 cells(2x 2 ml for each duplicate sample). HEPES was removed from the cover slips and carefully the samples (2ml /cover slip) were added.The dishes were incubated at 37 °C for 45 min to 1 h and then the cells were fixed with fixing solution (0.4% formalin in HEPES) for 15 min. The fixing solution was removed and the cells were stained with Coomassie blue (App II) for 15 min, and then rinsed with distilled water 2-3 times. The cover slips were air-dried, and mounted with clear mount on slides . The result were scored on Leitz ortholux microscope connected to a BEEB vidio digitizer interphase and BBC microcomputer. Data collected for 50 cells included the area dispersion and % scattering measured as described by Edwards *et al.* (1988) .

RESULTS

IDENTIFICATION AND CHARACTERIZATION OF ADENYLATE CYCLASE (AC)

Extraction of Adenylate Cyclase by Urea.

Extracts of whole organism and concentrated culture supernates have been used as starting material by different groups for the isolation and purification of *B. pertussis* AC.In the present study, extraction of AC from intact cells of the *B. pertussis* and cloned *E. coli* strains listed in Table 3&4 was carried out. High enzymic AC activity from the AC structural gene (*cyaA*) has been expressed in *E. coli* from cloned DNA (Brownlie *et al.*, 1988). A 10 kb *Bam* H1 fragment containing the open reading frames for *cyaA* , *B*, and *D* was cloned into the *E. coli* plasmid vector pIC20H to give a plasmid, pRMB3. Another 2.6 kb *Bam* H1 fragment containing the 5'end of the *cyaA* open reading frame was also cloned into pIC20H to give pRMB9. Both were expressed under the control of *lac* promoter (Brownlie *et al.*, 1988). Each plasmid was then transfered into Lon $^-$ (protease negative) *E. coli* H1469 (Rogel *et al.*, 1989).The 10 kb *Bam* H1 fragment was subcloned in another vector, pGLW11, in two orientations to give plasmids pRMB6 and pRMB7 . The insert in the former depended on the *tac* promoter in the vector. Production of AC was determined in these constructs.

AC was extracted by a 'urea extraction technique'. The method applied was an adaptation and modification of the procedures of Brownlie *et al.* (1988) and Confer and Eaton (1982) as described in Materials and Methods (see page 44). The cells after harvest were suspended in distilled water containing 0.5 mM PMSF (phenyl methyl sulphonyl flouride, a protease inhibitor) rather than in urea solution as described in the original protocols, and stored overnight at -20 °C. The cell suspension was thawed and urea was added to a final concentration of 4 M , which gave optimal results for extraction of AC from both *B. pertussis* and cloned *E. coli* strains. Moreover, this concentration of urea was easier to remove from the extracts in subsequent dialysis steps.

It is noteworthy to point out here that an extreme viscosity was observed when *B. pertussis* cells were harvested and treated with 4M urea in their early log phase (22 - 24 h), presumably due to the large amount of nucleic acid present in actively growing cells. The cell debris proved difficult to sediment in subsequent centrifugation steps. Therefore, cells were harvested after growth for 48 h (OD_{650} = 2) when the culture was in early stationary phase. This time of harvest solved the problem of viscosity and

improved the yield in terms of total protein obtained per batch in which the AC ratio was 2- times higher than the AC obtained from the cells harvested at 24 h . The *E. coli* cells were harvested after 24 h. This agrees with the findings of Glaser *et al* . (1989) who reported the maximum AC activity in recombinant *E. coli* cell extracts when the cells reached early stationary phase. Overall, these alterations of the methods led to an optimized production of crude AC by the urea extraction technique.

Isolation and Identification of AC in Urea Extracts by SDS-PAGE.

In order to detect the presence of AC in urea extracts of *B. pertussis* (wild type and recombinant) and *E. coli* (harbouring plasmids containing different fragments of the *B. pertussis cya* operon), the extracts were subjected to SDS-PAGE as described under Materials and Methods (see page 50). Coomassie blue staining of the gels showed a complex pattern of bands of various MWs ranging from 210-20 kDa (Fig7A)

The differences in gel profiles of *B. pertussis* BP348 (pRMB1) (lane 1) and *B. pertussis* BP348 (lane 2) were examined. Diffuse bands at 210, 110 and 95 kDa positions in urea extracts of the AC^+ strain of *B. pertussis* BP348 (pRMB1) were more prominent than in *B. pertussis* BP348 (AC^-). Bands visualized in the profile of *B. pertussis* BP348 (pRMB1) at 37 kDa and a few other low MW bands were missing from the gel profile of *B. pertussis* BP348.

The high MW bands were exclusively absent from the gel profile of a urea extract of a *vir* $^-$ transposon mutant *B. pertussis* BP347 (lane 3) which fits with the inability of this strain to produce *vir* regulated proteins, including AC . Urea extracts of another transposon insertion mutant *B. pertussis* BP349 Hly $^-$ AC $^+$ showed a gel profile (lane 4) not very much different from that of *B. pertussis* BP348 (pRMB1) AC^+ (lane 1) except that the 210 kDa band was less prominent in the gel profile of *B. pertussis* BP349.The profile of urea extract of the wild type *B. pertussis* Tohama (lane 5) showed a comparatively faint band at 210 kDa. Other bands in the range of 180 - 95 kDa, and a prominant band at 69 kDa, were observed.

Urea extracts of other Bordetellae i.e., *B. bronchiseptica* , *B. parapertussis* and *B. avium* were also investigated for the presence and absence of bands which could be related to AC.Lane 6, showing the gel profile of *B. bronchiseptica,* revealed a band at 220 kDa which did not corresponded to the bands in the gel profiles of urea extracts of *B. pertussis* strains (lanes 1- 5). The bands common to the gel profiles of urea extracts of the *B. pertussis* were 210 and 50 kDa. Another band at 110 kDa was comparable to that in the gel profile of wild type *B. pertussis* Tohama (lane 5) . The profile of the urea extracts of *B. parapertussis* (lane 7) showed a comparatively

reduced number of bands. In lane 8, representing the profile for *B. avium* (AC^-), a band at 110 kDa comparable to the bands visualized in the gel profiles of *B. bronchiseptica* and *B. pertussis* Tohama was observed. The possibility that this band was AC in *B. bronchiseptica* and in *B. pertussis* Tohama was therefore unlikely.

Urea extracts of *E. coli* harbouring various plasmids i.e., *E. coli* H1469 (pANN202), *E. coli* H1469 (pANN202, pRMB3), *E. coli* H1469, *E. coli* H1469 (pRMB9),and *E.coli* H1469 (pRMB3), were investigated. The relevant characteristics of these strains are listed in Tables 4 & 5 (see pages 47 &48) and pANN202 is described fully on page 75. Fig.7 B shows that none of the AC^+ *E. coli* urea extracts showed high MW 210 or 200 kDa band in the profile of Coomassie blue- stained gels. Comparison of gel profiles of urea extracts of AC^+ *E. coli* strains with AC^- strains revealed a common band of 95 kDa in all the AC+ preparations (lane 3= *E. coli* H1469 (pANN202, pRMB3), lane 5 =*E. coli* H1469 (pRMB9) and lane 6 = *E. coli* H1469 (pRMB3).

In order to investigate whether the absence of high MW bands of AC from Coomassie blue - stained gel profiles of urea extracts of *E. coli* was due to less AC protein being expressed in these strains, a more sensitive method, silver staining (detection limit for protein = 2 ng) was carried out. Fig. 8 shows the gel profile after silver staining , revealing a single band of 210 kDa , along with several other bands in the lane representing the urea extracts of *E. coli* H1469 (pRMB3) AC^+ (lane 4).This single band was correspondingly absent from the *E. coli* H1469 AC^- (lane 5). The extract of the strains with two plasmids i.e., *E. coli* H1469 (pRMB3, pANN202) did not show any band at high MW position . However, the bands at 50, and 95 kDa were observed (lane 2) which were missing from the profile of the control preparation *E. coli* H1469 (pANN202) (lane 3).It was therefore assumed that the bands observed at high MW position in AC^+ *B. pertussis* and *E. coli* urea extracts represented adenylate cyclase. The overall view was that the stained profiles of SDS-PAGE - separated urea extracts , either of *B. pertussis* or *E. coli* , displayed some differences among the profiles of AC^+ and AC^- strains, but it seemed difficult to identify these bands as AC at this stage.

Fig. 7

SDS-PAGE Analysis of Crude Preparations (Urea Extracts) of Adenylate Cyclase (AC) from *B. pertussis* and *E. coli* .

Samples were separated by SDS - PAGE using 7.5 % acrylamide resolving gels. The gels were stained with Coomassie blue .Arrows indicate major band difference between profiles.

Panel A) Urea extracts of *B. pertussis* and other Bordetellae

1= *B. pertussis* BP 348 (pRMB1) (AC^{++})
2= *B. pertussis* BP348 (AC^{-})
3= *B. pertussis* BP 347 (AC^{-})
4= *B. pertussis* BP349 (AC^{+}, HLY^{-})
5= *B. pertussis* Tohama (AC^{+})
6=*B. bronchiseptica* 214
7=*B. parapertussis* 5952
8=*B. avium* 4148

Panel B) *E. coli* urea extracts harbouring *B. pertussis cya* gene and *hly C* gene from *E. coli.*

1 = SDS-6H (MW standard)
2 =*E. coli* H1469 (pANN202) (AC^{-})
3= *E. coli* H1469 (pANN202 , pRMB3) (AC^{+})
4= *E. coli* H1469 (AC^{-})
5= *E. coli* H1469 (pRMB9) (AC^{+})
6= *E. coli* H1469 (pRMB3) (AC^{+})

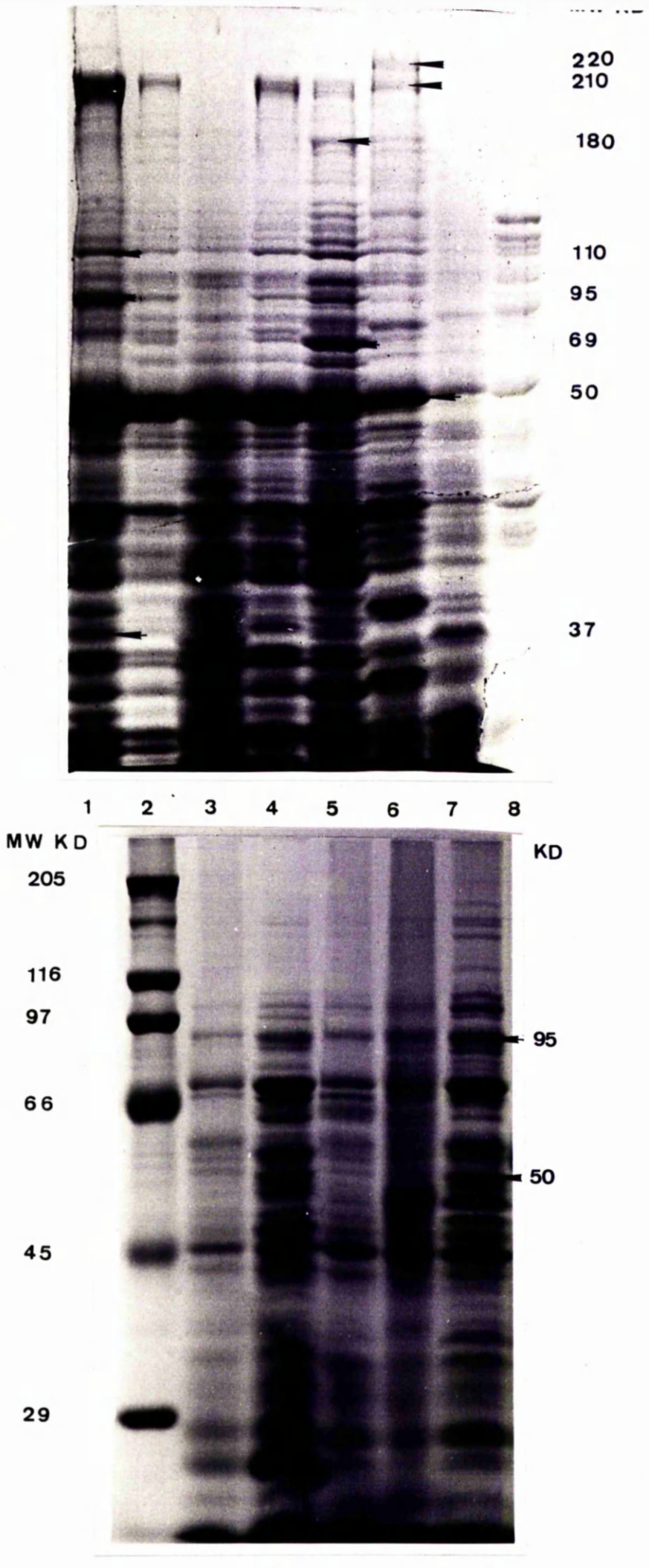
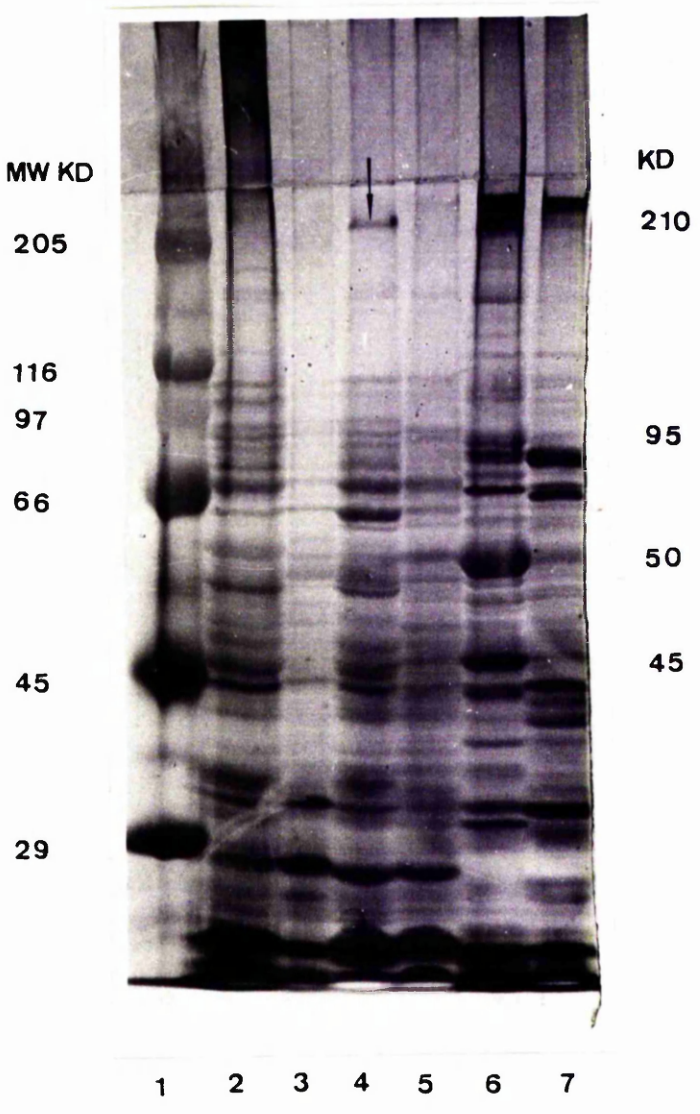

A
220
210
180
110
95
69
50
37
1 2 3 4 5 6 7 8
MW KD
205
116
97
66
45
29
KD
95
50
B

Fig. 8

SDS-PAGE Analysis of Crude Preparations (urea extracts) of AC from *B. pertussis* and *E. coli* by Silver Staining Technique.

Samples were separated by SDS - PAGE using 7.5 % acrylamide resolving gels. The gels were stained by silver staining. Arrows indicate major band difference between profiles.

1= SDS-6H (MW standard)
2= *E. coli* H1469 (pANN202, pRMB3) (AC^+)
3= *E. coli* H1469 (pANN202) (AC^-)
4= *E. coli* H1469 (pRMB3) (AC^+)
5= *E. coli* H1469 (AC^-)
6= *B. pertussis* BP348 (pRMB1) (AC^{++})
7= *B. pertussis* BP348 (AC^-)


MW KD
205
116
97
66
45
29
KD
210
95
50
45
1
2
3
4
5
6
7

Detection of AC by the Immunoblotting Technique.

A number of anti- AC antibodies, ranging from polyclonal to monoclonal were used to detect AC unequivocally using the immunoblotting technique.The antibodies applied in these studies comprised anti - AC polyclonal PcU raised in the rabbit against a 45 kDa fragment of AC, mono-specific antibodies R164 raised in mice against a purified 50 kDa fragment of AC, anti-AC monospecific antibodies Ms1 and monoclonals 9D4, 1H6 and McU. The relevant characteristics of these antibodies are listed in Table. 6. Urea extracts (20 μg protein/ lane) of *B. pertussis* and *E. coli* were resolved by SDS-PAGE and transferred to nitrocellulose as described under Materials and Methods (see page 50). Fig. 9A shows the immunoblot profile of urea extracts of different Bordetellae including recombinants and mutants, probed with anti-AC monoclonal 9D4. Generally, a profile with 2 major bands at MW 210 and 200 kDa was observed with *B. pertussis*, rather than a single band of 216 kDa (Hewlett *et al .*, 1989), or 200 kDa (Rogel *et al,* 1989) as previously reported. Lane 1, illustrating the immunoblot profile obtained from urea extracts of *B. parapertussis* showed a single band at 210 kDa . Lane 2, representing *B. avium* (AC^-) did not reveal any band in the whole profile, nor did a urea extract of *B. pertussis* BP348 (lane 3) a transposon insertion mutant, deficient in AC and HLY. This indicated the absence of AC from these strains and agreed with previous reports (Weiss *et al.*, 1983; Brownlie *et al.*, 1988) . Another mutant, *B. pertussis* BP349 (AC^+, HLY^-) showed a very faint band at 210 kDa (lane 4) suggesting the production of AC at a very low level, which agreed with finding of Weiss *et al* . (1983) who reported that *B. pertussis* BP349 produced a reduced level of AC.Comparison of the immunoblot profiles of wild type *B. pertussis* 77/18319,165, 18323, and Tohama (lanes 5, 8, 9, and 10 respectively) showed different intensities of the reaction, indicating variable amounts of AC in these strains. *B. pertussis* 165 and 18323 showed a similar level of reaction. A weakly reactive profile was observed in the lane representing *B. pertussis* Tohama, as compared to other wild type *B. pertussis* , showing a faint band at 210 kDa and less prominent band at 200 kDa . *B. pertussis* 77/18319 revealed a moderate reaction and both bands i.e., 210 and 200 kDa were observed. When other transposon insertion mutants were compared with *B. pertussis* (wild type and recombinant) for the presence of AC, the immunoblot profile of urea extracts of BP357 (PT^-) showed an equal level of intensity of reaction (lane 7) to that of the recombinant *B. pertussis* BP348 (pRMB1) (AC^{++},HLY^+) (lane11).This recombinant has been reported to produce 5 - fold higher AC toxin and 3- fold higher AC enzyme compared to the wild-type *B. pertussis* Tohama (Brownlie *et al .*, 1988).

On the other hand, another transposon mutant, *B. pertussis* BP353 (lane 6) , deficient in FHA displayed a low level of reaction,comparable to *B. pertussis* Tohama.

These data suggest that despite preparing all the urea extracts under the same set of conditions, the levels of production / expression of AC varied from strain to strain in *B. pertussis* and that AC produced by these strains, by order of magnitude, was as follows :

B. pertussis BP348 (pRMB1) = BP357 > 165=18323 >77/18319 >*B. parapertussis* >BP353 > Tohama .

Polyclonal antiserum raised against a 45 kDa fragment of AC (PcU, Table 6) was used to recognize AC in the urea extracts of *B. pertussis* and *E. coli* on immunoblots.

The antiserum recognized a complex pattern of bands on immunoblots (Fig.9B). When the profile of urea extracts of AC^+ strains : *E. coli* H1469 (pRMB3), *E. coli* H1469 (pRMB3, pANN202) and *B. pertussis* BP348 (pRMB1) (lanes 2, 4, and 6 respectively) were compared with those of AC^- strains , only high MW AC was differentiable and was distinguished in *E.coli* H1469 (pRMB3) as a single band of 210 kDa, and in *B. pertussis* BP348 (pRMB1) as two major bands at 210 and 200 kDa and a minor band of 195 kDa with a few other faint bands presumably due to some degradation of high MW AC. Other faint bands at low MW positions were also observed in the profiles of urea extracts of *E.coli* H1469 (pRMB3) and *E.coli* H1469 (pRMB3, pANN202) at 47 - 50 kDa. These bands were missing from the profile of AC- strains used as controls i.e., *E.coli* H1469 (lane 3) and *E.coli* H1469 (pANN202) (lane5). Common cross- reacting bands visualized in AC^+ and AC^- preparations may be due to the use of polyclonal antibodies which may contain antibodies against group- specific antigens.

Urea extracts of *E. coli* H1469 (pRMB 3), and *E. coli* H1469 (pRMB 9), were tested for the presence of AC by using monospecific antiserum R164, raised in mice against purified fragment of 50 kDa AC. Fig. 10 A shows that urea extracts of *E. coli* H1469 (pRMB3) (lane1) showed a single sharp band of 210 kDa which was not present in AC^- *E. coli* H1469 (lane 3). *E. coli* H1469 (pRMB 9) carrying a 2.6 kb fragment of 5' end of the open reading frame of *cyaA* (Brownlie *et al.*, 1988), revealed a 95 kDa band (lane 2) not present in lanes 1 and 3. The band at 50 kDa was also visualized in AC^- *E. coli* H1469 (lane 3) and could not therefore be associated with *B. pertussis* AC. In addition, other faint bands at lower MW positions were found in all the three profiles, and may be due to other non - specific reactions.

In order to confirm that the high MW 210 - 200 kDa band visualized by probing with monospecific antiserum were AC, anti - AC monoclonal McU (Table 6) was used to detect the AC on immunoblots. Fig.10 B shows that *B. pertussis*

BP348 (pRMB1) (lane 1) revealed the 2 band profile at 210 and 200 kDa (plus a couple of bands, possibly degradation products) which was consistent with the previously visualised bands observed after probing the extracts with monospecific and polyclonal antisera.Immunoblot profile of wild type *B. pertussis* strain 18323 showed two bands (lane 2).The immunoblot profile of *B. pertussis* BP348 (AC^-) revealed a band reacting at 67 kDa MW position (lane 3) which was not seen with monoclonal 9D4 (Fig. 9A). The profile of *E. coli* H1469 (pRMB3) revealed a single band of 210 kDa (lane 4) and no other band was observed. The urea extract of *E. coli* H1469 was used as a control and the immunoblot profile showed no band in the whole lane (lane 5), confirming that the bands observed in the profiles of AC^+ and missing from the profiles of AC^- *B. pertussis* and *E. coli* after probing with anti - AC polyclonals and monospecific antisera were in fact AC.

Fig. 9

Identification of *Bordetella* AC by the Immunoblotting Technique with Anti-AC Monoclonal and Polyclonal Antibody.

Urea extracts of *Bordetella* were separated by SDS-PAGE using 7.5% acrylamide. The resolved proteins were then electrophoretically transferred from gels to nitrocellulose. The nitrocellulose blots were probed with anti-AC monoclonal 9D4 (1:1000) or anti-45 kDa AC polyclonal PcU (1:500).

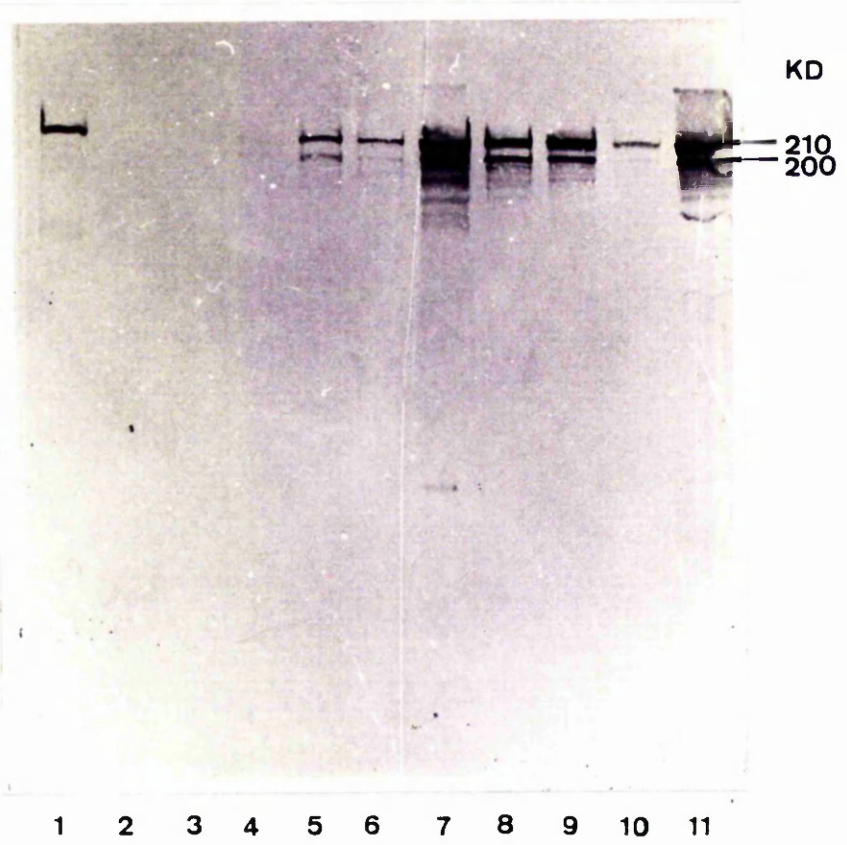
Panel A) Immunoblot of *Bordetella* AC, probed with monoclonal 9D4.

1= *B. parapertussis* (AC^+)
2= *B. avium* (AC^-)
3= *B. pertussis* BP348 (AC^-)
4= *B. pertussis* BP349 (AC^+, HLY^-)
5= *B. pertussis* 77/18319 (AC^+)
6= *B. pertussis* BP353 (AC^+, FHA^-)
7= *B. pertussis* BP357 (AC^+, PT^-)
8= *B. pertussis* 165 (AC^+)
9 = *B. pertussis* 18323 (AC^+)
10 = *B. pertussis* Tohama (AC^+)
11=*B. pertussis* BP348 (pRMB1) (AC^{++})

Panel B) Immunoblot of *B. pertussis* and *E. coli* probed with polyclonal PcU.

1= MW standard SDS-6H
2= *E. coli* H1469 (pRMB3) (AC^+)
3= *E. coli* H1469 (AC^-)
4= *E. coli* H1469 (pANN202, pRMB3) (AC^+)
5= *E. coli* H1469 (pANN202) (AC^-)
6= *B. pertussis* BP348 (pRMB1) (AC^{++})
7= *B. pertussis* BP348 (AC^-)

A



B

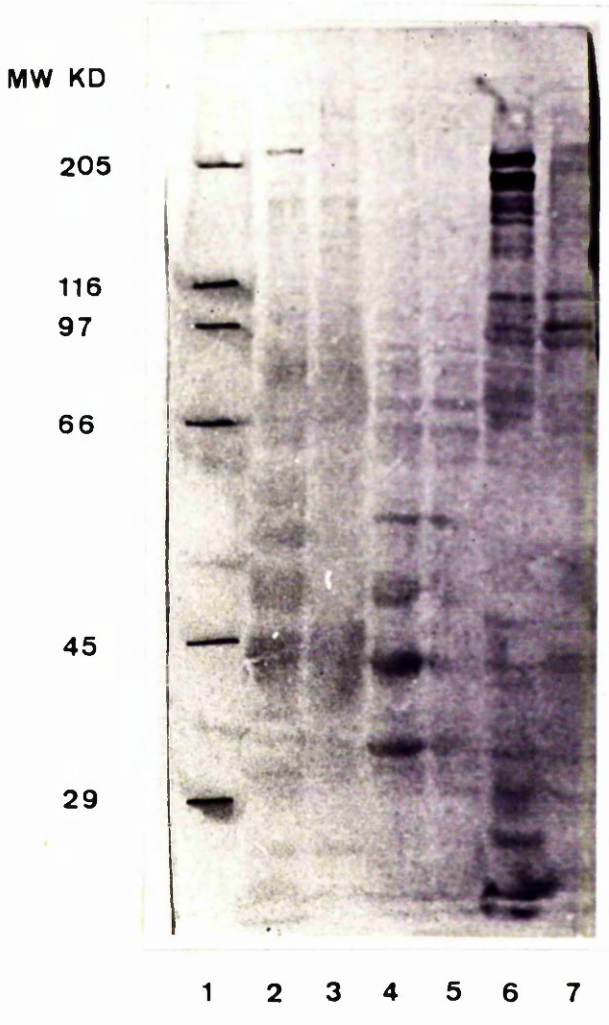


Fig.10
Identification of Cloned *B. pertussis* AC in *E. coli* by the Immunoblotting Technique.

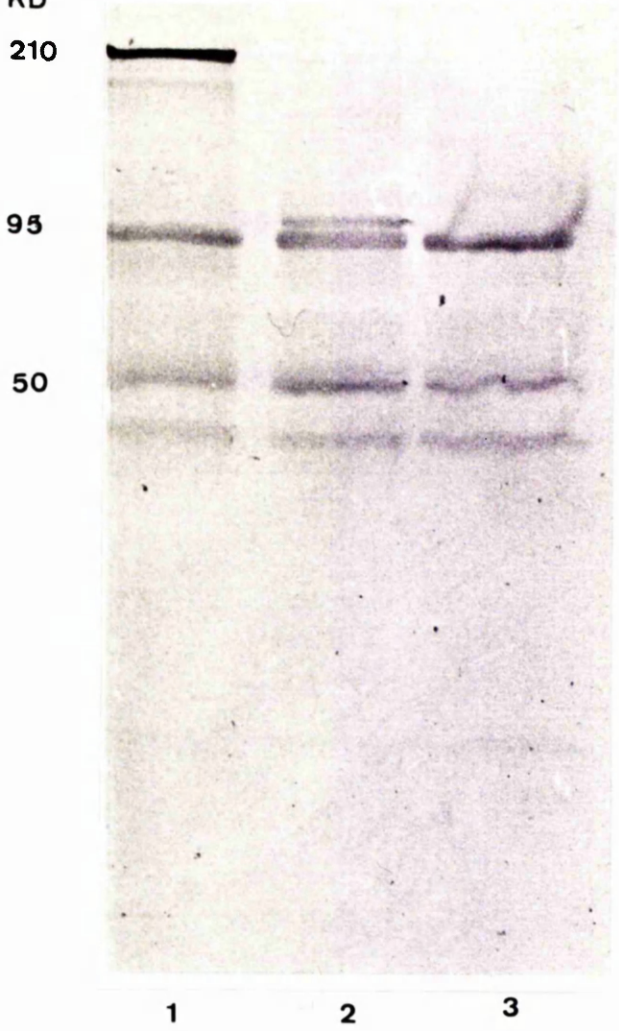
4M urea extracts of *E. coli* containing the cloned AC gene were subjected to SDS-PAGE in 12.5% (panel A) and 7.5% (panel B) acrylamide resolving gels and electrophoretically transferred to nitrocellulose. The blots were probed with mono-specific anti-AC antibodies R164 raised against 50 kDa AC., and monoclonal anti-AC antibodies McU. The dilutions of antibody were 1: 500, and 1: 4000 respectively.

Panel A) Immunoblot of *E. coli* urea extracts, probed with anti-AC monospecific antibodies R164

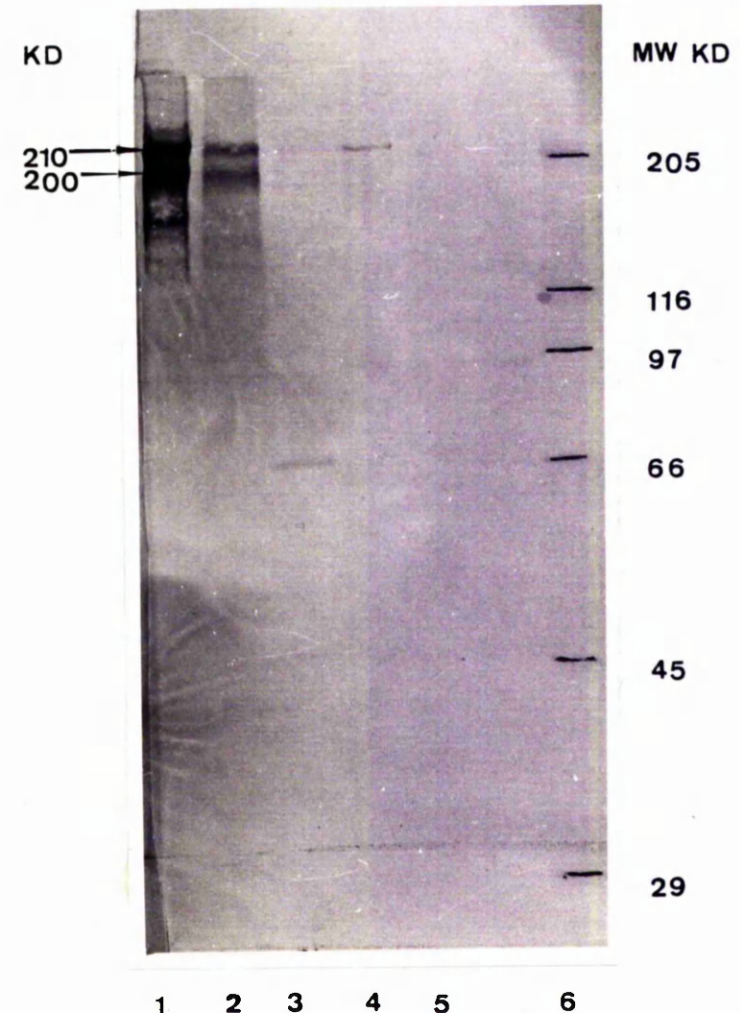
1= *E. coli* H1469 (pRMB3) (AC^{+})
2= *E. coli* H1469 (pRMB9) (AC^{+})
3= *E. coli* H1469 (AC^{-})

Panel B) Immunoblot of *E. coli* urea extracts, probed with anti-AC monoclonal antibodies McU

1= *B. pertussis* BP348 (pRMB1) (AC^{++})
2= *B. pertussis* 18323
3= *B. pertussis* BP348 (AC^{-})
4= *E. coli* H1469 (pRMB3) (AC^{+})
5= *E. coli* H1469 (AC^{-})
6=MW standard



A



B

Calmodulin - Affinity Probing of AC

Taking advantage of the affinity of AC for calmodulin (CaM), an alternative method was sought for the detection of AC on Western blots. The binding site for CaM is a part of the N-terminal portion of the toxin , adjacent to the catalytic site (Glaser *et al.*, 1988).

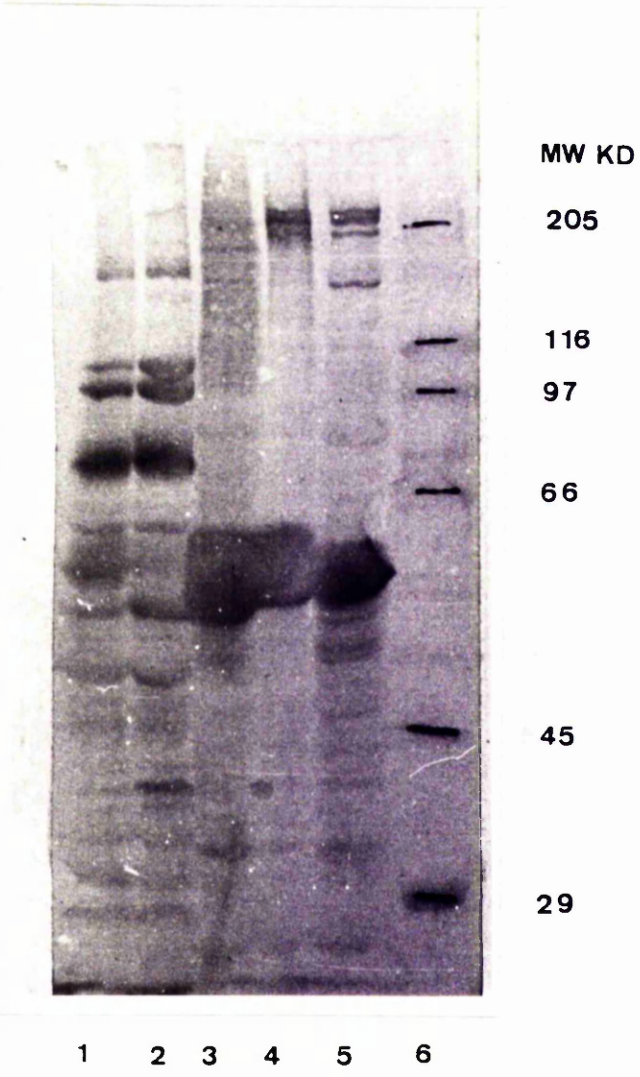
Urea extracts of *B. pertussis* BP348 (pRMB1), and *E. coli* H1469 (pRMB3) containing the high MW AC (as confirmed by its positive reaction with ant- AC monoclonal antibody), and the AC^- BP348 and *E. coli* H1469 (controls) were separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose. Tranferred proteins were probed with bovine brain calmodulin (50 units/lane) for 1h. Bound calmodulin was detected by probing the complex with goat anti- calmodulin, and finally with HRP-anti goat IgG. Substrate detection was carried out using DAB (diaminobenzidine substrate solution) as described in Materials and Methods under the procedure for immunoblotting (see page 50).

Fig.11 shows the profile obtained after using calmodulin as a probe. Two major bands at 210 and 200 kDa and a minor band at 195 kDa were observed in the profiles representing two different urea extracts of *B. pertussis* BP348 (pRMB1) (lanes 4 and 5) , and a single faint band at 210 kDa in the profile of *E. coli* H1469 (pRMB3) (lane 2). This was consistent with the previous profiles obtained by probing the *B. pertussis* BP348 (pRMB1) and *E. coli* H1469 (pRMB3) with monoclonal and polyclonal antibodies (see Fig.9 & 10). These bands were missing from the profile of *B. pertussis* BP 348 (lane 3) and *E. coli* H1469 (lane1), which were used as AC^- controls. Various other common bands between AC^+ and AC^- *B. pertussis* and *E. coli* were observed possibly due to the presence of calcium-binding proteins in the crude AC preparations. Since non - fat dried milk was used as a blocking agent, calmodulin may have coupled with these proteins via calcium present in milk and subsequently recognized by anti - calmodulin antibodies. However, the presence of high MW 210 and 200 kDa bands, similar to those observed by probing with monoclonal , polyclonal, and monospecific anti-AC antibodies in the urea extracts of AC^+ strains, and their parallel absence from the urea extracts of AC^- strains of both *B. pertussis* and *E. coli* confirmed that the bands observed in the profiles were AC.Therefore, this novel method could be proposed as an alternative, although less sensitive technique for the detection of AC where anti- AC antibodies for the detection of AC are not available.

Fig. 11
Detection of ACT in Urea Extracts by a Calmodulin - Affinity Probing Technique.

Urea extracts of *B. pertussis* and *E. coli* were resolved on SDS-PAGE with 7.5% acrylamide gel and transferred electrophoretically to nitrocellulose. The blots were probed with bovine brain calmodulin, followed by anti - calmodulin , and finally with anti- IgG conjugate.

1= *E. coli* H1469 (AC^-)
2 =*E. coli* H1469 (pRMB 3) (AC^+)
3= *B. pertussis* BP348 (AC^-)
4= *B. pertussis* BP348 (pRMB1) (AC^{++})
5= *B. pertussis* BP348 (pRMB1) (AC^{++})
6= SDS-6H (MW standard)


MW KD
205
116
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6

EXPRESSION OF *B. PERTUSSIS* AC IN *E. COLI*

Analysis of adenylate cyclase production from the *E.coli* strains, H1469 (pRMB3), and H1469 (pRMB9) showed a loss of AC production when repeatedly subcultured, as determined by immunoblotting (data not shown) although the antibiotic selection was maintained. This problem was not observed when freshly transformed cells were used. This finding suggested that, since pIC20H is a high copy number plasmid, the *lac* repressor protein (chromosomally encoded in *E. coli* strain H1469) might be limiting and expression of AC would therefore be constitutive. Overproduction of AC might have caused a metabolic burden or toxicity for the plasmid-bearing strain resulting in a slow and steady loss of the insert expressing AC by internal rearrangements of the plasmid. The *E. coli* strain harbouring double plasmids (*E. coli* H1469 pRMB3, pANN202) failed to produce full length AC. However, bands at 45, 47 , and 50 kDa were detected by anti- AC polyclonal antibodies PCU (see Fig. 10) and AC enzymic activity was detected when these samples were tested by the microplate assay (Table 8, see page 82).

In order to overcome these problems, a new construct H1469 (pRMB6) was prepared by Dr. Gareth Westrop . Here the same *Bam* H1 fragment was cloned into vector pGLW11 carrying a *lac* I gene, encoding the *lac* repressor (Brownlie *et al.*, 1988). Expression of AC in this strain is under the *tac* promoter and is inducible, requiring 1mM IPTG. Restoration of the plasmid stability was thus achieved by placing *cyaA* expression under the control of the inducible promoter. The new construct was characterized for the production of AC (Fig.12A). Expression of *cyaA* in the presence of IPTG showed a stablized production of AC (lane 5, panel A) when compared with the AC production in the absence of inducer (lane 4, Panel A) which did not reveal any band. However, the AC produced by the strain *E. coli* H1469 (pRMB6) was found non-toxic in the CL assay (toxicity assay) as shown in Table 17.

It has been recently reported that *cyaC* , which is located upstream of *cyaA,* is required for activation of the *cyaA* to its cell invasive form and the *cyaC* is homologous to *hlyC* of *E. coli* (Barry *et al.*, 1991). In order to activate the *cyaA* gene product in cloned *E. coli* , plasmids carrying either *hlyC* of *P. vulgaris* (pKIM1) or *hlyC* of *E. coli* (pANN202), or *cyaC* of *B. pertussis* (pANGE3) were cloned in a *E. coli* H1469 (pRMB6) background by transformation. These strains were donated by Dr. G. Westrop, Deparment of Microbiology, University of Glasgow.The relevant characteristics of these strains are outlined in Tables 4 & 5 (Materials and Methods, see pages 47 &48).Stabilized production of AC by these strain namely : *E. coli* H1469 (pRMB6, pKIM1) , *E. coli* H1469 (pRMB6, pANN202), and *E. coli* H1469

(pRMB6, pANGE 3) was observed . Fig.12 B shows the immunoblot profile of urea extracts of these strains probed with anti-AC monoclonal 9D4. The high MW AC (210 kDa) and a few minor bands were observed in the profile of all these strains (lanes 3-5) which was comparable to the positive control, *B. pertussis* 18323 (lane 2). *B. pertussis* BP348 (pRMB1) showed a family of bands ranging from 180- 45 kDa because the protein content was normalized in order to apply equal protein per well for *B. pertussis* and *E. coli* . The strong reaction of the bands reflects a 3 - 4 fold more production of AC by this strain which supports the previous reports for the overproduction of AC by this strain. Urea extracts of *E. coli* H1469 (pANGE 3) (lane 6) and *E. coli* H1469 (pKIM1) (lane 7) were included in this study as controls which did not show any band reacting to the anti-AC monoclonal 9D4 .Lane 8 shows the production of AC in *E. coli* in the presence of pRMB6 alone.These data indicate that high MW AC (210 kDa) was expressed by these strain in the presence of two compatible plasmids. Further characterization of the activation of CyaA by HlyC was determined by toxicity assays as described on page 128.

Fig.12
Expression and Induction of Cloned *B. pertussis* AC in *E. coli* : Immunoblot Assessment .

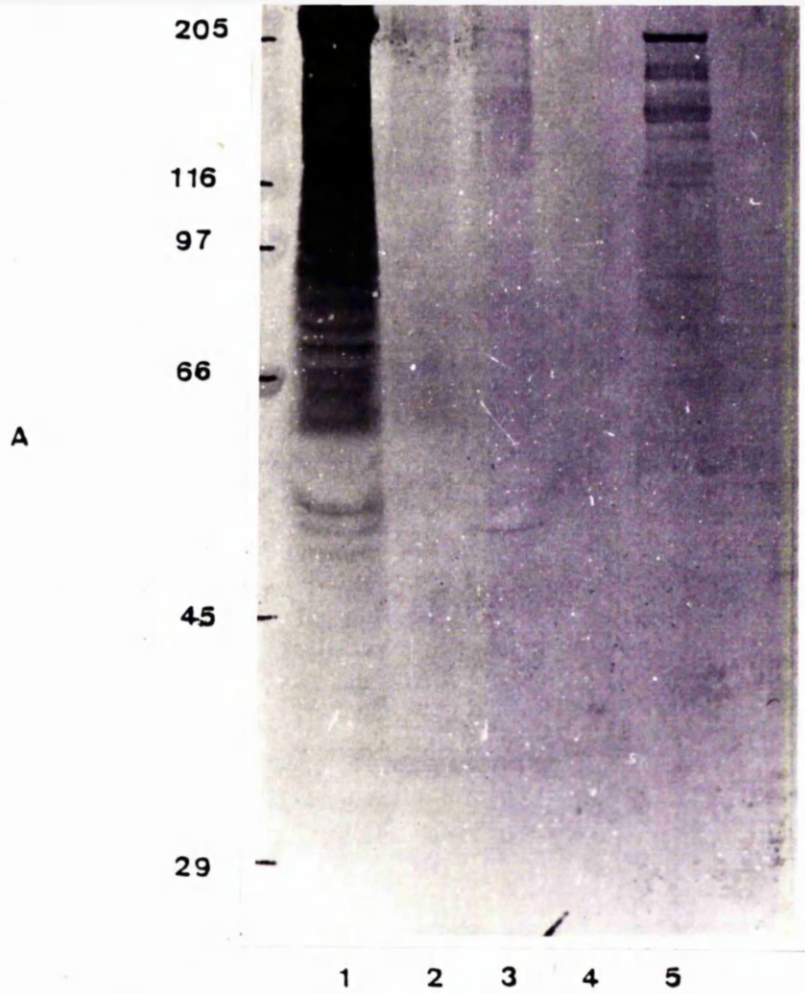
Urea extracts of *B. pertussis* strains and *E. coli* H1469 (pRMB6) (AC production under *tac* promoter and induced with IPTG) were separated by SDS-PAGE using 7.5% acrylamide resolving gels, and transferred to nitrocellulose.The immunoblot was probed with anti-AC monoclonal 9D4 (1:1000 dilution).

Panel A)

1= *B. pertussis* BP348 (pRMB1)
2= *B. pertussis* BP348
3= *B. pertussis* BP349
4= *E. coli* H1469 (pRMB6) not induced with IPTG
5= *E. coli* H1469 (pRMB6) induced with IPTG

Panel B) Immunoblot assessment of production of AC by *B. pertussis* strains and by *E.coli* strains harbouring single or double plasmids. All strains containing pRMB6 were induced with IPTG.
Urea extracts were resolved by SDS-PAGE and transferred to immunoblots. Probing was carried out with anti-AC monoclonal 9D4 (1:1000)

1= *B. pertussis* BP348 (pRMB1)
2= *B. pertussis* 18323
3= *E. coli* H1469 (pRMB6, pKIM1)
4= *E. coli* H1469 (pRMB6, pANN202)
5= *E. coli* H1469 (pRMB6, pANGE3)
6= *E. coli* H1469 (pANGE3)
7= *E. coli* H1469 (pKIM1)
8=*E. coli* H1469 (pRMB6)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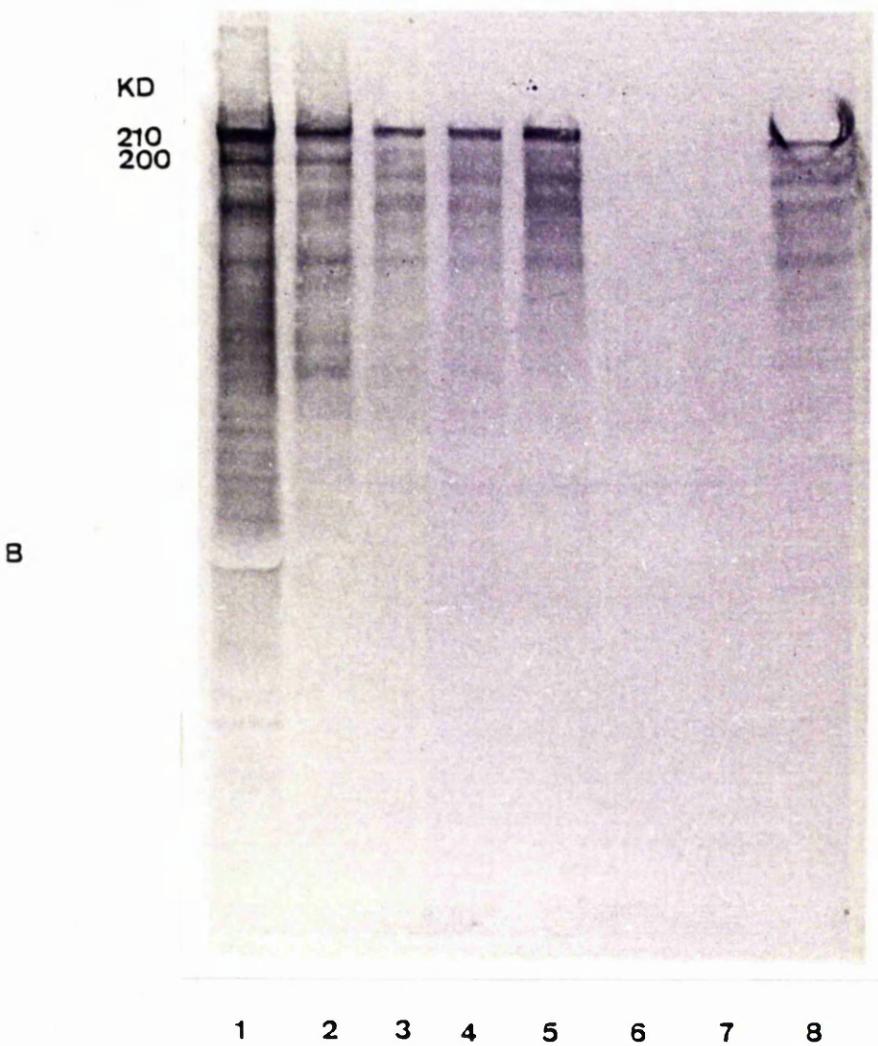
KD
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DETERMINATION OF ADENYLATE CYCLASE ENZYMIC ACTIVITY

Estimation of AC Enzymic Activity by Binding Protein Assay (Amersham Assay).

Adenylate cyclase enzyme assay was performed with urea extracts of *B.pertussis* and *E. coli* strains using a cyclic AMP assay kit as described under Materials and Methods (see page 56).The assay was based on the competition between unlabelled cAMP of the test sample and a fixed quantity of tritium-labelled compound for binding to a protein which has affinity for cAMP. The detection limit for this assay was 0.2 - 16 pmol and due to the narrow range for detection, the urea extracts of *B. pertussis* and *E .coli* were used as neat and 10 - fold diluted (10^{-1}, 10^{-2}, 10^{-4}) samples. The urea extracts from *B. pertussis* BP348 (pRMB1), *B. pertussis* BP348, *E.coli* H1469 (pRMB3), and *E. coli* H1469 were assayed. The samples were mixed with reaction mixture and incubated for 15 min at 30 ^{o}C , and the reaction was terminated by boiling. Test samples were stored at - 20 ^{o}C at this stage. The cAMP assay was performed as per manufacturer's instructions and, finally, the samples were added to scintillation fluid. Tritiated cAMP was counted in a scintillation counter.

The results showed that the amount of cAMP produced by AC from urea extracts of *B. pertussis* BP348 (pRMB1) was off the scale in neat and 10^{-2} dilution and it was measurable only in 10^{-4} dilution , in which the AC enzyme activity was scored as 20 µmol cAMP/min/mg of protein. Measurable AC enzymic activity was observed in urea extracts of *E.coli* H1469 (pRMB3) in 10^{-2} dilution equilvalent to 6.8 µmol cAMP/min/mg of protein. *E.coli* H1469 and BP348 showed < 0.002 µmol cAMP/min/mg activity. The diffculty encountered in this assay was due to its narrow detection limits, since it required a range of dilutions of the samples which increased the number of tubes 4 - 5 -fold for each sample. The assay itself was uneconomical due to the short half life of the cAMP binding protein used in this assay. Therefore, another assay, based on the method of Salomon *et al* . (1974) was performed for the detection of AC enzyme activity.

Determination of Adenylate Cyclase Enzymic Activity by Salomon Assay.

Adenylate cyclase enzymic activity was measured by conversion of [alpha -^{32}P] ATP to [^{32}P] cAMP in a cell- free assay as described under Materials and Methods (see page 56) . The AC enzyme activity of urea extracts from various strains of

B.pertussis and *E.coli* is given in Table 7. As is evident from the Table, the enzymic activity obtained varied from strain to strain. The recombinant strain *B. pertussis* BP348 (pRMB1) showed the highest level of enzyme activity. This agrees with the findings of Brownlie *et al.*, (1988) who reported a 3-fold increased enzymic activity over the wild type *B. pertussis* in this strain . The level of enzymic activity in the extract of a transposon insertion mutant *B. pertussis* BP357 (PT $^-$) was comparable to that of *B. pertussis* BP348 (pRMB1) which corresponds to the data from the immunoblot study (see Fig 9A).

On the other hand, the extracts of wild type *B. pertussis* : Tohama, 165, 18323, and 77/18319 displayed variable enzymic activities ranging from as low as 36.7 nmol cAMP/min/mg protein activity in the case of *B. pertussis* Tohama to 413 nmol cAMP /min/mg protein enzymic activity in case of *B. pertussis* 165. Comparable to the activity of extract of *B. pertussis* 165 was the activity detected in the extract of *B. pertussis* 18323 . The level of enzymic activity obtained by *B. parapertussis* was comparable to *B. pertussis* Tohama which agrees with the data from the immunoblot study.

Enzymic activity obtained from extracts of other transposon insertion mutants of *B. pertussis* also differed from strain to strain . *B. pertussis* BP353 (FHA$^-$) did not show high levels of AC activity like BP357 and a 94 nmol cAMP /min/mg protein enzymic activity was found in this strain. Enzymic activities obtained from *B. pertussis* BP348 (AC $^-$, HLY$^-$) and *B. pertussis* BP349 (AC$^+$, Hly $^-$), which is reported to have a low level of enzymic activitiy (Weiss *et al.*, 1983), were not different from each other and in both cases < 0.02 nmol cAMP/min/mg protein enzymic activity was observed.

When urea extracts of *E. coli* strains with single and double plasmids were tested for their AC enzymic activity, *E. coli* H1469 (pRMB6,pANN202) showed the highest level of enzymic activity (1652 nmol cAMP/min/mg protein) as compared to *E. coli* H1469 (pRMB6,pANGE3), *E. coli* H1469 (pRMB6,pKIM1) and *E. coli* H1469 (pRMB6). The data presented here indicate that the AC enzymic activity of cell extracts, despite preparation under the same set of conditions, seems to vary from strain to strain.

Table 7

Determination of AC Enzymic Activity of Urea Extracts by Salomon Assay.

Strain	Protein content (mg/ml) of urea extracts	Enzymic activity (nmol cAMP/min/mg protein)
Bordetellae •		
B. pertussis BP 348 (pRMB1)	2	1450.68
B. pertussis Tohama	2	36.70
B. pertussis 18323	2	315.68
B. pertussis 165	2	413.65
B. pertussis BP357	2	1267.27
B. pertussis BP353	2	94.65
B. pertussis 77/18319	2	40.96
B. pertussis BP349	2	< 0.02
B. pertussis BP348	2	< 0.02
B. parapertussis 5952	2	40.13
B.bronchiseptica 214	2	541.37
E.coli ••		
H1469 (pRMB6)	2.59	416.88
H1469 (pRMB6, pANN202)	1.36	1652.73
H1469 (pRMB6, pKIM1)	2.29	713.49
H1469 (pRMB6, pANGE3)	1.62	1136.11
H1469	1.62	< 0.02

•• = Average of three determinations

• = Average of two determinations

Determination of AC Enzymic Activity by Microtitre Plate Assay.

A plate assay was developed for the detection of enzymatic activity of AC. The principle of the assay involves cross feeding of an AC $^-$ strain of *E. coli* (indicator strain) with cAMP to activate its *lac* operon for ß- galactosidase production in the presence of X-gal. Utilization of this chromogenic substrate by the indicator strain results in a blue colour. This assay was performed successfully with urea extracts and culture supernates of both *B. pertussis* and *E.coli* (with cloned AC gene). The results indicate that the AC enzymic activity is detectable from both species . Examples of the assay used qualitatively are shown in Fig.13 A and Table 8. In order to quantify the levels of cAMP required to produce a clear blue colour, the detection limits of the assay were worked out. Controls with exogenous cAMP at different concentrations were set up in triplicate . A standard curve of cAMP concentration plotted against A_{620} suggests that AC enzymic activity could be conveniently detected between the range of 4-32 micromol cAMP (Fig.13 B, Table 9). It was observed , however, that the A_{620} plateaued below 4 µmol and above 32 µmole cAMP suggesting rather narrow detection limits of the assay. When calmodulin affinity-purified AC was investigated for its specific enzymic activity in the microplate assay, and with the same assay conditions (i.e., 15 min incubation of the sample at 37 ° C with reaction mixture and subsequent boiling for 15 min to stop the reaction) as were used with the Salomon assay, 33 µmol cAMP / min / mg protein specific activity was detected. This level of AC activity was comparable with the activity obtained by Salomon assay for purified AC (Table 11, see page 90).

These findings suggest the possibility of the use of this assay as a tool for the rapid and safe investigation of AC activity in any sample, including urea extracts.

Table 8

Determination of Enzymic Activity of AC by Microplate Assay

Sample	Strain								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Urea extracts	++	++	-	+	++	+	+	-	-
Culture supernates	+	+	-	+	ND	ND	ND	ND	-

Dialyzed urea extracts (25 µl) and culture supernates (25 µl) of *B. pertussis* and *E. coli* were incubated in duplicate with reaction mixture (25 µl) for 1h at 37 °C . Test culture (150 µl) i.e., test suspension previously inoculated with the indicator strain *E. coli* G802 and grown at 37°C to an OD_{650} of 0.4, was added and the plates were incubated at 37 °C for 12 h.

1= *B. pertussis* BP348 (pRMB1)
2= *B. pertussis* 18323
3= *B. pertussis* BP348
4= *B. pertussis* Tohama
5= *E. coli* H1469 (pRMB3)
6= *E. coli* H1469 (pRMB3,pANN202)
7= *E. coli* H1469 (pRMB9)
8= *E. coli* H1469
9=Control (reaction mixture and test suspension only)
ND= Not determined
++ = Strongly positive
+ = Positive
- = Negative

Table 9 **cAMP Standard Curve for AC Enzymic Activity**

Sample	A_{620}
cAMP (micromol/50µl)	
64	1.96
32	1.80
16	1.27
8	1.10
4	1.00
2	1.99
1	0.94
0.5	0.92
Unknown	
Neat	1.469
1/2	1.290
1/4	1.068
1/8	0.981
1/16	0.964
1/32	0.931
1/64	0.930

Different concentrations of cAMP ranging from 64 micromol to 0.5 micromol in a final volume of 50 µl were dispensed in a microplate in triplicate. For the sample with unknown activity, 50 µl of the sample was incubated with 50 µl of the reaction mixture for 15 min at 37 °C and then boiled for 15 min. This sample was diluted 2-fold in distilled water in the same plate and to all the wells, 150 µl of test culture i.e., test suspension previously inoculated with the indicator strain *E. coli* G802 and grown at 37 °C to an OD_{650} of 0.4, was added. Contents were mixed and the plate was incubated at 37 °C for 12 h, A_{620} was measured on an automated ELISA reader and the standard curve was plotted. The data are a mean of four determinations.

Fig.13 Determination of AC Enzymic Activity by Microplate Assay.

Panel A) AC enzymic activity in urea extracts.

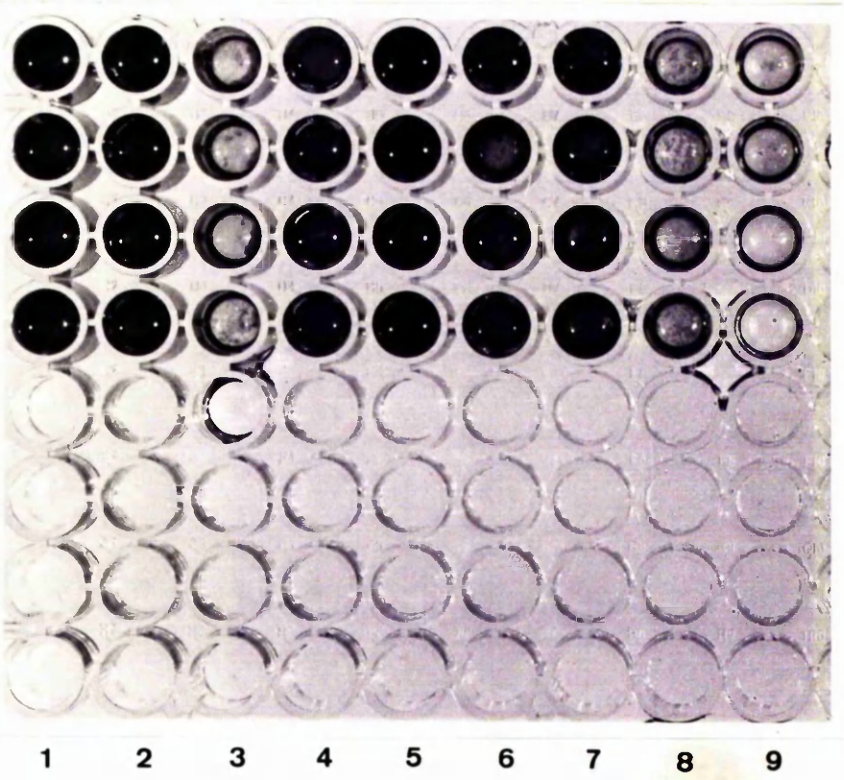
Dialyzed urea extracts (25 μl) of *B. pertussis* and *E. coli* were incubated in quadruplicate with 25 μl of reaction mixture for 1h at 37 ^{o}C . Test culture (150 μl) was added and the plates were incubated at 37 ^{o}C for 12 h .

1=*B. pertussis* BP348 (pRMB1)
2=*B. pertussis* 18323
3=*B. pertussis* BP348
4=*B. pertussis* Tohama
5=*E. coli* H1469 (pRMB3)
6 =*E. coli* H1469 (pRMB3, pANN202)
7=*E. coli* H1469 (pRMB9)
8= *E. coli* H1469
9= Control (Reaction mixture and test suspension only)

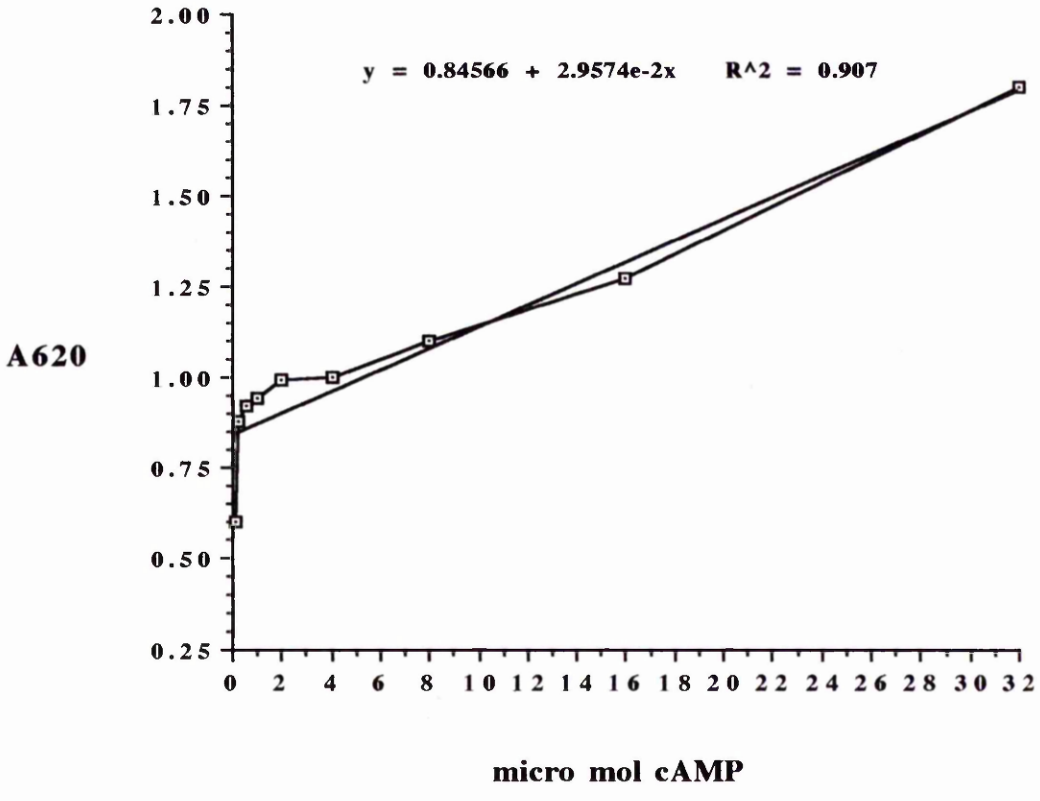
Panel B) cAMP standard curve for estimation of AC enzymic activity by microplate assay.

Different concentrations of cAMP ranging from 64 micromol to 0.5 micromol in a final volume of 50 μl were dispensed in a microplate in triplicate. 150 μl of test culture was added to the wells. Contents were mixed and the plate was incubated at 37 ^{o}C for 12h, A_{620} was measured on an automated ELISA reader and the standard curve was plotted.

A


1
2
3
4
5
6
7
8
9

B


y = 0.84566 + 2.9574e-2x R^2 = 0.907
A620
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
micro mol cAMP

EFFECT OF DIALYSIS ON AC

The effect of extensive dialysis on AC in urea extracts of *B. pertussis* BP348 (pRMB1) was investigated as described in Materials and Methods (see page 45). SDS-PAGE analysis of dialyzed and undialyzed urea extracts showed a decrease in intensity of some protein bands , including AC, by about 25% in dialyzed urea extracts (Fig.14A). Protein estimation of urea extracts before and after dialysis (extensive) yielded 10-20% less protein (mg/ml) in dialyzed urea extracts, possibly due to the sticking of protein onto the inner wall of the dialysis membrane or possibly due to dilution of the sample since the volume sometimes increased slightly during dialysis.

Stablity of enzymic activity of dialyzed and undialyzed urea extracts was then compared; a 40-50% loss of activity was observed within 2 months in dialyzed urea extracts as determined by adenylate cyclase enzymic assay (binding protein assay). The results obtained are outlined in Table 10.These results suggest that extracts could be stored in 4-8M urea at -20 ^{o}C for up to 2 months with only 15-20% loss when stored with urea. A substantial loss was observed when urea was dialyzed out (Fig.14 B) These findings correspond with those of Gentile *et al.* (1990) who reported the similar loss of activity in dialyzed urea extracts.

EFFECT OF PROTEASE INHIBITOR ON AC

The heterogeneity of AC revealed by SDS-PAGE and immunoblotting signified that it might be susceptible to degradation by endogenous proteases and suggested that at least some low MW polypeptides present in urea extracts are degraded forms of AC. In order to protect AC from degradation, a protease inhibitor, phenyl methyl sulphonyl flouride (PMSF), effective against serine proteases, was used at 0.5 mM concentration . Addition of PMSF immediately after harvest of the cells (Materials and Methods, see page 44) appeared to reduce degradation as determined by SDS-PAGE and immunoblotting with polyclonal antibodies.Fig. 15 A&B shows the SDS-PAGE and immunoblot profiles of PMSF- treated and untreated urea extracts of *B. pertussis* BP348 (pRMB1)(AC^{+}) , It is evident from the profiles that PMSF-treated urea extracts of *B. pertussis* BP348 (pRMB1) showed less degradation bands then PMSF-untreated *B. pertussis* BP348 (pRMB1). The PMSF was used routinely after these observations, However, the samples prepared for the toxicity assays did not recieve PMSF in order to avoid the possiblity of interference of this product with the assays.

Table 10 **Effect of Dialysis on AC in Urea Extracts**

Urea extract BP348 (pRMB1)	Time (months)	Protein (mg/ml)	Activity (nmolcAMP/min/mgprotein)
Undialyzed	0	2.71	4378.3
Dialyzed	0	2.53	3816.2
Undialyzed	2	2.58	3483.5
Dialyzed	2	2.47	2146.5

The values are mean of two determinations

Fig. 14

Effect of dialysis on AC

Urea extracts (dialyzed and undialyzed) were resolved by SDS-PAGE and stained with Coomassie blue . Panel A represents the effect of dialysis on fresh urea extracts.

Panel B represents the effect of dialysis on AC after storage.

Panel A) Effect of dialysis on urea extracts of *B. pertussis*

1= SDS-6H (MW standard)

2= Undialyzed urea extract of *B. pertussis* BP348 (pRMB1)

3= Dialyzed Urea extract of *B. pertussis* BP348 (pRMB1)

4= Dialyzed Urea extract of *B. pertussis* BP348.

Panel B) Effect of dialysis on AC after storage.

1= Undialyzed *B. pertussis* BP348 (pRMB1) urea extract.

2= Undialyzed urea extracts of *B. pertussis* BP348 (pRMB)1 after 2 months storage at - 20 °C

3= Dialyzed urea extract of *B. pertussis* BP348 (pRMB1) after 2 months storage at - 20 °C

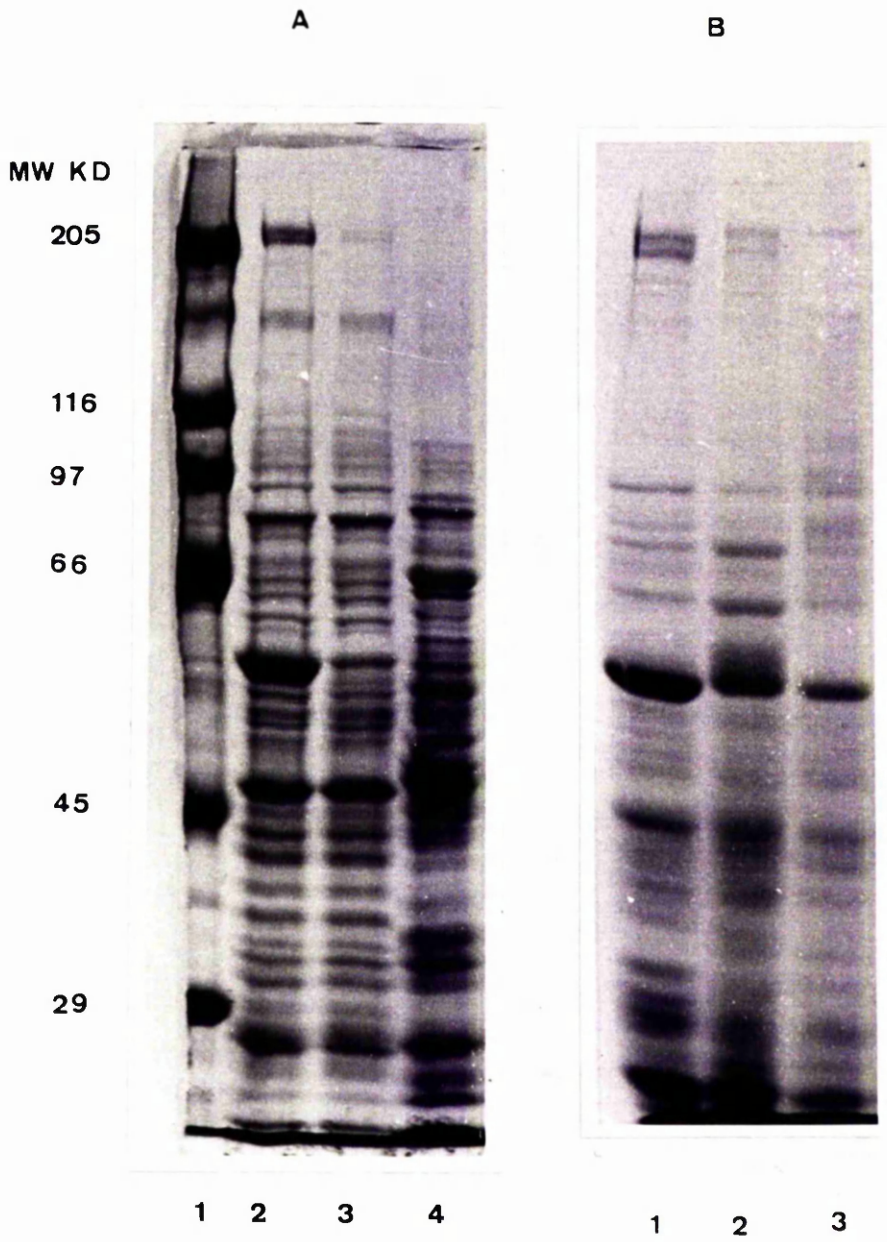

A
B
MW KD
205
116
97
66
45
29
1
2
3
4
1
2
3

Fig.15

Effect of Protease Inhibitor (PMSF) on AC

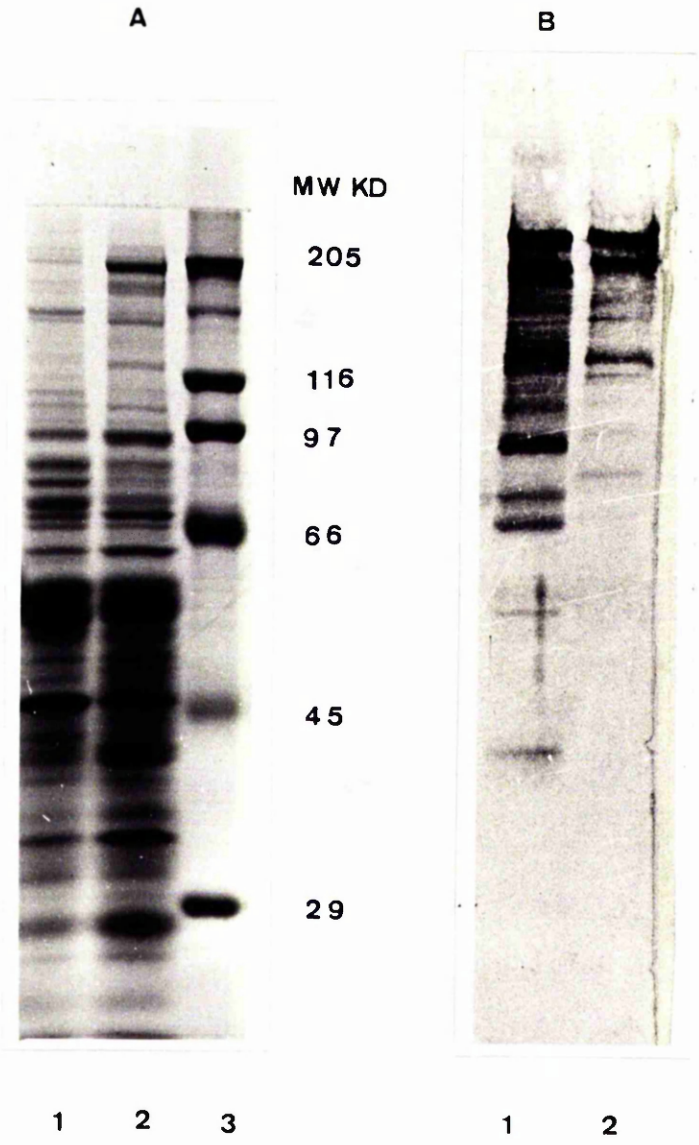
Crude preparations of adenylate cyclase (urea extracts) were used to investigate the effect of protease inhibitors on AC. A urea extract containing 0.5 mM PMSF was kept at 4 ^{o}C for 48 h. A control of untreated sample was also included in this study.

Panel A) Coomassie blue - stained gel of PMSF-treated and untreated urea extracts.

1= *B. pertussis* BP348 (pRMB1) without PMSF
2= *B. pertussis* BP348 (pRMB1) with PMSF
3= MW standard

Panel B) Immunoblot of PMSF- treated and untreated urea extracts.
The blots were probed with anti-AC monoclonal 1H6 at a 1:1000 dilution .

1= *B. pertussis* BP348 (pRMB1) without PMSF
2= *B. pertussis* BP348 (pRMB1) with PMSF


A
B
MW KD
205
116
97
66
45
29
1
2
3
1
2

PURIFICATION OF ADENYLATE CYCLASE BY CAM-AFFINITY CHROMATOGRAPHY.

Enzymatically and biologically active AC had been shown previously to be recoverable by one-step chromatography (Friedman, 1987 a ; Bellalou *et al* .,1990 a), a convenient and reproducible method of purification . Adenylate cyclase from *B. pertussis* BP348 (pRMB1) and *E. coli* with cloned AC was purified by calmodulin-affinity chromatography as described by Bellalou *et al.* (1990 a).

Urea extracts of *B. pertussis* BP348 (pRMB1), BP348, and *E. coli* strains listed in Tables. 3&4 (see pages 46 &47) were diluted 1 in 4 and adsorbed onto CaM-agarose. The gel was washed and eluted with 8 M urea as detailed under Materials and Methods (see page 49). The purified enzyme was stored at - 20 ^{o}C in the undialyzed state . Adenylate cyclase activity was measured using the procedure of Salomon *et al.* (1974) and the purification factor was calculated. The purification of AC is demonstrated in Fig. 16 and Table 11.The *B. pertussis* BP348 (pRMB1) preparation (lanes 2 - 5) but not the control *B. pertussis* BP348 (lane 1) displayed two bands of 210 and 200 kD that were recognized by the anti-AC monoclonal antibodies. The intensity and thickness of the AC bands (in mm) was considerably greater than in the crude preparations (not shown) and only trace amounts of lower MW bands were seen

The two bands observed on immunoblots possibly indicate the existence of AC in two forms as has been reported by Rogel *et al.* (1991) who found that the purification of AC resulted in two forms after separation by SDS-PAGE. These were isolated from *B. pertussis* BP348 (pRMB1) , were both of 200 kDa yet one form was toxic, capable of generating cAMP in the target cells, whereas the other was not.

Table.11

Purification of AC from Urea Extracts by Calmodulin - Affinity Chromatography.

Sample	Activity* (crude)	Activity* (Purified)	Purification factor
E.coli			
H1469 (pRMB6)	0.37	2.39	6.45
H1469 (pRMB6, pANN202)	0.22	5.02	22.82
H1469 (pRMB6, pKIM1)	0.27	3.84	14.22
H1469 (pRMB6, pANGE3)	0.40	6.36	15.90
B. pertussis			
BP348 pRMB1	5.03	30.57	6.08
18323	1.43	20.6	14.03
BP 348	<0.001	-	-

* Specific activity , µmol cAMP / min / mg protein, mean of 4 determinations.

Fig.16

Immunoblotting of Calmodulin - Affinity Purified ACT

ACT purified from urea extracts of *B. pertussis* BP348 (pRMB1) by calmodulin - affinity chromatography ; bound AC was eluted and sequential 0.5 ml fractions were collected. Each fraction was subjected to SDS-PAGE and the resolved protein was electrophoretically transferred to nitrocellulose membrane.Immunoblot was probed with anti-AC monoclonal antibody McU (1:1000)

1= BP348 (Third fraction)
2= First fraction of *B. pertussis* BP348 (pRMB1)
3= Second fraction of *B. pertussis* BP348 (pRMB1)
4= Third fraction of *B. pertussis* BP348 (pRMB1)
5= Fourth fraction of *B. pertussis* BP348 (pRMB1)


KD
210
200
1
2
3
4
5

ISO - ELECTRIC FOCUSING OF ACT

Analytical iso-electric focusing of calmodulin-affinity purified ACT in polyacrylamide was carried out in the presence of 6 M urea in an attempt to isolate and characterize the two high MW forms seen by SDS-PAGE and immunoblotting . Ampholines were used with a wide pH range (3.5-10) as described under Materials and Methods(see page 51). The sample was applied at three different sites to detect the iso-electric point of the ACT. Fig.17 shows the focusing pattern of ACT when the sample was applied at the cathode (lane 1), in the middle (lane 2) and at the anode side (lane 3) . Lane 4 shows the migration profile of a CaM-treated urea extract of *B. pertussis* BP348 (pRMB1). p*I* standards were also applied at the anode and the cathode sides (lane 5 and 6 respectively). The migration profile from the cathode exhibited a single diffused band at p*I* of approximately 7 (lane 1) after staining with coomassie blue.This coincides with the previously reported p*I* for AC (Leusch *et al.*, 1990). Application of ACT at the other sites did not reveal any focussed band and only a precipitation of the sample at the site of application was observed .

Attempts were made to transfer and detect the iso-electric focused AC on nitrocellulose, by Western blotting but this did not prove successful. This may be because the non-denaturing conditions used in iso-electric focusing were not favourable for transfer of the high MW, aggregated form of AC. In order to provide the denaturing conditions to the focused AC, 0.0375 % SDS was added to the transfer buffer for the Western blotting but the same problem was faced; that is poor transfer and negative reaction after probing with monoclonal anti-AC antibodies. This indicated that the SDS in the transfer buffer did not denature the ACT well enough for it to be transferred to the membrane . Another possibility could be that the ampholines interfered with the transfer.

Fig.17
Iso-Electric Focusing (IEF) of ACT

Calmodulin-purified ACT (10 μg) from urea extract of *B. pertrussis* BP348 (pRMB1) was subjected to IEF in a polyacrylamide gel in the presence of pH range 3.5 -10 ampholines, and 6M urea . The samples were applied at the cathode, middle and anode sites. The gel was stained with Commassie blue and destained until the background was clear.

1= CaM - purified ACT applied at cathode

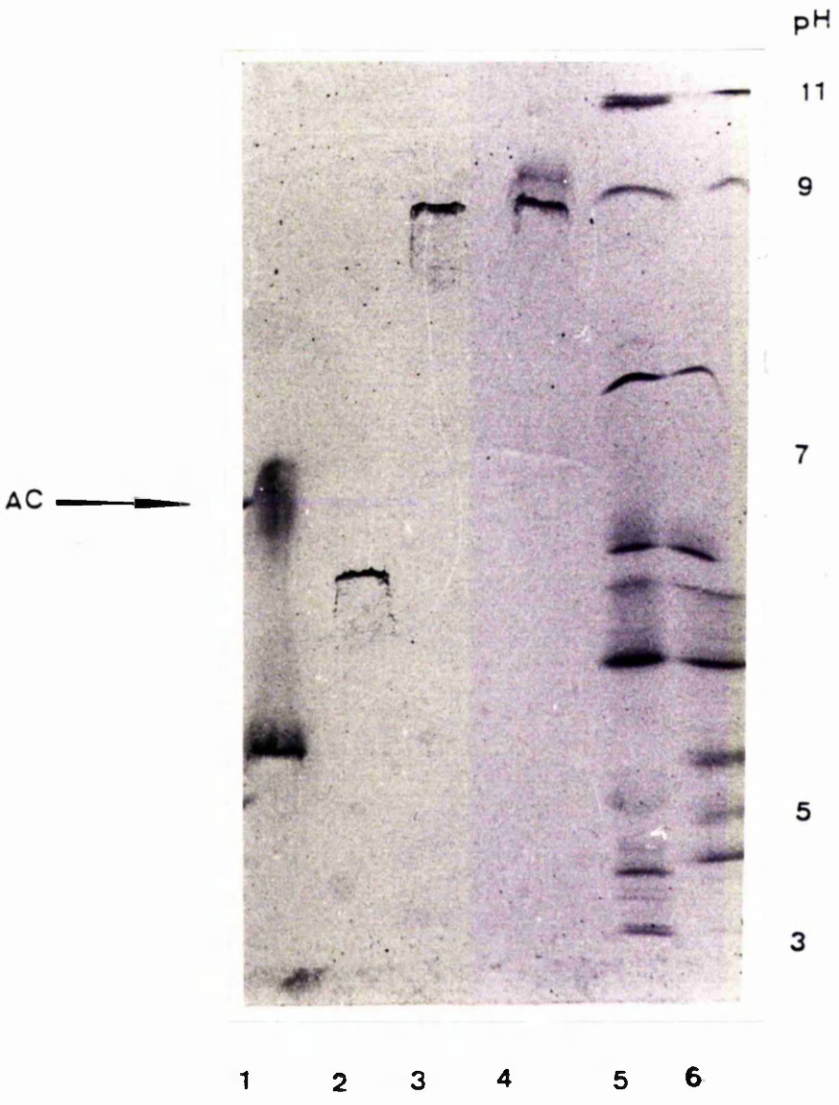
2= CaM - purified ACT applied in middle

3= CaM - purified ACT applied at anode

4= CaM- treated extract of *B. pertussis* BP348

5= p*I* standard applied at cathode

6= p*I* standard at anode


pH
11
9
7
5
3
AC
1
2
3
4
5
6

DETECTION OF HOMOLOGY BETWEEN ACT AND OTHER RTX -

RTX (Repeat in toxin) is a designation given to a family of 102-177 kDa cytotoxins produced by a number of Gram-negative pathogenic bacteria. The important and common feature of these toxins is the presence of a series of glycine -rich repeat units at the C-terminal end of each protein. The members of this family include : *Escherichia coli* haemolysin (HlyA), *Pasteurella haemolytica* leukotoxin (LktA), *Actinobacillus actinomycetemcomitans* leukotoxin (Akta) and *Bordetella pertussis* adenylate cyclase toxin (ACT or CyaA) (Welch, 1991; Coote, 1992).

In order to search for any antigenic homology between these toxins, an immunoblotting investigation was performed. Toxins preparation is given in the Materials and Methods (see page 53). The toxins were separated by SDS-PAGE and transferred to nitrocellulose. Probing was carried out with anti-AC monoclonal antibodies and polyclonal antibodies .

Fig.18 A shows the monoclonal 9D4 reacted positively with all the four toxins tested at their respective positions on the blots. Leukotoxin of *P. haemolytica* (S/C 82/ 1) was recognized at the MW 106 kDa, alpha-haemolysin of *E.coli* (582) was recognized at 110 kDa, and *A.actinomycetemcomitans* (JP2 and 29523) exotoxin at 116 kDa. It was observed, however, that the exotoxin of *A. actinomycetemcomitans* was extremly unstable when extracts from whole cells were used. Cross- reactive high MW bands at 116 kDa were observed only in fresh preparations and low MW bands (presumably the breakdown products of the toxin) were observed when the samples were used after freezing and thawing. LktA failed to be recognized by polyclonal anti-AC antibodies as is evident in Fig. 18 B,a , although it was recognized by its homologous hyperimmune serum (Fig 18 B, b) and by anti-AC monoclonal 9D4. *B. pertussis* ACT however, was not recognized by the anti-LktA hyperimmune serum. Failure of the recognition of epitopes by polyclonal antisera but not with the monoclonal antibody has also been observed by Dr.R. Welch, (personal communication). Other cross-reactive bands revealed in the urea extracts of *B. pertussis* BP348 (pRMB1) after probing with anti-AC polyclonal antibodies (PcU) and anti-*P. haemolytica* hyperimmune serum (Fig. 18 panels B,a and B,b) could be due to the presence of preexisting cross-reacting antibodies against *B. pertussis* components.

Fig.18
Investigation of Homology Between RTX Toxins by Immunoblotting.

Urea extracts of *B. pertussis* , *A. actinomycetemcomitans,* and culture supernates of *P. haemolytica* and *E. coli* were separated by SDS-PAGE , transferred to nitrocellulose and probed with various sera.

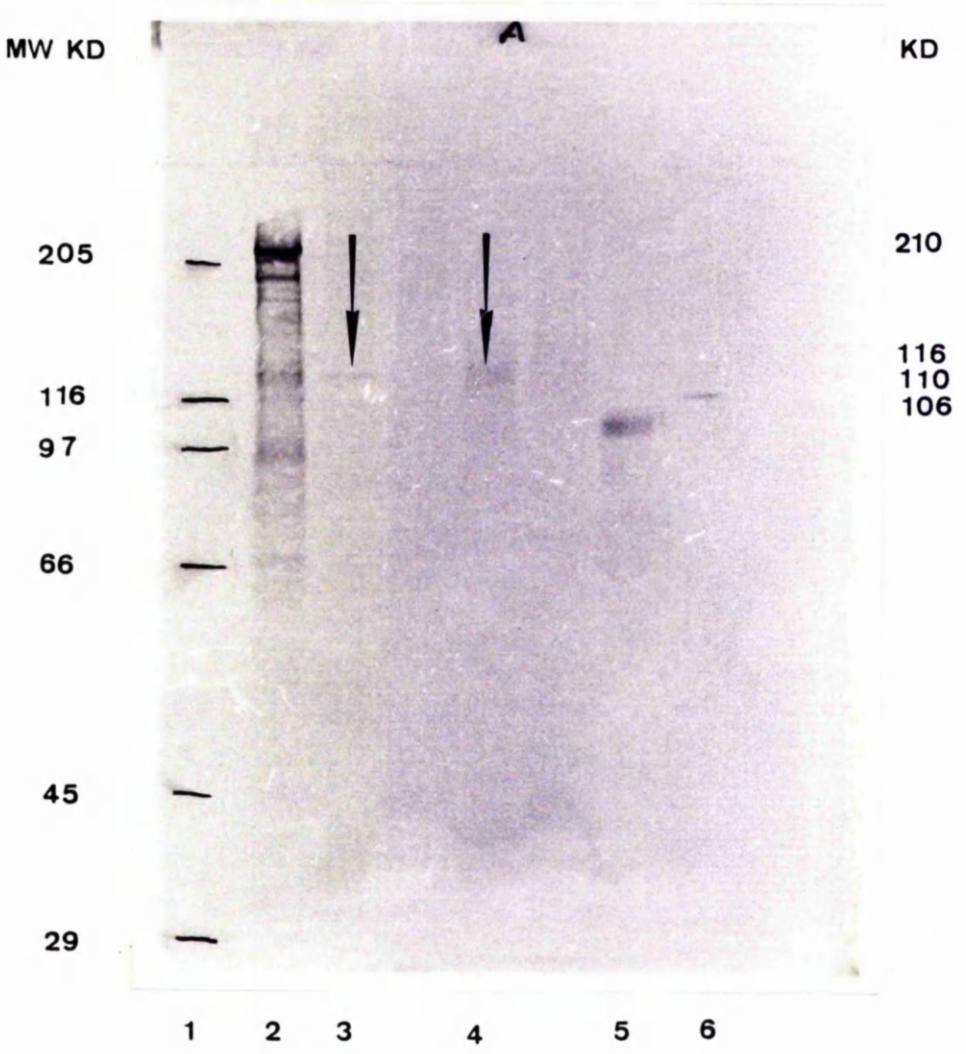
Panel A) Immunoblotting of RTX toxins , probed with anti- AC monoclonal 9D4 (1: 1000).

1= MW standard
2= *B. pertussis* BP348 (pRMB1)
3= *A. actinomycetemcomitans* JP2
4= *A. actinomycetemcomitans* 59524
5=Lkt (*P. haemolytica*)
6= alpha -haemolysin (*E.coli* 582)

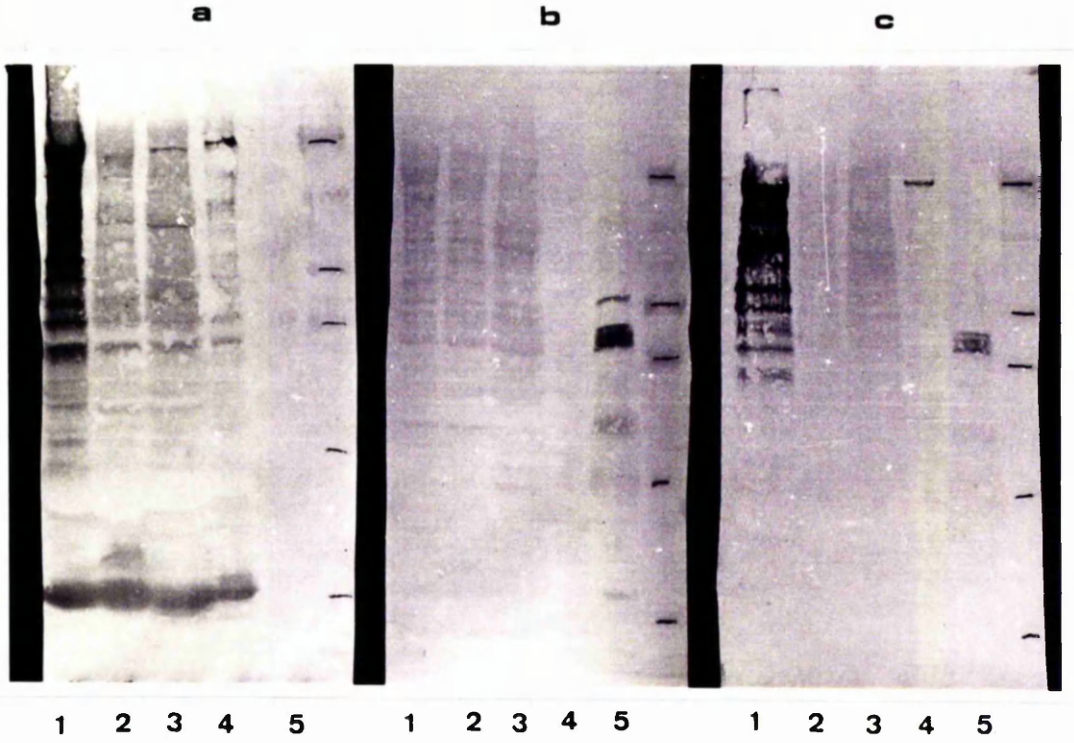
Panel B) Cross reaction of Lkt and ACT
Samples were run in triplicate and probed with a) Anti-AC polyclonal PcU (1: 500) , b) anti-Lkt hyperimmune serum (1: 500) , and c) anti-AC monoclonal 9D4.

1= *B. pertussis* BP348 (pRMB1) (AC^{++})
2= *B. pertussis* BP348 (AC^{-})
3= *B. pertussis* BP349 (AC^{+} HLY^{-})
4= *B. pertussis* 18323 (AC^{+})
5= LktA (*P. haemolytica*)

A


MW KD
A
KD
205
116
97
66
45
29
210
116
110
106
1
2
3
4
5
6

B


a
b
c
1
2
3
4
5
1
2
3
4
5
1
2
3
4
5

DETERMINATION OF AC TOXIC ACTIVITY.

The aim of this study was to establish a simple, reliable, sensitive and convenient assay for the detection of toxicity of the AC . Toxic AC is capable of penetrating into target cells where it is activated by a host cell protein, calmodulin, and catalyzes the generation of uncontrolled cAMP by using host cell ATP (Confer and Eaton., 1982). Previous studies described the toxic effect of AC on various cell types, including human PMNs and macrophages (Confer and Eaton., 1982), lymphocytes, (Hanski and Farfel., 1985), monocytes, CHO cells , mouse S49 lymphoma cells, isolated rat pituitary cells (Hewlett *et al.*, 1985 ; Gentile *et al.*, 1988) , and red blood cells (Rogel *et al.*, 1991) . The bases of evaluation of toxicity in these assays involved the demonstration of high levels of cAMP in the target cells, which, although reliable, is not simple and convenient. Therefore, a more direct approach was sought to detect the toxic activity of AC by using a variety of alternative assays.

Nitroblue Tetrazolium Reduction Assay (NBTR Assay) for detection of AC toxic activity.

Nitroblue tetrazolium (NBT) is a useful marker of normal redox capabilities of polymorphonuclear neutrophils (PMNs) in which it is reduced to a blue formazan derivative. Nitroblue tetrazolium reduction is an indicator of neutrophil superoxide production for which NBT serves as an artificial electron acceptor (Baehner*et al.*,1976). NBT in its oxidized state is colourless and soluble, but when reduced, is precipitated into insoluble blue formazan.The reduction occurs due to oxygen-dependent oxidase activity in the form of excitable O_2 products i.e., superoxide anion, hydrogen peroxide , and singlet oxygen.
The overall reaction is:

$$NADH\ (NADPH) + O_2 \text{ ----------> } NAD(NADP) + O_2 + 2H^+$$

$$O_2 + NBT + 2H^+ \text{ ----------> } O_2 + NBTH_2 \text{ (Formazan)}$$

This property of NBT was used to detect the toxic effects of AC on PMNs.
Rabbit neutrophils were pre-incubated in a microtitre tray with the dialyzed urea extracts of *B. pertussis* and *E. coli* (20 µg protein / well and its 2-fold dilutions as described under Materials and Methods (see page 58) and induced with PMA (phorbol myristate acetate). Controls included PMA-induced and uninduced neutrophils without the extracts . The reaction in the microplate was examined visually. Control cells

induced with PMA showed the purple- blue colouration as a sign of their metabolic activity and NBT reduction while uninduced cells remained colourless suggesting their resting state. Extracts from *B. pertussis* Tohama and *B. pertussis* BP348 (pRMB1) were able to inhibit the NBT reduction when used neat and 1 in 2 dilution respectively. *B. pertussis* BP348, *E. coli* H1469 (pRMB3), *E. coli* H1469 (pRMB9) and *E. coli* H1469 failed to inhibit the NBT reduction even when used neat (Fig. 19). These data support the previous reports of the absence of toxicity of the *E. coli* AC extracts (Brownlie *et al.*, 1988 ; Rogel *et al.*, 1989), but indicate that *B. pertussis* ACT can inhibit the NBT reduction of phagocytes, in the present case, rabbit neutrophils.

Determination of Haemolytic Activities of ACT on Sheep and Rabbit RBCs.

Haemolytic activity of calmodulin - affinity purified ACT was determined according to the method of Bellalou *et al.* (1990 b), except that 2% RBCs were used in this assay. Sensitivity of two species of RBCs towards ACT was investigated. Purified ACT (100 nmol cAMP / min / ml AC enzyme activity) and 2-fold dilutions of this activity were incubated with 2% SRBCs as detailed under Materials and Methods, (see page 60).

The results (Fig. 20 A) showed a clear linear relationship between % haemolysis and the toxin dilutions . The rabbit RBCs were found fragile and some autolysis was observed in control cells. Sheep RBCs , on the other hand were found to be sensitive enough to detect haemolytic activity of ACT down to the less than 6.25 nmol cAMP /min /ml AC enzymic activity. The haemolytic activity of ACT was found to be dose -dependent.

In order to determine the blocking effect of anti - AC antibodies on the haemolytic activity of ACT, calmodulin affinity-purified ACT (60 nmol cAMP / min / ml AC enzyme activity and its 2-fold dilutions) were incubated with neat anti-AC monospecific antibodies (Ms1). A 60% reduction of haemolysis was observed in the cells which were incubated with Ms1-treated ACT as compared to the cells, incubated with ACT alone (Fig.20 B) . This coincides with the findings of Rogel *et al.* (1991) who reported the neutralization of haemolytic activity of ACT by anti-AC polyclonal antibodies when incubated with ACT for 1h prior to the addition to sheep RBCs.

Fig.19

Nitroblue Tetrazolium Reduction Assay (NBTR assay) of ACT

Rabbit peritoneal neutrophils ($5x10^4$ / well) were incubated with neat (20µg protein / well , row A) and 2- fold dilutions (rows B ---- F) of dialyzed urea extracts of *B. pertussis* and *E. coli* and induced with PMA.

Column 1 = *B. pertussis* BP348 (AC^-)

Column 2 = *B. pertussis* Tohama (AC^+)

Column 3 = *B. pertussis* BP349 (AC^-)

Column 4 = *B. pertussis* BP348 (pRMB1) (AC^{++})

Column 5 = *E. coli* H1469 (pRMB3) (AC +)

Column 6 = *E. coli* H1469 (AC^-)

Column 7 = Control (Cells + PMA)

Column 8 = Control (Cells - PMA)

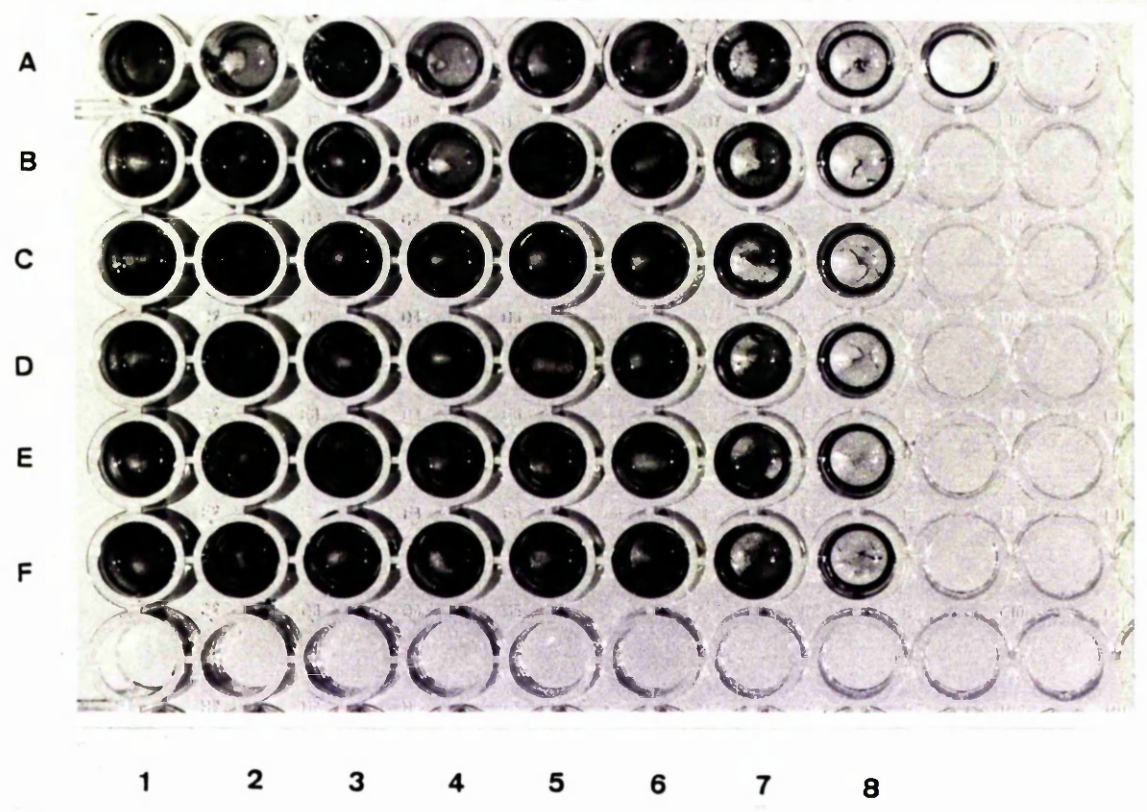

A
B
C
D
E
F
1
2
3
4
5
6
7
8

Fig. 20

Determination of Haemolytic Activity of ACT on Sheep RBCs and its Neutralization by Mono specific anti-AC antibodies (Ms1)

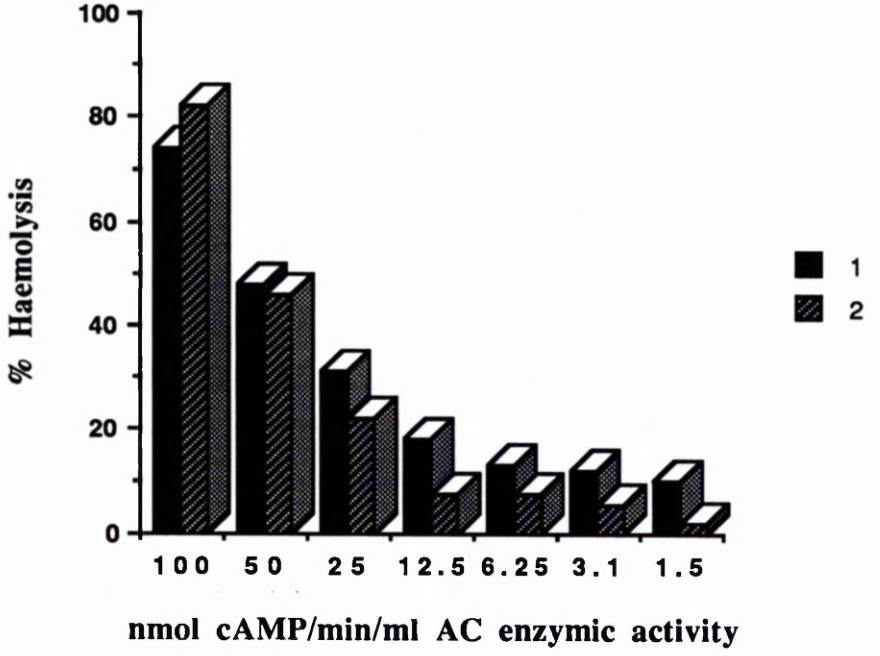
Panel A) Comparison of sheep and rabbit RBCs for their sensitivity towards ACT

(2%) rabbit (1) and sheep (2) RBCs were incubated for 12h at 37 °C with AC of *B. pertussis* BP348 (pRMB1) (100nmol cAMP/min/ml AC enzymic activity and its 2-fold dilutions. The haemolysis was measured on an automated ELISA reader at A_{540}.

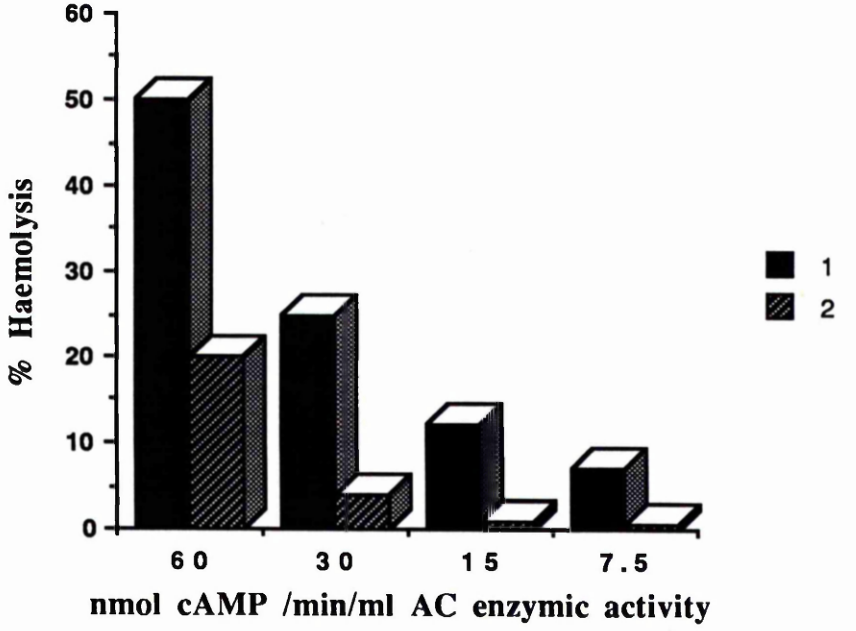
Panel B) Neutralizing effect of Ms1

Calmodulin - affinity purified AC (60 nmol/min / ml AC enzymic activity) , neat and 2 - fold dilutions were incubated with 2% sheep RBCs (1). The same amount of the AC was incubated with anti-AC mono-specific antibodies (1:1) at room temp for 1h and 2% sheep RBCs were added (2). The haemolysis was measured on an automated ELISA reader at A_{540}.

A


100
80
60
40
20
0
% Haemolysis
1
2
100 50 25 12.5 6.25 3.1 1.5
nmol cAMP/min/ml AC enzymic activity

B


60
50
40
30
20
10
0
% Haemolysis
1
2
60 30 15 7.5
nmol cAMP /min/ml AC enzymic activity

Chinese Hamster Ovary Cell Assay (CHO Cell Assay)

Morphological changes of chinese hamster ovary cells induced by ACT were investigated. CHO cells have been used extensively as a bio-assay system for studying a number of toxins, especially the heat - labile enterotoxins (Guerrant *et al.*, 1974) and pertussis toxin (Hewlett *et al.*, 1983). The assay involves the elongation of CHO cells due to increased intracellular cAMP levels (Sandefur and Peterson,1977).

Approximately $5x10^4$ cells were incubated with different concentrations of CaM-affinity purified ACT as detailed under Materials and Methods (see page 61).The effect was evaluated after observing the cells by microscopy and assessed by counting the number of elongated cells /100 cells / dilution. The assay was found to be sensitive enough to detect 12.5 nmol / min / ml AC enzymic activity applied at which 25% cells were found elongated. Below this level an elongation was difficult to detect was considered nill since some of the control cells also showed some elongation-like effect, probably due to squeezing of the cells. The effect at 50 nmol /min/ml AC enzymic activity applied is shown in Fig.21. Higher concentrations of toxin (>200 nmol/min/m1 AC enzymic activity) were found either ineffective or deleterious for the CHO cells and apparently disturbed their growth. The morphological effects were prevented when AC was incubated with anti-AC monospecific antibodies (not shown). This results support the data showing that the morphological changes were caused by an increase in intracellular cAMP. However, the use of this assay to determine the AC toxin activity depended on a rather subjective approach in which the possibility of error seemed to be high.

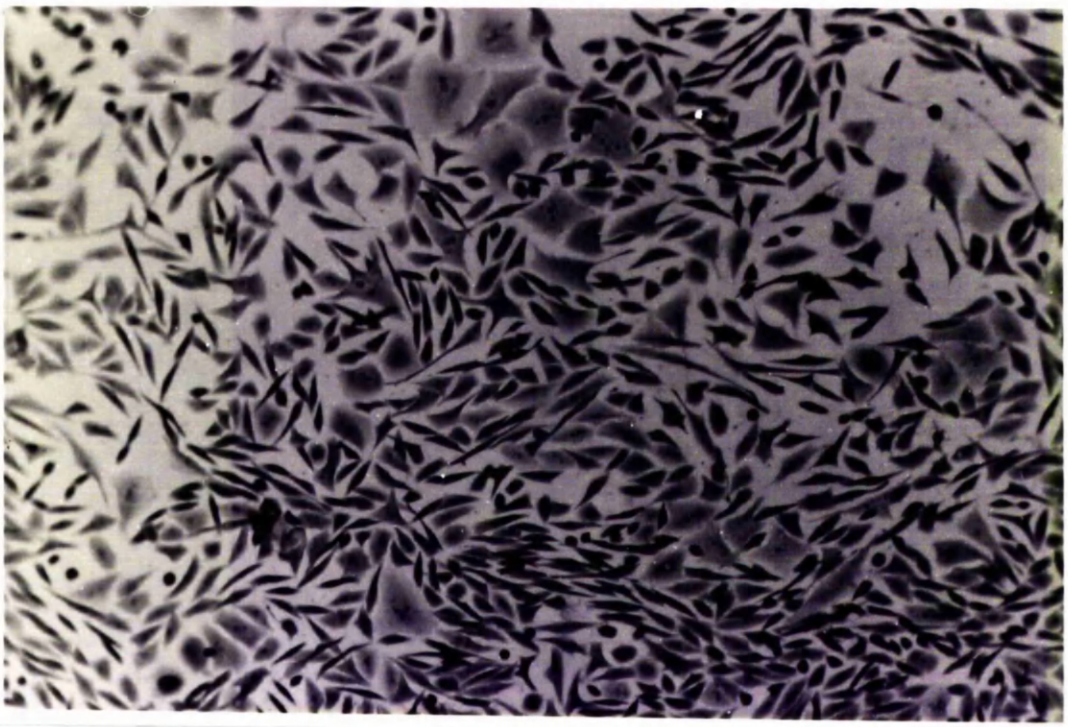
Fig.21 Effect of ACT on CHO Cells.

CHO cells ($5x10^4$ cells/well) were incubated with CaM-affinity purified ACT. 2-fold dilution of 200 nmol /min/ml AC enzymic activity was applied. The cells were incubated in 5% CO_2 at 37°C for 15 -20 h in a humidified chamber.

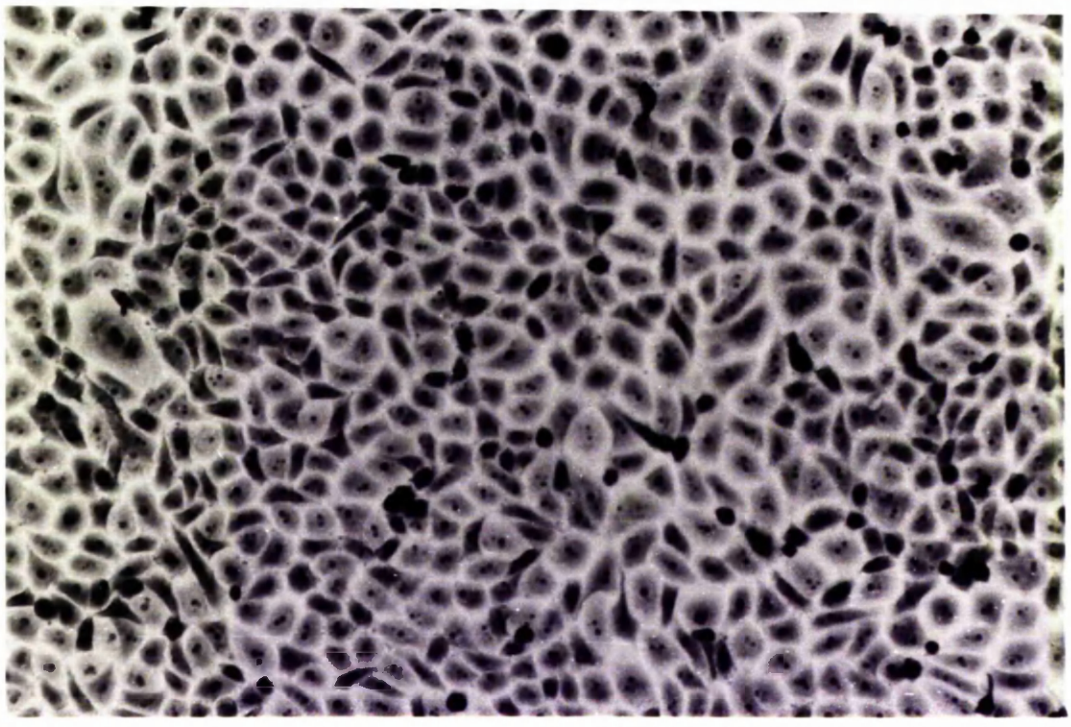
Panel A) The photograph shows CHO cells affected with 50 nmol /min / ml AC enzymic activity

Panel B) Control CHO cells

A



B



Baby Hamster Kidney Cell Assay (BHK Cell Assay)

The effect of crude ACT from *B.pertussis* and *E.coli* was investigated on baby hamster kidney cells (BHK cells) with the collaboration of Dr. John Edwards, Department of Cell Biology, University of Glasgow. BHK cells in presence of high cAMP, tend to change their shape from regular to a complex , dendritic form. The effect of crude ACT (dialyzed urea extracts) on BHK cells was recorded by measuring the mean area and % scattering of 50 randomly - picked affected cells with the help of Leitz ortholux microscope connected to a computer.Fig.22 shows the effect of dialyzed urea extracts *B. pertussis* BP348 (pRMB1) (AC^{++}) and *B. pertussis* BP348 (AC^{-}) on the BHK cells. *B. pertussis* BP348 (pRMB1) showed a positive effect on the cells resulting in the deshaping of the cells, and the effect was approximately comparable to that of the positive control (exogenous dibutyryl cAMP). On the other hand, *B. pertussis* BP348 failed to produce any effect on the cells indicating the specificity of the assay for ACT.

The effects of dialyzed urea extracts from various *B. pertussis* strains and from *E. coli* H1469 (pRMB6,pKIM1) are given in Table 12 .It was observed that the toxicity levels of *B. pertussis* BP348 (pRMB1) (AC^{++}) and *B. pertussis* BP357 (PT^{-}) were comparable to each other, which agrees with their enzymic activity (Table 7, see page 80) . *B. pertussis* BP349 (AC, HLY^{-}) failed to produce any toxic effect. AC expressed in *E. coli* H1469 (pRMB6,pKIM1), a strain harbouring separate plasmids expressing AC and HlyC (*P. vulgaris*) showed an effect comparable to that of *B. bronchiseptica* when three times more protein than that used for *B. bronchiseptica* was applied in the assay.

These data suggest another possible assay for the detection of AC toxic / invasive activity.

Table 12 **Effect of ACT on BHK Cells**

Sample	Protein µg protein / $5x10^4$ cells	Enzymic activity nmol cAMP/min/mg protein	Mean area	% Scattering
HEPES º	-	-	2286	740
cAMP •	-	-	950	300
Urea extracts				
B. pertussis				
BP348 (pRMB1)	75	1450	1240	430
BP348	100	< 0.0 2	1861	603
BP357	100	1267	1330	426
BP349	100	< 0.0 2	2288	746
B. bronchiseptica	100	40	1720	556
E. coli H1469 (pRMB6,pKIM1)	730	270	1700	550

º Negative control

• Dibutyryl cAMP(0.1mM) = positive control.

Fig.22

Effect of ACT on BHK cells.

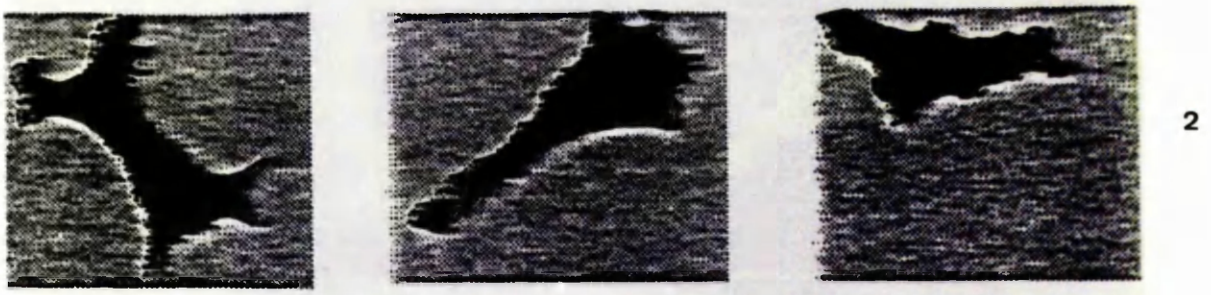
BHK cells ($5x10^4$) were treated with dialyzed urea extracts of *B. pertussis* BP348 (pRMB1) (AC^{++}) and *B. pertussis* BP348 (AC^-). Results were recorded by measuring the mean dispersion and % scattering of the cells in the presence and absence of the toxin. Positive controls (dibutyryl cAMP) and negative controls (HEPES buffer) were also included in the assay.

Rows 1&2= Negative Control (Buffer)

Rows 3&4= 1/20 Dilution of Dialyzed urea extract of *B. pertussis* BP348 (pRMB1)

Rows 5&6= Positive Control

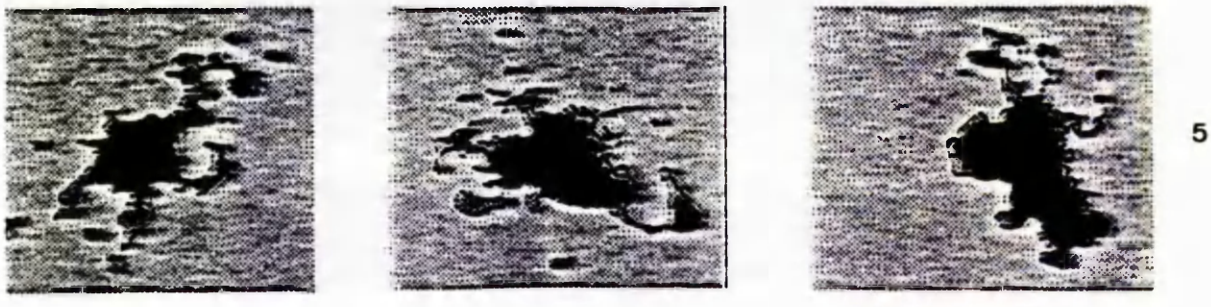
1



2

3

4



5

6

Chemiluminescence Assay

In this study, the chemiluminescence properties of PMNs were exploited with ACT.The rabbit peritoneal neutrophils were selected as target cells as they have been previously used for chemiluminescence assays for pertussis toxin (Craig *et al.*, 1988). Moreover, the rabbit is susceptible to *B. pertussis* aerosol infection and therefore constitutes a relevant model for the disease in humans (Ashworth *et al.*, 1982). To exclude the possible influence of pertussis toxin on the assay of crude ACT, PMA was used as a stimulant (unless otherwise stated) since pertussis toxin does not have an effect on PMA-induced events of the respiratory burst (Becker *et al.*, 1985) . PMA affects the reactions of the respiratory burst initiated via the activation of protein kinase C and increases the cytoskeletal actins in phagocytosis. Pertussis toxin does not have any effect on this sequences of events (Schefcyk *et al.*, 1985; Becker *et al.*, 1985) .

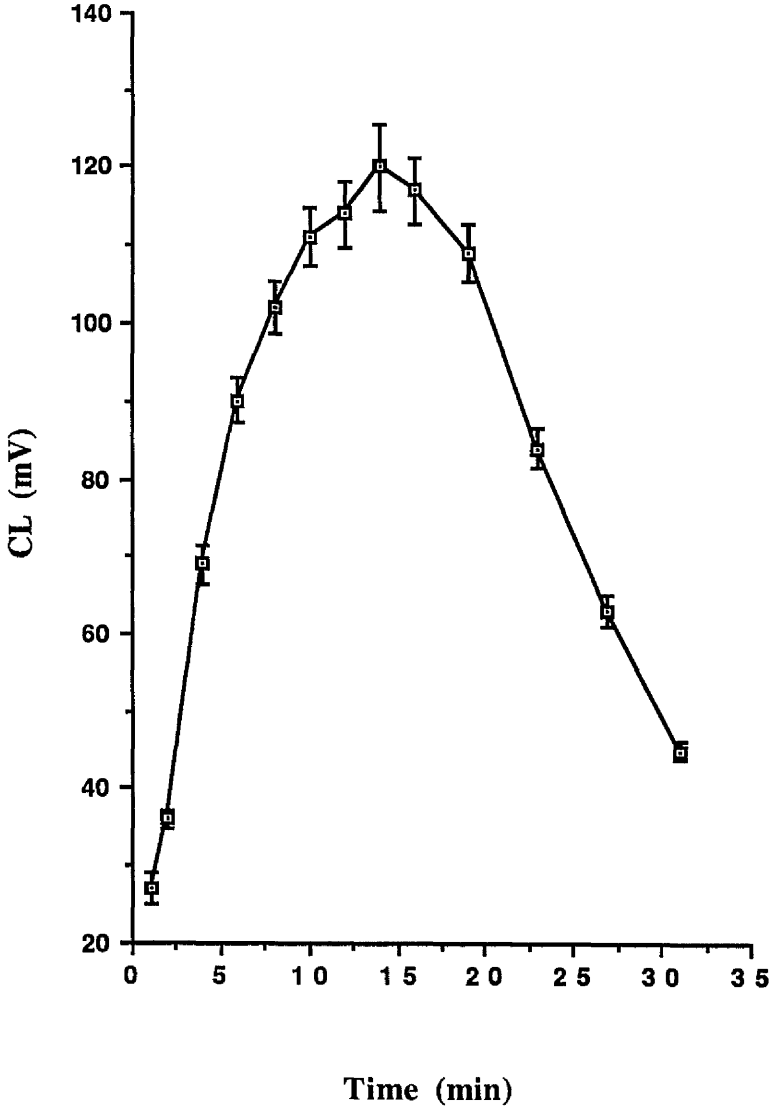
Determination of the PMA-Induced CL Response of Rabbit Peritoneal Neutrophils.

The dose response curve to find the optimal concentraion of PMA needed to stimulate 10^6 PMNs was determined.Various concentrations of PMA ranging from 0.01 to 2.0 μg /ml were used to induce the metabolic burst in the rabbit PMNs. The PMA-induced response was noted to be dose dependent .The increase in CL was very rapid and the peak response occurred at less then 3 min with the higher doses of PMA. At 0.01 μg of PMA/ml, the response was sluggish and the peak CL occurred later, at 30-40 min. An optimal response was observed at a concentration of 0.1 μg/ml (Fig.23) in which the peak CL occurred in 10-15 min. It is noteworthy that no CL response was observed in the absence of DNDH, showing that a detectable PMA response is dependent upon the presence of a suitable substrate. This agrees with the finding of Westrick *et al.* (1980).

Fig. 23

CL Response of Rabbit Neutrophils to PMA.

Rabbit peritoneal neutrophils (10^6 cells / ml of HEPES) were prewarmed at 37 °C for 5 min and then stimulated with 0.1 μg PMA. The figure shown is a mean of four observations and representative of six separate experiments. Each point is a mean and SEM of four observations.


140
120
100
80
60
40
20
CL (mV)
0
5
10
15
20
25
30
35
Time (min)

Influence of Pre-Incubation Time on AC Toxic Activity

For evaluation of AC toxic activity on rabbit PMNs at different time exposures prior to PMA-stimulation, the effect of crude (20 μg protein) and calmodulin -affinity purified ACT (50 nmol/min/ml enzymic activity applied) was investigated. Fig. 24 shows the CL response of PMA-induced rabbit peritoneal neutrophils, after different pre-incubation times at 37 ^{o}C. A 2-fold increase of CL response was observed when cells were treated with crude ACT for 1min, indicating a possible role for other components of *B. pertussis*, like FHA and endotoxins, which are reported to enhance the CL response of rabbit peritoneal neutrophils to fMLP (Craig *et al.*, 1988).With CaM-affinity purified ACT, however, the CL response was inhibited by up to 50% within 1 min. Hewlett *et al.* (1989) also reported a rapid AC toxin activity when CHO cells were treated with ACT at 30 °C; an accumulation of cAMP was observed without any lag period. A reduction in CL response was observed by up to 99% with pure toxin and up to 96% with crude toxin when pre-incubated with neutrophils for 15 min,before the addition of PMA (stimulant) (Fig. 24).

Effect of Purified PT and ACT on CL Response of Rabbit PMNs to PMA or Zymosan.

In order to determine the influence of PT on the CL inhibition response of ACT, purified PT (20μg/10^6 cells) was used to inhibit CL response induced by two different stimuli (PMA, and zymosan). Fig.25 A shows that the PMA - induced response of PMNs was not inhibited by PT (kindly provided by Miss. F Mirza, Department of Microbiology, University of Glasgow) and there was a massive stimulation after 15 min incubation of PT and PMNs. Increasing the pre-incubation time of PT with PMNs to 1h did not show any sign of inhibition but a reduction in stimulation was observed. This result agrees with the findings of Becker *et al.* (1985). On repetition of the same experiment with a particulate stimulus, zymosan, about 98% inhibition of the response was recorded with PT after 15 min pre-incubation, and complete inhibition was observed with 1h incubation with PMNs. PT appeared to be inhibitory with 15 min pre-inubation time (Fig. 25B). When the effect of ACT was compared in the presence of each stimulus, the PMA- induced CL response was markedly inhibited whereas zymosan induced CL response was inhibited by 40-50 % after 15 min pre-incubation.

Fig.24

Influence of Pre-incubation Time on the Effect of Purified and Crude ACT on Neutrophil Chemiluminescence Response to PMA.

Rabbit peritoneal neutrophils were pre-incubated with dialyzed urea extracts or purified AC for 1min and 15 min before the addition of PMA. The figure shown is a mean of four observations and representative of six separate experiments.

Panel A) Effect of crude ACT after 1min and 15 min

1= 1 min

2= Control (No ACT)

3= 15 min

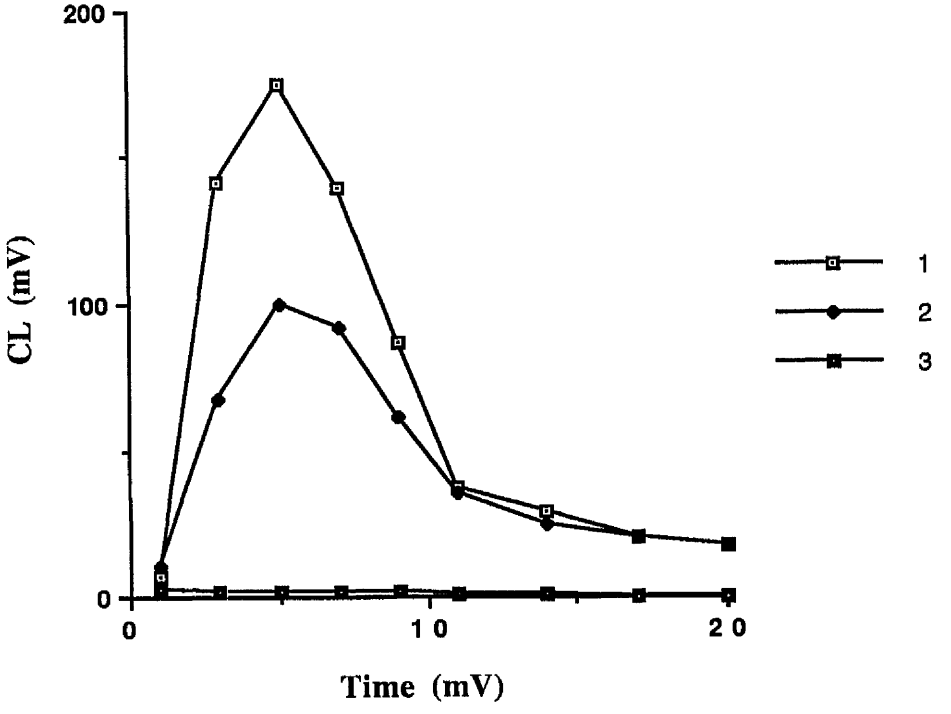
Panel B) Effect of purified ACT

1= 1min

2= Control (No ACT)

3= 15 min

A


200
100
0
CL (mV)
0
10
20
Time (mV)
1
2
3

B

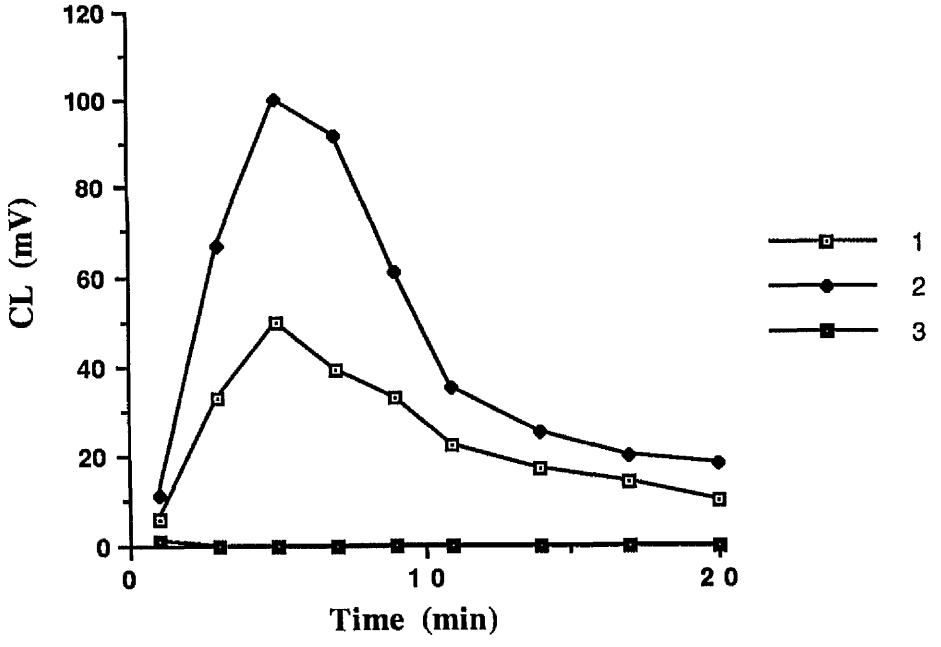

120
100
80
60
40
20
0
CL (mV)
0
10
20
Time (min)
1
2
3

Fig.25

The Effect of PT and ACT on CL Response of PMNs to PMA and Zymosan.

Rabbit peritoneal neutrophils were pre-incubated at 37 °C with purified PT (20 μg/ assay tube) or ACT (50 nmol/min/ml enzymic activity) for 15 min or 1h before the addition of stimuli. Pre-incubation time is given in brackets. The figure shown is a mean of four observations and representative of six separate experiments.

Panel A) Effect of PT and ACT on PMA- induced CL response.

1= Control (No ACT or PT)

2= ACT (15 min)

3= PT (15min)

4= PT (1h)

Panel B) Effect of PT and ACT on zymosan -induced CL response

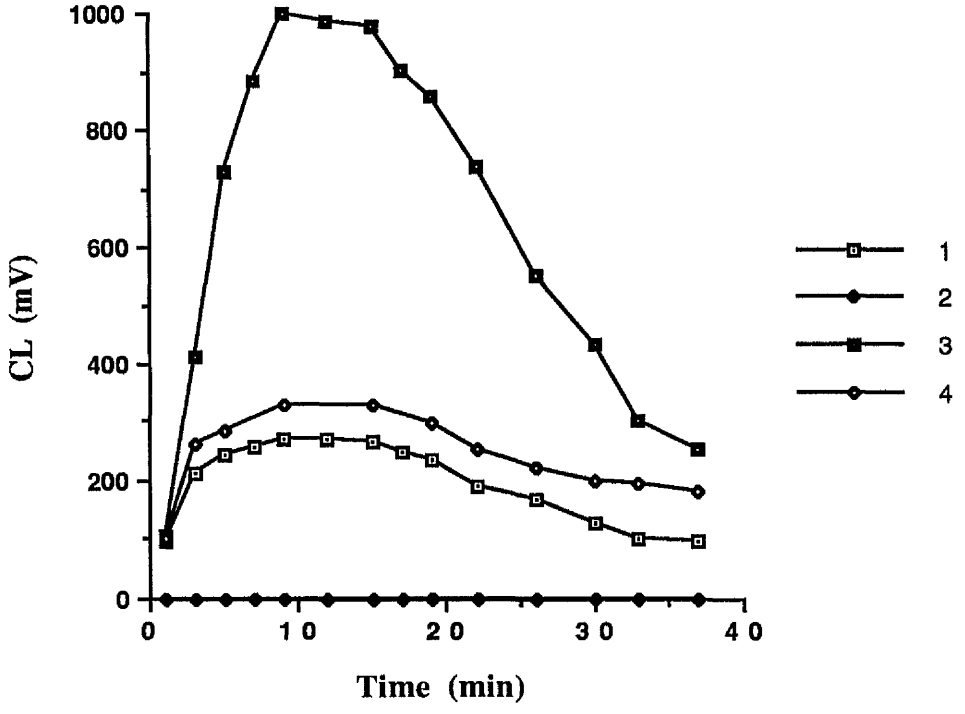
1= ACT (15 min)

2= Control

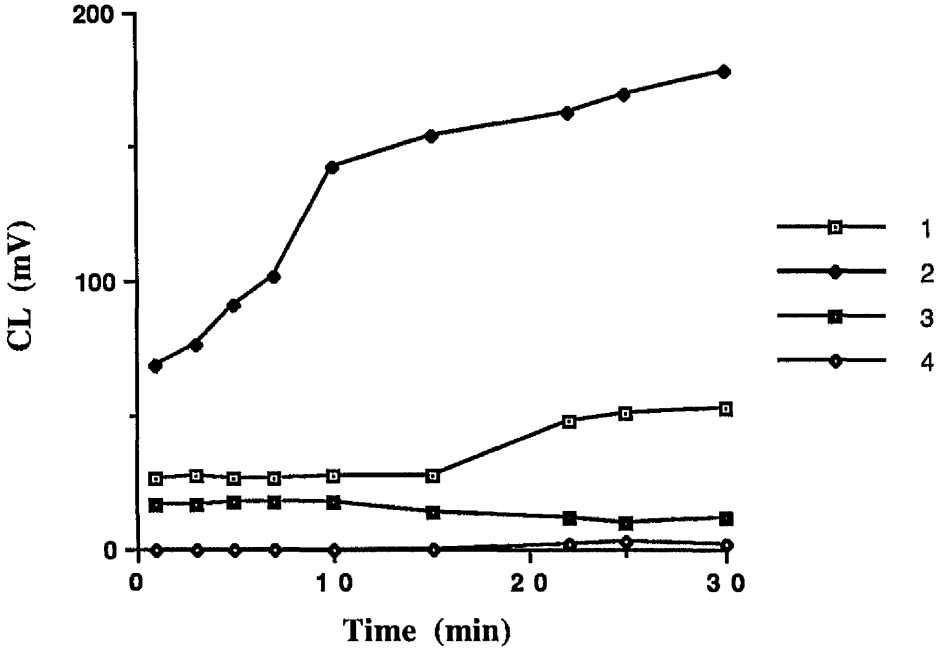
3=PT (15 min)

4= PT (1 h)

A


1000
800
600
400
200
0
CL (mV)
0
10
20
30
40
Time (min)
1
2
3
4

B


200
100
0
CL (mV)
0
10
20
30
Time (min)
1
2
3
4

Effect of Crude ACT from Different Bordetellae on CL Response

The aim of this study was to determine the effects of ACT produced by different Bordetellae under the same set of growth and extraction conditions . Dialyzed urea extracts (20 μg of protein / assay tube) of the strains of wild type *B. pertussis* 18323, *B. pertussis* Tohama, recombinant *B. pertussis* BP348 (pRMB1) (AC^{++}) and transposon insertion mutants *B. pertussis* BP348 (AC^-), *B. pertussis* BP349 (HLY^-), *B. pertussis* BP353 (FHA^-), *B. pertussis* BP357 (PT^-), *B. pertussis* BP347 (Vir -) and other Bordetellae including *B.bronchiseptica* and *B. parapertussis* were tested for their ablity to inhibit the CL response. The results are summarized in Table 13. An extract from the AC^- strain *B. pertussis* BP348 showed a 26% stimulation of CL response compared to the control cells. This possibly indicates the interfering effect of FHA and LPS in the crude preparations. Extracts of *B. pertussis* BP349 (AC^+, HLY^-) showed a 29% stimulation of the CL response. This mutant is reported to have low (7.1nmol cAMP/min/mg protein) AC enzymic activity (Weiss *et al.*, 1983) although this was not confirmed in this study (Table 7, see page 80). No toxicity was observed even with high doses (60 μg protein / assay tube) of extracts of *B. pertussis* BP349 . Extracts of *B. pertussis* BP353, a Tn5 mutant for FHA, exhibited inhibition of CL similar to that of *B. pertussis* Tohama (wild type). Another Tn5 mutant, BP357 (PT^-), showed inhibition of CL similar to BP348 (pRMB1) (AC^{++}) which is reported to have five-fold higher toxic activity than wild type *B. pertussis* (Brownlie *et al.*, 1988). *B. parapertussis* showed a very low level of toxin activity (15% inhibition). This toxicity was however increased when high doses of protein were used. *B bronchiseptica* was found equally as inhibitory as *B. pertussis* 18323.

Table.13
CL Inhibition Effect of Urea Extracts from Different Bordetellae on CL Response of Rabbit PMNs to PMA.

Source of urea extracts	Total CL	Peak	CL (mV) ± SEM * % Inhibition	% Stimulation
Control	1084 ± 32.4	120 ± 3.58	0	100
B. pertussis				
BP348 (pRMBI)	4 ± 0.11	1 ± 0.02	99.6 ± 2.97	-
Tohama	259 ±7.74	38 ± 2.13	76 ± 2.34	-
BP349	1408 ± 33.11	172 ± 5.14	-	129
BP348	1372 ± 36.32	150 ± 4.48	-	126
18323	10 ± 0.29	1 ± 0.03	99 ± 2.9	-
BP357	12 ± 0.35	1 ± 0.04	98 ± 2.9	-
BP353	236 ± 6.65	32 ± 1.21	78 ± 3.3	-
B. bronchiseptica	14 ± 0.41	1 ± 0.03	98 ± 2.9	-
B. parapertussis	920 ± 25.35	89 ± 2.44	15 ± 0.51	

* Individual CL counts were summed over the total assay time and the mean value was obtained for each sample (n=4). Each point is a mean and ± SEM of four observations.The assay was done for 30 min with each vial being counted approximately every 60 sec.The % inhibition or stimulation data relate to the total CL values.

Determination of AC Toxic activity of Live Opsonized *B. pertussis*

The effect of ACT from live, phase I, intact cells of *B. pertussis* was determined. Approximately $5x10^6$ cells of *B. pertussis* BP348 (pRMB1) and *B.pertussis* BP348 were opsonized with normal fresh rabbit serum as described in Materials and Methods (see page 59). The ratio of bacteria to PMN was approx 40 : 1. Fig.26 shows a time-dependent reduction in CL response of neutrophils treated with *B. pertussis* BP348 (pRMB1) (AC^{++}). An initial burst was observed due to interaction of opsonized bacteria and PMNs, but it was reduced by up to 60% -70% within 30-40 min compared to *B. pertussis* BP348 (AC^-) . This result indicates the effect of ACT, which may be exposed at the bacterial surface *or* the ingestion of the virulent, AC^+ *B. pertussis* by PMNs.

Determination of ID_{50} Dose of Crude ACT and Dose Response Curve for Purified ACT.

Crude and CaM-affinity purified ACT were used to determine the dose responsible for 50% inhibition of PMA-induced CL response of rabbit neutrophils (ID_{50}), and to create a dose response curve. Dialyzed urea extract (20 μg protein) and its dilutions and purified ACT (50 nmol cAMP/min/ml AC enzymic activity) and its 10-fold dilutions were pre-incubated with 10^6 PMNs for 15 min at 37 ^oC. Fig.27 and Table 14 shows a 49% inhibition of PMA-induced CL response of PMNs at the dose of 3.3 μg protein of crude ACT from *B. pertussis* BP348 (pRMB1).When CaM-affinity purified ACT of the same strain was used, an interesting response was observed as demonstrated in Fig.27 &Table 15. The results indicate a three stage toxic effect on PMNs . At a concentration of 50 nmol cAMP /min/ml enzymic activity dose, the ACT showed 99% inhibition of CL response.A 64% inhibition was observed at a dilution of 10^{-1} and a small inhibitory effect (5%) was seen with 10^{-2}.The dilutions 10^{-3} -10^{-5} showed a slight stimulation and when the dilution shifted from 10^{-5} -10^{-6}, a massive stimulation rather than an inhibition of the CL response was observed. This stimulation of the oxidative burst could be attributed to the action of ACT at sub-toxic doses.It is been reported that human neutrophils treated with sub-lytic doses (picograms) of *E. coli* alpha-haemolysin created a 14-fold increase in superoxide generation (Keane *et al.*,1987). Bhakdi and Martin (1991) have very recently reported the stimulatory effect of alpha-haemolysin of *E. coli* . Since ACT exhibits 25% homology with *E. coli* alpha-haemolysin, (Glaser *et al.*,1988b) and is a member of the RTX family (Welch,1991),the stimulatory effect at low doses might be anticipitaed.

Fig.26

Determination of Neutrophil Chemiluminescence Response to Live *B. pertussis*

Rabbit peritoneal neutrophils were treated with opsonized , live , *B. pertussis* BP348 (pRMB1) or BP348. The approximate ratio of bacteria to neutrophils was 40:1. The figure shown is a mean of four observations .

1= *B. pertussis* BP348 (pRMB1) (AC^{++})

2= *B. pertussis* BP348 (AC^{-})

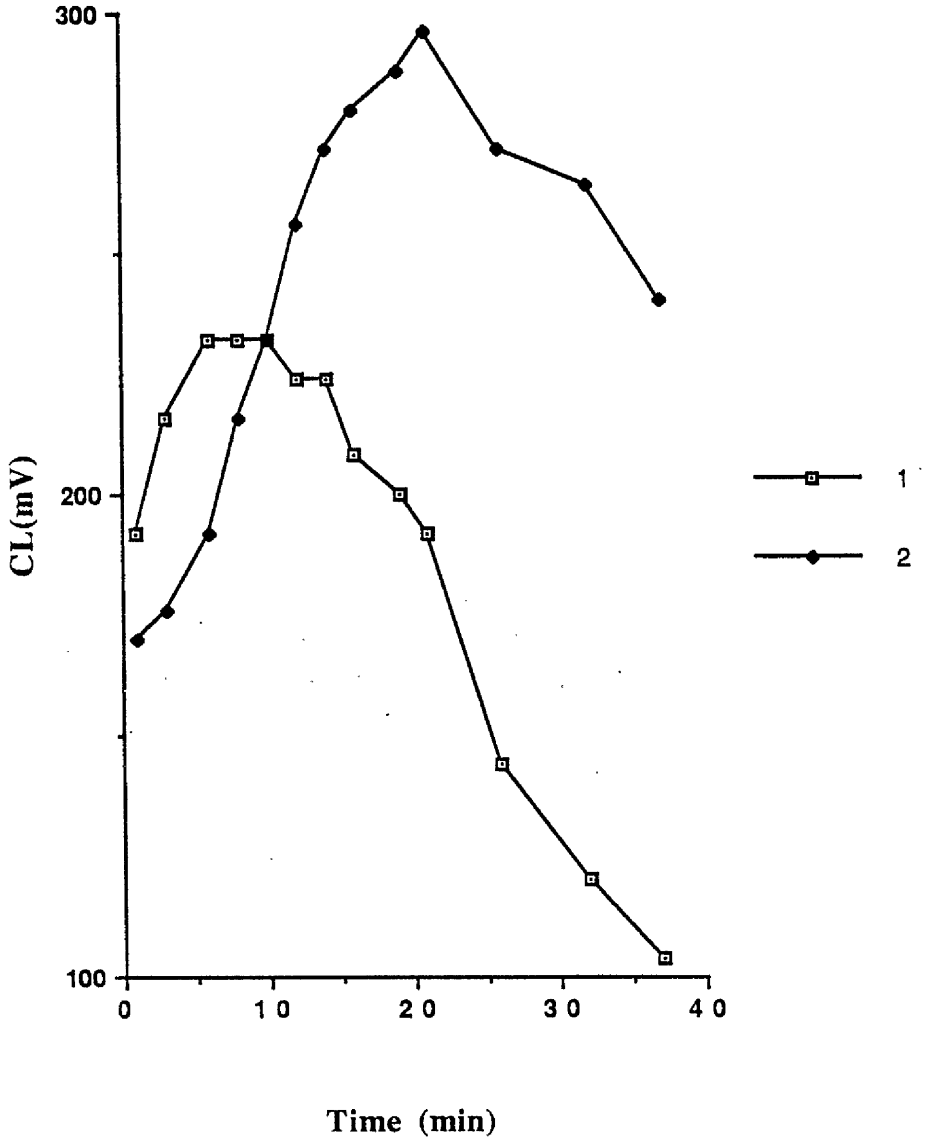

300
200
100
CL(mV)
0
10
20
30
40
Time (min)
1
2

Table 14

Determination of 50% Inhibition Dose for Crude ACT

Dilution	Total CL	CL(mV) ± SEM* Peak	%inhibition	Protein(μg)
Control	2305 ± 46.44	210 ± 4.22	0	-
1:100	231 ± 4.65	34 ± 0.68	89 ± 1.81	20
1:200	528 ± 10.63	66 ± 1.32	77 ± 1.55	10
1:300	687 ± 13.84	82 ± 1.65	70 ± 1.41	6.6
1:400	755 ± 15.12	88 ± 1.77	67 ± 1.34	5
1:500	975 ± 23.65	102 ± 2.05	57 ± 1.15	4
1:600	1174 ± 23.64	120 ± 2.14	49 ± 0.99	3.3

* Derived as in Table 13 ; n = 4

Table 15

Dose Response Curve for Purified ACT Activity.

Dilution	Total CL	Peak	CL (mV) ± SEM * % inhibition	% stimulation	Dose•
Control	2221 ± 69.37	256 ± 7.98	0	100	0
Neat	15 ± 2.34	8 ± 0.24	99.62	-	50
10^{-1}	790 ± 24.64	114 ± 3.41	64.32	-	5
10^{-2}	2106 ± 65.68	248 ± 7.44	5.1	-	0.5
10^{-3}	2265 ± 70.64	264 ± 7.92	0	101.98	0.05
10^{-4}	2315 ± 72.20	256 ± 7.62	0	104.23	0.005
10^{-5}	2449 ± 76.37	262 ± 7.86	0	110.26	0.0005
10^{-6}	4612 ± 143.82	589 ± 17.67	0	207.65	0.00005

* Derived as in Table 13 ; n = 4.

• nmol cAMP/min/ml AC enzymic activity/ vial .

Fig.27

Panel A) Determination of ID_{50} for Crude ACT.

Rabbit peritoneal neutrophils were pre-incubated with neat (20 µg protein / assay tube) and different dilutions of dialyzed urea extract of *B. pertussis* BP348 (pRMB1) at 37 °C for 15 min before the addition of PMA.
The individual counts of CL over 30 min were summed and % inhibition of the control by each dilution was plotted.
The figure shown is a mean of four observations and representative of six experiments.

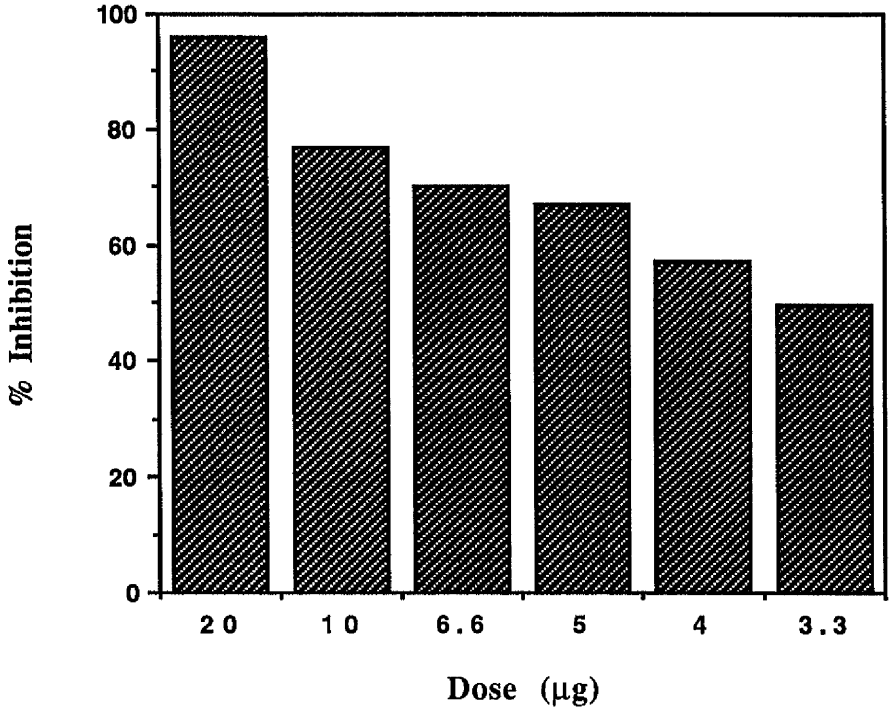
Panel B) Dose response curve

Rabbit peritoneal neutrophils were pre-incubated with neat (50 nmol cAMP /min/ml enzymic activity) and 10-fold dilution of CaM-affinity purified ACT of *B. pertussis* BP348 (pRMB1) at 37°C for 15 min before the addition of PMA.
The individual counts of CL over 30 min were summed and % inhibition of the control by each dilution was plotted.
The figure shown is a mean of four observations and representative of six experiments.

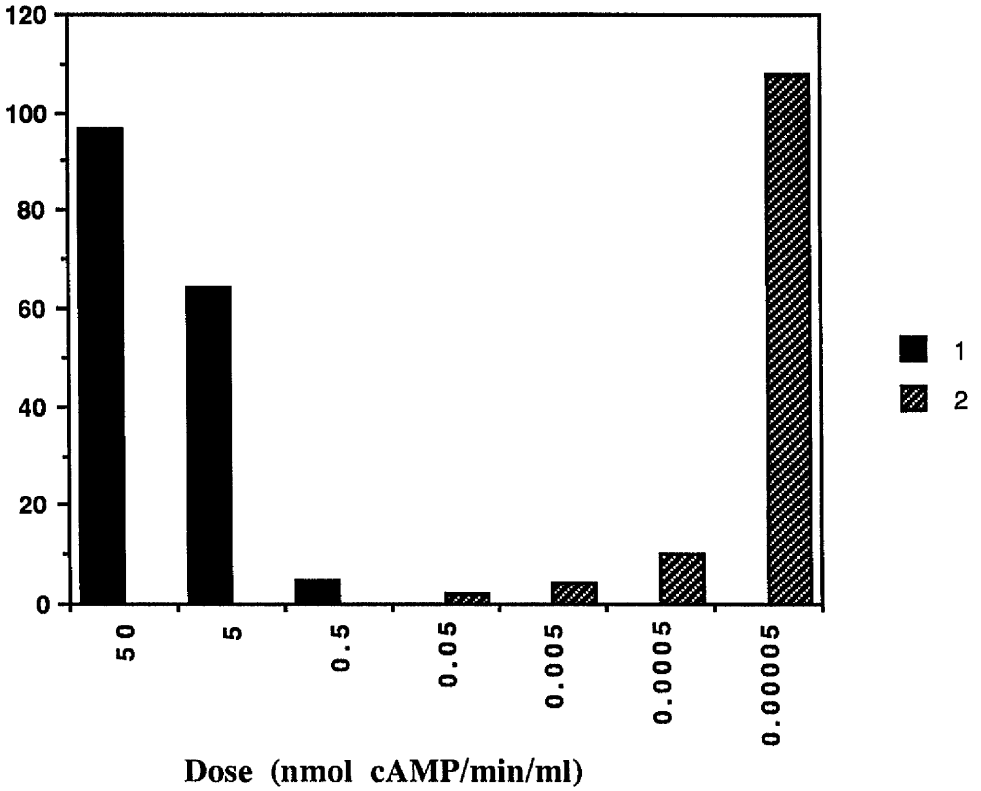
1=% Inhibition

2=% Stimulation

A


100
80
60
40
20
0
% Inhibition
20
10
6.6
5
4
3.3
Dose (μg)

B


120
100
80
60
40
20
0
50
5
0.5
0.05
0.005
0.0005
0.00005
Dose (nmol cAMP/min/ml)
1
2

Effect of Exogenous Calcium on the CL Inhibition Effect of ACT

The influence of exogenous calcium on CL inhibition due to ACT was investigated by CL assay on rabbit peritoneal neutrophils. The calcium concentration required for maximum ACT activity was evaluated by treating the calcium- deprived PMNs with known amounts of exogenous calcium (mM) as detailed under Materials and Methods (see page 60). Chelation of calcium in the presence of 0.8 mM EDTA and EGTA caused 95% reduction in CL inhibition effect of ACT toxicity . An interesting response of AC towards calcium was that the effect on ACT was not linear with increasing concentrations of calcium but was bi -modal i.e; the exposure of cells to 20 μg of AC in presence of 0.1 and 0.3 mM calcium did not show a significanct effect on CL response but as soon as the concentration of calcium was shifted from 0.3 to 1 mM, a rapid shift in toxicity was observed (Table16). On the other hand, the CL produced in PMNs in response to PMA rose with an increase in the calcium concentration . This result agrees with the findings of Janah *et al.* (1990) who reported a higher CL response in 1-2 mM calcium as compared to 0.1-0.5 mM calcium . Fig .28 and Table 17 summarizes the influence of calcium on the toxicity of AC. These findings correlate with the reports of Hewlett *et al.* (1990, 1991), who observed a conformational change in ACT at low levels of calcium (1 - 10 μM) and half maximal toxicity was observed at 500 μM. According to the data presented here, extracellular calcium is absolutely required for toxic activity of ACT and toxicity is blocked in the presence of calcium chelators in the medium. The minimal concentration of calcium to achieve 90% inhibition of PMA- induced CL response of 10^6 rabbit peritoneal neutrophils by crude ACT is 3 mM.

Table 16

Influence of Calcium on Chemiluminesence Inhibition Effect of ACT

		CL(mV) ± SEM*	
Sample	**Ca++(mM)**	**Peak value**	**Total**
Buffer	0.1	78 ± 3.22	565 ± 15.56
ACT	0.1	72 ± 3.10	528 ± 12.88
Buffer	0.3	76 ± 3.16	560 ± 18.61
ACT	0.3	60 ± 2.91	485 ± 16.25
Buffer	1.0	88 ± 21.34	686 ± 19.11
ACT	1.0	18 ± 2.23	148 ± 7.42
Buffer	3.0	120 ± 3.12	734 ± 18.23
ACT	3.0	4 ± 2.14	58 ± 4.83
Buffer	10.0	284 ± 9.32	2030 ± 41.67
ACT	10.0	4 ± 1.4	36 ± 5.72

* Derived as in Table 13 ; n = 4. .

Fig.28

Influence of Calcium on the CL Inhibition Effect of ACT.

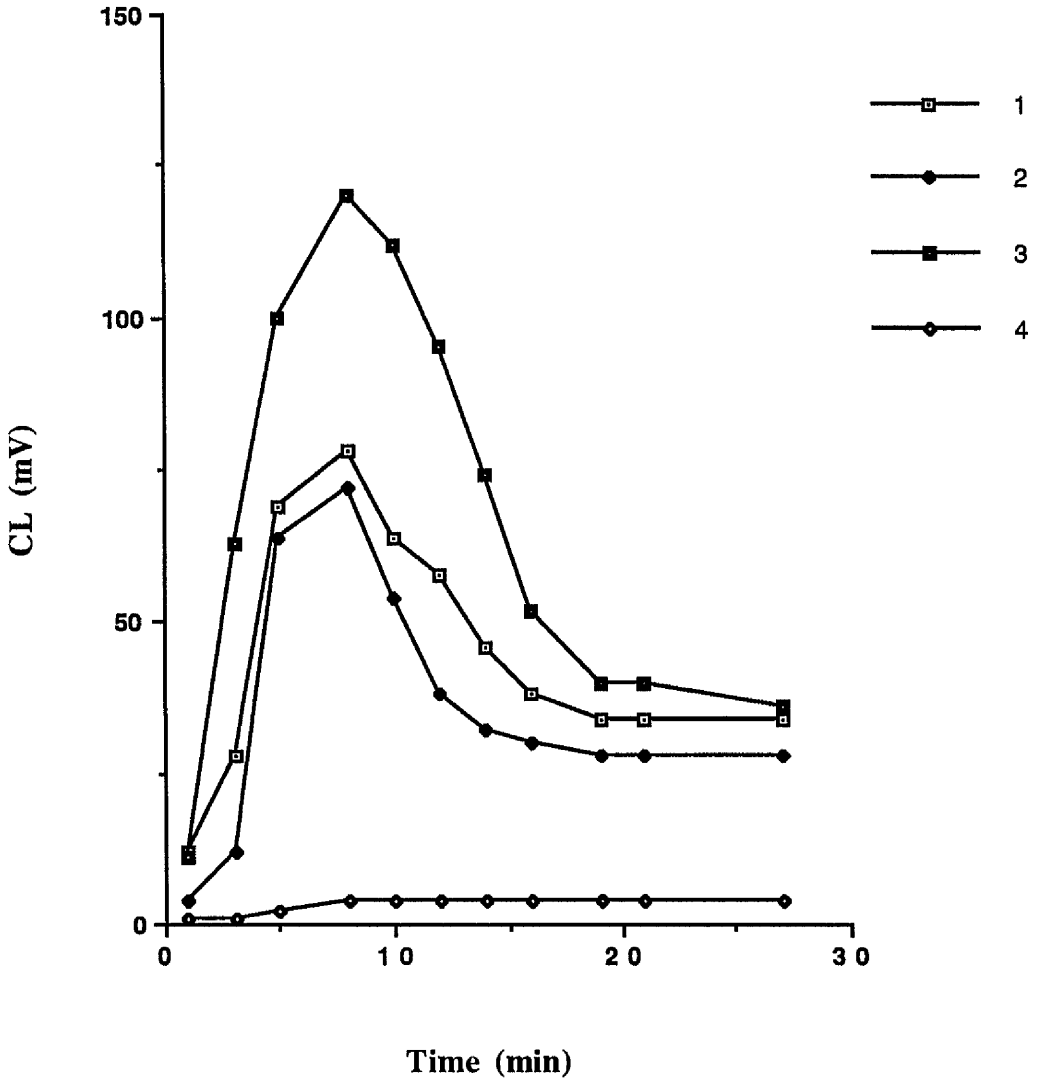
Rabbit peritoneal neutrophils were pre-incubated with dialyzed urea extracts of *B. pertussis* BP348 (pRMB1) (20 µg protein / 10^6 cells) in presence of different concentrations of calcium for 15 min at 37 °C before the addition of PMA.The figure shown is the mean of 4 observations .

1= 0.1 mM calcium

2= 0.1 mM calcium + ACT

3= 3 mM calcium

4= 3 mM calcium + ACT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50
0
0
10
20
30
CL (mV)
Time (min)
1
2
3
4

Influence of Calmodulin on the CL Inhibition Effect of ACT.

The effect of exogenous calmodulin (CaM) on the toxicity of ACT was determined by CL assay. Exogenous CaM at μM concentrations has been reported to inhibit the cAMP response of cells to ACT (Rogel *et al.*, 1988). Crude ACT (dialyzed urea extract (6 μg protein/ assay tube) of *B. pertussis* BP348 (pRMB1) was pre-incubated with 3 μM bovine brain CaM at room temp for 1h. The controls of CaM with buffer and ACT with buffer were also incubated at room temp for 1h.As shown in Fig.29, a 50% reduction in CL inhibition activity of ACT was observed, suggesting a role for calmodulin in conformational changes of ACT which consequently change its toxic activity. These data agree with the results of Shattuck and Storm (1985) who demonstrated the inhibition of cAMP accumulation in target cells when ACT (partially purified) was treated with CaM .

Neutralization of AC Toxic Activity by Human Convalescent Pertussis Antiserum and Anti-AC Monospecific Antibodies.

Neutralization of biological activity of ACT in the presence of human covalescent pertussis antiserum HS2 and anti-AC monospecific antiserum Ms1was investigated. Cude ACT (dialyzed urea extract of *B. pertussis* BP348 (pRMB1)) was preincubated with each antiserum at room temp for 1h in 1:1 ratio. Controls of ACT and buffer and antisera and buffer+ ACT were also included for incubation . The results (Fig .30) show a 50-60 % reduction in the CL inhibition effect of ACT in PMA-induced rabbit neutrophils. This result contradicts the report of Farfel and Hanski (1990) who reported the inability of post- infection antisera to neutralize the enzymic or toxic activities of AC. However the findings coincide with with those of Brezin *et al.* (1987) and Guiso *et al.* (1989) who reported the neutralization effect of anti-AC antibodies against the toxic and enzymic activity of ACT.

Fig.29

The Effect of Extracellular Calmodulin on CL Inhibition Effect of ACT

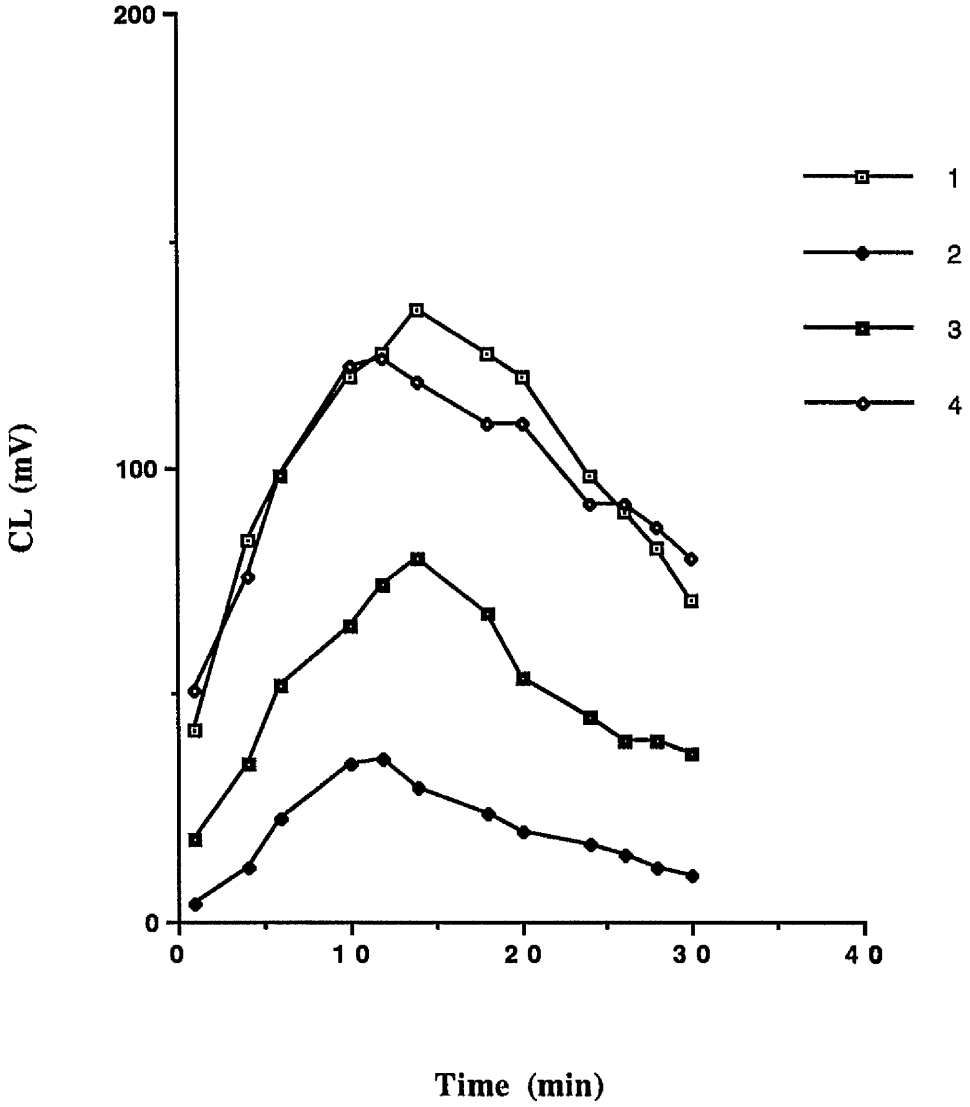
Bovine brain calmodulin (3 mM) was incubated with crude ACT (dialyzed urea extract of *B. pertussis* BP348 (pRMB1), 6 μg protein) for 1h at room temp . Rabbit peritoneal neutrophils were pre-incubated with CaM-treated and untreated ACT for 15 min at 37 ^{o}C before the addition of PMA.The figure shown is the mean of four observations .

1= Control (no ACT or CaM)

2= Untreated ACT

3= CaM - treated ACT

4= CaM only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0
CL (mV)
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10
20
30
40
Time (min)
1
2
3
4

Fig.30

Neutralization of Toxin Activities of ACT in Presence of Human Convalescent Pertussis Antiserum and Anti-AC Monospecific Antibodies (Ms1)

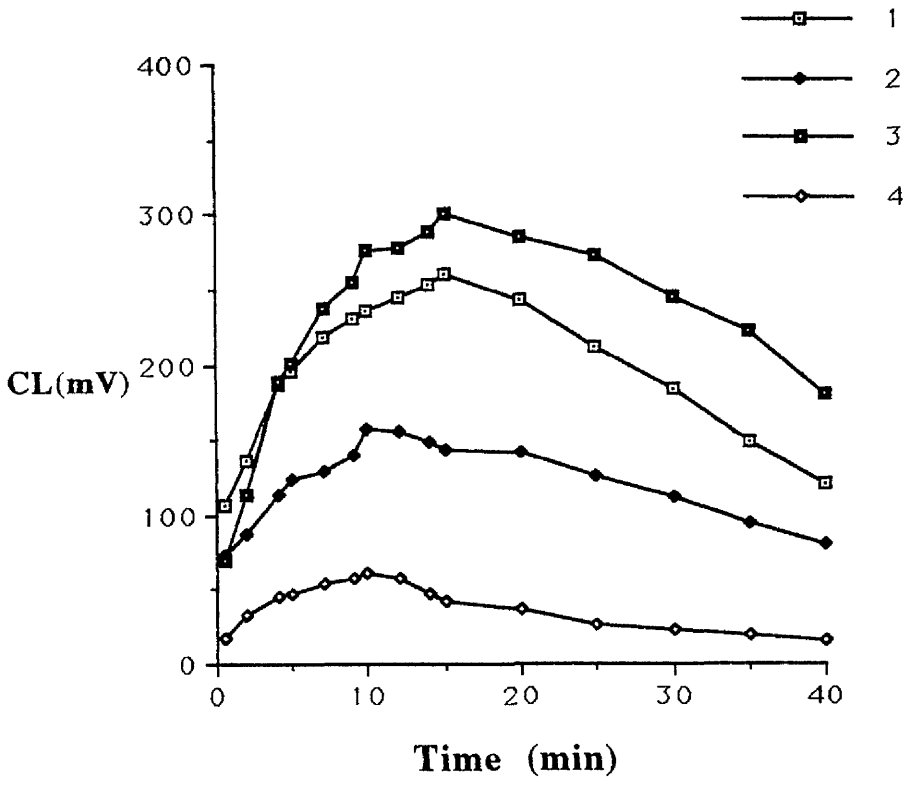
Dialyzed urea extract of *B. pertussis* BP348 (pRMB1) (4 μg protein) was incubated with human convalescent pertussis antiserum HS2 or anti-AC monospecific antibodies Ms1 (1:1) at room temp for 1h . Rabbit peritoneal neutrophils were then added and incubated for 15 min at 37°C. Controls consisted of antisera alone and ACT. The neutrophils were stimulated with PMA.

Panel A) Neutralizing activity of human convalescent pertussis antiserum HS2

1= Control (PMA only)
2= ACT + human convalescent pertussis antiserum
3= Human convalescent pertussis antiserum only
4= ACT

Panel B) Neutralizing activity of anti-AC monospecific antiserum Ms1

1= Anti-AC monospecific antiserum only
2= ACT + anti-AC monospecific antiserum
3= Control
4= ACT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CL(mV)
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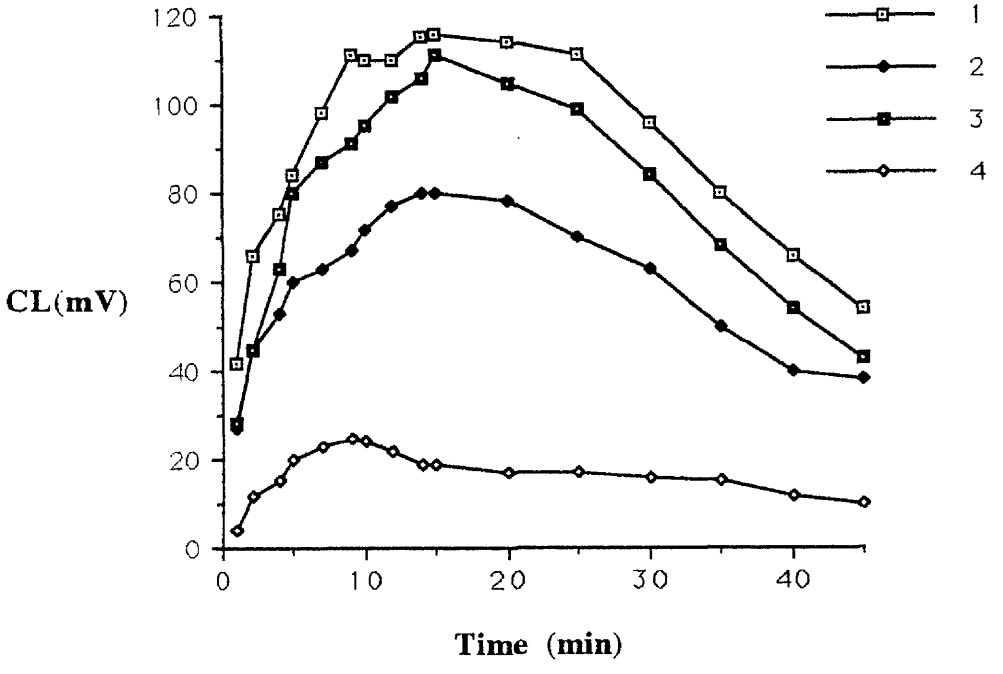
10
20
30
40
Time (min)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2
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40
20
0
CL(mV)
0
10
20
30
40
Time (min)

Effect of Cloned *B. pertussis* AC on PMA- Induced CL Response of Rabbit Peritoneal Neutrophils.

Strains of *E. coli* harbouring single plasmids which expressed the *cyaA* gene under the control of the *tac* promoter and compatible plasmids which expressed the *cyaA* gene and the *hlyC* gene of either *P. vulgaris* or *E. coli* or the *cyaC* gene of *B. pertussis* were constructed by Dr. G. Westrop. The relevant characteristics of these constructs are summarized in Table 4 (see page 47).

Urea extracts of these strains were prepared as described in Materials and Methods (see page 44) and examined for the presence of AC by immunoblotting (see page 77). The activation of *cyaA* in *E. coli* was determined by CL assay as described in Materials and Methods (see page 59). Rabbit neutrophils were incubated with dialyzed urea extracts and stimulated with PMA. Fig. 31 indicates a 98% inhibition of CL response by *B. pertussis* BP348 (pRMB1) and a stimulation rather than inhibition of CL response with all the *E. coli* extracts. The result shows that the stimulation was independent of *cyaA* gene expression since one of the controls (*E. coli* H1469 pRMB7) showed the same level of stimulation. This suggests that the stimulation may be caused by a contaminating product of *E. coli* , possibly, LPS . It has been reported that LPS enhances the CL response of PMNs (Guthrie *et al.*, 1984; Aida and Pabst ,1991). Before other *E. coli* extracts were tested, it was decided to try to remove the stimulatory component.

Fig. 31
Effect of Cloned AC on CL Response

Rabbit peritoneal neutrophils were pre-incubated with dialysed urea extracts of *E. coli* and *B. pertussis* strains for 15 min before the addition of PMA. The Fig. shown is the mean of four observations and is representative of six experiments.

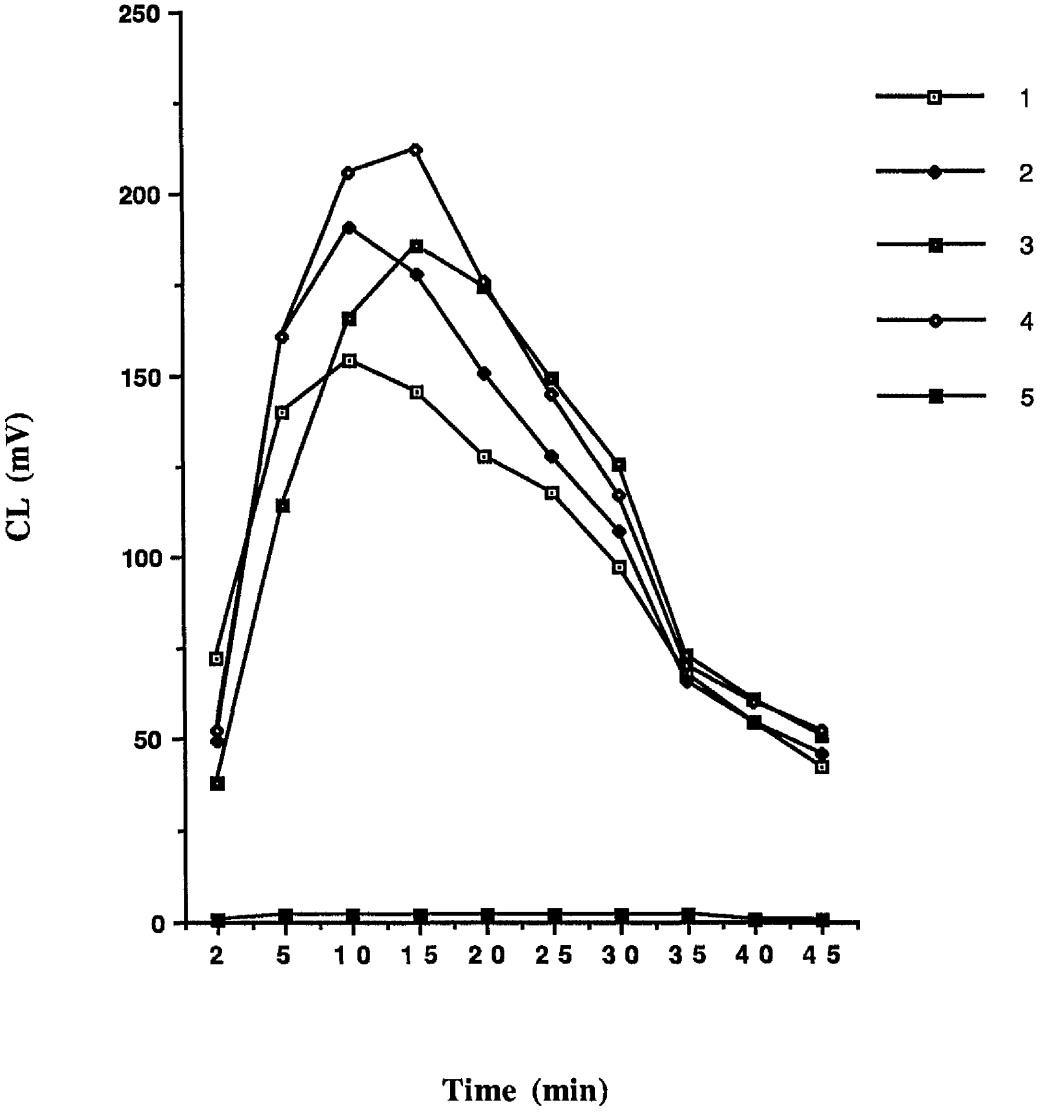
1= Control

2= *E. coli* H1469 (pRMB6)

3= *E. coli* H1469 (pRMB6, pKIM1)

4= *E. coli* H1469 (pRMB7)

5= *B. pertussis* BP348 (pRMB1)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200
150
100
50
0
CL (mV)
2 5 10 15 20 25 30 35 40 45
Time (min)
1
2
3
4
5

Removal of LPS by Detoxi-Gel Chromatography .

In order to investigate whether LPS enhances the PMA induced CL response of rabbit peritoneal neutrophils, the effect of commercially available LPS from *E. coli* was determined. The control PMNs were induced with PMA. When the CL response of PMA- induced PMNs in presence of LPS was compared with that of CL response of PMNs induced with PMA only, a 5- fold stimulation was observed in the response of cells treated with LPS (Fig. 32 A). These data indicate that the enhancement of PMA induced response of PMNs observed in the previous experiments may well have been due to LPS in the urea extracts of the *E. coli* strains.

Attempts were made to remove the LPS using a commercially available Detoxi-Gel chromatography system (Pierce,USA). A sample containing 20 μgLPS/ml was subjected to the gel as per manufacturer's instructions. The eluate was used to determine the removal of LPS from the sample . CL assay was performed with Detoxi Gel-treated and untreated LPS. Fig. 32 B shows the comparison of the effects and indicates that this method removed 98% stimulatory activity. The same method was applied on the urea extracts of *E. coli* strains. Dialyzed urea extracts from *E. coli* were passed through Detoxi-Gel and the effect was measured by CL assay. The comparative CL effect of Detoxi- Gel treated and untreated extracts is shown in Fig.33 A. The data indicate that no inhibition of PMA - induced CL response was observed with the extract , although the stimulatory effect was reduced by 98 %. This suggested that either the *hlyC* gene product from *P. vulgaris* was incapable of activation of ACT or the activity may have been lost during the purification steps. The second possibility was explored. Dialysed urea extract of *B. pertussis* 18323 was subjected to Detoxi-Gel and the eluate was collected. The effect of this eluate was determined by CL assay . Fig. 33 B shows the Detoxi-Gel -treated and untreated urea extracts of *B. pertussis* 18323. The result indicates that a 50% loss of AC toxic activity occured during purification with Detoxi-Gel treatment so presumably the ACT becomes bound to the column.

Fig.32

Determination of Stimulatory Effect of LPS on PMA - Induced CL Response of Neutrophils.

A) Effect of LPS on CL response of rabbit peritoneal neutrophils.
Commercally available LPS (20 μg) from *E. coli* (Sigma) was incubated with rabbit peritoneal neutrophils for 15 min at 37 °C before the addition of PMA. LPS was not added to the control.The figure shown is a mean of four observations .

1= Control

2= LPS

B) Removal of LPS effect by Detoxi-Gel .

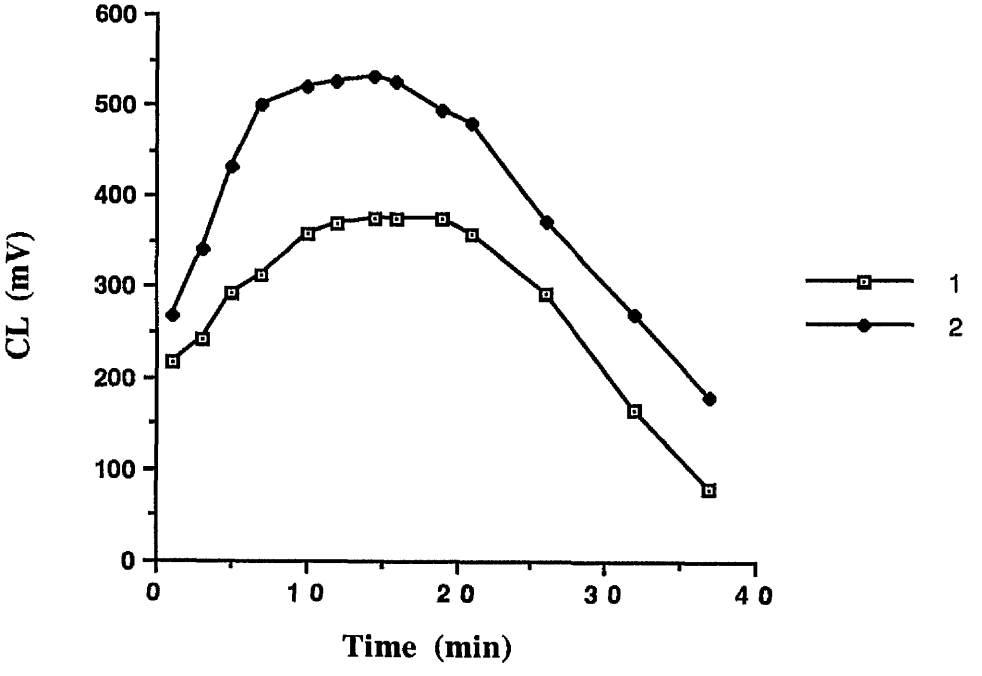
A sample containing 20 μg of commercially available LPS from *E. coli* (Sigma) was passed through the Detoxi-Gel system. The eluate was incubated with rabbit peritoneal neutrophils for 15 min at 37 °C before the addition of PMA. LPS was not added to the control. Untreated LPS was also included to compare the effect of Detoxi-Gel treatment. The figure is a mean of four observations .

1= Control

2= Detoxi-Gel treated LPS.

3= LPS only

A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Time (min)
1
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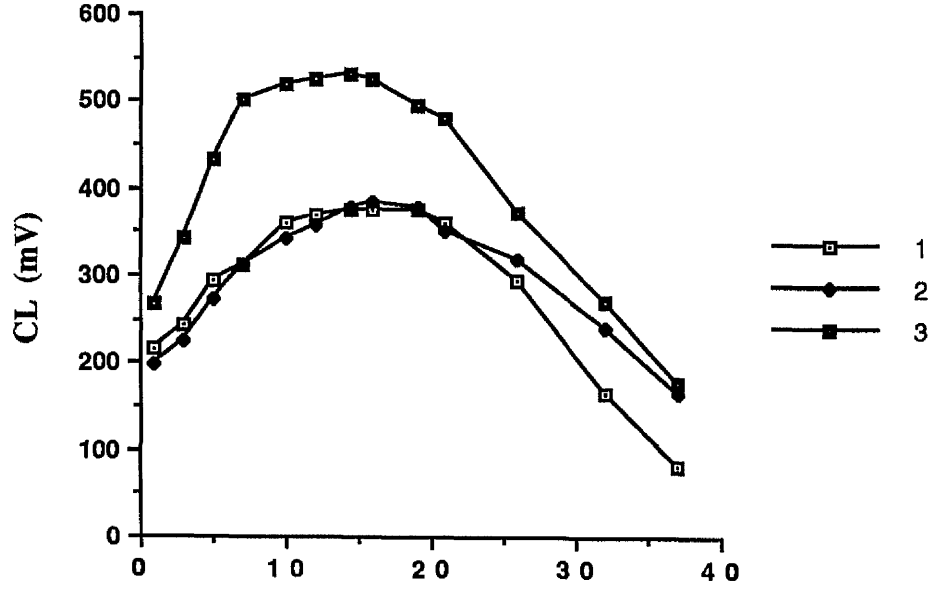
B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CL (mV)
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Fig.33

A) Removal of Stimulatory Activity from *E. coli* Urea Extract by Detoxi-Gel Chromatography.

Dialyzed urea extract (20 μg protein) of *E. coli* H1469 (pRMB6, pKIM1) was passed through the Detoxi-Gel and the eluate was collected. Rabbit peritoneal neutrophils were incubated with this eluate at 37 °C for 15 min prior to the addition of PMA. The figure shown is a mean of four observations .

1= Control

2= *E. coli* H1469 (pRMB6, pKIM1) , Detoxi-Gel -treated

3= *E. coli* H1469 (pRMB6, pKIM1), Detoxi-Gel -untreated

B) Effect of Detoxi-Gel treatment on the extract of *B. pertussis* 18323

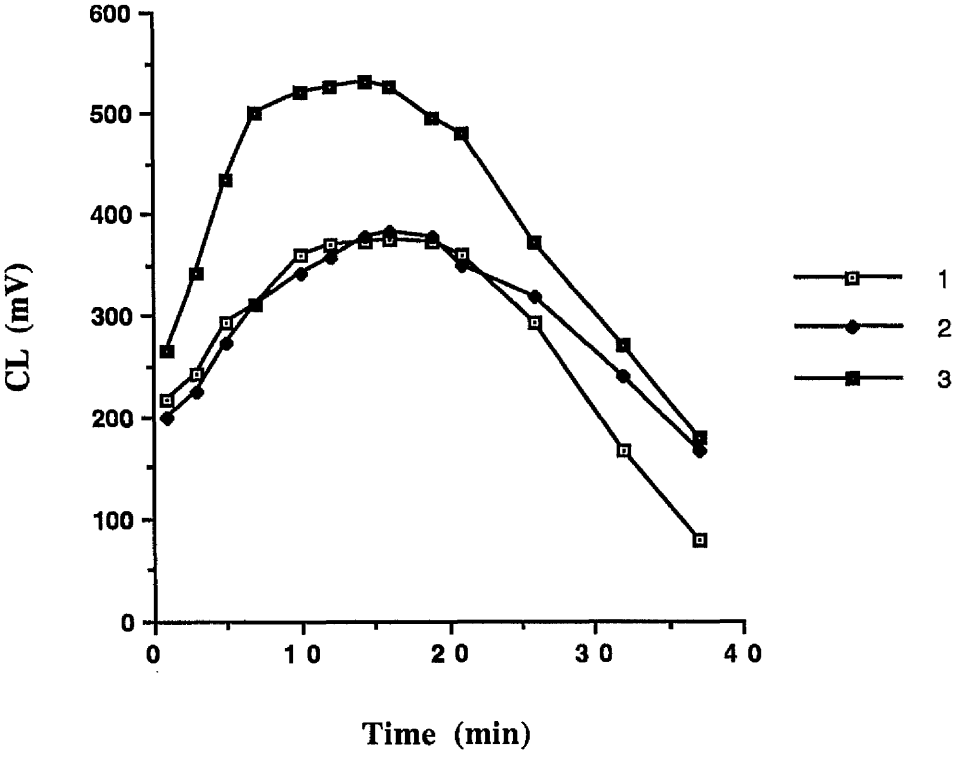
Dialyzed urea extracts of *B. pertussis* 18323 (20 μg protein) was passed through the Detoxi- Gel and the eluate was collected. Rabbit peritoneal neutrophils were incubated with this eluate at 37 °C for 15 min prior the addition of PMA. The figure is a mean of four observations.

1= Control

2= *B. pertussis* 18323, Detoxi-Gel-treated

3= *B. pertussis* 18323, Detoxi-Gel -untreated.

A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Time (min)
1
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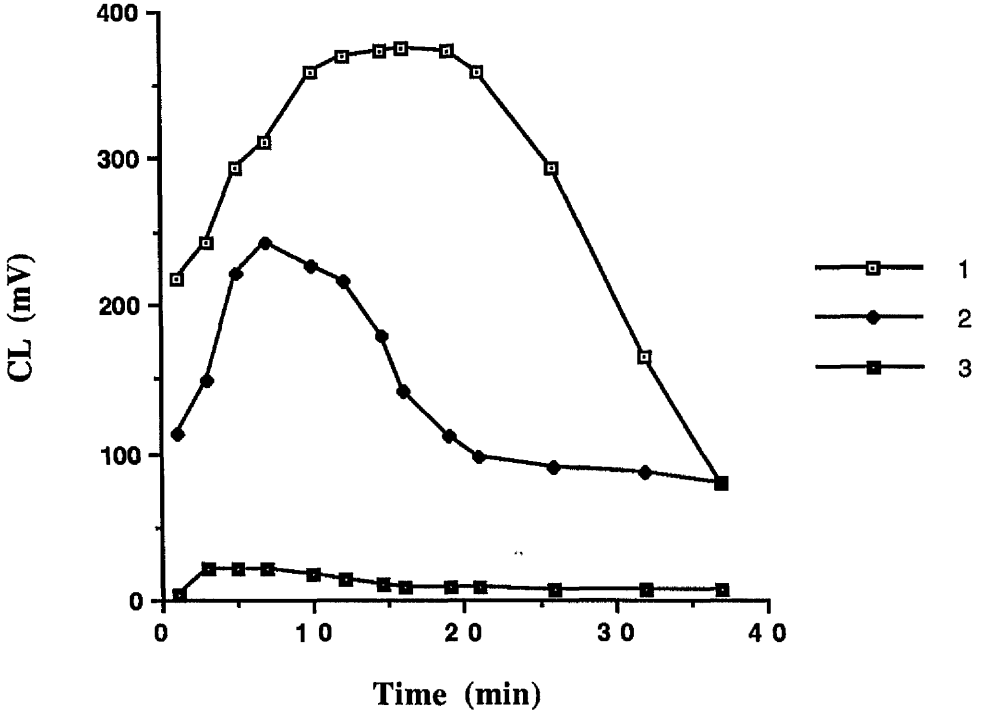
B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10
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40
Time (min)
1
2
3

Effect of Cloned AC from *E. coli* Urea Extracts Purified by Phenyl-Sepharose Chromatographty

After encountering the problems of Detoxi-Gel chromatography , attempts were made to separate the stimulatory effect of urea extracts from *E. coli* by hydrophobic interaction chromatography using phenyl- sepharose 4 B (hydrophobic matrix) . This method of separation depends on the hydrophobic interactions of surface hydrophobic groups with the gel.

Dialysed urea extracts were subjected to commercially available phenyl- sepharose 4 B according to the method of Hewlett *et al.* (1989) as detailed in Materials and Methods (see page 49).The resultant eluate was dialyzed and used for CL assay of PMA-induced CL reponse of rabbit neutrophils.However, passage through phenyl-sepharose columns was not successful in removing the stimulatory effect from the *E. coli* urea extracts.

Effect of CaM-Affinity Purified AC from *E. coli* on CL Response of Rabbit Neutrophils.

One-step purification of *E.coli* urea extracts was carried out by CaM-affinity chromatography as described under Materials and Methods (see page 49).Urea extract of *B. pertussis* BP348 (pRMB1) was included as a positive control. The eluates were dialyzed and their enzymic activity was measured by the assay of Salomon *et al.*, (1974) . The purification profile of these extracts is given in Table11. The effect of purified AC (50 nmol cAMP/min /ml AC enzymic activity) from extracts of *E. coli* H1469 (pRMB6), *E. coli* H1469 (pRMB6, pKIM1), *E. coli* H1469 (pRMB6, pANN202), *E. coli* H1469 (pRMB6, pANGE3) and their controls : *E. coli* H1469 (pKIM1), *E. coli* H1469 (pANGE3) and *E. coli* H1469 (pRMB6) was determined by CL assay on PMA- induced rabbit neutrophils. It is evident from Table. 17 and Fig. 34 that AC purified from extracts of *E. coli* H1469 (pRMB6, pKIM1), containing *hlyC* from *P. vulgaris,* showed 51% inhibition of CL response whereas its controls i.e., *E. coli* H1469 (pKIM1) containing *hlyC* alone and *E. coli* H1469 (pRMB6), containing *cyaA* alone were unable to inhibit or reduce the CL reponse. This supplements the result of the effect of *E. coli* H1469 (pRMB6,pKIM1) extract on BHK cells (Table 12).AC purified from other constructs also showed a similar level of toxicity . AC from *E. coli* H1469 (pRMB6, pANN202) containing *hlyC* from *E. coli* inhibited the PMA-induced CL response by 41% and *E. coli* H1469

(pRMB6, pANGE3) having *cyaC* from *B. pertussis* reduced the CL response by 45% . The controls for each preparation showed stimulation of CL response rather than inhibition suggesting that the stimulating component from the extracts of *E. coli* strains may not be removed by one -step chromatography.

These data suggest that CyaA produced alone in *E. coli* has no CL inhibition activity (toxic activity) and that the *C* gene, either from *P. vulgaris* , *E. coli* or *B. pertussis* activates CyaA to its toxic form which is responsible for inhibition of CL response of PMA-induced rabbit neutrophils.

It is important to point out here that despite normalizing the enzymic activity per vial for CaM-affinity purified AC from *E. coli* and from *B. pertussis* extracts, the CL inhibition effect was not comparable, i.e., *cyaA* in *E. coli* in the presence of *C* genes was found 50-60% less toxic than in *B. pertussis* . However, despite the fact that the *E. coli* extracts contained stimulatory, interfering activities, the active ACT, with CL inhibitory activity, could still be detected.

Table.17

Effect of Cloned AC on CL Response of Rabbit Neutrophils.

	CL (mV) ± SEM*			
Source	**Total**	**Peak**	**% In•**	**% Stº**
Control	1029 ± 38.1	183 ± 15.4	-	100
E. coli H1469 (pRMB6),	1368 ± 42.5	262 ± 28.2	-	132
E. coli H1469 (pKIM1)	1351 ± 39.1	238 ± 23.1	-	131
E. coli H1469 (pRMB6, pKIM1)	495 ± 11.6	95 ± 8.2	51	-
E. coli H1469 (pRMB6, pANN202)	607 ± 10.3	153 ± 14.7	41	-
E. coli H1469 (pANGE3)	1138 ± 31.5	219 ± 18.3	-	110
E. coli H1469 (pRMB6, pANGE3)	563 ± 22.0	140 ± 13.8	45	-

50 nmol cAMP/min/ml AC enzymic activity was applied per vial . Controls were normalized by adding volume equal to their coresponding AC positive samples.

* = Derived as in Table 13 ; n = 4

% In• = % inhibition of total CL

% Stº = % stimulation of total CL

Fig.34

Determination of Activation of CyaA in *E. coli* by CL Assay

Rabbit peritoneal neutrophils were pre-incubated with Calmodulin - affinity purified AC (50 nmol cAMP/min/ml AC enzymic activity / vial) from *E. coli* H1469 (pRMB6,pKIM 1), *E. coli* H1469 (pRMB6,pANN202), and *E. coli* H1469 (pRMB6, pANGE3) and the controls : *E. coli* H1469 (pRMB6), *E. coli* H1469 (pKIM1), *E. coli* H1469 (pANGE3) for 20 min at 37 °C before the addition of PMA. The figure shown is a mean and SEM of 4 observations using two different batches of neutrophils.

1= Control (Buffer only)

2= *E. coli* H1469 (pRMB6)

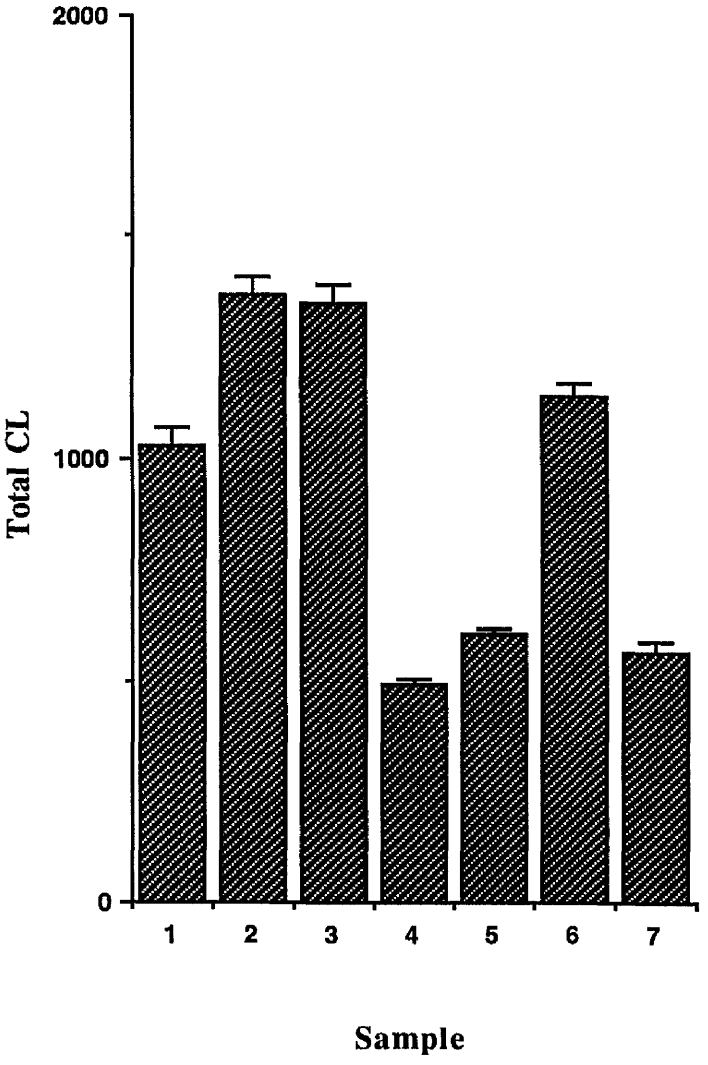
3= *E. coli* H1469 (pKIM1)

4= *E. coli* H1469 (pRMB6, pKIM1)

5= *E. coli* H1469 (pRMB6, pANN202)

6= *E. coli* H1469 (pANGE3)

7= *E. coli* H1469 (pRMB6, pANGE3)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Total CL
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3
4
5
6
7
Sample

DETECTION OF ANTI-AC ANTIBODIES IN PERTUSSIS CONVALESCENT HUMAN SERUM.

Pertussis convalescent sera from 4 patients (adults) were tested for the presence of anti-AC antibodies by the immunoblotting technique. Fig.35 A demonstrates the reaction profiles of *B. pertussis* BP348 (pRMB1) after probing with the sera HS1, HS2, HS3, HS4 (lanes 2-5) and monospecific anti-AC antiserum R164, (lane 1) which was included in this study as a positive control. All were found positive for ACT. The titre (the highest dilution of the antisera which could specifically recognize AC on immunoblots) for each antiserum was determined by probing the extracts with 1:100, 1: 300, 1: 500 , and 1:1000 dilutions of the antisera . HS2 showed the highest titre i.e., 1:1000 where HS3 and HS4 gave equal titres of 1:500. HS1 was the lowest and reacted at 1:100 dilution only.

Further confirmation of the presence of anti-AC antibodies in these sera was done by probing the cloned *B. pertussis* AC expressed in *E. coli* . Urea extracts of AC^+ *B. pertussis* BP348 (pRMB1) and *E. coli* H1469 (pRMB3) and their AC^- controls *B. pertussis* BP348 and *E. coli* H1469 were probed with HS2. Fig. 35 B shows that the antisera reacted positively at high MW position with AC^+*B. pertussis* and *E. coli* strains and not with AC^- *B. pertussis* BP348 and *E. coli* H1469. ACT of *B. pertussis* was recognized as 2 major bands of 210 and 200 kDa and a minor band of 195 kDa . Cloned AC from *E.coli* showed a single band of 210 kDa . The reaction profile with pertussis convalescent sera was consistent with the previous profiles recognized by anti-AC polyclonal , monoclonal, monospecific and calmodulin-affinity probed profiles of ACT. The reactions seen with other bands are presumably due to other immunogenic components of *B. pertussis* which are also present in these crude AC preparations.The reactions with *E. coli* are due to cross reacting antibodies and / or to pre- existing antibodies to the ubiquitous commensal.

These results clearly demonstrated the presence and production of anti-AC antibodies in high amounts during the course of the disease and they confirm the findings of Farfel *et al.* (1990).

Fig.35
Immunoblot Assessment of *B. pertussis* Antibodies in Human Convalescent Pertussis Sera.

The urea extracts of *B. pertussis* and *E. coli* were resolved by SDS-PAGE using 7.5% acrylamide resolving gel, electrophoretically transferred to nitrocellulose membrane and probed with different dilutions of human convalescent pertussis antiserum.

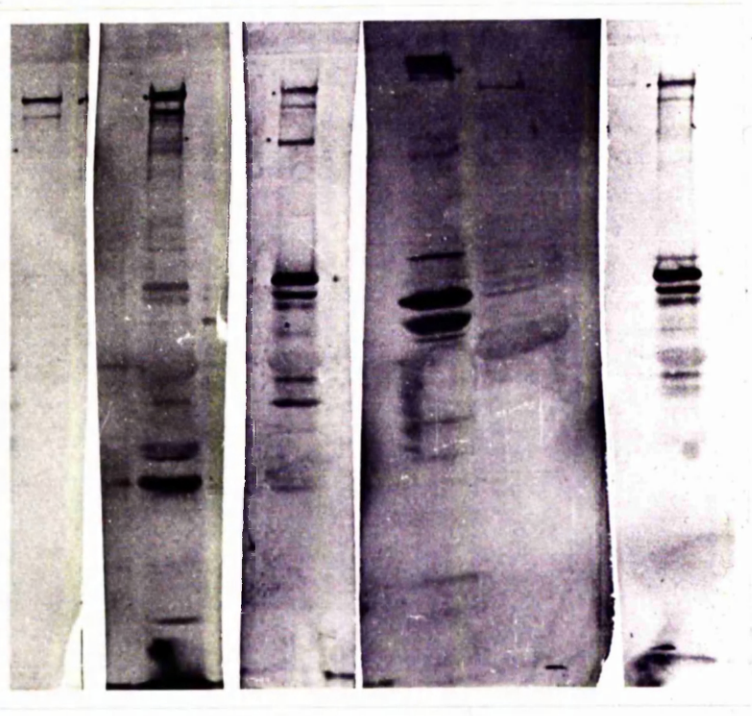
Panel A) Immunoblot of *B. pertussis* BP348 (pRMB1) AC (urea extracts), probed with human convalescent pertussis antisera (HS1, HS2, HS3, HS4)

1= Monospecific antiserum R164 (1:500)
2= HS2 (1:1000)
3= HS3 (1:500)
4= HS1 (1:100)
5= HS4 (1:500)

Panel B) Immunoblot of *B. pertussis* and *E. coli* urea extracts probed with HS 2 (1:1000)

1 = *E. coli* H1469 (AC^-)
2 = *E. coli* H1469 (pRMB3) (AC^+)
3 = *B. pertussis* BP348 (AC^-)
4 = *B. pertussis* BP348 (pRMB1) (AC^+)
5 = *B. pertussis* BP348 (pRMB1) (AC^+)
6 = SDS-6H (MW standard)

A



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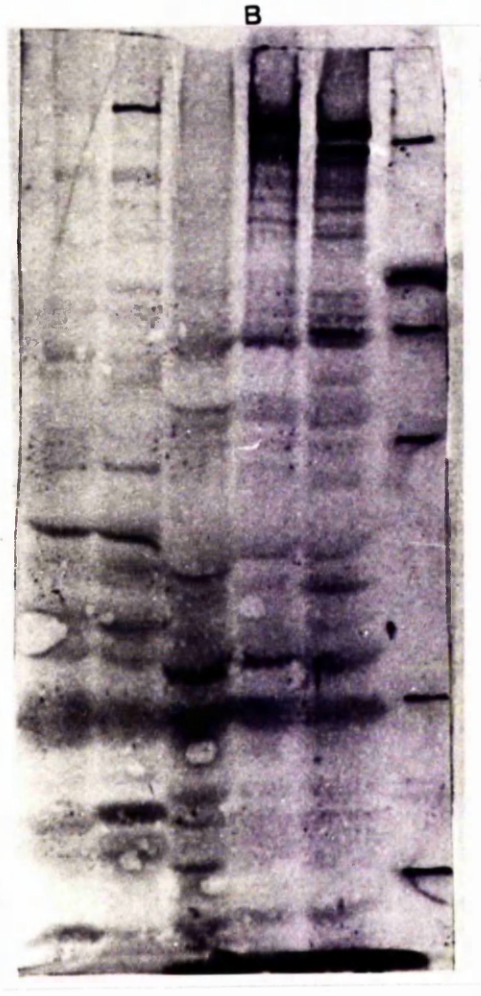
B



1 2 3 4 5 6

PRODUCTION OF ANTI-AC MONOSPECIFIC ANTIBODIES AGAINST NITROCELLULOSE- BOUND ACT.

ACT from urea extracts of *B. pertrussis* BP348 (pRMB1) was purified by the calmodulin -affinity purification method as described under Materials and Methods (see page 49). Purified protein was resolved by SDS-PAGE and transferred to nitrocellulose. The 210 and 200 kDa bands were excised, solubilized by sonication in PBS and used as immunogen in rabbits with Freunds incomplete adjuvant. The procedure is detailed in Materials and Methods (see page 53). Antiserum collected after two booster doses was designated as mono-specific1 (Ms1) and characterized by immunoblotting. Fig.36 clearly demonstrates the production of highly homologous antibodies against ACT. The Ms1 was used in 1:1000 dilution.The titre of the antiserum was evaluated by ELISA, with CaM-affinity purified AC (4 μg protein / well) and was determined by reference to the monoclonal anti-AC antibody 1H6 (Table 6). The unitage was calculated with a parallel-line bio-assay described by Manclark and Meade (1986) .

To ensure that the serum recognizes only the proteins injected, *B. pertussis* BP348 (AC $^-$) was included as a control in this study. A cross reacting, 50 kD band was found in preimmune serum, which was successfully adsorbed out by using the urea extracts of AC $^-$ *B. pertussis* BP348. The specificity of Ms1 was compared with the anti-AC monoclonal antibody (McU) which showed a similar reaction profile. Crude preparations of AC from *B. pertussis* and *E. coli* were used to analyse the Ms1 in order to ensure the specificity of the antiserum and the purity standard of the affinity -purified ACT used for immunization. FHA, a 220 kDa protein of *B.pertussis* resolves closely on gels with ACT. Leusch *et al.* (1990) reported co-purification of ACT with FHA in one-step calmodulin-affinity purification. However, in the present study, the presence of FHA in purified ACT preparations was not detected when probed with anti-FHA monoclonal antibody (not shown). Moreover, if FHA had been copurified with ACT, antibodies should have been generated against it , which in turn could have recognized the FHA in the urea extracts in BP348 (AC $^-$, FHA).

Taken together, these findings suggest that highly specific, homologous antibodies could be raised by this novel and simple method. Although monoclonal antibodies against ACT offer higher specificity, they are more difficult and time-consuming to produce.

Fig.36

Immunoblot Assessment of Monospecific Antibodies Raised Against Nitrocellulose - bound ACT

Urea extracts were separated by SDS-PAGE using 7.5% acrylamide resolving gel, and transferred to nitrocellulose. Probing was carried out with anti-AC monospecific antibodies Ms1 (1:1000) and anti-AC monoclonal antibody McU (1:4000).

Panel A) Urea extracts probed with pre-immune serum.

1=*B. pertussis* BP348 (pRMB1)
2=*B. pertussis* B348
3= *B. pertussis* Tohama

Panel B) Urea extracts probed with monospecific antiserum (unabsorbed)

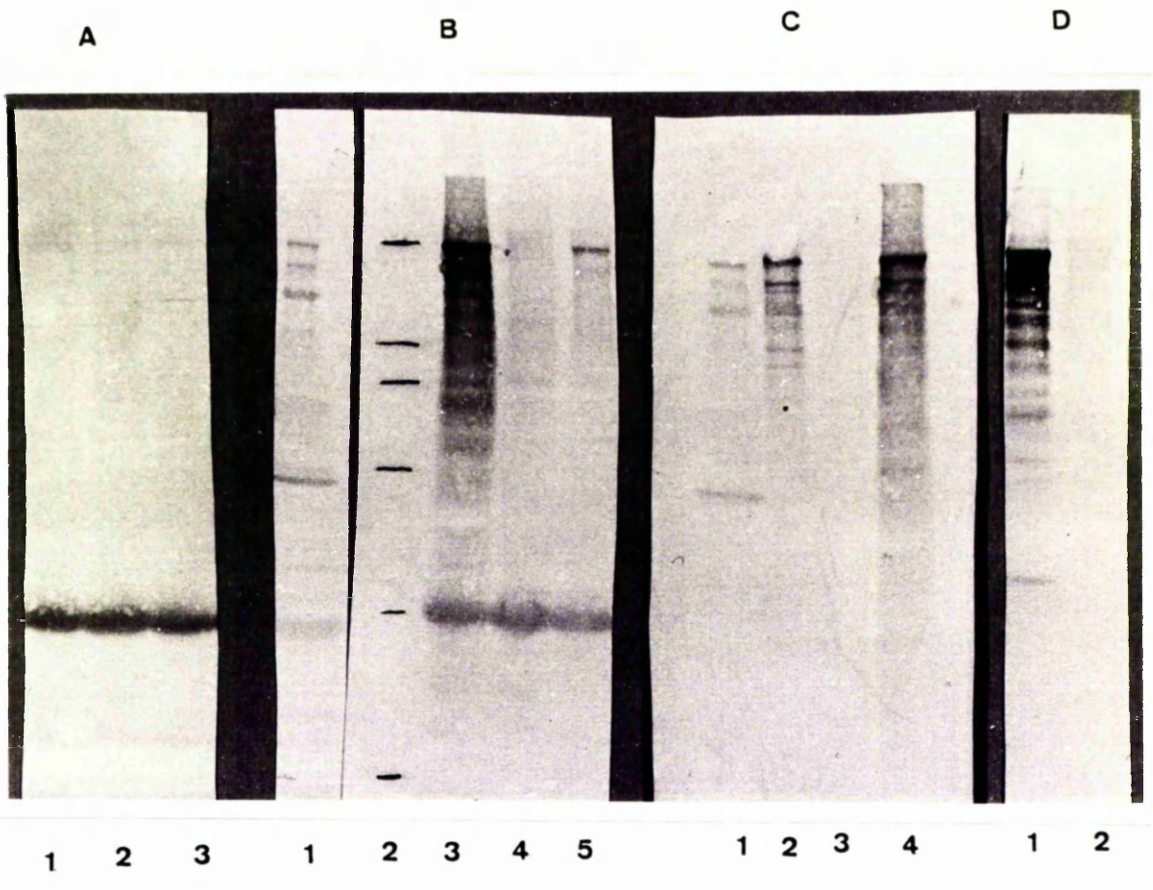
1=*E.coli* H1469 (pRMB6)
2=SDS6H (MW standard)
3= *B. pertussis* BP348 (pRMB1)
4= *B. pertussis* BP348
5= *B. pertussis* Tohama

Panel C) Urea extracts probed with monospecific antiserum (absorbed with extracts of BP348))

1= *E.coli* H1469 (pRMB6)
2= *B. pertussis* Tohama
3= *B. pertussis* BP348
4= *B. pertussis* BP348 (pRMB1)

Panel D) Urea extracts probed with monoclonal antibody.

1= *B. pertussis* BP348 (pRMB1)
2= *B. pertussis* BP348


A
B
C
D
1 2 3
1 2 3 4 5
1 2 3 4
1 2

INTRA- NASAL MOUSE PROTECTION TEST

The protective activity of monospecific anti-AC serum (Ms1) was tested in mice against intranasal *B.pertussis* challenge. Ms1 was raised in rabbits against nitrocellulose- bound ACT as described in Materials and Methods, (see page 53) and was tested on immunoblots for its specificity for AC (see page 135). This antiserum was used for a mouse passive protection test. Ms1 was heated at 56 °C for 20 min to eliminate the possibility of complement mediated killing of the challenge . *B. pertussis* 18323 were mixed in 1:1 ratio with the heat inactivated Ms1and incubated at room temp for 1h. Controls included cells suspended in 1% casamino acid solution in PBS for each dilution (positive control) and 1% casamino acid solution in PBS (negative control). At this stage, before instillation, the viable count was performed for each preparation by plating the 10-fold serially diluted challenge doses on BG and the plates were incubated at 37 ° C for 72h in a humidified box.

The sublethal intranasal challenge of *B. pertussis* 18323 was given to 3- 4 week old, ether-anaesthetized mice in groups of 10. The protective effect of Ms1 was monitored against two challenge doses i.e., high challenge dose (5 x 10^6 cfu / mouse) and low challenge dose (2.5x 10^6 cfu/ mouse) . The mice were inspected and weighed every alternate day for 14 days and the deaths were recorded. On day 14, the remaining mice were killed, weighed individually and the lungs were inspected for the signs of consolidation i.e., grayish discoloured areas. Healthy lungs were scored as 0 and infected lungs from 1+ signifying few small areas of consolidation to 4+ for extensive consolidation in all lobes of lungs. Oedema was recorded and the weight of the lungs as % body weight was calculated. Lung culture was performed by spreading aseptically a small peice of infected lung onto a BG plate. The total leukocyte count (TLC) was also performed.The data shown in Table 18 represents the mean of two identical experiments . As is evident from the data, a low level of protection was achieved when the protective effect of Ms1 was monitored by observing loss in weight gain (Fig.37 A), although lung pathology, lung culture and total leukocyte counts were very similar.

Examination of the lungs revealed a moderate pathology of lungs infected with both, high and low challenge doses with mean of 3 and 2.3 scores respectively. In mice challenged with Ms1-mixed high and low challenge doses, the lung scores were lower at 2.2 and 1.4. Similarly, the mice challenged with Ms1-treated bacteria, showed a reduction in loss of weight gain though this was not very significant. Lungs as % body weight, again, showed a difference of a narrow margin (Fig 38A) The total

did not differ significantly between those of control mice challenged with CAA only or those which recieved high and low challenge doses. The reason for this could be the lack of consistent and reproducible infection in all mice since ether as an anaesthetizing agent was not found satisfactory in the present studies and challenge instillation was found difficult. In a further experiment, halothane was used as anaesthetizing agent in an attempt to improve the infection rate. In order to more clearly demonstrate a passive protection effect, Ms1 antiserum which had been concentrated 2-fold by salting-out technique (see Materials and Methods, page 54) was used.

As is evident from Table 19, concentrated Ms1(CMs1) partially protected the mice against infection. Deaths occured in mice, treated with high the challenge dose, between 12th and 13th day but not in the mice treated with the low challenge dose or with either doses mixed with CMs1. The weight gain in CMs1-treated mice was almost at control levels whereas that in challenge-alone groups was reduced (Fig.37 B). Similarly, there were clear differences in the lung weights of the challenge-alone groups compared with the CMs1- treated groups (Fig. 38B). Total leukocyte counts did reveal differences although again these were not very significant. Viable counts of Ms1- treated and un-treated challenge were approximately equal confirming the viablity of the challenge suspensions showing that the CMs1 itself was not bactericidal. Recovery from lung culture (Fig. 39) was positive in high challenge dose and low challenge dose+ CMs1. These data indicate that ACT could possibly act as one of the major virulence factors and protective antigens during infection with *B. pertussis* since its neutralization by specific antibodies (CMs1) caused a reduction in the severity and rate of infection and passively protected the mice.

Table18

The Protective Effect of Ms1 in Mouse Intranasal Passive Protection Test

Challenge	Deaths /20	Mouse wt (g) Mean ± SEM	Lungs as% body wt (g) Mean (SEM)	Score LP••	TLC log^{º}	Lung culture
CAA	0	23.5 ± 22.6	1.2 ± 0.06	0	3.3	-
HC	0	22.8 ± 18.8	2.4 ± 0.44	3	3.4	+
HC +Ms1	0	23.2 ± 21.5	2 ± 0.38	2.2	3.3	+
LC	0	23.0 ± 20.7	1.6 ± 0.17	2.3	3.3	+
LC + Ms1	0	23.7 ± 23.2	1.3 ± 0.18	1.4	3.3	+

^{º} and •• are the mean values of two replicate experiments (2x10 mice per group).
CAA = Casamino acids (1% v/w in PBS)
HC= High challenge dose of *B. pertussis* 18323 ($5x10^6$ cfu / mouse)
LC= Low challenge dose of *B. pertussis* 18323 ($2.5 x10^6$ cfu / mouse)
LP= Lung pathology
TLC=Total leukocyte count of WBCs / mm^3 blood
Ms1= Anti-AC monospecific antiserum raised in rabbits (see page 53)
+= Positive

Table.19

The Protective Effect of Concentrated Anti-AC Monospecific Antibodies in Mouse Intranasal Challenge Test.

Challenge 10 mice	Deaths (/10mice)	Mouse wt (g) Mean ± SEM	Lungs as % body wt (g) Mean (SEM)	Score º LP	TLC •• (log)	Lung culture
Control CAA	0	22.5 ± 13.6	0.74 ± 0.01	0	4.0	0
HC	2	17.4 ± 9.4	3.11 ± 0.52	3.3	4.5	+
HC+CMs1	0	22.0 ± 10.6	2.18 ± 0.19	3.2	4.3	+
LC	0	21.6 ± 11.3	2.3 ± 0.17	3.3	4.6	+
LC+CMs1	0	22.3 ± 12.4	1.2 ± 0.26	2.4	4.3	+

Mean and ± SEM values are from 10 mice

º and •• are the mean values of 10 mice

CAA = Casamino acids (1% v/w in PBS)

HC= High challenge dose of *B. pertussis* 18323 ($5x10^6$ cfu / mouse)

LC= Low challenge dose of *B. pertussis* 18323 ($2.5 x10^6$ cfu / mouse)

LP= Lung pathology

TLC=Total leukocyte count of WBCs / mm^3 blood

CMs1= Anti-AC monospecific antiserum raised in rabbits (see page 53) and concentrated by salting-out technique (see page 54).

+= Positive

Fig. 37

The Effect of Ms1 on Loss of Weight Gain

The sublethal intranasal challenge of *B. pertussis* 18323 was given to 3- 4 weeks old, ether-anaesthetized (A) and halothane-anaesthtized (B) mice in groups of 10. The protective effect of Ms1 (A) and Concentrated Ms1 (B) was monitored against two challenge doses i.e., high challenge dose (5 x 10^6 cfu / mouse) and low challenge dose (2.5x 10^6 cfu/ mouse) . The mice were inspected and weighed every alternate day for 14 days and the deaths were recorded.

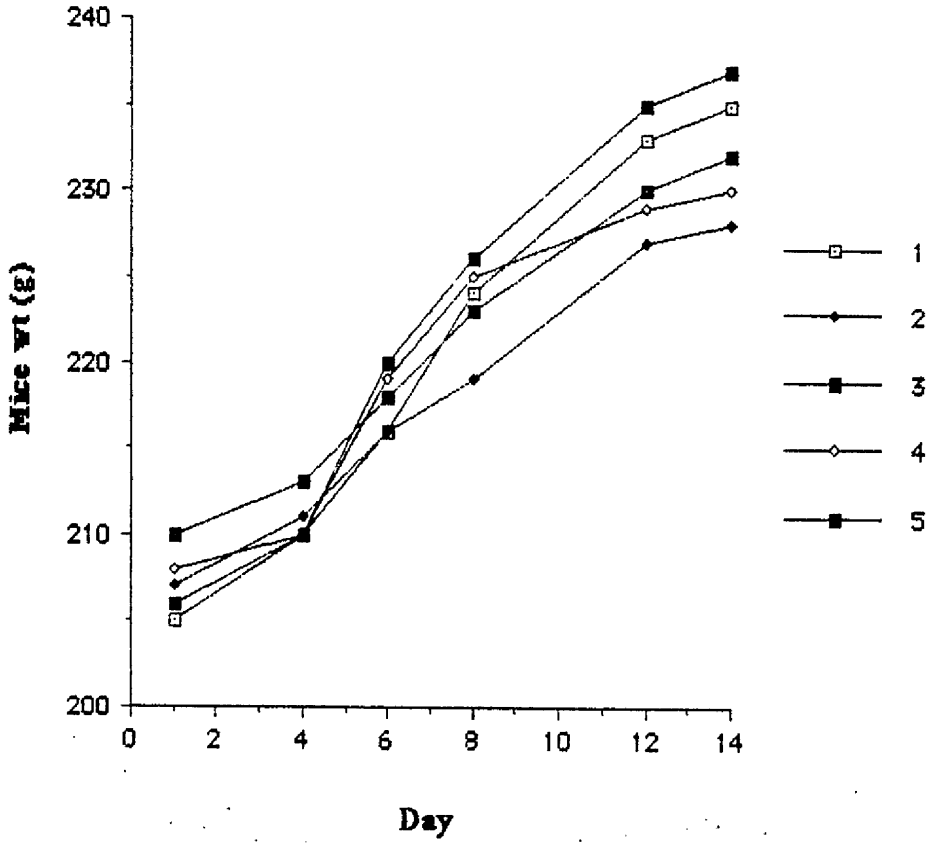
Panel A) Effect of Ms1

1= Casamino acid only
2= High challenge dose (5x10^6 cfu)
3= High challenge dose+ anti-AC monospecific antiserum
4=Low challenge dose (2.5x10^6cfu)
5= Low challenge dose+ anti-AC monospecific antiserum

Panel B) Effect of concentrated Ms1

1= Casamino acid only
2=High challenge dose (5x10^6 cfu)
3=Ms1= High challenge dose+ anti-AC monospecific antiserum
4=Low challenge dose (2.5x10^6cfu)
5= Low challenge dose+ anti-AC monospecific antiserum

A


240
230
220
210
200
Mice wt (g)
0
2
4
6
8
10
12
14
Day
1
2
3
4
5

B

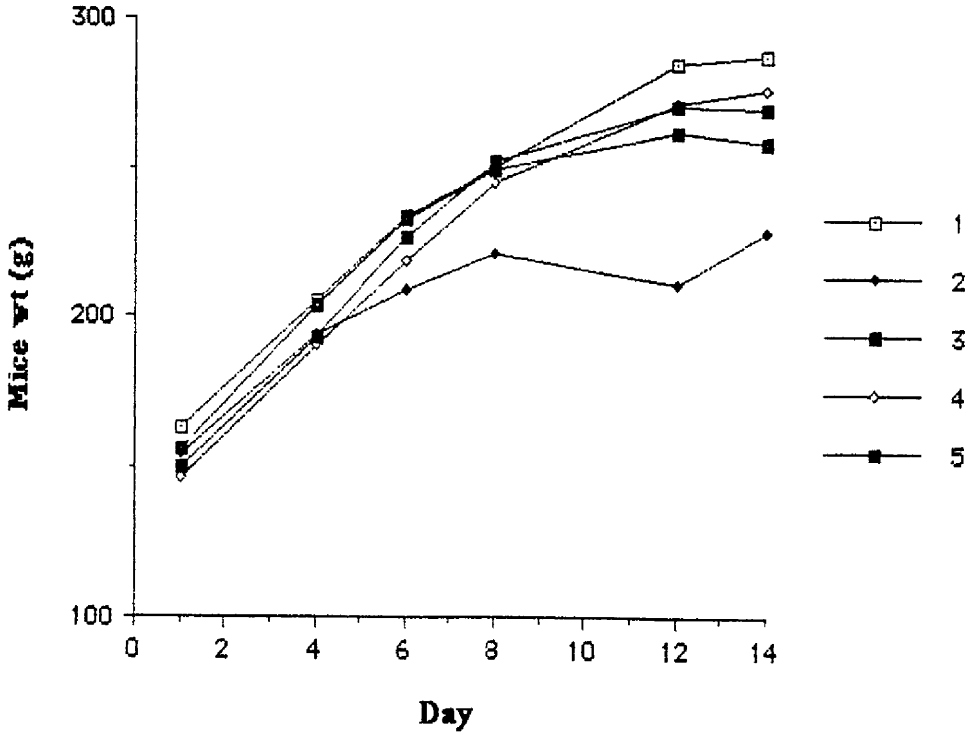

300
200
100
Mice wt (g)
0
2
4
6
8
10
12
14
Day
1
2
3
4
5

Fig.38

Mouse - Protective Effect of Monospecific anti - AC Antibodies.

Lungs as % body weight.

Groups of mice were challenged with *B. pertussis* 18323 at high challenge (HC=$5x10^6$ cfu) and low challenge (LC=$2.5x10^6$cfu) doses, treated and untreated with anti-AC monospecific antibodies Ms1 (Panel A) and concentrated Ms1 (Panel B), On the day 14, the mice were sacrificed, weighed individually, lungs were removed and related to body weight of mice.
Bars indicate SEM of the data of 10 mice.

CAA= Casamino acid only

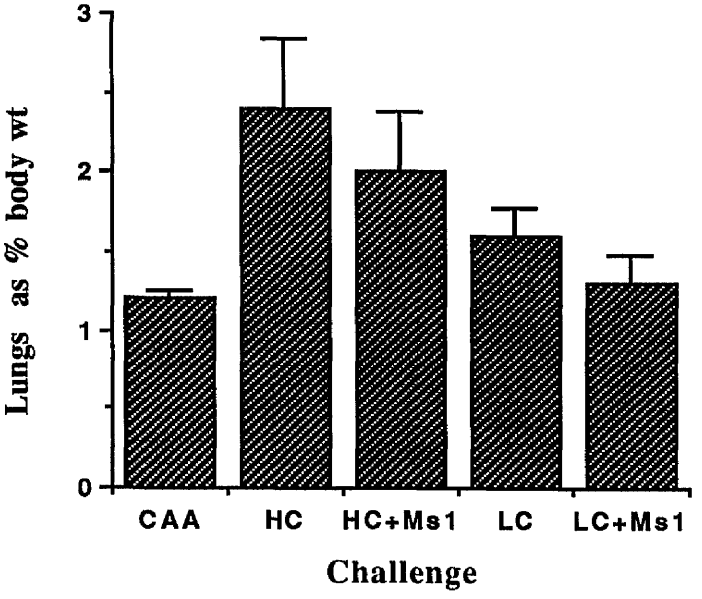
HC=High challenge dose ($5x10^6$ cfu)

HC+Ms1= High challenge dose+ anti-AC monospecific antiserum

LC=Low challenge dose ($2.5x10^6$cfu)

LC+Ms1= Low challenge dose+ anti-AC monospecific antiserum

A


Lungs as % body wt
0
1
2
3
CAA
HC
HC+Ms1
LC
LC+Ms1
Challenge

B

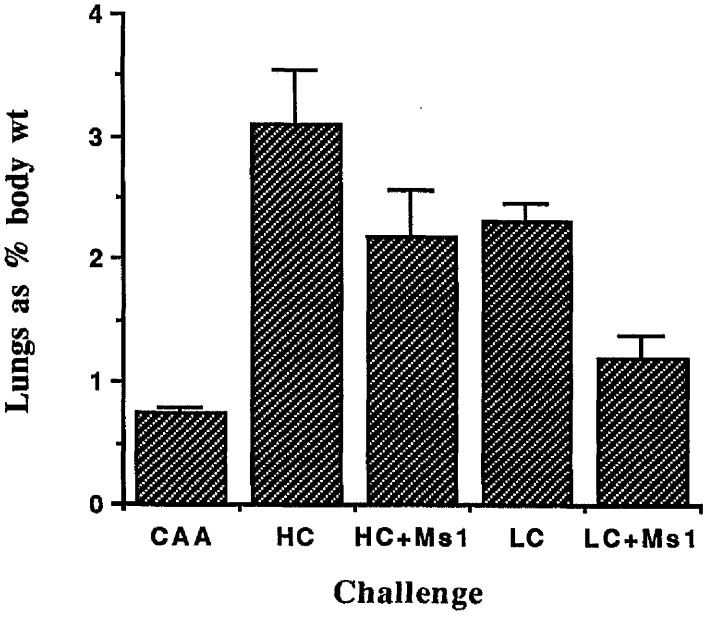
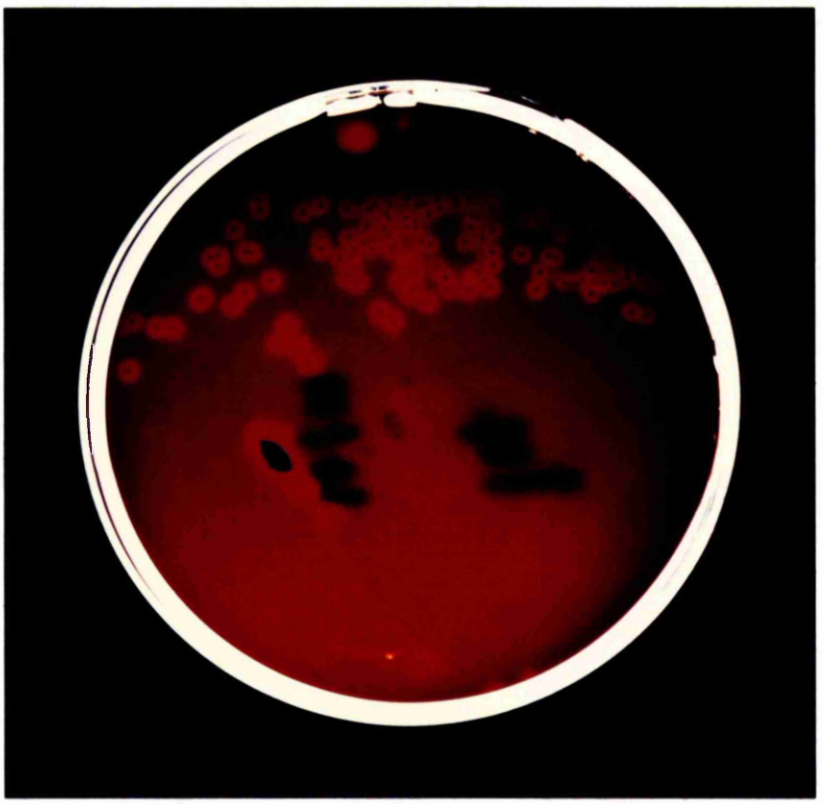

Lungs as % body wt
0
1
2
3
4
CAA
HC
HC+Ms1
LC
LC+Ms1
Challenge

Fig.39

Recovery of *B. pertussis* 18323 (Challenge Strain) from the Infected Lung.

Lung culture was performed by spreading aseptically a small peice of infected lung onto a BG plate and the plate was incubated at 37 °C for 72 h in a humidified box.



DISCUSSION

ISOLATION OF ACT

Utsumi *et al.* (1978) showed that AC could be solubilized from *B. pertussis* cells by urea extraction and that AC activity was relatively stable under these conditions. In this way, Confer & Eaton (1982) were able to extract AC which had both enzymic and toxic activities and they reported that this activity was heat-stable.

The majority of AC enzyme has been shown to be cell-associated and 90% of the activity resides on the outer surface of the bacterial cell (Hewlett and Wolff, 1976; Masure & Storm, 1989).

Since the time of the discovery of AC in the mid-70s, urea extracts of *B. pertussis* organisms and concentrated culture supernates have been used for preparation of AC and ACT. In the present study, cell-associated AC from various strains of *B. pertussis* (including wild-type, recombinant and mutant) and *E. coli* strains harbouring the *B. pertussis cyaA,B,D* genes and the related *C* genes from other bacteria was extracted by modification of the techniques used by Confer & Eaton (1982) and Brownlie *et al.* (1988). The modifications introduced were as follows:

The cells after harvest were stored at -20 ^{o}C prior to addition of 4M urea . The crystalline urea was added after thawing the cell suspension. A low speed centrifugation before dialysis was found to be beneficial since removal of cell debris before dialysis facilitated the efficiency of dialysis. Brownlie *et al.* (1988) reported that more vigorous conditions were needed to obtain AC from *E. coli* strains, and used 8M urea. In the present work, this was found to be unnessesary because the cell storage overnight at -20 ^{o}C made it possible to extract AC from *E. coli* strains even in the presence of 4 M urea and no obvious difference was observed between 8 M or 4 M urea for recovery of AC.The reduction of the urea concentration was convenient for removal of urea in subsequent dialysis steps which was a requirement for the biological assays. These data indicate that osmotic pressure exerted on the cell walls of the organisms or rupture of the cells by ice crystals formed at -20 °C presumably facilitated the recovery of AC from these compartments.

CHARACTERIZATION OF AC BY IMMUNOBLOTTING.

The immunoblotting technique has been used previously by Hewlett *et al.*,(1989) and Bellalou *et al.*, (1990 a) to analyse the composition of crude and purified AC, and a detailed study was done during the work presented here. Urea extracts of wild-type

B. pertussis, recombinant *B. pertussis* strain BP348 (pRMB1), Tn5 mutant strains deficient in different individual virulence factors (Weiss *et al.*, 1983) and the other *Bordetella* species, *B. parapertussis, B. bronchiseptica* and *B. avium.*, were examined. In addition, urea extracts from *E. coli* strains containing either the *cya* genes of *B. pertussis* or harbouring both the *cya* genes and one of the C genes either from *E. coli* (*hlyC*), *P. vulgaris* (*hlyC*) or *B. pertussis* (*cyaC*) were examined . Immunoblotting with two polyclonal sera raised against AC in rabbits and guinea pigs against 45 kDa AC gave a number of bands which were also present in *B. pertussis* strain BP348 (AC $^-$). However, these sera recognized the high MW holotoxin molecule of ACT in both *B. pertussis* and *E. coli.*. The other bands may have been due to cross- reacting antibodies present in these antisera since they were raised against crude AC.These sera could have been absorbed with extracts of AC $^-$ *B. pertussis* strains if sufficient quantity of these sera had been available.

Despite preparation of the urea extracts of all *B. pertussis* strains under the same set of conditions, they showed differences in immunoblot profiles.An important observation was the production of comparable levels of ACT by a Tn*5* mutant lacking a functional PT gene, *B. pertussis* BP357 (Weiss *et al.*, 1983), and recombinant *B. pertussis* strain BP348 (pRMB1) which was reported to produce 5-fold higher toxin activity and 3-fold higher enzyme activity than wild type *B. pertussis* Tohama (Brownlie *et al.*, 1988). This property of strain BP348 (pRMB1) was attributed to a gene dosage effect by the authors. The high level of ACT shown on immunoblot s by BP357 may reflect a possibility of metabolic competition between PT and ACT molecules for synthesis and transport, in which case absence of one may facilitate the production and transport of the other. However, another Tn*5* mutant BP353, deficient in FHA, produced a lower level of ACT than the wild type *B. pertussis* in urea extracts as revealed by immunoblotting . This is consistent with the reports of Weiss *et al.* (1983) who observed a lower production of cell-associated AC in this strain than in the parent strain BP338. However, they found higher AC activities in culture supernates of the FHA $^-$ mutant and ascribed this to differences in compartmentalisation of AC and not as a reduction in total AC activity. Another possibility may be that since AC and FHA are both surface- associated proteins, they could compete for transport machinery. Consequently, in the mutant BP353, more sites would be available to ACT, which would result in high levels of ACT in the culture supernate rather than the intact cell. Since both FHA and AC are extracytoplasmic proteins, the loss of one may result in more efficient release of the other by either an active or passive transport process. Bassford and Beckwith (1979)

reported that the levels of secretion of extracellular proteins are interrelated. When synthesis of a ß -galactosidase fusion protein of *E. coli* was induced, secretion of other proteins was impaired. A similar phenomenon could be suggested for BP353.Leusch *et al.* (1990) found the majority of AC enzymic activity in culture supernate fractions. This may explain the less cell-associated AC enzymic and toxic activity obtained in the present study.

The different behaviour of the two mutants (BP357 and BP353) regarding production of ACT may reflect differences in the regulatory mechanism involved in transcriptional activation of individual virulence determinants.Roy *et al.* (1989) showed that the *fha* promoter was active in *E. coli* in the presence of the *bvg* operon but the *ptx* promoter was not active under the same conditions , providing strong evidence for two different mechanisms of transcriptional control. Goyard & Ullmann (1991) have also suggested an indirect regulation by *bvg* of *cya* and *ptx* , and reported that the *bvg* locus was not sufficient for their activation in *E. coli*.. Additional promoter-specific factors seem to be required for transcriptional activation of these operons. This common requirement of the *cya* and *ptx* operons may account for the competition observed: lack of expression of one would allow the formation of more efficient promoter complexes and better expression of the other. Further work is required to confirm these suggestions.

The immunoblot study of crude ACT of all *B. pertussis* species showed not a single band of 216 kDa as reported by Hewlett *et al.* (1989), or of 200 kDa as reported by Rogel *et al.* (1989), Hanski, (1989) and Bellalou *et al.* (1990 a) , but two bands at MW 210 kDa and 200 kDa reacting with monoclonal antibodies. The family of lower MW bands reacting with the monoclonals (9D4, McU) appeared only in the case of ageing of ACT and after repeated freeze-thaw procedures. A protease inhibitor, PMSF, was used to investigate whether the degradation was due to the proteases present in these preparations. Use of PMSF showed a reduction in proteolytic cleavage but the degradation was not completely prevented. The partial prevention suggests that other protease inhibitors may be necessary and perhaps used as a cocktail to prevent the proteolytic cleavage of ACT during storage. A similar study on degradation of FHA has been reported by Irons *et al.* (1983) in which they found that the degradation was totally abolished by the presence of 1mM PMSF in the solution. These data support the view that the other bands observed in the crude and purified ACT preparations by previous investigators and in the present study were mainly the degradation products of the high MW form of ACT. This suggests that the major cell-associated ACT is the MW 210 and /or 200 kDa form. The two band pattern obtained by immunoblotting

could be explained by the partial degradation of the 210 kDa form, but the CyaA protein expressed in *E. coli* did not reveal the 200 kDa component. Thus the presence of two bands of similar MWs, with more or less similar intensities may not not be a mere problem of degradation.

Further investigation was performed on purified ACT. The recombinant *B. pertussis* strain BP348 (pRMB1) was selected as a source for purification of ACT due to its high production of both enzymic and toxic activities (Brownlie *et al.*, 1988).The high expression of *B. pertussis* ACT by this recombinant strain facilitated the purification of the AC by CaM-affinity chromatography of urea extracts. The AC was detected by immunoblotting with anti-AC monoclonal antibodies, which revealed that AC was again composed of two bands of 210 and 200 kDa. The 200 kDa form was found less stable on storage and this was consistent with the immunoblot study performed with crude AC. Rogel *et al.* (1989) reported the presence of toxic and non-toxic 200 kDa AC which resolved as two peaks by gel filtration. However, SDS-PAGE studies performed by these investigators showed only a single band of 200 kDa. In an attempt to investigate further this observation , isoelectric focusing of CaM - affinity purified ACT from *B. pertussis* BP348 (pRMB1) was performed. The results showed a single diffuse band at p*I* approx. 7 rather than two bands suggesting that the two forms observed by immunoblotting belong to the same polypeptide. These two different electrophoretic forms of AC may represent the toxic and non-toxic forms of AC observed by Rogel *et al.* (1989) as the AC protein expressed in *E. coli* was non-toxic.

Raptis *et al.* (1989) reported that exogenous CaM was capable of protecting the catalytic activity of AC against inactivation by trypsin but was unable to protect invasiveness. They proposed that two different domains on the same protein or on different proteins were responsible for each activity. Furthermore, they predicted that the penetration domain would have to be downstream (toward C-terminus) from the catalytic domain. Recently Bellalou *et al* . (1990 b) were able to show the production of non-invasive AC by in-phase deletions of the 3'-end of CyaA. Truncated AC showed markedly reduced haemolytic activity and no invasive activity. Bellalou *et al.* (1990 b) proposed that the structural integrity of the full length protein (CyaA) was necessary for the toxin activity and that distinct structural determinants within the CyaA protein were involved in secretion, pore formation and entry of ACT into target cells.

Very recently, the haemolytic and invasive activities have been differentiated on the basis of inactivation with exogenous CaM. The invasive activity was found inhibited in the presence of CaM, while haemolytic activity was unaltered or potentiated

(Rogel *et al.*, 1991), again suggesting that two distinct domains or structural determinants are responsible for these two activities.

In the present study, it was observed that those extracts of *B. pertussis* , *E. coli* and other Bordetellae showing reduced or no 200 kDa in immunoblot profiles were less toxic or non- toxic as assesed by toxicity assays (which will be discussed later). These included, *B. pertussis* Tohama (wild-type), *B. pertussis* Tn*5* mutants BP349, BP353, *B. parapertussis* and *E. coli* expressing the *cyaA* gene . As evident from the data presented in the Results section, urea extracts of *B. pertussis* Tohama , and Tn5 mutant BP353 (FHA^-) were less toxic than *B. pertussis* 18323, *B. pertussis* BP357, and *B. pertussis* BP348 (pRMB1) in CL assays, although equal amounts of total protein were assayed. *B. pertussis* BP349 which showed only a faint 210 kDa and no 200 kDa was non-toxic in CL assays and BHK cell assays even if twice the normal level of protein was assayed. Similarly, extracts of *E. coli* H1469 (pRMB3) were non-toxic. *B. parapertussis* was also very weakly toxic.

Controversial data have been reported on the structural-functional relationship of *B. pertussis* AC. Both enzymic and toxic activities have been linked with a variety of molecular forms (Hanski and Farfel,1985; Shattuck and Storm, 1985; Kessin and Franke, 1986; Ladant,1986; Hewlett *et al.*, 1989; Rogel *et al.*, 1989; Masure and Storm, 1989) depending on whether the AC has been extracted from the cells or culture supernates.

In the present study , a 50 - 45 kDa form was observed in culture supernates which reacted on immunoblots with both polyclonal and monoclonal AC antibodies and was assumed to be a degradation product . This form had AC enzyme activity and therefore presumably represents an N-terminal moiety of CyaA (Glaser *et al.*, 1988). This form was further degraded after storage of culture supernate into lower MW forms ranging from 37-25 kDa. Characterization of the cell- associated ACT by immunoblotting revealed that ACT was synthesized as high MW 210 and 200 kDa holotoxin forms by *B. pertussis* and *B. bronchiseptica* and mainly as a 210 kDa form in *B. parapertussis*. However Bellalou *et al.* (1990 a) have reported the secretion of the 200 kDa form into culture supernates of *B. pertussis* under certain growth conditions. They grew *B. pertussis* in filter - sterilized SS medium with 2% BSA instead of the autoclaved medium which was used in the present study. Under the growth conditions provided during this study, the holotoxic ACT was not found in culture supernates even after 48 h growth. .

When AC was expressed in *E. coli* (pRMB3) it was synthesized as a 210 kDa molecule . In *E. coli* (pRMB6) a few more bands, probably break -down products

were observed along with the 210 kDa form.These results support the findings of Bellalou *et al.* (1990 a) that the *cyaA* gene product appears as a 200 kDa protein that corresponds to the major intracellular form of the AC and that those forms present in the culture supernate are proteolytic cleavage products of the holotoxin resulting in catalytically active fragments.

The MW of 210 and 200 kDa for AC is substantially larger than the the MW weight calculated from the deduced amino acid sequence (177 kDa) of the protein (Glaser *et al.*, 1988 a). The difference between calculated MW and the apparent MW of the protein may be due to post-translational modification or due to incomplete unfolding of the molecule in the solublizing buffer used for SDS-PAGE .

DETECTION OF AC BY CALMODULIN-AFFINITY PROBING.

Wolff *et al.* (1980) discovered that *B. pertussiss* ACT was stimulated by calmodulin (CaM) and the stimulation of cell- bound AC was 100-fold higher than the stimulation of AC recovered from culture supernates. AC is synthesized as a large precursor of 1706 amino acids (Glaser *et al.*, 1988 a). Under the usual growth conditions the N-terminal moiety of this precursor is processed to low molecular weight forms of 43 to 50 kDa and secreted into the medium (Shattuck *et al.*, 1985, Ladant *et al.*, 1986). Glaser *et al.* (1989) showed that the N-terminal portion of this 43 kDa (tryptic) fragment of ACT from 1-235/237 residues harbour the active, ATP binding site whereas the C-terminal region of this fragment from residues 235/237-399 corresponds to the main CaM-binding domain of the enzyme. These data established the fact that AC is a CaM-sensitive protein and has a strong affinity for it. On the basis of this information, an alternate method for detection of AC on Western blots was devised by probing the AC with CaM.

The data presented in this study clearly demonstrate that although not as specific as monoclonal anti-AC antibodies, this method still has its advantages in that it is reproducible and that detection by Western blotting could be performed without the aid of anti-AC antibodies. This result is the first report on the detection of ACT with CaM providing direct evidence of the affinity of ACT with CaM.

MICROPLATE ASSAY FOR DETERMINATION OF AC ENZYMIC ACTIVITY.

A microplate assay developed for the determination of AC enzymic activity from *B. pertussis* and *E. coli* showed a detection range from 4 - 32 micromol cAMP.
The principle of the assay is based on the cross-feeding of a *cya* defective mutant, *E. coli* G802 with cAMP to initiate the transcription of ß-galactosidase which then hydrolyzes a chromogenic substrate in the medium, producing a blue colour. *E. coli* is able to use the disaccharide lactose as its sole carbon source provided that lactose can enter the cells and be broken down into its component saccharides i.e., glucose and galactose. The proteins that carry out these functions are specified by the structural genes of the *lac* operon (Gale, 1943). In the absence of lactose, the *lac* operon genes are not transcribed due to the presence of a 'repressor' which is attached to the operator site to block the transcription. When lactose is supplied, derepression occurs by binding of lactose to the repressor, resulting in production of ß-galactosidase and permease for lactose. The mechanism is called metabolic induction and occurs in *E. coli* (Reznikoff,1992). Another regulation mechanism 'catabolite repression' (Magasanik,.1961) is also active in *E. coli*. In the presence of glucose, production of enzymes by the *lac* operon is switched-off and efficient transcription is prevented even when lactose is present. RNA polymerase does not bind directly to the promoter unless a special protein factor called CAP (catabolite activator protein) and cAMP are present.

These two form a complex, CAP undergoes allosteric changes (Kumar, 1975) and binds to the promoter after which RNA-polymerase can recognize and bind to the promoter to initiate transcription (Pastan and Adhya, 1976). The strongest evidence of involvement of cAMP in catabolite repression is that mutants deficient in *cya* and *cap* are defective in AC and CAP and are unable to utilize certain carbohydrates (Perlman and Pastan, 1969). The ability can be restored in *cya* mutants by addition of exogenous cAMP or dibutyryl cAMP (Aono *et al.*, 1979). cAMP uptake in *E. coli* occurs by facilitated diffusion (Goldenbaum and Hall, 1979).

The results show that this assay could be used for detection and semi-quantification of AC. Despite its narrow detection limits, a significant quantity of AC enzymic activity could be detected as is evident from the activity detected in CaM- affinity purified AC from *B. pertussis* BP348 (pRMB1). The microplate assay described here offers many advantages since it allows the analysis of many samples, is reproducible, simple to perform, and economic in requirements of time and chemicals. The assay does not require the use of radioisotopes and can be easily assessed by spectrophotometric

means for quantification. Although not as sensitive as the Salomon assay, the microplate assay may be a good choice in future for the preliminary screening of samples for the presence of AC, including clinical specimens of pertussis and semi-quantification of AC enzymic activity from cell extracts, culture supernates, or purified preparations .

DETERMINATION OF AC TOXIC ACTIVITY BY DIFFERENT ASSAYS.

The purpose of this study was to devise a simple, reliable, convenient and safe assay for determination of toxic activity of ACT. Increased levels of intracellular cAMP have been reported to inhibit various phagocytic functions. Confer & Eaton (1982) reported that ACT entered mammalian cells such as polymorphonuclear leukocytes (PMNs), was activated by host cell calmodulin (CaM), and induced increased levels of intracellular cAMP which impaired the bactericidal functions of these cells. The fact that ACT penetration raises the intracellular cAMP concentration provided a sensitive assay for ACT. Several studies have reported that ACT increases the cAMP concentrations in a wide range of eukaryotic cells. It is because ACT has little target cell specificity (Hanski *et al.*, 1985; Hewlett and Gordon, 1988). Target cells have included : monocytes, lymphocytes, mouse S49 lymphoma cells, isolated rat pituitary cells, CHO-cells, (Hewlett *et al.*, 1985); HeLa 229 cells (Ewanowich, 1989); J774 cells (Hewlett *et al.*, 1989); red blood cells (Bellalou *et al.*, 1990; Rogel *et al.*, 1991) and mouse neuroblastoma cells (Masure *et al.*, 1991).

The most extensively studied category of target cells is that of immune effector cells in which ACT has been found capable of inhibiting the activities involved in defences against microbes, such as : chemotaxis, phagocytosis, oxidative activity and killing (Confer & Eaton, 1982; Hewlett *et al.*, 1983 a; Friedman *et al.*, 1987 b; Pearson *et al.*, 1987; Galgiani *et al.*, 1988).

ACT also inhibited the cytotoxicity of non-phagocytic immune effector cells, e.g., natural killer (NK) cells (Hewlett *et al.*, 1983 a). In cells in which cAMP is a positive signal, e.g., rat pituitary cells, ACT promoted the secretion of growth hormone (prolactin), luteinizing hormone and adrenocorticotropic hormone (Cronin *et al.*, 1986). The basis of determination of ACT in other cells is the measurement of the accumulation of intracellular cAMP. This involves the use of radioactive isotopes in the form of radiolabelled cAMP in a competitive binding assay (Gilman, 1970).The assay provides a standard and sensitive determination of ACT toxic activity , but the

methodology involved is laborious and time-consuming. Moreover, involvement of radioactive isotopes make its use expensive and potentially hazardous and confined to authorized persons.

The data presented here have taken some of the other consequences of ACT toxin activity as parameters to measure the effect of the toxin on target cells, such as : elongation effect on CHO cells; haemolytic effect on red blood cells; arborization of baby hamster kidney cells (BHK cells) ; inhibition of nitroblue tetrazolium reduction by PMNs; and inhibition of the chemiluminescent response of PMNs to chemotactic stimuli.

CHO Cell Assay.

The elongation effect induced in CHO cells by cAMP has been shown with a number of toxins which either stimulate host cell AC or increase intracellular cAMP levels directly. The effect has been shown with heat labile enterotoxins of *Vibrio cholerae* and *E. coli* (Guerrant *et al.*, 1974; Merson *et al.*, 1979), cholera toxin (CT) and adenylate cyclase toxin (Hewlett *et al.*, 1983 b; Hewlett *et al.*, 1985). Pertussis toxin (PT) did not produce the elongation, but had a clustering effect on CHO cells (Hewlett *et al.*, 1983 b). The sensitivity and detection limits of this system for CT and PT have been reported to be < 1ng/ml (Hewlett *et al.*, 1983 b).

The use of CHO cell elongation as a parameter to detect ACT with sensitivity and specificity was explored during this study. When ACT was used in high doses (200 nmol cAMP/min/ml) no effect was observed. This agrees with the observations of Hewlett (personal communication) who also observed a similar effect with high doses of ACT . With lower doses, however, elongation was observed and the limit of detection was 12.5 nmol cAMP/min /ml enzymic activity. These data indicate that there may be a dissociation between cAMP accumulation elicited by ACT and morphological change in CHO cells and it may be assumed that at high levels of ACT, the cells may undergo a physiological stress due to depletion of ATP resulting in paralysis or dormancy of the cell functions, including shape-change. The elongation response may be used to monitor AC toxin at appropriate dilutions, but the assay did not show the sensitivity comparable to that reported for PT and CT. As far as the specificity was concerned, this assay was found satisfactory since no cluster pattern indicative of PT was observed with CaM-affinity purified ACT. Furthermore, when crude extract of BP348 (AC^-,PT^+) was tested, only a clustering effect was observed suggesting that the elongation effect shown in CHO cells by CaM - affinity purified

AC was due to ACT.

The underlying mechanism involved in this elongation effect has not been fully elucidated. It has been recognized by membrane-membrane interaction studies that various membrane components of CHO cells change with cell density (Porter *et al.*, 1973). In a recent study, incubation of respiratory epithelial tissue with *B. pertussis* culture filtrate showed cytotoxic effects in the epithelial cells which produced mitochondrial swelling and cell surface cytoplasmic blebbing (Wilson *et al.*, 1991). This effect could be due to ACT activity in the culture filtrates.

Although the CHO cell assay offered a specific assay for detection of AC, it was not found to be very sensitive and the effect was detectable only within a narrow range, since high doses did not induce elongation at all. Moreover, the assay required aseptic conditions and was found to be time-consuming, taking 16-24 h to observe the morphological changes. Above all, the results were recorded by microscopy which was purely subjective and likely to involve human bias in the scoring of results.

Haemolytic Assay.

ACT has been reported as a bi-functional protein exhibiting both CaM-sensitive ACT enzymic and haemolytic activities (Glaser *et al.*, 1988 b; Bellalou *et al.*, 1990 b; Rogel *et al.*, 1991). A single Tn*5* insertion mutation in the *cya* operon resulted in a mutant BP348, deficient in both AC enzymic and haemolytic activity (Weiss *et al.*, 1983). Both activities were restored when BP348 was complemented with plasmids carrying the complete *cya* operon (Brownlie *et al.*, 1988). Sequence analysis of *cyaA* revealed a domain between residues 640 and 910, displaying a particularly high amino acid homology with an internal part of the alpha -haemolysin of *E. coli* (Glaser *et al.*, 1988 b).Bellalou *et al.* (1990 b) have recently shown that in-phase deletions in this region resulted in a mutant with markedly reduced haemolytic activity. Moreover, the mutant AC was completely devoid of invasive activity. These data indicate that the haemolytic activity has a direct impact on toxicity and that this activity could be used to determine the toxicity of ACT. RBCs are the most convenient cells for assaying cytolytic toxins because they are readily available, and contain a built-in marker in the form of haemoglobin (Bernheimer, 1988). Species of RBCs used in an assay might be of importance since human RBCs have been shown to be an excellent target for internalization of AC (Masure *et al.*, 1991) but not for haemolysis (Rogel *et al.*, 1991).

The data presented in this study concerned an investigation of the haemolytic activity as an assay for toxicity of ACT. Haemolytic activity of ACT on sheep and

rabbit RBCs was explored and sheep RBCs were found to be more sensitive. This does not agree with a recent study by Rogel *et al.* (1991) who found rabbit RBCs more sensitive but the discrepancy may be explained by different assay conditions. 80% haemolytic activity was observed with a dose of 60 nmol cAMP/min/ml AC enzymic activity which is considerably less than that applied by Rogel *et al.* (1991). This difference may be due to the the difference in incubation times which was 12 h in the case of this study whereas Rogel *et al.* (1991) incubated cells for only 3h.

The haemolytic activity of ACT was blocked when it was incubated with monospecific anti-AC antiserum which agrees with the data of Rogel *et al.* (1991). Taken together, these findings suggest that haemolytic activity of ACT could be used as an assay for determination of toxic activities in *B. pertussis* ACT. However, in the case of ACT expressed and activated in *E. coli* , only a weak haemolytic activity was observed by Sebo *et al.* (1991). This activity was 5 times less than *B. pertussis* haemolytic activity which is already 100 times lower than that of *E. coli* alpha -haemolysin. (Bellalou *et al.*, 1990 b).

BHK Cell Assay.

A variety of mammalian cells have been used to detect the toxic effects of *B. pertussis* ACT, but the morphological change as a parameter has been reported only in CHO cells (Hewlett *et al.*, 1985).These cells, after intoxication with ACT, exhibit an elongation or stretching effect, thought to be due to the intracellular elevation of cAMP. An effect of elevated intracellular levels of cAMP in baby hamster kidney (BHK) cells has been observed by Dr. John Edwards, Department of Cell Biology , University of Glasgow. These cells are fibroblasts and, after incubation with exogenous dibutyryl cAMP, tend to change their shape from regular to dendritic (tree -like) forms. When the effect of ACT on these cells was assessed, a similar change in the BHK cells was observed, indicating the intoxication of these cells by ACT and suggesting an alternative assay for ACT. This effect was not seen with crude extracts of ACT-deficient Tn*5* mutant BP348. This encouraging study suggests the specificity of this assay for ACT in a dose- dependent manner. Furthermore, this assay was not time consuming, required less time than the CHO cell assay and was found more reliable since unlike CHO cells assay, assessment was not purely subjective and the results were scored by computer analysis.

Inhibition of Nitroblue Tetrazolium Reduction .

A series of redox reactions takes place in PMNs and monocytes during phagocytosis. These create increased O_2 consumption and stimulation of the hexose monophosphate shunt . Products of the reduction of oxygen include hydrogen peroxide and several highly reactive unstable intermediates of oxygen i.e., superoxide, singlet oxygen, and hydroxyl radical (Allen *et al.,* 1972).

Nitroblue tetrazolium reduction (NBTR) is an indication of neutrophil superoxide production and can serve as a useful marker of the normal redox capabilities of the PMNs to convert NBT to a blue formazan derivative after stimulation with PMA, a stimulus which promotes a variety of leukocyte responses including degranulation, superoxide generation, and aggregation (DeChatelet *et al.,* 1976). In the present study this stimulus was used because PT inhibits neutrophil response to fMLP but not to PMA (Becker *et al.*, 1985; Schefcyk *et al.*, 1985) and it thus made the assay suitable for both crude or purified ACT preparations. PMA was therefore selected to exclude the possibility of any effect PT may have had on the assay.

The microplate assay, described for the first time in this study for detecting ACT activity, allowed the analysis of toxic activity of ACT in many samples simultaneously, was reproducible and simple to perform. This assay did not require the use of radioisotopes and was able to be assessed visually. The suppression of NBT reduction by ACT presumably occurred by impairment of cell functions due to increased levels of intracellular cAMP . No decrease in viability of cells was observed in ACT - treated PMNs by the trypan blue dye exclusion test. Inhibition of leukocyte reduction of NBT after stimulation by a chemotactic peptide fMLP has been reported for the assay of pertussis toxin (Craig & Parton, 1988) and leukotoxin of *P. haemolytica* (Craig *et al.*, 1990). The inhibition of nitroblue tetrazolium reduction test was simple to perform and rapid, but the sensitivity for ACT was low and furthermore, the results were scored subjectively. Spectrophotometric assesment of the results was difficult because of uneven settlement of the insoluble formazan particles in the microplate wells. However, for rapid screening of ACT activity either in crude or purified preparations, the NBTR-microplate assay was found quite suitable. Moreover, with correct choice of target cells, appropriate stimuli, and high doses of toxin, it is possible that this assay may be useful for screening other toxins which suppress PMNs functions.

Chemiluminescence Inhibition Assay.

Chemiluminescence (CL), or the emission of light by phagocytosing PMNs is believed to result from the generation of oxidative species i.e., superoxide, singlet oxygen, and hydrogen peroxide (Allen *et al.*, 1972). It is an indirect measure of the bactericidal ability of phagocytes. Addition of chemical and enzymic inhibitors of these metabolites and the enzymes involved in their generation, causes a decrease in bactericidal activity of PMNs (Johnston *et al.*, 1975) suggesting their importance in microbiocidal functions. The addition of the non-particulate stimulus, PMA, to resting human PMNs also results in metabolic events similar to those seen during phagocytosis (De Chatelet *et al.*, 1976). Thus the alteration of neutrophil response to PMA may indicate an alteration of the normal neutrophil responses to infection.

The CL inhibitory effect of ACT on human macrophages (Confer & Eaton 1982), neutrophils (Friedman *et al.*, 1987 b) and monocytes (Pearson *et al.*, 1987) has been reported. In fact, the inhibition of the CL response of PMA-induced human macrophages in the presence of crude urea extracts (Confer & Eaton, 1982) was the first report to reveal toxin activity of *B. pertussis* ACT. Another study by Friedman *et al.* (1987 b) demonstrated the CL-inhibitory effect of affinity-purified ACT on human neutrophils stimulated with heat-killed opsonized *S. aureus*.

The CL inhibition assay for detection of AC toxic activity applied in this study was found to be more sensitive than previously reported CL assays. It is evident from the data presented in this study that this assay was sensitive enough to detect as little as 0.5 nmol cAMP/min/ml AC enzymic activity applied /vial.

PMA was used as a stimulus to avoid any possible interference by PT. PMA appears to exert its effects by activating the protein kinase C in target cells (Nishizuka, 1984). Interestingly, PT inhibits neutrophil response to fMLP, C5a, and leukotriene B4, but has no influence on neutrophil response to PMA (Becker *et al.*, 1986). Moreover, fMLP-induced PMNs showed enhanced CL in the presence of other components of *B. pertussis*, such as FHA and LPS (Craig *et al.*, 1988). However, because PMA is a carcinogen, its use could be substituted with fMLP when working with purified preparation of ACT in CL assays.

In the present work, the PMA-induced, CL response of rabbit PMNs was impaired by both crude and CaM-affinity purified ACT. This suggests that myeloperoxidase-dependent production of hydrogen peroxide was being inhibited since luminol-enhanced CL detects mainly this activity (Briheim *et al.*, 1984).The minimum time to achieve clear inhibition of the CL response by crude ACT was 15

min. On incubation of PMNs with crude ACT for less than a 5 min, marked enhancement of response was observed. The summed levels of neutrophil CL generated in the presence of PMA and crude ACT was higher than the summed levels of neutrophils CL generated in the presence of PMA alone.This may indicate the enhancement of CL by FHA or LPS in the presence of PMA. The rough LPS of *E. coli* J5, and smooth LPS of *E. coli* 0111:B4 are reported to stimulate human neutrophil PMNs (Henricks *et al.*, 1983). LPS is also found to serve a role of priming and stimulation of neutrophils (Aida and Pabst, 1991).

On the other hand, affinity- purified ACT did not show a similar time course of activity.The inhibitory effect of purified ACT on PMA-induced neutrophils was evident within 5 min and no early enhancement of CL was seen. Hewlett *et al* (1989) reported that purified ACT entered CHO cells and started production of cAMP without any lag period and this was supported by the data here. Studies of Friedman *et al.* (1987 b) have shown a dose-dependent effect of ACT on human neutrophils. The present results revealed an interesting biphasic response to ACT. Dose response studies showed that purified ACT was able to suppress PMA-induced CL response at doses above 0.5 nmol cAMP /min /ml AC enzymic activity per vial. Doses of approx: 0.05 -0.0005 nmol cAMP /min /ml AC enzymic activity per vial did not inhibit CL response. With lower doses, a significant stimulation of CL response was observed. This effect of ACT on CL response has not been reported previously but related effects have been reported with low doses of other RTX toxins.Since ACT shares amino acid sequence similarities with other RTX toxins (Glaser *et al.*, 1988 b), it could be proposed that ACT at subtoxic doses may produce changes in the target cell membrane which lead to their altered function. In the case of alpha-haemolysin of *E. coli* , which is the most extensively studied toxin among all the pore forming RTX toxins, cytolytic events are attributed to the rapid formation of cation selective channels in host cells that lead to osmotic lysis (Bhakdi *et al.*, 1986). Apart from lysis there are data suggesting that it can probably cause more subtle effects by impairment of host-cell activities. A 14-fold increase in superoxide production has been reported with sublytic (picogram) doses of *E. coli* haemolysin in rat renal tubular cells (Keane *et al.*, 1987). This supports the view that alteration of the cell functions of animal cells could occur upon exposure to subtoxic or sublytic doses of RTX toxins, including ACT. A similar effect at sublytic concentrations of *E. coli* alpha haemolysin has been reported recently by Bhakdi and Martin (1991). The stimulatory effect at subtoxic concentration of ACT in the CL reponse could possibly provide an extremely sensitive assay for the detection of very low concentrations of the toxin in purified preparations.

The respiratory burst is a distinguishing property of phagocytes. It reflects the activation of events with the formation of products essential for the killing of microorganisms, but these products are also a cause of tissue damage and inflammation (Baggiolini and Wymann, 1990). It seems possible therefore, that at high doses, ACT impairs phagocytic functions and facilitates *B. pertussis* survival, and at lower doses, it may cause the respiratory burst on a massive scale in PMNs and other target cells which may lead to local tissue damage and inflammation, thereby ensuring proliferation of *B. pertussis* in respiratory epithelial cells.

In conclusion,the inhibition PMA-induced, luminol/DNDH- enhanced CL response of rabbit peritoneal neutrophils was found to be a sensitive assay for ACT toxicity . This assay detected AC toxin activity in crude and purified preparations. With crude AC , approx 50 % inhibition of CL was observed at 3.3 μg protein/ml whereas Friedman *et al.* (1987 b) found 50 % inhibition of CL at a dose of 36 μg /ml even when they used purified ACT. Purified AC in the present study detected a CL inhibitory effect down to 0.5 nmol cAMP/min/ml AC enzymic activity / vial and a 50 - 60 % inhibition of CL was observed at dose of 5 nmol cAMP/min/ml AC enzymic activity /vial. This sensitivity may be achieved due to the assay conditions used in the present study. Although both PMA and heat-killed *S. aureus* initiate the respiratory burst, the PMA -induced CL response is totally dependent upon the presence of a suitable substrate e.g., luminol in the reaction medium to permit detection of the light emission (Westrick *et al.*, 1980), although particulate stimulants such as zymosan or a bacterium itself may serve as the substrate for secondary light producing reactions (McPhiel *et al.*, 1979). The use of a particulate stimulus also may results in a more strong enhancement of the burst and may last longer than the burst induced by the luminol and PMA .

Another important variable to consider is the PMNs. Friedman *et al.* (1987 b) used human neutrophils isolated from peripheral blood whereas in the present study, rabbit peritoneal neutrophils were used. It has been reported that PMNs from different species and different sources may differ in their response to stimuli.De Chatelet *et al.* (1975) showed superoxide formation by rabbit peritoneal neutrophils when incubated with opsonized zymosan but rabbit peritoneal macrophages did not generate measurable quantities of superoxide. PMNs from peripheral blood and peritoneal exudate may also differ in their responsiveness.Takamori and Yamashita (1980) have reported two-times higher generation of superoxide anion in peritoneal neutrophils than in blood neutrophils. Blood neutrophils were found more resistant to hypotonic lysis.Furthermore, the activity of the enzyme alkaline phosphatase, a specificic marker enzyme for granules of rabbit PMNs was about 1.5 times higher in

peritoneal PMNs than in blood PMNs. Therefore, it seems reasonable to suggest that rabbit peritoneal PMNs may have a more sensitive response towards CL events induced with PMA than the blood PMNs. Thus the use of rabbit peritoneal neutrophils and PMA in the CL assay seems to have advantages over the use of peripheral blood neutrophils and *S. aureus.*

Rabbit peritoneal neutrophils are relatively easily obtainable, survive longer during storage at 4 °C and containment conditions are not required for their use, whereas human blood is potentionally hazardous and less stable. Moreover, the preparation may contain contaminating erythrocytes and other leukocytes if not properly prepared.On the other hand, rabbit peritoneal neutrophil preparation is relatively simple and gives > 90% neutrophils. Furthermore, the rabbit is susceptible to *B. pertussis* aerosol infection and constitutes a relevant model for the disease in humans (Ashworth *et al.*, 1982). Therefore, rabbit neutrophils may also serve as a substitute for human neutrophils for the assays.

Detection of AC Toxic Activity in Different Bordetella by CL Assay

Crude preparations of ACT from different *Bordetella* showed different CL-inhibition effects. Urea extracts from AC- negative strains showed stimulation rather than inhibition. Since urea extracts are crude preparations from the cells, they presumably contain other components of *B. pertussis* e.g., FHA, LPS which enhance the PMA-induced response of neutrophils. LPS and FHA have been reported to enhance the fMLP - induced CL response of rabbit peritoneal neutrophils by Craig *et al.* (1988). In preparations containing ACT however, the stimulatory response was overidden by the inhibitory effect.

One of the important points to come out of this study was the strain variation that occurred between different *Bordetella* species and among *B. pertussis* strains for the production of ACT. *B. pertussis* Tohama was not comparable to other wild-type strains for the production of cell-associated ACT as assessed by the inhibition effect of PMA-induced CL response of rabbit PMNs. Furthermore, this strain also showed weak reactivity on immunoblots with monoclonal anti-AC antibodies, and had reduced AC enzymic activity. One possible explanation could be that the production of ACT in this strain follows a different time course . Leusch *et al.* (1990) have reported the earlier production of cell-associated AC in the case of one strain in comparison with other strains of *B. pertussis.*

Tn*5* mutants of *B. pertussis* deficient in a individual virulence factors (Weiss *et al.*, 1983) were tested in the CL assay in an attempt to determine the impact of the

presence and absence of these factors on AC toxic activities. The FHA mutant BP353 showed reduced toxicity in CL assay like *B. pertussis* Tohama. This strain also displayed less prominent AC bands on immunoblots with monoclonal antibody and contained less enzymic activity than another Tn*5* mutant BP357 (PT mutant) or other wild-type strains used in this study. The lower enzymic activity in this strain has also been reported by Weiss *et al.* (1983) and Leusch *et al.* (1990) but reduced toxic activity has not been reported. This difference in ACT (cell-associated) production by this strain could possibly be explained in several ways as discussed previously.

In contrast, another Tn*5* mutant BP357 (PT $^-$) showed high level production of AC which was comparable to the recombinant strain *B. pertussis* BP348 (pRMB1) which was shown to produce 3-fold higher enzymic activity and 5-fold higher toxin activity when tested on S49 lymphoma cells (Brownlie *et al.*, 1988). This behaviour of BP357 was noted in the immunoblot study, enzymic assay and CL assay. A possible reason for the high level expression of ACT in BP348 (pRMB1) is a gene dosage effect (Brownlie *et al.*, 1988). High level expression of ACT in BP357 (PT $^-$) may reflect mechanisms already discussed on page 144 .

Although PT is widely regarded as one of the major protective antigens of *B. pertussis* , it may also be responsible for some of the untoward effects of *B. pertussis* and pertussis whole-cell vaccine, Moreover, BP357 (PT $^-$) has been found protective in mouse protection test (Olander *et al.*, 1986). It is of interest to speculate if the enhanced level of ACT contributes to the protective capacity of this strain and, if so, then this strain might be an interesting vaccine candidate.

In order to determine the specificity of the assay for ACT , the influence of calmodulin and calcium on the CL-inhibition effect of ACT was determined. The data agree with previous reports. It has been demonstrated that addition of μM CaM prevented the formation of cAMP catalyzed by partially purified AC in target cells (Shattuck and Storm, 1985). Rogel *et al.* (1989, 1991) have reported the CaM-mediated inhibition of purified ACT activity and shown that the inhibitory effect of CaM was dose- dependent. The effect has been attributed to the altered configuration of the N-terminus of ACT after binding with CaM which may than prevent the access of ACT to the target cell interior (Rogel *et. al.*, 1991; Ehrman *et al.*, 1991). Blocking effect however, seems to be target cell specific, depending upon the type and nature of the cells, since in Chinese hamster ovary (CHO) cells, accumulation of cAMP was unaffected when CaM was added (Gentile *et al.*, 1990).

In the present study, this blocking effect of CaM for AC toxic activity was demonstrated by reduction of the CL-inhibition effect of ACT suggesting that the toxic effect was specifically caused by ACT.

The role of calcium in the catalytic and invasive activities of ACT has been investigated by several workers. Masure *et al.* (1988) reported that calcium influenced several properties of ACT including its chromatographic behaviour, the Stokes radius of the enzyme, the mobility of catalytic activity on SDS-PAGE, and the effectiveness of ACT to elevate cAMP in target cells. Although not an obligatory requirement for the enzymic activity (Greenlee *et al.*, 1988; Gentile *et al.*, 1990), calcium has been reported as an essential feature and indispensible cofactor for the invasive activity of ACT (Gentile *et al.*, 1990; Hewlett *et al.* 1990; 1991). Calcium might be involved in attachment of ACT to target cells since the presence of 11 tandemly-repeated sequences each consisting of 9 amino acids have been found responsible for the binding of *E. coli* haemolysin to the erythrocyte membrane and these sequences appear to bind calcium (Boehm *et al.*, 1990). Similar sequences have been identified on *B. pertussis* ACT (Glaser *et al.*, 1988 b). Hewlett *et al.* (1990 ;1991) have also shown that calcium binding caused a conformational change in ACT. The exposure of purified ACT to free calcium at concentrations between 1-10 μM resulted in conformational alteration as viewed by negative staining and electron microscopy. The present study supports the findings of Hewlett *et al.*, (1991) that at low concentrations of calcium, the toxic effect of ACT could not be detected and that increasing the calcium concentration caused a visible effect. The maximum effect of toxin was seen with 3 mM calcium / 10^6 cells. The bi-modal response observed in the present study could be explained as the effect of ACT at sub-optimal and then optimal concentrations of calcium which could be those described by Hewlett *et al.* (1991) i.e., unfolding/ conformational changes and then penetration into the target cells.

Suppression of CL Response of Neutrophils by Live B. pertussis

Suppression of the CL response of rabbit peritoneal neutrophils with live opsonized *B. pertussis* BP348 (pRMB1) but not with the ACT deficient Tn*5* mutant BP348 suggests that this suppression was caused by ACT. Since the bacteria were coated with fresh rabbit serum so as to provide opsonins to facilitate phagocytosis (Allen,1977) the possibility exists that the whole bacteria were ingested rather than the ACT being released from the bacteria and then taken up by the neutrophils. Furthermore, the slightly delayed time for the suppression of CL may be due to the time required for entry of the bacteria and their possible processing within the host or both.

Hewlett *et al.* (1988) found intoxication of J744 cells with *B. pertussis* but not with culture supernates. They suggested that cell to cell contact may have resulted in

the accumulation of cAMP in these cells. Recently, Mouallem *et al.* (1990) have shown the intoxication of CHO cells by *B. pertussis* Tohama. The entry of whole bacteria was revealed by transmission electron micrographs. Very recently, Bromberg *et al.* (1991) have suggested the intracellular appearance of *B. pertussis* in alveolar macrophages of patients with human immunodeficiency virus infection. Saukkonen *et al.* (1991) have shown an integrin - mediated localization of *B. pertussis* within rabbit macrophages.

A similar interaction of human neutrophils with virulent and avirulent strains of *Salmonella typhi* were examined by Kossack *et al.* (1981) . They observed equivalent phagocytosis of the strains when quantitated by uptake of radiolabelled virulent and avirulent strains of *S. typhi* but found a decrease in postphagocytic PMN oxidative metabolism, by measuring oxygen consumption and by decline in chemiluminescence response, in those PMNs ingesting virulent *S. typhi* .

These studies suggest that *B. pertussis* whole cells may invade host cells of various types and induce increased intracellular cAMP. The present results show a time-dependent decline in CL response of rabbit peritoneal neutrophils indicating that the whole cell of *B. pertussis* may have entered the neutrophils via phagocytosis and caused the suppression of CL. This effect shown by AC -positive *B. pertussis* but not by the AC - negative *B. pertussis* has not been reported previously.

The results from different assays for determination of AC toxic activity are summarized in Table 20. Comparison shows that the CL assay appears to be more sensitive .

Table 20
Comparative Study of Toxicity Assays for *B. pertussis* ACT

Assay	Detection limit		Advantages	Disadvantages
	Crude (μg protein)	**Purified** (nmol cAMP/min/ml)		
NBTR	20 / $5x10^4$ cells	50/ $5x10^4$ cells	Simple,rapid, economical	Qualitative, less sensitive
Haemolytic	20 / $2x10^8$ cells	6.25/ $2x10^8$ cells	Simple,rapid, economical, quantitative	Less sensitive
CHO cell	ND	12.5/ $5x10^4$ cells	Specific	Laborious,time-consuming, narrow range, less sensitive.
BHK cell	75 / $5x10^4$ cells	ND	Specific, quantitative?	Time-consuming
CL	1 / 10^6 cells	0.5/ 10^6 cells	Sensitive, simple,rapid, quantitative, economical.	Stimulation by undesirable factors

NBTR= nitroblue tetrazolium reduction; CHO=chinese hamster ovary ; BHK= baby hamster kidney; CL = chemiluminiscence inhibition; ND= not determined

DETECTION OF ANTIBODIES AGAINST AC IN HUMAN CONVALESCENT SERA OF PERTUSSIS PATIENTS.

The human antibody response to *B. pertussis* ACT after pertussis infection and immunization has been recently reported (Farfel *et al.* 1990, Arciniega *et al.*, 1991). To gain a broader understanding of the human immune response to *B. pertussis* AC as a step towards determining a definite role of AC antibodies in protection, convalescent sera from adult individuals with culture-confirmed pertussis were examined. Although pertussis is mainly considered to be a disease of children it is becoming observed more frequently in adults (Linneman & Nasenberry, 1977), presumably due to waning immunity after vaccination.

Immunoblot analysis of convalescent sera from four patients of pertussis showed clearly the presence of anti-AC antibodies strongly recognizing both the native ACT in *B. pertussis* urea extract and cloned AC in *E. coli* on immunoblot profiles. It has been reported that FHA antibodies appear in sera of pertussis patients (Redd *et al.*, 1988). Since FHA has a closely similar MW (220) kDa to ACT, the possibility is there that the recognized bands could represent FHA.To exclude this possibility, extracts of Tn5 mutant BP348 deficient in ACT (Weiss *et al.*, 1983) were probed with the same sera but did not show the presence of high MW protein in these extracts, confirming that the antigen recognized by human sera was in fact ACT.

In a separate study of CL assays, these same human sera were found to neutralize the toxic effect of AC. This result contradicts the findings of Farfel *et al.* (1990) who reported that the post-pertussis infection sera did not neutralize the enzymic and penetrative ability of the AC. The studies of Brezin *et al.* (1987) showed that both AC enzymic activity neutralizing and non-neutralizing antibodies protected mice against infection with *B. pertussis* which suggests that the lack of neutralizing capacity may not indicate that these antibodies are insignificant. Raptis *et al.* (1989) however, reported that hyperimmune sera could neutralize *B. pertussis* AC activity. The data presented in this study therefore indicate that neutralizing antibodies are produced against ACT during the course of infection, and their presence in convalescent patients may reflect their role as protective antibodies. The presence of neutralizing antibodies against PT in whooping cough have been shown to be important in long term immunity against pertussis (Granstrom *et al.*, 1985).

The differences in titre observed among the sera for recognition of *B. pertussis* AC on immunoblots may have been due to the timing of serum sampling or to the severity of the disease which could have been severe and prolonged in those cases where high titre AC antibodies were obtained.

In a very recent study by Guiso *et al.* (1991), the antibodies in the mouse after active immunization with 200 kDa AC holotoxin (ACT) appeared very early in pertussis, which suggests that AC antibodies may play a role against initial pathogenesis i.e., colonization , as has been proposed by Goodwin & Weiss (1990).

AC TRANSFERRED TO NITROCELLULOSE FOR USE AS AN IMMUNOGEN.

Highly specific (monospecific) antibody against ACT was prepared by using affinity-purified ACT (210 and 200 kDa) , transferred to nitrocellulose and used as an immunogen in rabbits.

Antibodies have become a useful reagent for the identification, localization and purification of biologically interesting molecules (Knudsen, 1985). However their usefulness in providing reliable and creditable information depends upon their specificity. While monoclonal antibodies offer higher specificity, they sometimes can be difficult to produce because the procedure involved is expensive and time-consuming and requires well-equipped tissue culture facilities. Moreover, hybridoma cell lines are frequently unstable due to either chromosome loss or to tissue culture contamination (Goding, 1986).

An alternative is the production of polyclonal antibodies from antigen separated by SDS-PAGE, although the separated protein will contain both acrylamide and SDS, which may have detrimental consequences. However, transfer of SDS-PAGE-separated ACT to nitrocellulose in the present case, proved successful for raising monospecific antibodies against ACT. This antibody, although raised against denatured AC, was capable of neutralizing AC enzymic and toxic activities as assessed by Salomon assays and CL-assays showing that it was able to recognize epitopes in both native and denatured molecules. This makes the antibody useful for a variety of purposes e.g., passive protection tests against ACT and detection of ACT on immunoblots and ELISA.

INTRANASAL PASSIVE PROTECTION TEST.

It is now well known, at least from animal studies, that ACT is one of the important virulence factors of *B. pertussis* because Weiss *et al.* (1983) showed by Tn*5* -induced mutagenesis, that a *B. pertussis* strain deficient in AC was avirulent in a mouse model, and virulence of this strain was restored when this mutant harboured a recombinant plasmid expressing ACT activity (Brownlie *et al.*, 1988). Recent reports further substantiated the role of AC in virulence as a colonizing factor (Goodwin & Weiss, 1990). Besides its role as a virulence factor, a protective role of ACT has also been elaborated since both these roles are interrelated. Brezin *et al.* (1987) and Guiso *et al* . (1989) have reported an efficient protection of mice after passive immunization with monoclonal and polyclonal antibodies against ACT. A protective role of AC holotoxin (200 kDa) and its degradation product, 45 kDa AC, against colonization has recently been reported by Guiso *et al.* (1991).

The results presented in this study support the previous findings in that groups of mice treated with monospecific anti-AC antibodies (MS1) were partially protected when compared with those which did not receive this treatment (controls) . Increased lung weight and oedema was reduced by the use of antibody but lung pathology was still present although it was not very prominent. The lung consolidation may have been due to the other factors produced by *B. pertussis* such as LPS. The leukocyte count did not show any difference. Preston (1988) described this characterization of infection as a less reliable diagnostic guide since in many cases he found that true pertussis did not develop a significant increase in circulating lymphocytes.

The possibility that MS1 may have a bactericidal effect on 18323 was clearly ruled out since the viable count performed before and after incubation with MS1 showed almost equal c.f.u./ml as those in MS1 untreated, control doses. Furthermore, recovery of *B. pertussis* 18323 from the infected lungs proved that the mice were infected with *B. pertussis.*

In conclusion, passively administered anti- *B. pertussis* ACT antibodies protected mice against *B. pertussis* 18323. The reason why a complete protection was not achieved may be due to the antibodies (MS1) used or that they were raised against denatured ACT. However, SDS-denatured ACT has been shown to renature under hydrophobic conditions (Masure & Storm, 1989). Some renaturation could have occured on removal of SDS. The other possibility could be the titre of antibody which may not have been high enough to neutralize the ACT produced by *B. pertussis* 18323, which has been reported to produce three times more AC activity and be more virulent than other wild-type *B. pertussis* (Guiso *et al.* 1991) . The data presented in

this study supports this view. In the present work this strain had approximately 10 times more enzymic activity and a 70% increased toxic activity than *B. pertussis* Tohama.

The partial protection also reflects the role of other virulence factors of *B. pertussis*, since, of course, the MS1 neutralized the effect of AC, but *B. pertussis* has other factors involved in colonization of the host. However, in combination with the other components proposed for acellular vaccines, e.g., FHA, PT, P-69, ACT may play a protective role against infection as suggested by Weiss *et al.* (1989) and Guiso *et al.* (1991).

IMMUNOBLOT STUDY OF RTX TOXINS

The immunoblot study of RTX toxins investigated a cross- reaction with monoclonal anti-AC antibodies . This study included *Bordetella pertussis* adenylate cyclas toxin (ACT), *Pasteurella haemolytica* leukotoxin (LktA), *Actinobacillus actinomycetemcomitans* leukotoxin (AktA), and *E. coli* alpha - haemolysin (HlyA). The *E. coli* alpha -haemolysin is the most thoroughly studied cytolysin. Genetic studies have revealed that haemolysins of the RTX family are encoded by an identical number of genes which are organized in an operon. The nucleotide sequences have shown a high degree of homology and, as a consequence, activation of the haemolysins,their secretion, and mode of action share similar mechanisms. These toxins contain tandemly-repeated amino acid sequences of 9 residues which are involved in the calcium- dependent binding of the cytolysin to target cells. The close amino acid homology of the C-terminal portion of CyaA with HlyA of haemolysin provides a model for the entry of CyaA into the target cell . It has been reported that *E. coli* HlyC can function in place of the equivalent protein from *P. haemolytica* to activate LktA expressed in *E. coli* (Forestier and Welch, 1990) .

In present study, these similarities were assesed by the immunoblot study. Monoclonal antibody raised against the holotoxin AC of *B. pertussis* seemed an appropriate tool to search for similarities between RTX toxins from different organisms.The immunlogical relatedness among RTX toxins has not been examined previously. The results suggest that the RTX toxins share common epitopes or antigenic determinants and the demonstration of a cross-reaction between ant-AC monoclonal 9D4 and the AktA may prove to be highly useful for the isolation and study of this recently described toxin. Further studies are required to determine what role this relatedness plays in the structure-function relationship and in the diagnosis of the disease since the possibility exists that the antibodies detected by serological tests

of patients sera may have been raised against, for example, Hly of *E. coli* present in the host. The genetic and immunological relatedness between RTX toxins indicate that these toxins might have a common evolutionary origin.

EXPRESSION OF CYA IN *E. COLI*

The gene (*cyaA*) encoding ACT has been shown to be part of an operon consisting of additional genes *cyaB cyaD cyaE* (Glaser *et al.*, 1988 b) and *cyaC* (Barry *et al.*, 1991). Complementation studies have shown that *cyaB* , *cyaD* , *cyaE* are required for the secretion of ACT from *B. pertussis* (Glaser *et al.*, 1988 b), and share a large degree of sequence similarity with *hlyA, hlyB,* and *hlyD* genes (Glaser *et al.*, 1988 b). Like ACT, secretion of HlyA also needs HlyB and HlyD. Regarding the homology between *E. coli* HlyA and *B. pertussis* CyaA, export of CyaA from *E. coli* has been reported, although only 2% of total AC activity was released into the culture medium as compared to *B. pertussis* Tohama (Masure *et al.*, 1990).

Previous studies from this laboratory have described the expression of the *cyaA* gene in *E. coli* harbouring plasmid pRMB3. Cell extracts from this strain showed AC enzymic activity which was stimulated in the presence of exogenous CaM (Brownlie *et al.*, 1988). Further studies revealed a 200 kDa protein expressed in a protease-defective *E. coli* strain (H1469) harbouring plasmid pRMB3. This product had AC enzymic activity and was recognized by anti-*B. pertussis* ACT monoclonal antibodies (Rogel *et al.*, 1989). Barry *et al.* (1991) reported that an additional gene, *cyaC,* was required for the activation of CyaA to its cell-invasive form in *B. pertussis* . Very recently, Sebo *et al.* (1991) were able to show the activation of CyaA in *E. coli* in the presence of *cyaC* from *B. pertussis*. In the present study, expression of cyaA in *E. coli* strain H1469, harbouring either plasmids pRMB3 or pRMB6 and/or double plasmids i.e., pRMB6 and a plasmid carrying *C* genes either from *E. coli, P. vulgaris,* or *B. pertussis* were examined for the production and activation of CyaA . The results obtained from the *E. coli* H1469 (pRMB3) showed enzymic activity equivalent to wild-type *B. pertussis* which reacted with anti-ACT polyclonal and monoclonal antibodies and post-infection human sera of pertussis. The protein was also detected by the CaM -affinity probing method for detection of CyaA. However the extracts from this strain were non-toxic in toxicity assays. This agrees with the previous reports by Brownlie *et al.* (1988) and Rogel *et al* .(1989).

Cloned *C* genes from other RTX toxins were introduced into *E. coli* on plasmids which resulted in the constructs: H1469 (pRMB6,pANN202); H1469 (pRMB6, pKIM1); and H1469 (pRMB6, pANGE3). These constructs revealed the production of

210 kDa CyaA with AC enzymic activity comparable to wild-type *B. pertussis* which reacted with monoclonal and polyclonal anti-AC antibodies on immunoblots.AC toxic activity in CL assays of crude preparations was determined. There was a stimulatory factor present in crude preparations of these constructs which was possibly LPS . This was determined by treating the neutrophils with purified LPS which gave a similar enhancement of the CL response . Efforts to remove the LPS from these preparations by Detoxi -Gel chromatography also removed the toxic activity of the ACT since a 50-60 % reduction in CL inhibition effect was observed in a urea extract of *B. pertussis* 18323 after Detoxi-Gel treatment. The reason for this reduction remained unclear. Phenyl-sepharose chromatography was also not able to remove the stimulatory effect from these extracts.

CaM - agarose chromatography was performed and the dialysed eluate was assessed for the toxic effect on CL response. A low level of toxicity was observed in the purified AC from cloned constructs indicating the activation of *cyaA* in the presence of *hlyC* from *E. coli* and *P. vulgaris* and *cyaC* from *B. pertussis* . The level of activation by *hlyC* from *P. vulgaris* seemed to be higher than that by the *hlyC* from *E. coli* or *cyaC* from *B. pertussis* because only 51% inhibition of CL response was observed with the dose of enzymic activity which gave 99% inhibition when measured with the *B. pertussis* ACT. The low toxicity observed in *E. coli* - expressed ACT may be due either to the presence of stimulatory factor(s) which may not have been removed completely by CaM-affinity chromatography, or may reflect the low level production of activated CyaA. This may be due to a number of factors. It has been reported for *E. coli* haemolysin that an additional factor, cytosolic activating factor, is required for conversion of pro-toxin to activated toxin and that the HlyC alone cannot activate pro-HlyA to activated toxin (Hardie *et al.*, 1991).The HlyC proteins in the absence of this factor, which has been identified as acyl carrier protein -like factor (Issartel *et al.*, 1991) may not function properly with CyaA and CyaA may not function satisfactorily in *E. coli* as the activation process requires an acyl carrier protein as donor of a fatty acid molecule which is specific to *B. pertussis* . Both of these moieties may be different in *E. coli* from the equivalent components by *B. pertussis*. Sebo *et al.* (1991) also found less haemolytic activity in ACT, expressed and activated in *E. coli* .

APPENDICES

APPENDIX I: MEDIA

(i) Bordet-Gengou Medium (BG)

Bordet -Gengou agar base obtained from Difco was used in this study since the agar base obtained from Oxoid failed to show haemolytic colonies of *B. pertussis* . This was important from the point of view of selecting ACT $^{+}$ from the ACT $^{-}$ strains of *B.pertussis* . The base was prepared in 500 ml aliquots. Prior to autoclaving, the base was dissolved fully in distilled water containing 1% glycerol by heating at 90 °C for 20 min in a steamer after which the dissolved agar was autoclaved at 121 °C for 15 min . At this stage the autoclaved agar was either stored or cooled to 56 °C in a water bath and used for pouring by adding 100 ml defibrinated sheep blood under a laminar flow cabinet.

BG with kanamycin (40 μg/ml) was also used for selection of Tn*5* mutants. BG with tetracycline (10 μg/ml) was used for the recombinant strain *B. pertussis* BP348 (pRMB1). The antibiotics were added to the agar base just before adding the blood and well mixed by rotation of the bottle to avoid frothing. Stock solutions of the antibiotics were filter-sterilized, using 0.45 μ pore size Acrodisc syringe filter (Gelman).

(ii) Cyclodextrin Liquid Medium (CDL)

	g
Mono sodium glutamate	10.7
L-Proline	0.24
NaCl	0.5
KH_2PO_4	0.5
$MgCl_2.6H_2O$	0.1
$CaCl_2$	0.02
Tris	6.1
Casamino Acids	0.5
Me βCD	1.0

Contents were mixed in 700 ml distilled water and dissolved, pH was adjusted to 7.4 with 2N HCl and the medium was autoclaved at 121°C for 15 min at 15 p.s.i.The volume was made up to 990 ml with distilled water.

Supplement preparation for 1 litre CDL	*g*
L-cysteine	0.04
Fe SO_4 $7H_2O$	0.01
Ascorbic acid	0.02
Niacin	0.004
Glutathione (reduced)	0.15

Contents were dissolved in 10 ml distilled water and filter- sterilized by passage through a 0.45 μ pore size Acrodisc syringe filter (Gelman) and added to 990 ml of the cooled autoclaved medium.

APPENDIX II: SODIUM DODECYL SULPHATE POLY ACRYLAMIDE GEL ELECTROPHORESIS (SDS- PAGE).

(i) Acrylamide/Bis

Acrylamide	30.0 g
N, N-methylene bis acrylamide	0.8 g
Distilled water	100 ml

(ii) Lower Gel Buffer (pH 8.8)

Tris	18.1 g
SDS	0.4 g
Distilled water	50.0 ml

The pH was adjusted to 8.8 with 2N HCl and the final volume made up to 100 ml with distilled water.

(iii) Upper Gel buffer (pH 6.8)

Tris	6.0 g
SDS	0.4 g
Distilled water	50.0 ml

The pH was adjusted to 6.8 with 2N HCl and the final volume made up to 100 ml with distilled water.

(iv) TEMED (Undiluted stock)

(v) Ammonium Persulphate Solution

A 10% solution was made up freshly (50 mg in 0.5 ml distilled water).

(vi) Solubilizing Buffer

Glycerol	10 ml
2-mercaptoethanol	5 ml
SDS	3 g
Bromophenol Blue	0.01 g
Upper buffer , 1in 8 dilution of (iii) to 100 ml	

(vii) Running Buffer (pH 8.3)

Tris	3.03 g
Glycine	14.4 g
SDS	1.0 g
Distilled water	1000 ml

(viii) Staining Solution

Coomassie Blue R 250	1.525 g
50% (v/v) methanol	454 ml
Glacial acetic acid	46 ml

(ix) Destaining Solution

Methanol	50 ml
Glacial Acetic Acid	75 ml
Distilled water	875 ml

Slab Gel Preparation

(i) Lower (Separating) Gel	**7.5% acrylamide** (0.8 mm)
Lower buffer	5 ml
Distilled water	10 ml
Acrylamide/Bis	5 ml
Ammonium persulphate solution	100µl
TEMED	20µl

(ii) Upper (Stacking) Gel	**4.5% acrylamide**
Upper gel buffer	2.5 ml
Distilled water	6 ml
Acrylamide/ Bis	1.5 ml
Ammonium persulphate solution	30µl
TEMED	20µl

APPENDIX III: BUFFERS AND REAGENTS

NOTE: Deionized, distilled water was used to prepare all reagents.

(i) HEPES Buffered Saline (HBS)

NaCl	8.0 g
KCl	0.4 g
$CaCl_2$	0.14 g
Glucose	1.0 g
HEPES	2.388 g

Ingredients were dissolved in 900 ml of water and the pH adjusted to 7.4 with 1 N NaOH. The volume was made up to 1 litre with water and the preparation was autoclaved at 121 °C for 15 min. The divalent cation-free HEPES solution was

prepared by the omission of the calcium.

(ii) Phosphate- Buffered Saline (Dulbecco A)

Phosphate-buffered saline tablets (Dulbecco A, pH 7.3, Oxoid) were used. Each tablet was added to 100 ml of distilled water and the solution was autoclaved at 121°C for 15 min .

(iii) Immunoblotting/ Western Blotting

Transfer Buffer

Tris	3.03 g
Glycine	14.4 g
20% (v/v) methanol to	1000 ml

Diaminobenzidine (DAB) substrate solution

3,3'- diaminobenzidine (DAB)	0.05 g
1% $CoCl_2$ in H_2O	2 ml
PBS (Dulbecco A ,pH 7.3, Oxiod)	98 ml
30 % H_2O_2, added immediately prior to use	0.1 ml

Caution: Handle DAB with care. It is a carcinogen

Electroblotting buffer (20 mM Tris/ 150 mM glycine, pH 8)

Tris base (sigma)	15.5 g
Glycine	17.4 g
methanol	1200 ml
Distilled water to	6000 ml

Ponceau -S solution

Ponceau- S	0.5 g
Glacial acetic acid	1.0 ml
Distilled water to	100 ml

Horseradish peroxidase-anti-Ig conjugate

A variety of species-specific anti-Ig conjugates were purchased from Scottish Antibody Production Unit (SAPU). Diluted these as indicated by the manufacturers and stored frozen in 0.025 ml aliquots until use.

(iv) ELISA

Coating Buffer

Na_2CO_3	1.59 g
$NaHCO_3$	2.92 g

Reagents were dissolved in 1litre of distilled water and kept at 4 °C. The pH of the solution was approximately 9.6.

Washing/ Incubation Buffer

NaCl	8.0 g
KH_2PO_4	0.2 g
Na_3HPO_4	1.15 g
KCl	0.2 g

All the ingredients were dissolved in 1litre of distilled water. Tween 20 (0.5 ml) was then added to a final concentration of 0.05 %. The pH of the solution was 7.4.

Citrate-Phosphate Buffer

Solution A: 0.1 M citric acid	21.01 g
Solution B: 2M Na_2HPO_4	28.3 1 g
of distilled water	

To prepare a 0.15 M solution of citrate-phosphate pH 5.0, 49 ml of solution A and 51 ml of solution B were added together just before use.

OPD Substrate Solution

OPD (O-phenylenediamine, Sigma)	34 mg
Citrate phosphate buffer (pH5) to	100 ml
30 % H_2O_2 (Sigma)	100 µl

(v) Iso- Electric Focusing

Fixing solution

Trichloroacetic acid	57.5 g
Sulphosalicylic acid	17.25 g
Distilled water	500 ml

Staining solution

Coomassie Blue R 250	0.46 g
Destaining solution	400 ml
Filtered through Whatman paper 1.	

Destaining solution

Ethanol	500 ml
Acetic acid	160 ml
Diluted with distilled water up to	2000 ml.

(vi) Reaction Mixture for Microplate Assay for AC Enzymic Activity

1 M Tris-HCl pH8	300 µl
1 M M_gCl_2	50 µl
ATP (Sigma)	2.5 mg
1 M $CaCl_2$	50 µl
Calmodulin (bovine brain, Sigma)	2 µM
Make up to 5ml with distilled water	

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