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Interaction of *Bordetella bronchiseptica* and different *Bordetella* factors with sheep bone marrow mast cells and other cell types

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DECLARATION

Unless otherwise stated, this thesis is the original work of the author.

Ismail Al-Turkestany

DEDICATION

This study is dedicated to the spirit of my grand mother Rehana, she died in May-2000, before I came to Glasgow in July-2000.

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LIST OF CONTENTS

LIST OF FIGURES

CHAPTER ONE: INTRODUCTION

CHAPTER TWO: MATERIALS AND METHODS

CHAPTER THREE: RESULTS

Section One: Differentiation of sheep bone marrow mast cells (Sheep BMMC)

Section Two: Application of bioluminescent bacteria as an in vitro reporter of the interaction of *B. bronchiseptica* and sheep BMMC

LIST OF TABLES

LIST OF ABBREVIATION

µg	micrograms
48/80	compound 48/80
A23187	calcium ionophore A23187
AC	adenylate cyclase
ACV	acellular vaccine
Amp	ampicillin
ATP	adenosine triphosphate
BapC	Bordetella autotransporter protein C
BG	Bordet-Gengou
BMMC	bone marrow-derived mast cell (s)
BrkA	Bordetella resistance to killing protein A
BrkB	Bordetella resistance to killing protein B
BSA	bovine serum albumin
Bvg	Bordetella virulence gene
CAA	casamino acids
CaM	clmodulin
cAMP	adenosine3',5'-cyclic monophosphate
CD	cluster determinant
CDNA	complementary DNA
cfu	colony forming unit
CL	cyclodextrin liquid
CTMC	connective tissue mast cell(s)
CyaA	adenylate cyclase toxin
dH_2O	distilled water
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
FHA	filamentous haemagglutinin
Fim	fimbriae
g	gram

h	hour
HBSS	Hank's Balanced Salt Solution
Ig	immunogloblin
IL	interleukin
IMDM	Iscove's modification of Dulbecco's medium
L	litre
LB	Luria Bertani
mg	milligrams
min	minute
ml	millilitre
MMC	mucosal mast cell(s)
MMCP	mouse mast cell proteinase
MRI	Moredun Research Institute
MTT	3-(4,4-dimethylthazol-2,5-diphenyl tetrazolium bromide)
MW	molecular weight
OD	optical density
PBS	phosphate buffered saline
PPi	pyrophosphate
PPiase	inorganic pyrophosphatase
PRN	pertactin
PT	pertussis toxin
r.p.m.	revolutions per minute
RMCP	rat mast cell proteinase
RNA	ribonucleic acid
rOvIL-3	recombinant ovine interleukin-3
rOvSCF	recombinant ovine stem cell factor
SD	standard deviation
SDS	sodium dodecyl sulphate
sec	second
SEM	standard error of the mean
SP	substance P
SS	Stainer-Scholte
TEM	transmission electron micrograph
v/v	volume in volume

vag	virulence-activated gene
Vag8	virulence-activated gene 8
vrg	virulence-repressed gene
w/v	weight in volume

Abstract

A number of studies have reported that mast cells (MCs) play a critical role in host immune defence against bacterial infections. These studies have shown that mast cells have the ability to recognize and engulf bacteria. This project aimed to investigate the effect of MCs on selected microbial pathogens and the effect of these pathogen and their products on MCs and other cell types (RBL-2H3 and J774A.1 cell lines).

The phagocytic activity of sheep BMMC was studied by using bioluminescent strains of *B. bronchiseptica* and *E. coli,* and degranulation was followed by estimating β-hexosaminidase release. The bioluminescent method is based on the assumption that the level of light output from bacteria is directly proportional to the number of viable bacterial cells. Comparison between light output of bioluminescent *E. coli* and *B. bronchiseptica* (Lux 95) in a short-run experiment (< 20 h) suggested that the correlation between bioluminescence output and optical density of the bioluminescent *E. coli* was not as good as with *B. bronchiseptica* (Lux 95). The difference is probably due to the fact that *E. coli* is a much more rapidly growing organism than *B. bronchiseptica* and will therefore reach stationary phase in a much shorter time.

A problem encountered in this study with *B. bronchiseptica* (Lux 95) was that it had been derived from a laboratory strain that may have partially lost its virulence attributes. Comparison of *B. bronchiseptica* (Lux 95) strain with *B. bronchiseptica* (Bvg^+) and *B. bronchiseptica* (Bvg^-) strains revealed that it had more properties in common with the Bvg^- than the Bvg^+ mode and may not, therefore, have been an ideal strain for this study. Construction of further bioluminescent derivatives of a wild-type (Bvg^+) and avirulent (Bvg^-) strains should be considered for future work.

The bioluminescent method however, was shown to offer an easy and simple method of measuring the viability of the bacteria in real-time. Based on *in vitro* invasion assays, the kinetics of interaction between sheep BMMC and *B. bronchiseptica* (Lux 95) was followed. The data indicated for the first time, the presence of viable bioluminescent *B. bronchiseptica* within sheep BMMC, and this observation was confirmed by both viable count and TEM. For the first time, viable intracellular *B. bronchiseptica* (Lux 95) were shown within sheep BMMC. These results establish baseline data for individual and age-related variation in sheep BMMC phagocytic function, and form a basis for further evaluation of other functions such as chemotaxis, oxidative burst and clearance of bacterial infection by mast cells, either in sheep or other animal models.

Interaction of *B. bronchiseptica* strains with sheep BMMC was studied *in vitro* and their cytotoxicity was compared with that of other bacterial species. Virulence of *B. bronchiseptica* is controlled by the BvgAS genetic system which is shared with

certain other species including *B. pertussis*. Comparison of *B. bronchiseptica* (Bvg^-) and (Bvg^-) strains indicated that only the Bvg^+ phase significantly reduced the viability of sheep BMMC and other cell types (RBL-2H3 and J774A.1 cells). Furthermore, a significant β-hexosaminidase release was occurred after incubation of sheep BMMC with *B. bronchiseptica* (Bvg^+) and this was much greater than the effect of the (Bvg^-) strain and certain other *Bordetella* species. However, it was observed for the first time, that *B. trematum* and *B. hinzii* could cause β-hexosaminidase release from RBL-2H3 cells, a model cell line for mucosal mast cells, similar to the effect of *B. bronchiseptica* (Bvg^+).

Cytotoxicity of different purified factors of *Bordetella* species (ACT, PRN, FHA and PT) towards sheep BMMC was investigated. The results showed that the cytotoxic effect of purified recombinant ACT depends on its enzymatic activity rather than on its cell-invasive activity alone. Inhibition of spontaneous β-hexosaminidase release was detected after incubation of RBL-2H3 cells with either rACT or PT. Also, both factors showed an increase in caspase-3/7 activity in RBL-2H3 cells, a novel finding. Caspase 3/7 and DNA fragmentation assays suggested that rACT could induce apoptosis in both sheep BMMC and RBL-2H3 cells, a novel finding.

The effect of rACT and PT on β-hexosaminidase release from RBL-2H3 cells induced by stimulation with *B. bronchiseptica* (Bvg^+) showed that these factors were working in different ways. rACT was able to inhibit β-hexosaminidase release from RBL-2H3 cells exposed to *B. bronchiseptica* (Bvg^+), but this effect was temporary (i.e. it decreased with increasing time of incubation). In contrast, the effect of PT on β-hexosaminidsae release from RBL-2H3 cells exposed to *B. bronchiseptica* (Bvg^-) appeared to be time-dependent. With increased time of pre-treatment a greater inhibitory effect was observed.

The J774A.1 mouse macrophage-like cell line was used to study the effect of PT and rACT on survival of these J774A.1 cells when infected by *B. bronchiseptica*. The results showed that pre-treatment with PT increased the survival of J774A.1 cells against *B. bronchiseptica* (Bvg^+), but a similar concentration of rACT showed an enhanced cytotoxic effect. However, with lower doses of rACT, an increase in intracellular cAMP may cause impotence of phagocytic function in J774A.1 cells and then their ability to take up *B. bronchiseptica* (Bvg^+) would be crippled and their survival enhanced.

The cytotoxic effect of *B. bronchiseptica* (Bvg^+) on mast cells and other cell types, either directly or by inducing degranulation or apoptosis may suggest how this organisms overcome the natural defences of hosts and causes disease.

CHAPTER ONE

INTRODUCTION

1.1 Introduction to *Bordetellae*

1.1.1 The genus *Bordetella*

On the basis of several criteria including morphological, physiological and antigenic properties and DNA hybridization studies, nine species are recognized to be closely related and, accordingly, are classified in the genus *Bordetella*: *B. pertussis*, *B. bronchiseptica*, *B. parapertussis*$_{hu}$ (human-adapted *B. parapertussis*), *B. parapertussis*$_{ov}$ (ovine-adapted *B. parapertussis*), *B. avium*, *B. hinzii*, *B. holmesii*, *B. trematum*, *B. petrii* and *B. ansorpii* (Fig. 1.1) (Ko *et al.*, 2005; Mattoo and Cherry, 2005). Members of these species are Gram-negative, minute (0.2 micron X 0.7 micron) coccobacilli, which are strictly aerobic (except *B. petrii*) and have a respiratory but never exhibit fermentative metabolism. Their optimal growth temperature is between 35 and 37 °C (Gerlach *et al.*, 2001). *B. avium*, *B. bronchiseptica* and *B. hinzii* are motile by pertrichous flagella (Parton, 2005).

1.1.1.1 *B. pertussis*

B. pertussis causes whooping cough in human. Bordet and Gengou reported the isolation of *B. pertussis* in 1906, although they had observed the organism microscopically in the sputum of a patient with pertussis in 1900 (Bordet and Gengou 1906, cited by Parton, 2005). The organism was originally classified as *Haemophilus pertussis* due to its growth requirement for blood but it was later found not to need the growth factors X (haemin) and V (nicotinamide adenine dinucleotide). The organism was later reclassified in its own genus *Bordetella* (Moreno-Lopez 1952, cited by Parton, 2005).

1.1.1.2 *B. bronchiseptica*

B. bronchiseptica was first isolated during the first decade of the 20th century (Ferry 1910, cited by Parton, 2005) from dogs suffering from distemper. It is also now known to infect many mammals, including humans, although infection in humans is relatively uncommon and usually occurs in immunocompromised hosts (Woolfrey and Moody, 1991). Some cases of humans infected with *B. bronchiseptica* have been related to contact with infected animals (Gueirard *et al.*, 1995).

1.1.1.3 *B. parapertussis* hu or ov

B. parapertussis was isolated originally from a mild case of human pertussis (Bradford and Slavin, 1937 cited by Parton 2005). More recently, ovine strains of *B. parapertussis*, distinct from human strains, have been identified from lambs showing signs of pneumonia (Cullinane *et al.*, 1987). It has been reported that *B. parapertussis* is capable of causing typical whooping cough with paroxysmal coughing, whooping and vomiting (Hoppe, 1999).

B. pertussis, *B. parapertussis* and *B. bronchiseptica* can cause similar diseases in the upper respiratory tract of man or animals. These disease involves the interaction of the bacteria with ciliated tracheal epithelial cells, resulting in ciliostasis and killing of the ciliated cells and damage to the respiratory epithelium (Preston, 2005). However, their host specificities vary dramatically. *B. pertussis* infects only humans; *B. parapertussis* strains can be classified in two groups, one of which infects only humans and one of which infects only sheep. *B. bronchiseptica* causes respiratory infections in a wide variety of mammals and birds but only rarely in humans (Mattoo and Cherry, 2005).

1.1.1.4 *B. avium*

B. avium is the aetiological agent of rhinotracheitis of birds, a disease of the upper respiratory tract which resembles pertussis infection in humans. It was reported that *B. avium* is similar to *B. pertussis* in that both species produce a dermonecrotic toxin and a tracheal cytotoxin, and production of dermonecrotic toxin is regulated by nicotinamide and $MgSO_4$ (Gentry-Weeks *et al.*, 1988).

1.1.1.5 *B. hinzii*

B. hinzii, a species similar to *B. avium*, can cause secondary or opportunistic respiratory infections in poultry. Bacteremia caused by *B. hinzii* has been described in an immunocompromised human without evidence of respiratory infection (Cookson *et al.*, 1994). *B. hinzii* was also isolated in four biliary specimens collected over 6 months from a liver transplant recipient with cholangitis. The isolates were resistant to most beta-lactam antibiotics and fluoroquinolones (Arvand *et al.*, 2004)

1.1.1.6 *B. holmesii*

B. holmesii isolates were originally classified as CDC non-oxidiser group 2 (i.e. vernacular name given to gram-negative, non-oxidizing, brown soluble pigment producing rods), but were found to conform to the description of the family *Alcaligenaceae* and the genus *Bordetella* (Weyant *et al.*, 1995). The first isolates were from the blood of patients that had underlying clinical conditions but, more recently, this species has been isolated from the sputum of immunocompromised patients and from nasopharyngeal specimens of patients suspected to have pertussis (Mazengia *et al.*, 2000).

1.1.1.7 *B. trematum*

B. trematum has been isolated from ear infections and skin wounds in human. It was found to be more closely related to the type species of the genus *Bordetella* (*B. pertussis*) than to the type species of the genus *Alcaligenes* (*Alcaligenes faecalis*) and had the general characteristics of members of this family (Vandamme *et al.*, 1996).

1.1.1.8 *B. petrii*

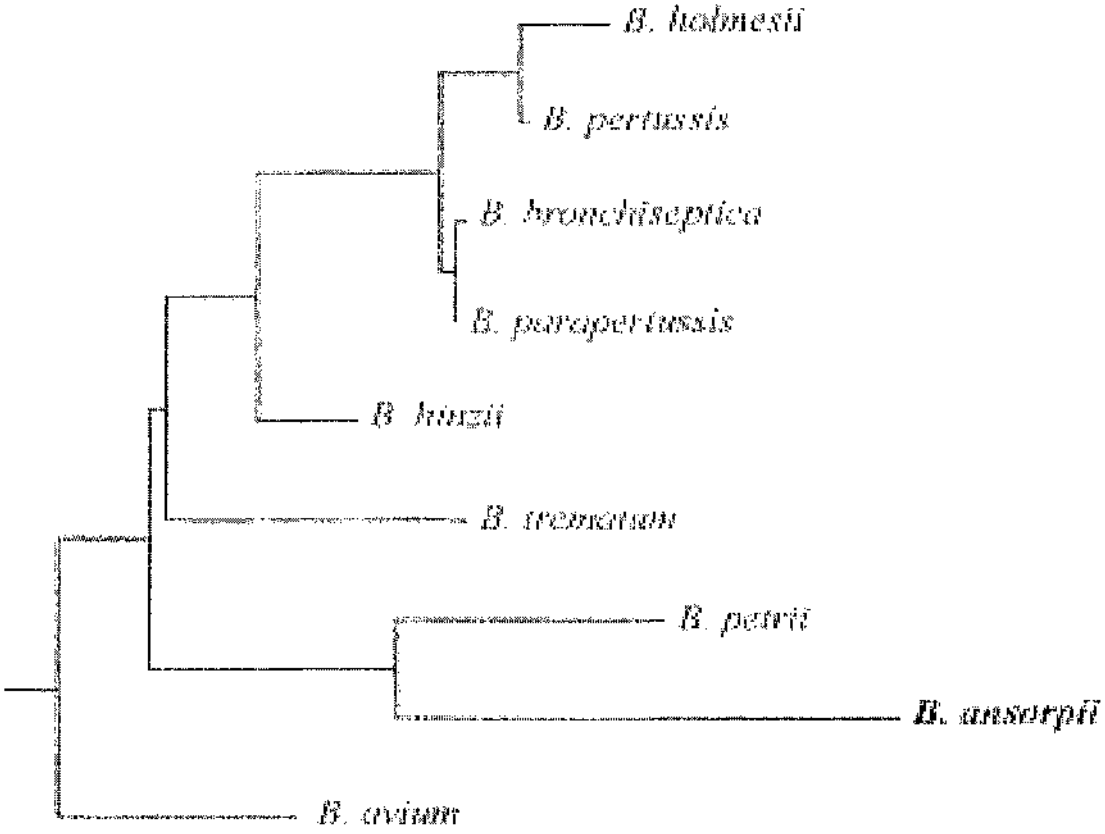
B. petrii strain was originally isolated from the environment and is capable of anaerobic growth (von Wintzingerode *et al.*, 2001). The only clinical isolate of *B. petrii* was from a patient with mandibular osteomyelitis (Fry *et al.*, 2005). *B. petrii* is suggested to be closely related to an environmental progenitor of *Bordetellae* and further characterisation of this species may provide interesting information on the early evolution of members of this genus (Gerlach *et al.*, 2001).

1.1.1.9 *B. ansorpii*

This is the most recently identified strain, based on the evaluated phenotypic and genotypic characteristics. It is a Gram-negative bacillus, originally designated SMC-8986^T, which was isolated from the purulent exudate of an epidermal cyst and was classified as a new *Bordetella* species, namely *Bordetella ansorpii* although its pathogenic significance remains unknown (Ko *et al.*, 2005).

Figure 1.1 Phylogenetic relationships among nine known *Bordetella* species

Schematic presentation of phylogenetic relationships among nine of the known *Bordetella* species inferred from 16S rRNA sequences (Ko *et al.*, 2005).


B. hinzii
B. avium

1.1.2 Growth of *Bordetellae*

Bordetellae are aerobic (except *B. petrii*) with the growth optimum temperature ranging between 35-37°C. Exposure to a temperature of 56°C is lethal within 30 min and the heat-labile toxin is destroyed. They are non-fermentative to carbohydrates. Primary isolation of the whooping-cough bacillus was first achieved by Bordet and Gengou (1906) from sputum. It was cultured in a glycerol-potato-extract agar medium without peptone but containing 50% defibrinated horse blood. A similar medium (Bordet-Gengou medium) is still commonly used. Various solid and liquid media without blood have also been developed for bulk growth of cells, for the production of toxin-containing supernates and for genetic studies. The amount of blood in Bordet-Gengou medium may be reduced to 15 – 20 % for easier detection of haemolysis around the colonies (Parton, 2005).

Stainer and Scholte (1970) developed a chemically-defined liquid medium containing glutamic acid, proline and cystine together with ascorbic acid, niacin, glutathione, salts and Tris buffer. A modified Stainer-Scholte medium was used to induce *B. pertussis* to release substantial quantities of PT and FHA into culture supernates and thereby facilitate the manufacture of an acellular vaccine containing these components (Sato *et al.*, 1984). However, Stainer and Scholte medium did not permit growth from small inocula (Stainer and Scholte, 1970). Imaizumi *et al* (1983) overcame this difficulty by adding dimethyl-β-cyclodextrin (DMβCD) after which it was solidified with agarose to constitute the first defined medium for *B. pertussis* to allow reliable the growth of separated colonies from diluted inocula (Imaizumi *et al.*, 1983).

Media suitable for *B. pertussis* growth are suitable for the other species. The time of visibility and size of the colonies differs between the species. For example, *B. pertussis* colonies appear after 3 days of growth on BG agar but *B. bronchiseptica* colonies become visible after 1 day (Parton, 2005). It was reported that both *B. bronchiseptica* and *B. avium* may be able to survive in the environment as they are capable of growing in low nutrient conditions such as natural waters or phosphate-buffered saline (Porter *et al.*, 1991). Modulation of many virulence factors of *B. bronchiseptica* in response to environmental signals has been shown (Passerini De Rossi *et al.*, 2001). For example, growth on nutrient agar and brain heart infusion broth induced the avirulent phase of *B. bronchiseptica* and growth on tryptic soy agar

induced an intermediate phase. Moreover, upon growth of the *Bordetella* in the laboratory, so-called phase variants may arise spontaneously with a strain-dependent frequency; these variants are no longer able to express the virulence factors owing to mutations in the virulence gene locus (Stibitz *et al.* 1988; Monack *et al.* 1989). Different *Bordetella* species can be distinguished from each other due to several phenotypic differences such as growth on blood-free peptone agar, motility, urease activity and nitrate reduction (Table 1.1).

Table 1.1: Properties of different *Bordetella* species. Adapted from Gerlach *et al* 2001, Preston, 2005 and Ko *et al* 2005.

Feature	*B. pertussis*	*B. bronchiseptica*	*B. parapertussis*	*B. holmesii*	*B. hinzii*	*B. avium*	*B. trematum*	*B. petrii*	*B. ansorpii*
Host	human	mammals	humans, sheep	humans?	birds, humans	birds, reptiles	humans?	environmental	humans?
Disease	whooping cough	various respiratory disease	mild whooping cough	respiratory illness	septicaemia	turkey coryza	unknown	unknown	unknown
Site of isolation	respiratory tract	respiratory tract & blood	respiratory tract	respiratory tract & blood	respiratory tract & blood	respirat-ory tract	wound, ear infection	river sediment	epiderm-al cyst
G+C contents (Mol%)	66 – 68	66 – 68	66 – 68	61.5 – 62.3	65 – 67	62	64 – 65	63.8	ND
Genome size (kbp)[a]	4,086,186	5,338,400	4,773,551	ND	ND	ND	ND	ND	ND
Growth on MacConkey agar	-	+	+	- / +	+	+	+	+	+
Urease production	-	+	+	-	+ / -	-	-	-	-
Oxidase	+	+	-	-	+	+	-	-	-
Nitrate reduction	-	+	-	-	-	-	- / +	-	-

(ND: not determined).

1.1.3 Properties of *B. bronchiseptica* cluster

Based on several criteria including morphological, physiological and antigenic properties, DNA hybridization studies and phage typing, *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* were recognized to be closely related and, accordingly, were classified in the genus *Bordetella* as the *B. bronchiseptica* cluster (Gerlach *et al.*, 2001). All three species cause upper respiratory disease which involves the interaction of the bacteria with ciliated tracheal epithelial cells, resulting in ciliostasis and killing of the ciliated cells (Mallory and Horner 1913, cited by Gerlach 2001). The three species can be distinguished from each other due to several phenotypic differences such as growth conditions, urease activity and nitrate reduction (Table 1.1). Furthermore, it was reported that the genetic diversity between these three *Bordetella* species is quite limited (Gerlach *et al.*, 2001).

1.1.3.1 Genetic variation

Recently, the genome sequences of *B. pertussis* (Tohama-I), *B. parapertussis* (12822, human strain) and *B. bronchiseptica* (RB50, rabbit strain) were successfully completed (Parkhill *et al.*, 2003). Comparison of these genomic sequences show that a large deletion event has occurred in the *B. pertussis* chromosome. A genetic locus in *B. bronchiseptica* which encodes several amino acid transporters is entirely missing in *B. pertussis*. In fact, in Southern blots this gene locus was found to be present in *B. bronchiseptica*, *B. parapertussis* and *B. avium* isolates, but no signal could be obtained with *B. pertussis* DNA (Schneider *et al.*, 2002). In addition, it has been reported that both *B. pertussis* and *B. parapertussis* are predominantly differentiated from *B. bronchiseptica* by large, species-specific regions of difference, many of which encode or direct synthesis of surface structures, including lipopolysaccharide which may be important determinants of host specificity (Cummings *et al.*, 2004). Importantly, the genomes of *B. pertussis* and *B. bronchiseptica* differ considerably in size and their size correlates with their gene coding capacities: *B. bronchiseptica* encodes 1191 more genes than *B. pertussis*. Pseudogenes, remnants of once active genes that have been silenced by mutation are common in *B. pertussis*. *B. bronchiseptica* has only 18 pseudogenes, whereas *B. pertussis* has 358. (Preston, 2005). Furthermore, *B. pertussis* and *B. bronchiseptica* contain a largely identical core genome. The genome sequencing project identified 114 genes as being unique to *B.*

pertussis. But genome analysis by microarray-mediated genomic typing revealed that 103 of the 114 genes were in fact found in other strains of *B. bronchiseptica* and only 11 genes in *B. pertussis* have been found to be species-specific (Cummings *et al.*, 2004).

Many of the genes that are present in *B. bronchiseptica* but absent from *B. pertussis* appear to encode surface proteins (those located in the bacterial outer membrane and thus exposed to the bacterium's environment). In addition, others encode proteins involved with small-molecule uptake and utilization; for example, a large number of proteins enable iron uptake. Both kinds of proteins are important for colonization of and persistence in a mammalian host, and it is likely that *B. bronchiseptica* has the ability to utilize a wide range of nutrients and to adhere to a wide repertoire of different molecules. This might explain the broad host range of *B. bronchiseptica* (Preston *et al.*, 2004).

It is well known that *B. pertussis* is a human pathogen and *B. bronchiseptica* is an animal pathogen. Indeed, *B. bronchiseptica* is rarely found associated with humans. However, preliminary analysis of microrray-mediated genomotyping data (Cummings *et al.*, 2004) has shown that there are no genes associated with adaptation to humans in *B. pertussis*. It is possible that this species expresses certain genes differently than *B. bronchiseptica* and that this difference is responsible for infection in humans. Furthermore, the genes expressed in *B. bronchiseptica* that are pseudogenes in *B. pertussis* are presumably responsible for functions required for existence in the environment or in a non-human host (Preston, 2005).

1.1.3.2 Antigenic differences

Many of the virulence factors characterized in the *Bordetellae* are common to both *B. pertussis* and *B. bronchiseptica*. These include adhesins, such as filamentous haemagglutinin (FHA), pertactin (PRN), tracheal colonization factor and fimbriae, and toxins, such as adenylate cyclase hemolysin, dermonecrotic toxin and tracheal cytotoxin. Other virulence factors are expressed by just one species, such as pertussis toxin and serum–resistance protein expressed by *B. pertussis* or a type-III secretion system expressed by *B. bronchiseptica* (Galan and Collmer, 1999; Smith *et al.*, 2001). The type-III secretion systems (i.e. specific mechanisms for the export of virulence factors into host cells) in *B. bronchiseptica* encode the full, intact operon, which is

expressed and the operon in *B. pertussis* does not seem to be expressed (Stockbauer *et al.*, 2003). Comparisons of many of these virulence factors in either *B. pertussis* or *B. bronchiseptica* identify differences that might bear on the different host ranges and virulence profiles of these organisms.

O-antigens, i.e. the membrane-distal domains of some lipopolysaccharides are involved in many bacterial species in evasion of host innate and acquired immunity mechanisms. The genetic region of the biosynthetic locus probably directs differential modification of the lipopolysaccharide molecule, possibly forming the genetic basis for O-antigenic variation. O-antigens have been reported to be expressed in *B. bronchiseptica* (Di Fabio *et al.*, 1992). The O-antigen biosynthesis locus is not expressed in *B. pertussis* (Preston *et al.*, 1999).

Regarding flagella (i.e. virulence factors for number of organisms), *B. bronchiseptica* is motile and encodes a full flagellar operon, whereas the flagellar operon of *B. pertussis* is inactivated by multiple pseudogenes, leading to a lack of motility (Parkhill *et al.*, 2003).

1.1.4 Phase variation

The variable expression of virulence factors in *B. pertussis,* known as phase variation, was originally divided into four phases. Phase I and phase II of *B. pertussis* were toxic for guinea pigs whereas phase III and IV were not (Leslie and Gardner, 1931, cited by Parton, 2005). Nowadays, only phase I and phase IV are generally recognised. Phase IV strains are avirulent-phase mutants and no longer express the virulence factors associated with phase I strain of *B. pertussis* (Parton, 2005). Phase variation indicates a reversible alteration in the genotype caused by frame shift mutations in which the virulent bacteria simultaneously lose the ability to synthesize toxins and other factors associated with pathogenicity (Weiss and Falkow, 1984). Another type of variation by *Bordetella* has been termed antigenic modulation (Lacey, 1960, cited by Lax, 1985). This involves changes similar to phase variation, but is readily reversed by changing the growth environment (Ezzell *et al.*, 1981). Thus phase I cells grown in the medium containing NaCl express all the above virulence characteristics and are termed X-mode; phase I cells grown in medium containing $MgSO_4$ do not express these characteristics and are termed C-mode. Therefore, expression of virulence factors in *Bordetella* is influenced by the growth conditions *in*

vitro (Idigbe *et al.*, 1981) which is called antigenic modulation, or may be due to genetic mutation (Weiss and Falkow, 1984). In this regard, it was known the transposon Tn*5* induces mutations by insertion of its DNA sequence into other DNA sequences. Tn*5* has been shown to cause polar mutations, that is, it disrupts synthesis of all gene products encoded at sites distal from the insertion site when there is more than one gene product encoded in an operon (Berg *et al.*, 1980). Insertion into a structural gene usually results in a nonfunctional gene product (Kleckner, 1981, cited by Weiss *et al.*, 1983). Tn*5* was introduced into *B. pertussis* by using a suicide plasmid vector (which is unable to replicate in this species). The advantage of transposon mutagenesis is that usually only a single gene is mutated and the site of the insertion is marked both physically by the transposon DNA and genetically with an antibiotic resistance. A transposon mutagen was used in order to produce a series of *B. pertussis* strains that were deficient in individual virulence factors including filamentous haemagglutinin, haemolysin and pertussis toxin and in a virulence regulatory locus (Weiss *et al.*, 1983). Hybridization experiments suggested that, with avirulent-phase mutants obtained by Tn5 mutagenesis, a single region located at a unique site in the *B. pertussis* chromosome controls the phase change. All recombinants which had reacquired the virulence-associated factors had also lost Tn5, indicating that loss of Tn5 was required to restore the virulent phenotype of *B. pertussis*. The Tn5 avirulent-phase mutants behave as if the insertion interrupted the function of a transacting gene product which is required for the expression of the other virulent-phase genes (Weiss and Falkow, 1984).

1.1.4.1 BvgAS signal-transduction system

Based on genetic analysis, evidence suggested that both phenotypic (antigenic) modulation and phase variation are under the control of a single genetic locus, which was originally designated as the *vir* (virulence) locus, that in turn was able to alter expression of virulence determinants. The *vir* locus was later termed the *b*ordetella *v*irulence *g*ene (*bvg*) locus (Arico *et al.*, 1989; Stibitz and Yang, 1991). The predicted products of this locus are homologous to a family of regulatory proteins that transmit sensory signals using a conserved two-component motif. Originally *bvgA*, *B* and *C* were described. The *bvgA* gene encodes a transcriptional activator and, in the absence of modulating conditions, it was proposed that BvgC activated BvgA by

phosphorylation (Arico *et al.*, 1989). It was later discovered that Bvg B and Bvg C were part of one larger protein and this was termed BvgS (sensor). BvgS is a 135-kDa membrane-bound protein with an N-terminal periplasmic region flanked by two transmembrane sequences that hold the protein in position within the cytoplasmic membrane (Stibitz and Yang, 1991). BvgA is a 23-kDa cytoplasmic protein. The 2-domain structure of BvgA (called the activator), consisting of a receiver at the N-terminus and C-terminal helix-turn-helix (HTH) motif, is characteristic of response regulators (Stibitz and Yang, 1991).

The BvgS periplasmic domains senses external stimuli (perhaps via dimerization) and triggers a series of phosphorylation steps starting with autophosphorylation in the transmitter domain at a conserved histidine residue. This is followed by the transfer of the phosphate group to a conserved aspartic acid in the receiver domain, which transfer it to a histidine at the C-terminal domain (Roy *et al.*, 1990; Uhl and Miller, 1996). BvgA is activated by the transfer of a phosphate group from the C-terminus of the BvgS to conserved aspartic acid in the BvgA N-terminal receiver domain. Upon phosphorylation, BvgA positively regulates the *bvgAS* operon and other virulence factor promoters, including *bvgR*, by binding to a heptameric target sequence (Karimova and Ullmann, 1997). Phosphorylated BvgA can bind DNA via its HTH domain and it functions as a transcriptional activator and repressor resulting in the expression of distinct phenotypic phases (Cotter and DiRita, 2000). Similar regulation of virulence factors is also known to occur in *B. parapertussis* and *B. bronchiseptica* and *B. avium* (Parton 2005). A number of virulence-*r*epressed *g*enes (*vrg*) have now been identified but, in most cases, their functions are unknown. They are regulated by the *bvg*-locus in reciprocal fashion to the *v*irulence-*a*ctivated *g*enes (*vag*) encoding the virulence factors (Parton, 2005). The regulation of virulence expression via the *bvg* locus is summarized in Fig. 1.2.

The virulent phenotype (Bvg^+ phase) is characterised by the expression of all Bvg-activated adhesins and toxins. In contrast, the avirulent phase type (the Bvg^- phase) is characterised by the absence of all Bvg-activated adhesins and toxins. The signals to which Bvg responds *in vivo* are unknown but, *in vitro*, the system is activated by growth at 37°C and silenced either by growth below 27°C or by adding sulphate ions or nicotinic acid to the growth medium, regardless of growth temperature (Coote, 2001).

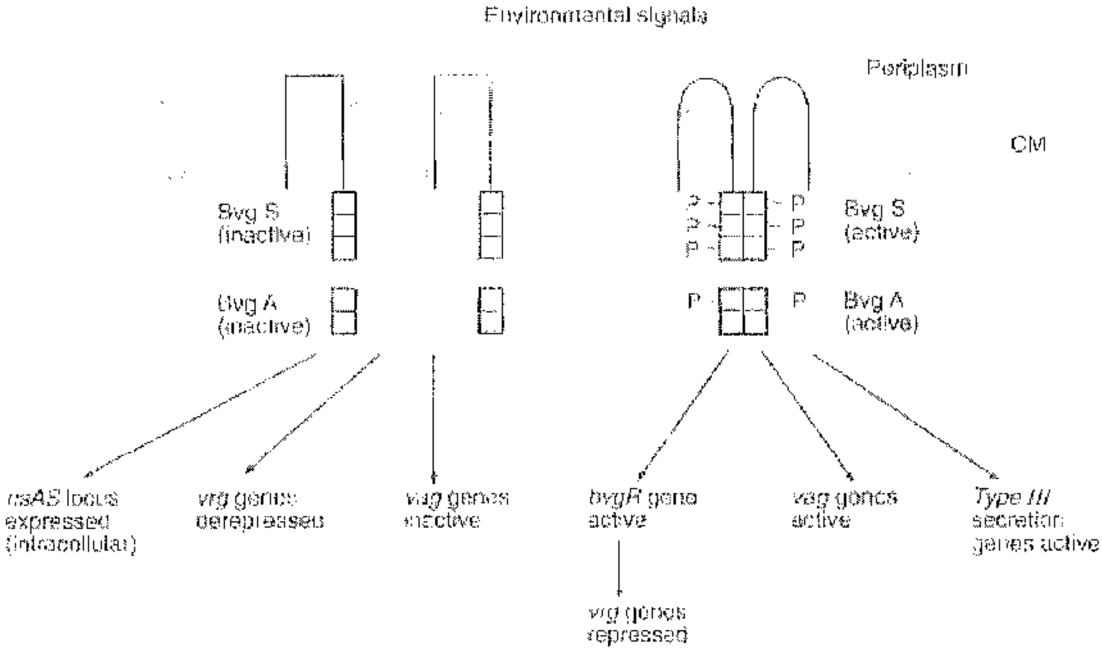
To directly address the role of Bvg-mediated signal transduction in *B. pertussis* pathogenesis, a Bvg $^-$ phase-locked mutant was constructed and compared with the wild type for its ability to colonize the respiratory tracts of mice. This experiment showed that the Bvg $^+$ phase of *B. pertussis* was necessary and sufficient for respiratory infection (Martinez de Tejada *et al.*, 1998). Therefore, the Bvg $^-$ phase appears not to play a role in virulence at least in the mouse (Akerley *et al.*, 1995). However, there is some indication of the presence of a Bvg^i (intermediate) phase and this has been assigned to wild type *B. bronchiseptica* able to grown in submodulating concentrations of magnesium sulphate or nicotinic acid. This phase is characterised by the absence of the Bvg-repressed phenotype and the presence of some but not all Bvg-activated virulence factors (Cotter and Miller, 1997; Cotter and DiRita, 2000).

The contribution of the BvgAS system to phenotypic differences in either *B. pertussis* or *B. bronchiseptica* was compared. Both *B. pertussis* and *B. bronchiseptica* contain a nearly identical BvgAS system that mediates a biphasic transition between virulent Bvg $^+$ and avirulent Bvg $^-$ phases. In the Bvg $^+$ phase, the two species express a similar set of adhesins and toxins (except PT which is restricted to *B. pertussis*) (Matto and Cherry 2005). The Bvg $^-$ phase of *B. bronchiseptica* is characterized by expression of motility and several metabolic processes invloved in redox reactions and amino acid transport (McMillan *et al.*, 1996) and survival under nutrient limiting conditions (Cotter and Miller, 1994). An environmental reservoir for *B. pertussis* seems less plausible, as these strains are more fastidious and appear to be confined to transmission by the respiratory droplet route (Mattoo and Cherry, 2005).

Recently, it was reported that the Bvg system also contributes to biofilm formation of *B. bronchiseptica* which is important for attachment of microorganisms to a surface (Irie *et al.*, 2004). It was shown that *B. bronchiseptica* forms the strongest biofilm phenotype *in vitro* in the intermediate (Bvg^i) phase. This was demonstrated by the formation of a strong biofilm phenotype by wild-type bacteria primarily in Bvg^i mode growth conditions and also by the consistently high biofilm levels formed by a Bvg^i-phase-locked mutant regardless of growth conditions. (Irie *et al.*, 2004).

Figure 1.2 *Bordetella virulence gene* (*bvg*) structure

Bvg-mediated virulence regulation. The *bvg* locus encodes two proteins BvgS and BvgA with homology to 2-component signal-transduction proteins. BvgS is a sensor protein spanning the inner membrane and BvgA is a cytoplasmic response regulator protein able to bind to the DNA. The virulent mode (Bvg^+) is due to incoming signals which are transmitted to the cytoplasmic portion of BvgS which autophosphorylates (P) and then activates BvgA. Then, allows transcription of the virulence-activated gene (*vags*) and causes repression of other, virulence-repressed gene (*vrgs*). In the avirulent (Bvg^-) mode, only the *vrgs* are expressed (adapted from Coote, 2001).



Environmental signals
Periplasm
CM
Bvg S
(inactive)
Bvg A
(inactive)
Bvg S
(active)
Bvg A
(active)
rsAS locus
expressed
(intracellular)
vrg genes
derepressed
vag genes
inactive
bvgR gene
active
vag genes
active
Type III
secretion
genes active
vrg genes
repressed

1.1.5 Virulence factors of *Bordetella*

Numerous bacterial factors important in the pathogenicity of *Bordetella* species and the process of this disease have been characterized (Table 1.2).

1.1.5.1 Adhesin factors

1.1.5.1.1 Filamentous Haemagglutinin (FHA)

FHA appears to be the major adhesin of *B. pertussis* and similar but not identical proteins are present in *B. bronchiseptica* (Spears *et al.*, 2003). Genes encoding other FHA-like proteins have been identified in the genome sequences of *B. bronchiseptica*, *B. pertussis* and *B. parapertussis* (Parkhill *et al.*, 2003). Also, it has a filamentous structure, supported by electron microscopy studies, giving the dimensions of the molecule as 2 nm wide and 45–50 nm long (Makhov *et al.*, 1994). Mature FHA (220 kDa) derives from a larger precursor, FhaB (367 kDa) that undergoes post-translational maturation (Guedin *et al.*, 2000).

FHA is both surface-associated and secreted from virulent *Bordetella*. It is efficiently secreted through a peptide-dependent pathway, with the help of one specific outer membrane accessory protein, FhaC (Guedin *et al.*, 2000). Most *B. bronchiseptica* isolates produce and secrete FHA at significantly lower levels than *B. pertussis*. This may reflect differences in strength of their respective FHA promoters, in the primary structure or function of FhaC accessory proteins, or in the overall cell envelop structure (Locht *et al.*, 1993).

Makhov and co-workers (1994) proposed that FHA consists of a polypeptide chain folded into a monomeric hairpin, comprising head, shaft and tail regions. The model predicts that whilst the head contains the terminal domains and the tail the important RGD (arginine-glycin-aspartic acid) sequence for adhesion, the shaft is composed of tandem 19-amino acid residue repeat regions R1 (38 cycles) and R2 (13 cycles) which maintain the structural integrity of the molecules. FHA also possesses a carbohydrate recognition domain (CRD), which has a unique affinity for glycolipids, and mediates attachment to ciliated epithelial cells as well as cultured Chinese hamster ovary cells (Prasad *et al.*, 1993).

Table (1.2): *Bordetella* virulence factors, adapted from Preston, 2005.

Bordetella factors	Expressed by	Function	Acellular vaccine component
Filamentous hemagglutinin	BP, Bpa, BB	Adhesin (mediates adherence of bacteria to host structures)	Yes
Pertactin	BP, Bpa, BB	Adhesin	Yes
Fimbriae	BP, Bpa, BB	Adhesin	Yes,:Type 2 & 3
Pertussis toxin	BP	Catalyse ADP ribosylation of host G-proteins which causes a number of effects	Yes: inactivated toxin
Adenylate cyclase	BP, Bpa, BB	Cytotoxin. Synthesizes cAMP in host cells, which disrupts host cell physiology. Anti-inflammatory effect through action on host immune cells.	NO
Type III secretion system	Bpa, BB	Alters host immune cell function. Important for chronic infection?	NO
Dermonecrotic toxin	BP, Bpa, BB	Toxin. Activates host GTP binding protein Rho. Causes changes in host cell morphology	NO
Tracheal colonization factor	BP	Adhesin?	NO
BrkA	BP. some BB	Resistance to serum-mediated killing	NO
Lipopolysaccharide	BP, Bpa, BB	Structural component of bacterial outer membrane. Proinflammatory activity. Resistance to host defence molecules	NO
Tracheal cytotoxin	BP, Bpa, BB	Cytotoxin. Contributes to damage to the respiratory epithelium	NO

BP: *B. pertussis*, **Bpa:** *B. parapertussis* and **BB:** *B. bronchiseptica.*

The tail region of FHA includes a binding moiety specific for complement receptor 3 (CR3) integrins present on the surface of macrophages. The RGD sequences in the tail region facilitates binding to CR3 and in turn leads to phagocytosis via CR3, which enables the bacteria to avoid triggering an oxidative burst and thereby permitting them to survive intracellularly whilst evading the immune system (Relman *et al.*, 1990). FHA is also known to bind the human serum protein C4BP. This protein is known as a regulator of complement activation and acts to inhibit the classical complement pathway, preventing the formation of the membrane attack complex (Berggard *et al.*, 1997).

A recent report suggested that FHA may be a critical virulence factor in facilitating pathogen persistence in the respiratory tract by suppressing or delaying the development of cell-mediated immunity (McGuirk and Mills, 2000). In this regard, it has been shown that purified FHA inhibited IL-12 secretion by a macrophage cell line or alveolar macrophages in response to *E. coli* or *B. pertussis* lipopolysaccharide. Antibodies to FHA or denaturation of FHA abrogated the inhibitory effect. The suppressive effect of FHA was specific for IL-12, since the production of TNF-α, IL-6 and IL-10 was not suppressed, and production of IL-6 and IL-10 was up-regulated. Antibody-blocking studies revealed that the inhibitory effect of FHA on IL-12 production was dependent on IL-10. Further work confirmed these findings by showing that FHA was able to stimulate secretion of TNFα and may induce apoptosis in human monocyte-like cells, macrophages and bronchial epithelial cells (Abramson *et al.*, 2001).

Recently, it has been demonstrated that FHA in *B. bronchiseptica* is both necessary and sufficient for mediating adherence to a rat lung epithelial cell line, and necessary but insufficient for colonisation of the tracheas of anaesthetised rats (Cotter *et al.*, 1998). Cotter *et al.* suggested that the role of FHA was to overcome the clearance mechanism of the mucociliary escalator.

It was reported that FHA alone will protect against *B. pertussis* infection. Mice that were immunised with FHA by intraperitoneal or intramuscular inoculation, then challenged with an aerosol of *B. pertussis*, had significantly reduced bacterial colonisation in the lungs and trachea (Kimura *et al.*, 1990). FHA is currently included in multicomponent acellular pertussis vaccines.

1.1.5.1.2 Fimbriae

Fimbriae (pili), also known as agglutinogens, are long filamentous protrusions which extend from the bacterial cell surface and facilitate a variety of binding capabilities. *Bordetella* species express fimbriae of at least four serotypes, Fim2, Fim3, FimX and FimA (Mattoo and Cherry, 2005). In both *B. pertussis* and *B. bronchiseptica* the required locus for fimbriae secretion and assembly (*fim*B, *fim*C and *fim*D) is located between *fha*B and *fha*C (Mattoo *et al.*, 2000). The BvgAS locus (Figure 1.2) encodes a two-component system which is responsible for the control of expression of *fim*2 and *fim*3. Fimbrial phase variation is characterised by switching between a high and a low level of expression of a particular *fim* gene. A region located 100 bp upstream of the fim3 gene is involved in the regulation, where a stretch of cytosines (Cs) upstream of the *fim* promoter would be subject to slip-strand mispairing during chromosome replication (Willems *et al.*, 1990).

It was suggested that fimbriae may mediate the binding of *Bordetella* to the respiratory epithelium via the major fimbrial subunits and to monocytes via the tip protein FimD. It was shown that purified *B. pertussis* fimbriae, with or without FimD, were able to bind to heparan sulphate, chondroitin sulphate, and dextran sulphate, sugars that are ubiquitously present in the mammalian respiratory tract (Geuijen *et al.*, 1996). Heparin-binding domains within the Fim2 subunit were identified and found to be similar to those of the eukaryotic extracellular matrix protein, fibronectin. Studies by Hazenbos et al. suggest that FimD mediates the binding of non-opsonized *B. pertussis* to VLA-5 on the surface of monocytes, which then causes activation of CR3, thereby enhancing its ability to bind FHA (Hazenbos *et al.*, 1995).

The role of fimbriae in the pathogenesis of *Bordetella* species has been investigated using mutant strains. A *B. bronchiseptica* mutant that was unable to express fimbriae was defective in its ability for efficient establishment and persistent colonisation of the rat trachea (Mattoo *et al.*, 2000). However, the FimD deficient mutant strain was found to produce 75% less FHA than the wild type and this may have been partly responsible for the difference in colonisation behaviour. The FHA mutant was far less affected in its colonisation ability than the FimD deficient strain so it was assumed that colonisation defects were due to the lack of this minor subunit (Geuijen *et al.*, 1997). Moreover, the serum antibody profiles of animals infected with Fim deficient *B. bronchiseptica* differ qualitatively and quantitatively from those of animals infected with wild type *B. bronchiseptica* suggesting that fimbriae may play

important roles, not only in adherence, but also in the nature and magnitude of the host immune response to *B. bronchiseptica* infection (Mattoo *et al.*, 2000).

Recently, it was demonstrated that the *B. pertussis* fimbriae protected mice against *B. parapertussis* infection although less efficiently than against *B. pertussis* infection. Furthermore, this result indicates that *B. pertussis* and *B. parapertussis* fimbriae are antigenically distinct (Willems *et al.*, 1998).

1.1.5.2 Autotransporters factors

Bordetella strains express a number of related surface-associated proteins belonging to the autotransporter secretion system. The autotransporter family includes functionally diverse proteins such as proteases, adhesins, toxins, invasins, and lipases, that appear to direct their own export to the outer membrane of the bacterium (Henderson and Nataro, 2001). Autotransporter proteins consist of an N-terminal region (passenger domain), which confers the effector functions, and a conserved C-terminal region (ß-barrel), which is required for the secretion of the passenger proteins across the membrane. It was reported that, after cleavage of the N-terminal signal sequence in the periplasm, the C terminus folds into a ß-barrel in the outer membrane, forming an aqueous channel for transport of the passaged domain to the cell surface (Mattoo and Cherry, 2005). The autotransporters of *Bordetella* form a homologous family defined on the basis of their conserved C termini and the possession of RGD motifs of the type implicated in integrin binding (Henderson and Nataro, 2001).

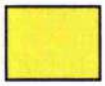
B. bronchiseptica encodes the greatest number of autotransporters and both *B. pertussis* and *B. parapertussis* have fewer autotransporter genes and more autotransporter pseudogenes (non-functional) than *B. bronchiseptica* (Parkhill *et al.*, 2003). The autotransporter factors that have been best characterised are pertactin (PRN), tracheal colonisation factor (Tcf), virulence-activated gene 8 (Vag8), *bordetella* resistance to killing protein A (BrkA) and *Bordetella* autotransporter protein C (BapC) (Figure 1.4).

Figure 1.3: *Bordetella* autotransporters factors

Schematic diagram showing similarities between the autotransporter proteins in *Bordetella*. Numbering relates to the amino acid position and Arginine-Glycine-Aspartatic acid (RGD) sites are indicated. Pertactin is also shown with its immunodominant region 1 (GGXXP) and immunodominant region 2 (PQP)x, adapted from Packard, 2004.

PRN: Pertcatin
BrkA: Bordetella resistance to killing protein A
Vag8: Virulence-activated gene 8
Tcf: Tracheal colonisation factor
BapC: Bordetella autotransporter protein C
RGD: arginine-glycine-aspartic acid

Outer membrane localisation signal



C-terminal region

Putative cleavage site

Signal sequence

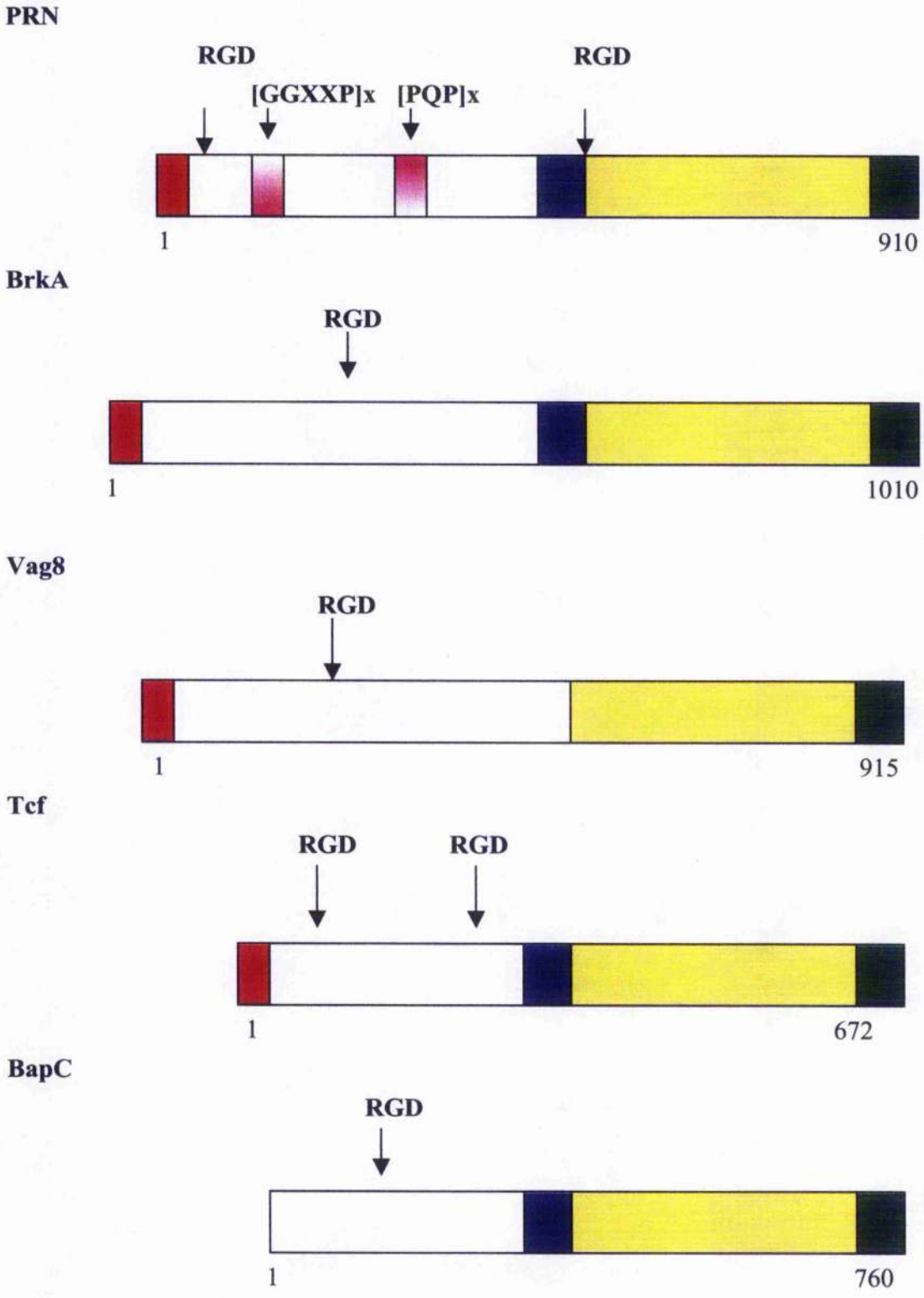


Figure 1.3: Different forms of Autotransporters factors. Adapted from Packard, 2004.

1.1.5.2.1 Pertactin

Pertactin (PRN) was the first autotransporter member of *Bordetella* species to be identified and studied. Mature pertactin is a 68- kDa protein in *B. bronchiseptica* (Montaraz *et al.*, 1985), a 69- kDa protein in *B. pertussis* (Charles *et al.*, 1989), a 70-kDa protein in *B. parapertussis* (Li *et al.*, 1991; Finn and Stevens, 1995). Pertactin has been proposed to play a role in attachment to mammalian cells such as Chinese hamster ovary (CHO) cells and this attachment involved the participation of RGD (Arg-Gly-Asp)-containing sequence within the molecule (Leininger *et al.*, 1991; Emsley *et al.*, 1994). The gene *prnA* which encodes PRN is controlled by the *bvg* locus (Charles *et al.*, 1994). Investigation of the role of pertactin in *B. bronchiseptica* pathogenesis revealed that pertactin was involved in the cytotoxicity of this organism for mononuclear phagocytic cells (Fleckenstein *et al.*, 1996). Furthermore, no evidence was found for the contribution of pertactin in *B. pertussis* adhesion to either bronchial or laryngeal cells (van den Berg *et al.*, 1999). PRN is the only autotransporter for which the crystal structure has been determined (resolution, 2.5 Å). The protein comprises a 16-stranded parallel β-helix with a V-shaped cross-section and is the largest β-helix known to date (Emsley *et al.*, 1996).

Pertactin has been shown to be an immunoprotective antigen, being used in subunit vaccines to protect mice from respiratory challenge with virulent *B. pertussis* (Novotny *et al.*, 1991; Roberts *et al.*, 1993). Pertactin is included in some of the multicomponent acellular pertussis vaccines that are currently in use (van Loo *et al.*, 2002).

1.1.5.2.2 *Bordetella* resistance to killing protein A

A *B. pertussis* mutant (BPM2041) which has Tn5 lac inserted in a *bvg*-regulated gene was found to be at least 10-fold more susceptible to human-serum killing than the wild type *B. pertussis*. This new locus was named brk (*bordetella* resistance to killing) (Fernandez and Weiss, 1994). The brk locus was cloned and sequenced, and it was found to encode two divergently transcribed open reading frames (ORFs), termed BrkA and BrkB and both ORFs were reported to be necessary for serum resistance. BrkA shows 29% identity to pertactin and has two RGD motifs in addition to a conserved proteolytic processing site and an outer membrane-targeting signal. Like pertactin, BrkA is involved in adherence and invasion (Fernandez and

Weiss, 1994). Despite the similarities, a pertactin mutant was found to be not as sensitive to serum killing as the BrkA or BrkB mutants.

In Southern blot analysis, *brkAB* sequences were found in *B. bronchiseptica* and *B. parapertussis* but not in *B. avium*. Clinical isolates of *B. bronchiseptica* and *B. parapertussis* were serum resistant, and wild-type strains possessing additional copies of the *brk* locus were two- to five fold more resistant to serum killing (Fernandez and Weiss, 1998). Although BrkA confers resistance to killing by complement in *B. pertussis*, interestingly, loss of BrkA in *B. bronchiseptica* did not confer sensitivity to complement-mediated killing (Rambow *et al.*, 1998).

1.1.5.2.3 Tracheal colonisation factor A

This protein is encoded by the *tcfA* gene. When a strain of *B. pertussis* 18323 lacking this protein was used to infect mice by an aerosol challenge, the number of bacteria isolated from the tracheas was decreased 10-fold when compared with the parent 18323 (Finn and Stevens, 1995). Furthermore, by southern blot analysis of chromosomal DNA from strains of *B. bronchiseptica* and *B. parapertussis*, using a probe derived from *tcfA*, strong hybridization was seen only to *B. pertussis* DNA. Thus, Tcf appears to be a unique virulence factor of *B. pertussis* (Finn and Stevens, 1995).

1.1.5.2.4 Virulence-activated gene 8

Vag 8 was initially discovered by transposon mutagenesis and shown to be positively controlled by the *bvg* locus (Finn and Amsbaugh, 1998). Vag-8 deficient *B. pertussis* was found to be significantly less able to colonise and persist in the lungs and tracheas of mice when compared to *B. pertussis* 18323^{T} (Finn and Amsbaugh, 1998). The complete gene is present in the *B. parapertussis* genome (Parkhill *et al.*, 2003).

1.1.5.2.5 Bordetella autotransporter protein C

BapC is a newly identified member of the autotransporter family. BapC has properties in common with the other members of the autotransporter family particularly BrkA (Blackburn, 2000; Bokhari, 2002). The *B. pertussis bapC* gene sequence was originally submitted to GenBank as a putative autotransporter protein gene (accession number AF081494) named *bap5* (Blackburn, 2000).

The potential role of *bapC* was identified using a *B. pertussis bapC* mutant strain. The mutant strain was more susceptible to complement-mediated killing *in vitro* than the parent strain and was less able to colonise the respiratory tract in a mouse model when compared to the parent strain (Bokhari, 2002).

1.1.5.3 Toxins

1.1.5.3.1 Adenylate cyclase toxin (ACT)

It was shown for the first time that *B. pertussis* in phase I produce a factor able to inhibit phagocytosis of opsonized targets and chemotaxis of PMN. The new factor was named PMN-inhibitory factor (PIF) (Utsumi *et al.*, 1978). Then, it was showed that *Bordetella* species are able to produce a soluble, heat-stable, and highly active adenylate cyclase. This enzyme is internalized by phagocytic cells and catalyzes the unregulated formation of adenosine 3',5'-monophosphate (cyclic AMP), thereby disrupting normal cellular function (Confer and Eaton, 1982). Transposon Tn5 was used to isolate mutants of *B. pertussis*. One haemolysin mutant had no detectable adenylate cyclase activity. The new deficient strain of *B. pertussis* was avirulent in an infant mouse model of infection. (Weiss *et al.*, 1983). The adenylate cyclase (AC) of *B. pertussis* was first shown to be associated with toxic activity by its ability to attenuate neutrophil superoxide production and bactericidal capabilities (Galgiani *et al.*, 1988). However, immune effector cells such as neutrophils, monocytes, macrophages, dendritic cells and natural killer cells are thought to be the primary target (Mattoo and Cherry, 2005). *B. parapertussis* and *B. bronchiseptica* have AC activity similar to that of *B. pertussis* but the toxins are antigentically distinct (Parton, 2005).

1.1.5.3.1.1 ACT structure

The adenylate cyclase toxin (or CyaA) (Figure 1.5) is a bifunctional protein with the calmodulin-stimulated adenylate cyclase activity residing in the amino-terminal, and haemolytic and invasive activity located in the carboxy-terminus (Glaser *et al.*, 1991). CyaA is encoded by the *cyaA* gene, and the *cya* operon (Fig. 1.6) is composed of *cyaA* and three accessory genes *cyaB*, *D* and *E*, that are located downstream and are necessary for the secretion of CyaA (Goyard and Ullmann, 1991). Furthermore, it was shown that the *cyaC* gene in *B. pertussis*, homologous to

the the *hlyC* gene in *E. coli* (Figure 1.6) was required for the haemolytic activities of the *cyaA* gene product (Barry *et al.*, 1991).

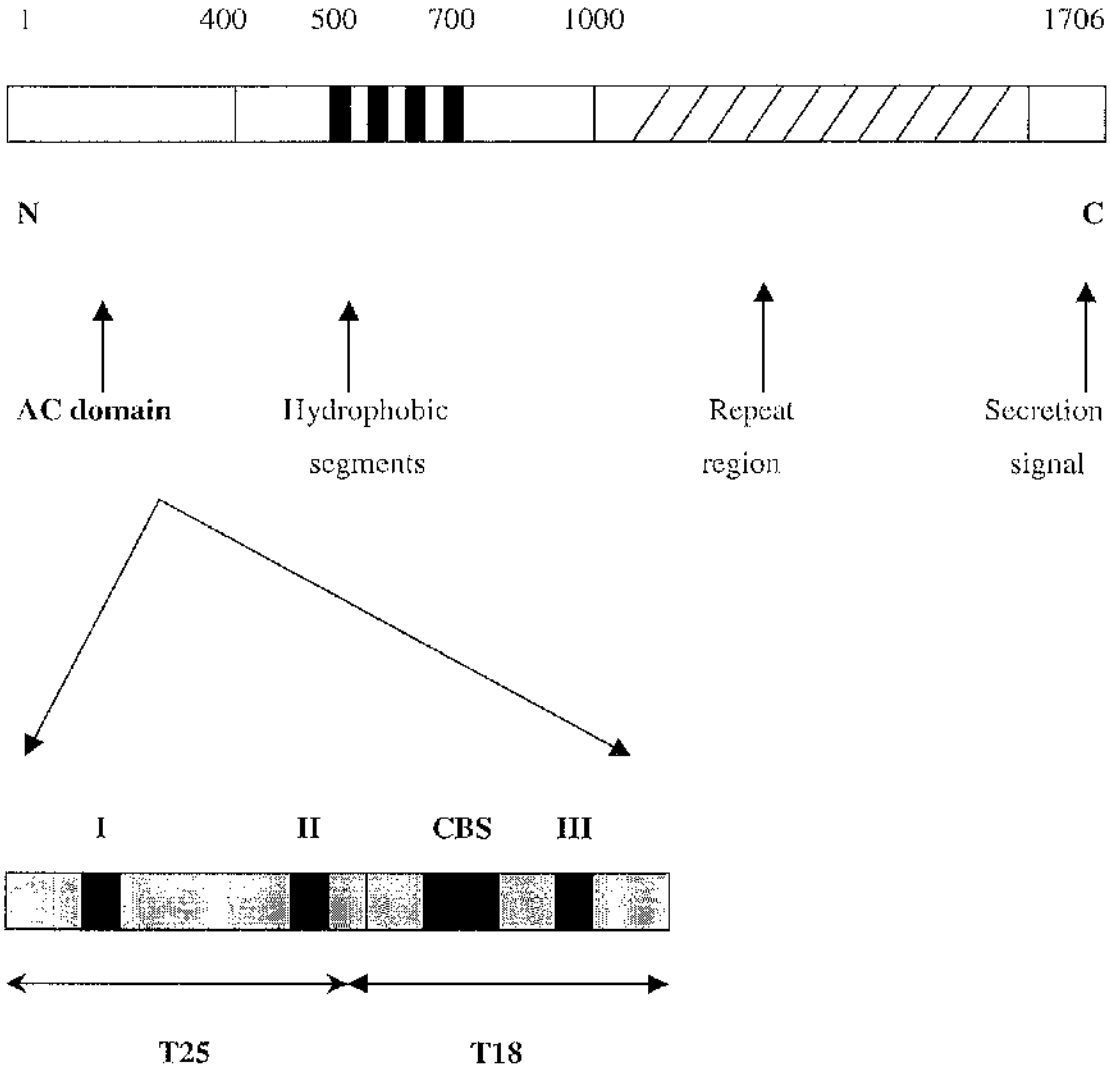
CyaA (or ACT) is a member of the repeats in toxin (RTX) family of calcium-dependent, pore-forming cytolysin (Coote, 1992). These toxins are characterised by a series of tandem repeats (41 repeats) that are rich in glycine (G) and aspartic acid (D) residues. The toxin (AC) has a molecular mass of 177 kDa, is a single polypeptide of 1706 amino acids composed of a 400 amino acids N-terminal domain, and responsible for the camodulin-simulated enzymatic activity (Ladant, 1988), and a 1307 amino acids C-terminal domain that is capable of forming pores, allowing the N-terminus to enter many eukaryotic cells where it catalyses the conversion of endogenous ATP into cAMP. Within the AC domain there are two subdomains designated T25 and T18 (Figure 1.4). The T25 domain contains the catalytic site where ATP is bound. T18 contains the calmodulin binding site (Mock and Ullmann, 1993b).

1.1.5.3.1.2 Activation of CyaA by CyaC

The *cya* locus of *B. pertussis* is composed of five genes: *cyaA* encodes the bifunctional CyaA protein, whose secretion requires the expression of the downstream *cyaB*, *D* and *E* genes. Activation of CyaA requires the expression of a fifth gene, *cyaC* located upstream from *cyaA* (Barry *et al.*, 1991). In this regard, mass spectrometry was used to study the activation of ACT from *B. pertussis*. It was shown that ACT requires the product of an accessory gene in order to express their biological activities. In this study, wild-type AC toxin was modified by amide-linked palmitoylation on the epsilon-amino group of lysine 983. This modification was absent from a mutant in which the accessory gene had been disrupted. A synthetic palmitoylated peptide corresponding to the tryptic fragment (glutamine 972 to arginine 984) that contained the acylation blocked AC toxin-induced accumulation of adenosine 3',5'-monophosphate, whereas the non-acylated peptide had no effect (Hackett *et al.*, 1994). These data suggest that palmitoylation at this site represents the modification that is required for the toxicity and haemolytic activity of CyaA.

Figure 1.4 Structural organisation of the *B. pertussis* adenylate cyclase toxin.
Structural organization of the *B. pertussis* adenylate cyclase (CyaA) toxin. Numbers represent amino acid residues. The catalytic domain (AC) is enlarged to show the T25 and T18 subdomains. CBS corresponds to the main calmodulin-binding site, and boxes I, II and III represent regions involved in catalysis, (Ladant and Ullmann, 1999).

Adenylate cyclase toxin (or Cya A) of *B. pertussis*

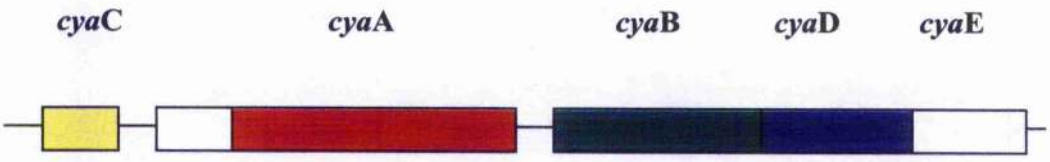


Enlargment structure of the catalytic domain (AC) to show T25 and T18

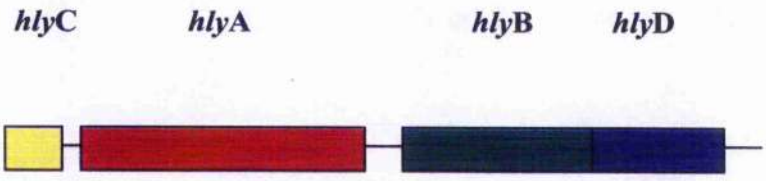
Figure 1.5 Comparison of the *B. pertussis* (*cya*) locus and the *E. coli* (*hly*) operon

The *cya* operon is composed of *cyaA* and three accessory genes *cyaB*, *D* and *E*, that are located downstream and are necessary for the secretion of CyaA, (Mock and Ullmann, 1993).

B. pertussis



E. coli



1.1.5.3.1.3 Recombinant ACT production by *E. coli*

Production of active CyaA was achieved in a reconstructed *E. coli* system by cloning the 5 different genes of the *cya* locus on appropriate vectors under the control of promoters and *E. coli*-specific translation initiation signals. This step was necessary because *B. pertussis* is a slow growing organism and its genetic analysis was still limited at the time of this work. It was shown that CyaA produced alone in *E. coli* had no invasive or haemolytic activity and that the *CyaC* gene product was required to render the CyaA holotoxin invasive and haemolytic in the reconstructed system (Sebo *et al.*, 1991). The aggregation of the CyaA proteins enabled a simple purification procedure by extracting with 8 M urea (Glaser *et al.*, 1989).

In another study, an efficient T7 RNA polymerase system was used for overexpression of *cyaA* and *cyaC* in *E. coli* (Westrop *et al.*, 1996). The *E. coli* host (BL21/DE3) strain was used for expression of different forms of AC. Generally, this strain contains chromosomally-located λDNA expressing the gene encoding T7RNA polymerase under the control of the inducible lacUV5 promoter. Addition of isopropyl-1-thio-β-D galactoside (IPTG) to a growing culture induces the polymerase which, in turn, transcribes the target DNA in the plasmid via a T7 promoter. It was demonstrated that *E. coli* (BL21/DE3) which contain plasmids pGW44 and pGW54 produced CyaA and CyaC in amounts that were similar to those obtained when the plasmids were carried separately (Westrop *et al.*, 1996). Furthermore, a third compatible plasmid (pGW 44/188) was constructed from pACM 188 (Ladant *et al.*, 1992) and encoded the CyaA protoxin that lacked enzymic activity due to a dipeptide amino acid insertion in the N-terminal end. pGW44/188 was co-expressed with pGW54 to produce a full-length invasive toxin with little enzymic activity (pGW 44/188,54). These constructs enabled the production of the different forms of AC (or CyaA) in *E. coli* without the inconvenience of growing *B. pertussis* and free from other *B. pertussis* components that could have displayed different effects.

Different forms of recombinant AC toxin produced in *E. coli* have been highly purified by using several methods. The most effective methods were a combination of DEAE sepharose column chromatography followed by phenyl sepharose column chromatography. Both methods were combined because it helped to remove contaminating proteins from *E. coli* (Lawrence *et al.*, 2002).

1.1.5.3.1.4 Interaction of ACT with target cells

AC toxin (or CyaA) can penetrate a wide range of cell types, including mammalian erythrocytes that lack membrane trafficking (Gray *et al.*, 1999). Entry of AC toxin into target cells is a two-step process. In the first step, CyaA binds to the cell surface, possibly to gangliosides. This requires both the integrity of the carboxyl-terminal region of CyaA and the CyaC-dependent palmitoylation of Lys983. Binding of toxin to erythrocytes will eventually lead to cell lysis, due to ability of CyaA to form pores (Gray *et al.*, 1998). The second step comprises the actual translocation of the catalytic domain of the bound CyaA through the plasma membrane of the cells. This process is dependent upon the temperature (it only occurs above 15-20°C), the membrane potential of the target cells and the presence of millimolar concentrations of calcium ions (Mock and Ullmann, 1993a). It was shown that extracellular calcium was absolutely essential for ACT entry into target cells, and the penetration was blocked by neutralising, anti-adenylate cyclase antibodies (Rogel *et al.*, 1989).

AC toxin (or CyaA) of *B. pertussis* uses the Mß2 integrin (CD11b/CD18) as a cell receptor. Binding of CyaA to the surface of various hematopoietic cell lines correlated with the presence of the Mß2 integrin on these cells. Moreover, binding of CyaA to various murine cell lines and human neutrophils was specifically blocked by anti-CD11b monoclonal antibodies (Guermonprez *et al.*, 2001). It was shown that increase of intracellular cAMP level and cell death triggered by CyaA intoxication was also specifically blocked by anti-CD11b monoclonal antibodies.

Interaction of AC toxin and target cells requires a close association of live bacteria with target cells and the active secretion of AC toxin and is not dependent on the surface-associated AC toxin (Gray *et al.*, 2004). Wild type *B. pertussis* (BP338) treated without or with gentamicin for 2 h at 37°C were tested for their ability to intoxicate J774 cells and intracellular cAMP levels were assessed. Treatment with gentamicin reduced the number of viable organisms and intoxication by *B. pertussis* was reduced by 98% without affecting the binding of these bacteria to target cells. The amount of measurable AC enzymatic activity on intact gentamicin-treated bacteria was 80% of untreated bacteria. This modest reduction is most likely because of the fact that untreated bacteria were growing normally for an additional 2 h during the gentamicin treatment and accumulating more surface-associated AC toxin. Also, it was shown that gentamicin was not having a detrimental effect on AC toxin. In addition, irradiation was applied to *B. pertussis* (BP338). It was found to behave

equivalently to those bacteria treated with gentamicin. Both numbers of viable bacteria and intoxication was decreased by 80%. However, there was no effect on binding of the bacteria to target cells or the amount of AC enzyme activity associated with the intact killed organism. These data show that viable bacteria are necessary for delivery of AC toxin to the target cell (Gray *et al.*, 2004).

Once inside the cell, AC toxin moiety is activated by the endogenous eukaryotic protein calmodulin, then causes unregulated synthesis of cAMP from adenosine triphosphate (ATP) which interfering with normal cell functions (Parton 2005).

1.1.5.3.1.5 Role AC toxin

It is important to mention that both *B. bronchiseptica* and *B. pertussis* produce AC toxin. It was reported that both a virulent *B. bronchiseptica* (9.73H+) strain and a virulent *B. pertussis* (Tohama) strain possessed AC, hemolytic and invasive activities that were not significantly different (Gueirard and Guiso, 1993). Immunological properties were tested by using Western bloting. Sera of *B. pertussis* infected mice collected 30 days post infection recognized the *B. pertussis* ACT but not the *B. bronchiseptica* ACT. Previous data suggest that immunological and protective properties of AC toxin from *B. bronchiseptica* are different from that of *B. pertussis* (Gueirard and Guiso, 1993).

The role that AC toxin may have in the pathogenicity of *B. pertussis* has been investigated. It was demonstrated that AC deficient *B. pertussis* had a reduced ability to cause lethal infection in infant mice (Weiss and Falkow, 1984). Furthermore, it was shown that an AC mutant *B. pertussis* 348, was rapidly cleared from mouse lungs with no viable bacteria remaining 10 days post challenge (Goodwin and Weiss, 1990). This observation suggested that AC toxin is critical for colonisation and initiation of infection. AC toxin may be involved in *B. pertussis*-induced lung inflammation and in apoptotic death of alveolar macrophages. The resulting apoptosis may play a role in the initiation of infection (Gueirard *et al.*, 1998). It was determined that both domains. AC and haemolysin, were necessary to induce macrophage apoptosis as a mutated protein containing only the haemolysin domain was unable to induce apoptosis (Bachelet *et al.*, 2002). It was found that CyaA targets one or more aspects of innate immune response, one of which is probably neutrophils. This was further investigated and it was demonstrated that CyaA inhibits both Fc receptor-mediated attachment and

phagocytosis of *B. pertussis* by neutrophil (Weingart *et al.*, 2000). Further study showed that FHA mediates attachment of *B. pertussis* to neutrophils, but adenylate cyclase toxin blocks phagocytosis (Weingart and Weiss, 2000).

1.1.5.3.1.6 Protective role of AC toxin

It was demonstrated that immunization with specific anti-*B. pertussis* adenylate cyclase antibodies or with purified *B. pertussis* secreted adenylate cyclase protected mice against a lethal respiratory challenge with *B. pertussis* or *B. parapertussis* (Guiso *et al.*, 1989). It has been shown that *B. pertussis* is killed following phagocytosis by neutrophils. Therefore, identifying the antigens that stimulate the immune response to promote phagocytosis and killing by neutrophils could help to improve pertussis vaccines. In these regards, it was reported that neutralizing antibodies to ACT, in conjunction with opsonzing antibodies, could be beneficial in promoting immunity to pertussis by enhancing the phagocytic defence mechanisms (Weingart *et al.*, 2000).

Unlike with most microorganisms, opsonization does not appear to promote phagocytosis of wild-type *B. pertussis* by neutrophils and the bacteria remain extracellular. Weingart et al. (2000) reported that opsonization with human immune serum inhibited both attachment and phagocytosis of the wild-type *B. pertussis* to neutrophils. But, when monoclonal antibodies (i.e. that had been demonstrated to be most potent in neutralizing ACT activity) was added to the opsonized bacteria caused a statistically significant increase in the number of extracellular bacteria attached to the neutrophils as well as in the number of phagocytosed bacteria relative to the number in opsonized controls. In contrast, monoclonal antibodies which inhibited hemolysin activity but not adenylate cyclase toxin activity did not elicit a significant increase in the numbers of bacteria attached or phagocytosed. These results indicate that antibodies which neutralize adenylate cyclase toxin activity mediate a beneficial effect on phagocytosis by neutrophils but only when combined with human opsonizing antibodies.

The effect of co-administration to mice of the fully functional acylated CyaA toxin or a toxin lacking adenylate cyclase enzymic activity (CyaA*) with other antigens from *B. pertussis* (PT, pertussis toxoid (PTd), FHA and PRN). CyaA* enhanced the serum IgG antibody responses to each of these antigens whereas, with acylated CyaA, only anti-PRN antibody titres showed a modest increase. Peritoneal

macrophages and spleen cells, collected at 2 weeks post-immunisation, were cultured and tested for nitric oxide (NO) and IFNgamma production, respectively, after stimulation in vitro with heat-killed *B. pertussis* cells or CyaA proteins. NO and IFN-γ production were higher in cells collected from mice immunised with CyaA or CyaA* in combination with a PT, FHA and PRN antigen mixture than from those taken from mice injected with antigen mixture alone, again with CyaA* acting as a better adjuvant than CyaA. The apparent enhancement of immune responses to the antigen mixture by CyaA* in particular was not paralleled by increased protection of mice against aerosol challenge with *B. pertussis* (Macdonald-Fyall *et al.*, 2004).

Recently, it was demonstrated that CyaA has adjuvant activity promoting Th2 (T helper type 2) and Tr1 (T regulatory type 1) responses and the enhancement of antigen-specific IL-10 producing cells (Ross *et al.*, 2004). Furthermore, it was shown that acylated AC toxin (A-CyaA) and nonacylated AC toxin (NA-CyaA) enhanced T-lymphocyte receptor (TLR-ligand) induced IL-10 and inhibited IL-12, and TNF-alpha production by macrophages and DC. Furthermore, both A-CyaA and NA-CyaA promoted the induction of murine IgG1 antibody, Th2, and regulatory T cells against coadministered antigens *in vivo* (Boyd *et al.*, 2005). This report examined the influence of acylation and enzymatic activity on the adjuvant effect of CyaA *in vivo* and it demonstrated that NA-CyaA and A-CyaA exert similar effects on innate cells and that CyaA with an inactive adenylate cyclase domain (iAC-CyaA), lacks immunomodulatory activity *in vitro*. BALB/c mice were immunized once or twice with keyhole limpet hemocyanin (KLH) alone or with A-CyaA, NA-CyaA, or iAC-CyaA. Analysis of antibody titers revealed that NA-CyaA and A-CyaA enhanced KLH-specific IgG responses to the same extent, whereas iAC-CyaA had significantly reduced adjuvant activity. The adjuvant effect was not thought to be due to residual LPS in the toxin preparations as enhancement of antibody response to KLH was also observed in TLR-4-defective C3H-HeJ mice. It was concluded from the data collected from this work that lack of acylation does not compromise the adjuvant properties of CyaA or its ability to direct the induction of T cell and antibody responses *in vivo* (Boyd *et al.*, 2005).

1.1.5.3.2 Pertussis Toxin

Pertussis toxin (PT) is an important virulence factor in *B. pertussis* and is actively secreted. Even if not expressed, the PT gene is also present in *B. bronchiseptica* and *B. parapertussis* but they appear to be transcriptionally silent due to defective promoters or activator (Bvg A) binding site (Arico and Rappuoli, 1987; Parkhill *et al.*, 2003). However, two atypical strains of *B. bronchiseptica* have been described that appeared to have produced the toxin during infection (Stefanelli *et al.*, 1997).

PT is an ADP-ribosylating toxin, and is structured as a hexamer composed of five subunits, designated S1–S5 relative to their electrophoretic mobilities in denaturing gels (Locht and Keith, 1986). All the genes for PT subunits are closely linked in an operon and are probably expressed in a precise ratio which is similar to that of other AB5 toxins such as, cholera and *E. coli* heat-labile toxins (Locht and Keith, 1986). Secretion of PT across the outer membrane requires a specialized transport apparatus the *p*ertussis *t*oxin *l*iberation, *ptl*, locus (Farizo *et al.*, 1996). In this process, PT and Ptl proteins form a complex for spanning the inner and outer membranes and passing through the peptidoglycan layer (Burns, 1999).

PT is important for virulence of *B. pertussis* in mouse models of infection. One probable target is the phagocytic cells, in limiting their migration to the site of infection in the respiratory tract and in reducing their phagocytic potential and killing mechanisms (Schaeffer and Weiss, 2001). Upon receptor binding, PT is taken into the eukaryotic cell by receptors-mediated endocytosis and undergoes vesicular trafficking and membrane translocation to its target proteins on the inner surface of the plasma membrane. PT needs to be activated to exert its toxic effects. This requires ATP and the reduction of a disulphide bond in the S1 subunit to liberate it from the B pentamer (Hazes *et al.*, 1996).

Many of the activities of PT depend on the enzymatic activity of the S1 subunit. Based on extensive *in vitro* characterization of PT, the S1 subunit in its reduced form has been shown to catalyze the transfer of ADP-ribose from NAD to the subunit of guanine nucleotide-binding proteins (G proteins) in eukaryotic cells. PT and their inactivation effects on G proteins such as Gi, Gt (transducin), and Go lead to different biological effects. Biological effects of PT attributed to the disruption of these signalling pathways include histamine sensitization, enhancement of insulin

secretion in response to regulatory signals, and both suppressive and stimulatory immunological effects (Mattoo and Cherry, 2005).

The precise role of PT and its action in the host cells still remain obscure (Hewlett, 1997). It was reported that PT enzymic activity was necessary for full colonisation of *B. pertussis* in the mouse model (Carbonetti *et al.*, 2003). Chemically-detoxified PT has been demonstrated to be protective in the mouse model against intracellular and aerosol challenges of *B. pertussis* (Sato *et al.*, 1984) and this molecule has been included in all acellular pertussis vaccine preparations (Hewlett, 1997). Plasmid DNA expressing PT (S1) subunit was evaluated for the ability to induce protection against PT challenge or *B. pertussis* infection in mice. This DNA vaccination induced anti-PT IgG production, inhibited leukocytosis-promoting activity and induced protection in mice against intracerebral challenge with *B. pertussis* (Kamachi *et al.*, 2003).

1.1.5.3.3 Dermonecrotic Toxin (DNT)

The dermonecrotic toxin is heat-labile at 56°C. The products of the *dnt* genes of *B. pertussis* and *B. bronchiseptica* are 1464 amino acids in length with a MW of 160.6 kD and there are only 11 residues different between the two polypeptides (Pullinger *et al.*, 1996). DNT activity is found in 4 species, and genetic studies showed that *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* produce DNTs which are essentially indistinguishable in their physicochemical, serological and biological properties whereas that of *B. avium* is similar (Walker and Weiss, 1994).

DNT (heat-labile) toxin induces localized necrotic lesions in mice and other laboratory animals when injected intradermally and is lethal for mice at low doses when administered intravenously (Parton, 1985). A *B. bronchiseptica* strain deficient in DNT production was associated with considerably less turbinate atrophy and pneumonia in pigs compared to the wild-type parent and also had reduced ability to colonize the nasal cavity (Brockmeier *et al.*, 2002). The various effects of DNT on target cells include stimulation of DNA and protein synthesis and formation of actin stress fibres and focal adhesions. These effects are thought to be mediated by its activity as a transglutaminase catalyzing the deamidation or polyamination of GTP-binding protein Rho and other members of the Rho family involved in regulation of the cytoskeleton. These result in constitutive activation of the Rho proteins (Masuda *et al.*, 2002). Furthermore, Cys 1305 has been identified as an essential amino acid for

the enzymatic activity of DNT, as the introduction of a mutation at Cys 1305 eliminated the activity (Kashimoto *et al.*, 1999).

The role of DNT in the pathogenicity of *B. pertussis* is still unclear. However, the dermonecrotising activity appears to be due to a specific constrictory effect on vascular smooth muscle tissue. This effect on similar tissue in the respiratory tract could induce a local inflammatory reaction and could account for some of the pathology of pertussis (Hodgson *et al.*, 2005).

1.1.5.3.4 Endotoxin (Lipopolysaccharides)

Endotoxins, or lipopolysaccharides (LPSs), are major components of the outer membrane of Gram-negative bacteria. They generally consist of complex mixtures of related lipopolysaccharides of which the lipid region (lipid A) is covalently linked to a polysaccharide comprising a core region to which may be linked an O-chain having a variable number of subunits. Different species of *Bordetella* express different LPS molecules, which may be a factor in the high level of species specificity demonstrated within this genus (van den Akker, 1998).

The endotoxin of *B. pertussis* has been serologically characterized, and the structures of the constituent lipid A and cores have been documented (Caroff *et al.*, 1994; Caroff *et al.*, 2000). *B. pertussis* endotoxin has no O-chains, unlike those of most other species of the *Bordetella*. Furthermore, LPS produced by *Bordetella bronchiseptica* is similar to that of *B. parapertussis*, as they both express temperature-dependent O antigen (Di Fabio *et al.*, 1992).

There is no clear understanding of the role of endotoxin in *Bordetella* infections although it may act in concert with other *Bordetella* toxins. However, its importance is suggested by the observation that changes in LPS structure in *B. bronchiseptica* are controlled by the BvgAS virulence regulatory system (van den Akker, 1998). *B. bronchiseptica* also produces host-specific LPS molecules, with isolates from dogs having more heterogeneous LPS structures than isolates from pigs (van den Akker, 1998). A human isolate of *B. bronchiseptica* displays a different LPS profile than that of a rabbit isolates. Subsequent isolation of *B. bronchiseptica* from the same human patient over 2 years has shown a variation in LPS profiles during the course of infection (Le Blay *et al.*, 1997).

The role of lipopolysaccharide in virulence of *Bordetella* species was studied. It was well known that the *wlb* gene cluster which is composed of 12 genes, is required for biosynthesis and addition of the trisaccharide in *B. pertussis* and *B. bronchiseptica* and the O-antigen-like repeat in *B. bronchiseptica* and *B. parapertussis* (Allen and Maskell, 1996). So, deletion of the *wlb* locus results in the loss of membrane-distal polysaccharide domains in the three subspecies of bordetellae. Different species of the *B. bronchiseptica* cluster with mutantions in *wlb* genes were used to investigate the roles of distal LPS structures in respiratory tract infection by bordetellae. Each mutant was defective compared to its parent strain in colonization of the respiratory tracts of BALB/c mice by *B. pertussis* and *B. parapertussis*. Interestingly, the *B. bronchiseptica* Deltawlb strain was defective, compared to the wt strain, in colonization of the respiratory tracts of BALB/c mice, but did not differ from the wt strain in its ability to colonize the respiratory tracts of B-cell- and T-cell-deficient mice, suggesting that wlb-dependent LPS modifications in *B. bronchiseptica* modulate interactions with adaptive immunity. These data show that biosynthesis of a full-length LPS molecule by these three bordetellae is essential for the expression of full virulence for mice. In addition, the data indicate that the different distal structures modifying the LPS molecules on these three closely related subspecies serve different purposes in respiratory tract infection, highlighting the diversity of functions attributable to LPS of gram-negative bacteria (Harvill *et al.*, 2000).

Mutants of *B. bronchiseptica* and *B. parapertussis* that were deficient in O-antigen biosynthesis both lacked the complement resistance associated with the wild type but only the *B. parapertussis* mutant had reduced ability to colonize the mouse respiratory tract (Burns *et al.*, 2003).

1.1.5.3.5 Tracheal cytotoxin

Tracheal cytotoxin (TCT) is released during log phase broth culture of *B. pertussis* and consists of 15 amino acid residues as well as two amino sugars. The selective biological activity of TCT has been studied in tracheal organ cultures by light and electron microscopy. A series of pathological changes precedes the eventual extrusion of ciliated cells, while all other epithelial cell types appear ultrastructurally normal. TCT also causes a dose-dependent inhibition of DNA synthesis in cultured hamster trachea epithelial cells (Goldman and Herwaldt, 1985). Tracheal cytotoxin is

an unusually small toxin with a mass of 921 Da. The toxin is known to be expressed by *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. avium*. TCT is a disaccharide-terapeptide derived from glycopeptide which is released from the cell envelope. Its structure is N-acetylglucosaminyl-1,6-anhydro-N-acetylmuramylalanyl-γ-glutaminyl-diaminopimelylalanine, which is identical to the *Neisseria gonorrhoeae* ciliostatic anhydropeptidoglycan (Cookson *et al.*, 1989).

The activities of TCT have been studied *in vitro* using in hamster tracheal epithelial cells. It has been demonstrated that the lactyl tetrapeptide part of the molecule is responsible for full toxic activity (Luker *et al.*, 1995). The abnormal release of large amounts of TCT by *Bordetella* spp. causes an exaggerated response that results in respiratory tract pathology (Flak *et al.*, 2000). The toxicity conferred by TCT is indirect, being caused by the induction of host cells to produce IL-1 which stimulates nitric oxide (NO) production. This activated host cell NO acts by destroying iron-dependent enzymes, eventually inhibiting mitochondrial function and DNA synthesis in nearby host cells (Heiss *et al.*, 1994). TCT also has a toxic effect on other cells, impairing neutrophil function at low concentrations and conferring toxic activity in larger quantities (Cundell *et al.*, 1994).

1.1.6 Type III secretion system

A large number of gram-negative pathogenic bacteria secrete virulence factors via the type III secretion system (TTSS) during infection of host cells (Lee, 1997). Type III secretion system allow bacteria to translocate bacterial effector proteins directly into the plasma membrane or cytoplasm of eukaryotic cells through a needle-like injection apparatus (Cornelis and Van Gijsegem, 2000), then these effector proteins alter normal host cell signalling cascades and other processes (Muller *et al.*, 2001). The *Bordetella* TTSS locus, termed the *bsc* locus, includes 22 genes, which encode a secretion apparatus that spans the bacterial cytoplasmic and outer membranes, as well as translocator proteins that form pores in the eukaryotic cell membrane and a type III secretion-specific ATPase required for apparatus assembly and secretion (Kimbrough and Miller, 2002).

A functional type III secretion system has been discovered in *B. bronchiseptica*. Wild type *B. bronchiseptica*, but not the TTSS mutant, was shown to induce cytotoxicity against L2 cells that were established from rat lung epithelial cells

(Yuk *et al.*, 1998). Biochemical and morphological analyses demonstrate that the *B. bronchiseptica* Type III secretion system induces a cell death pathway that resembles necrosis rather than apoptosis and is distinct from that of *Yersinia*, *Shigella* and *Salmonella* (Orth *et al.*, 1999). Furthermore, a TTSS mutant of *B. bronchiseptica* was unable to colonise over the long term in the tracheas of immunocompetent mice and this mutant was unable to induce cell death in J774 macrophage (Yuk *et al.*, 2000).

Recently, an additional locus consisting of four genes, *btrS*, *btrU*, *btrW* and *btrV* (*btr* encode for "Bordetella type III regulation"), situated 3' to the *bsc* locus was identified and shown to encode regulatory proteins for the Bordetella Type III secretion system. Like the *bsc* genes, these new genes are also transcriptionally activated by BvgAS. Deletions in *btrU* and *btrW* revealed an uncoupling of protein expression from secretion, since these mutants expressed type III proteins at normal levels but failed to secrete them (Mattoo *et al.*, 2004).

In addition, it was shown that TTSS in *B. bronchiseptica* participate in modulation of innate immune response (Legarda *et al.*, 2005). Wild type *B. bronchispetica* express TTSS, shows suppression of the activation of the signalling pathway that is induced upon LPS (lipopolysaccharide) stimulation, whereas a TTSS-defective strain did not. These results suggest a mechanism for bacterial evasion of the innate immune response in the airway, which could allow for the observed persistent colonization of *B. bronchiseptica* (Legarda *et al.*, 2005).

1.1.7 *Bordetella* infections

B. pertussis, *B. parapertussis* and *B. bronchiseptica* are highly related subspecies that colonize the respiratory tracts of mammals. *B. pertussis* has exclusively adapted to the human host while *B. parapertussis* comprises two distinct subspecies that infect either humans or sheep. In contrast to host-restricted members of the *Bordetella* genus, *B. bronchiseptica*, which appears to represent the evolutionary progenitor, infects the respiratory tracts of a broad range of mammals (Parton, 2005). *B. bronchiseptica* is associated with a variety of chronic respiratory diseases in laboratory animals. This contrasts with the acute human disease that results from *B. pertussis* and *B. parapertussis* infections.

Pertussis is primarily a disease of the respiratory tract and is a severe debilitating disease in young children. It still afflicts up to 40 million children

worldwide (Ivanoff and Robertson, 1997). Infection with *B. pertussis* is initiated by the attachment of organisms to the cilia of epithelial cells of the upper respiratory tract (Weiss and Hewlett, 1986) and most patients with fatal cases of pertussis have bronchopneumonia. The initial stages of infection with *B. pertussis* entail congestion and infiltration of the mucosa by lymphocytes and polymorphonuclear leukocytes. The lumens of the bronchi contain inflammatory debris. Various reports suggest that the initial pulmonary lesion in pertussis is a lymphoid hyperplasia of peribronchial and tracheobronchial lymph nodes (Lasfargues *et al.*, 1993). Pertussis or whooping cough is responsible for severe morbidity and mortality world wide mainly in unvaccinated communities. Fortunately, global immunization schemes are steadily reducing morbidity and mortality due to pertussis infection (WHO: www.who.int/vaccines-diseases/diseases/pertussisvaccine.htm) (Parton, 2005).

1.1.7.1 Infection and the immune response

The aim of the immune response against extracellular pathogens is to destroy and clear the pathogen and to neutralise its products. In vertebrates, immunity against pathogens or against other foreign substances is divided into two categories; innate immunity or acquired immunity (Roitt *et al.*, 1996). Innate immunity is present from birth and includes such functions as physiological barriers, cellular defences and inflammation, whereas acquired immunity develops as a consequence of an encounter with a foreign substances. B (develop in bone marrow) and T (develop in thymus) lymphocytes are responsible for the specificity exhibited by the acquired immune response. B-cells produce antibodies (Ab) which recognises to foreign bodies called antigens (Ag) and bind to them (i.e. humoral immunity). T-cells divided into different cells; cytotoxic T-cells which destroy the infected cells or helper T-cells which activate the infected cells by production of different chemical compounds such as cytokines (acquired immunity).

1.1.7.2 Immunity in pertussis

Immunity against pertussis infection could be developed either by natural infection or by vaccination. Natural infection with *B. pertussis* is followed by an increase in the concentrations in serum of IgA, IgG, and IgM antibodies to specific antigens as well as to preparations of the whole organism (Mattoo and Cherry, 2005).

Evidence of a role for antibodies has been shown in the murine model, particulary for PT and FHA. Early work showed the protective activities of FHA and PT and it was found that antisera containing antibodies to PT were able to protect mice against intracerebral challenge, whereas sera lacking anti-PT antibodies but containing anti-FHA antibodies was unable to protect mice (Munoz *et al.*, 1981). The role of antibodies in pertussis infection was studied by using Ig-defective mice (Ig $^{-/-}$ mice) (Mahon *et al.*, 1997). They demonstrated that B cell-deficient mice were unable to clear bacteria after aerosol infection when compared with the control mice. The Ig $^{-/-}$ mice failed to mount an IgG antibody response whereas the wild-type mice developed *B. pertussis*-specific serum antibody by the day 24 after aerosol challenge. The evidence for the importance of T cells in immunity to *B. pertussis* was provided by the demonstration that mice devoid of T and B cells failed to clear the bacterium from the respiratory tract, whereas BALB/c mice (immunocompetent) cleared the infection after 5 weeks (Mills *et al.*, 1993). Further evidence for a role of T cells (i.e. classified according to their cluster of differentiation (CD) into $CD4^+$ and $CD8^+$), was provided by the demonstration that $CD4^+$ T cell knockout mice could not be protected by intra-nasal challenge with inactivated bacteria whereas, in contrast, $CD8^+$-depleted mice did not differ from wild-type mice in their ability to control aerosol pertussis challenge (Leef *et al.*, 2000).

1.1.7.3 Pertussis vaccines and immunity

Killed *B. pertussis* whole-cell vaccines (Pw) have played an essential role in the prevention of pertussis infection (1959). These vaccines consist of heat- or formalin-inactivated virulent *B. pertussis*. Widespread vaccination significantly decreased the number of pertussis cases, but publicity in the 1970s directed to the possible adverse reactions to Pw presented a serious social problem for vaccination against pertussis (Gustafsson *et al.*, 1996). Therefore, the development of more efficacious pertussis vaccine became an urgent need. A major effort was undertaken to develop a new safer acellular pertussis (Pa) vaccine consisting of toxoided components of *B. pertussis* (formalinized pertussis toxin (PT) and filamentous haemagglutinin (FHA)) (Sato *et al.*, 1984). Thus, combined vaccine which is consisting of diphtheria toxoid (D), tetanus toxoid (T) and acellular pertussis (Pa) (Sato and Sato, 1999).

Regarding immune response against due to either Pw or Pa vaccines, induction of T cells and Ab production have been studied. The immune responses induced in mice due to Pa and Pw induced distinct T cell populations as in children. Pw vaccines have been shown to induce Th1 responses which are important for bacterial clearance. On the other hand, Pa has shown to produce Th2 responses and antibodies (IgG) which also play an important role in protective immunity against pertussis infection (Barnard *et al.*, 1996). Furthermore, it was reported that acellular vaccine induced a high level of antibody to PRN, Fim or PT and was associated with a lower risk of infection (Cherry *et al.*, 1998) and with a reduction in reactogenicity compared with Pw vaccine (Donnelly *et al.*, 2001).

1.1.7.4 Pertussis and asthma

In several studies, an inverse relationship between asthma and the overall burden of respiratory infections has been reported. Asthma is a continuing and growing problem world-wide (Umetsu *et al.*, 2002). The asthma cascade is initiated by allergic sensitisation dependent on T-cell activation, IL-4 production, IgE antibody synthesis and mediator release from mast cells.

Mast cells are well known as effector cells in allergic reaction (see section 1.2). They are widely distributed throughout the body especially at the host-environment interface and they can be activated to release various mediators in response to external stimuli via immunoglobulin E (IgE)-dependent or IgE-independent mechanisms.

Although pertussis is not generally viewed as an allergic disorder, pertussis-vaccinated animals have been used as models of allergic sensitisation, including asthma, and for investigating its pharmacological modulation (Nilsson *et al.*, 1998). Furthermore, associations have been reported between a history of pertussis infection and allergic sensitisation (Wjst *et al.*, 1994) and wheezing illness (Strachan *et al.*, 1996) and between pertussis vaccination and a risk of bronchial asthma (Odent *et al.*, 1994).

1.1.7.4.1 Pertussis infection and development of asthma

A prospective study on this subject was performed in 25 children aged 0.8-12.2 years. At the time of pertussis infection, the mean serum IgE concentration in the

study group was 62 +/- 30 kU/ml. At the follow-up visit, there was a significant increase in serum IgE to 137 +/- 51 kU/ml, which was also significantly higher than IgE in an age-matched control group. Children at a significantly higher risk for developing an IgE increase or new allergic sensitizations were those with a family history of allergy or potentially allergic disease in their personal history. These observations suggested that pertussis infection may induce IgE production in affected children (Schuster *et al.*, 1993).

The role of previous pertussis infection and the prevalence of allergic sensitization and allergic rhinitis in children has also been studied. A study was carried out on 13,937 10-year-old children from Germany and data (questionnaire and skin prick tests with six different allergens) were analyzed. It was found that pertussis was much more common in the western than in the eastern part of Germany. The allergic sensitization after pertussis infection was slightly increased in western Germany than in eastern Germany. These results suggested that infection with pertussis may have had a weak influence on allergic sensitization (Wjst *et al.*, 1994).

Furthermore, in a study of wheezing illness, it was reported that the cumulative incidence was 18% by age 7, 24% by age 16, and 43% by age 33. The incidence during childhood was strongly and independently associated with pneumonia, hay fever, and eczema. There were weaker independent associations with male sex, whooping cough, recurrent abdominal pain, and migraine (Strachan *et al.*, 1996). These results suggested that infection with pertussis seems may not have influence on wheezing illness.

1.1.7.4.2 Pertussis vaccine and development of asthma

It was reported in 1994 that, among 243 pertussis-vaccinated children at a mean age of 7.8 years, 10.7% were given a diagnosis of asthma compared with 1.97% of the 203 children who had not been immunized (Odent *et al.*, 1994). Furthermore, among a birth cohort of 1265 children that, at the age of 10 years, among those 23 children who had not received the pertussis component of the triple (diphtheria-pertussis-tetanus) vaccine, none had asthma or other allergic diseases, whereas among children receiving diphtheria-pertussis-tetanus vaccine, 23% had asthma and 30% had consultations for other allergic diseases (Kemp *et al.*, 1997).

A prospective cohort study was designed to examine the relation between pertussis vaccination and the prevalence of wheezing illnesses in 9444 young children

on whom data on wheezing symptoms and vaccination status was available. Unadjusted comparisons of the defined wheezing illnesses in vaccinated and non-vaccinated children showed no significant association between pertussis vaccination and any of the wheezing outcomes regardless of stratification for parental asthma or allergy. Wheeze was more common in non-vaccinated children at 18 months, and there was a tendency for late onset wheezing to be associated with non-vaccination in children whose parents did not have asthma, but this was not significant. The data suggested that no evidence was found that pertussis vaccination increased the risk of wheezing illnesses in young children (Henderson *et al.*, 1999).

In another study, it was shown that DTP vaccination appeared to increase the risk of allergies and related respiratory symptoms in children and adolescents. The data used were from the Third National Health and Nutrition Examination Survey on infants aged 2 months through to adolescents aged 16 years (Hurwitz and Morgenstern, 2000). DTP vaccination, lifetime allergy history, and allergy symptoms were recorded. Logistic regression modelling was performed to estimate the effects of DTP on allergy. The odds of having a history of asthma were twice as great among vaccinated subjects than among unvaccinated subjects. The odds of having had any allergy-related respiratory symptom was 63% greater among vaccinated subjects than unvaccinated subjects.

Recently, the effect of acellular pertussis vaccine Pa to enhance or prevent allergic sensitization was investigated. The effect of OVA sensitization upon the development of a protective response to *B. pertussis* infection in Pa-immunized and nonimmunized mice was also examined (Ennis *et al.*, 2005). BALB/c mice were used and divided into four groups; mice immunized with Pa only (group Pa); mice immunized with Pa and challenged with virulent *B. pertussis* via aerosol (group PaBp); mice immunized with Pa and sensitized with OVA (group PaOVA); and mice immunized with Pa, challenged with virulent *B. pertussis* via aerosol, and sensitized with OVA (group PaBpOVA). Sensitization with OVA did not impair the vaccine-mediated clearance of *B. pertussis* in this model. When the serum IgE responses of mice sensitized to OVA following Pa were examined, differences were observed in the induction of IgE antibodies to the different antigens, with the concentration of OVA-specific IgE typically being greater than the concentration of IgE induced to *B. pertussis*. Pa induced little *B. pertussis*-specific IgE, and this was not altered by OVA sensitization. The only increase in *B. pertussis*-specific IgE was observed when a

combination of immunization and infection prior to OVA sensitization was used. These data demonstrate that immunization with Pa did not enhance ovalbumin-specific serum IgE production.

The above information have shown that possible links between pertussis infection or pertussis vaccination and the development of asthma are controversial and need further investigation. Therefore, this project was based on the assumption that *B. pertussis* infection and pertussis vaccination might be linked to development of asthma and allergic sensitization.

1.1.7.5 Pathogenesis of *B. bronchiseptica*

B. bronchiseptica infects the respiratory tract of wild, domestic and laboratory animals (Parton, 2005). For example, it causes kennel cough disease in dogs (Bemis and Kennedy, 1981). Kennel cough is characterized by congestion of the mucosal lining of the trachea and bronchi and a mucoid or mucopurulent exudate (Mattoo and Cherry, 2005). In young piglets, it causes atrophic rhinitis via an interaction with *P. multocida* (Magyar *et al.*, 1988), and in laboratory animals such as guinea pigs it causes lesions include mucopurulent or catarrhal exudates (Mattoo and Cherry, 2005). Infection with this bacterium has also been shown to increase the frequency and severity of respiratory disease due to other viral and bacterial pathogens (Brockmeier *et al.*, 2001). *B. bronchiseptica* and its importance as a human pathogen have been investigated. Evidence suggests that *B. bronchiseptica* may be rarely encountered as a commensal or colonizer of the respiratory tract of humans and rarely in association with disease (Woolfrey and Moody, 1991).

1.1.7.6 Prevention of *B. bronchiseptica* infection

Whole-cell, chemically-inactivated *B. bronchiseptica* vaccine containing adjuvant protected mice and guinea pigs against death and bronchopneumonia resulting from laboratory induced or natural *B. bronchiseptica* infections (Little, T.W., 1975 cited by Goodnow, 1980). Furthermore, a group of guinea pigs were vaccinated with commercial porcine *B. bronchiseptica* vaccines, a human DPT vaccine (which includes a *B. pertussis* component), or an autogenous *B. bronchiseptica* bacterin. 21-days following vaccination, the animals were challenged with an intranasal dose of 10^6 virulent *B. bronchiseptica* cells. After a furher 15 days, the nares, nasopharynx,

distal trachea and lungs were cultured. All non-vaccinated control animals developed acute signs of pneumonia, while none of the vaccinated animals developed clinical signs of disease or gross lesions. The frequency of *B. bronchiseptica* isolation from the lungs of animals in each vaccine group was reduced (Matherne *et al.*, 1987).

B. bronchiseptica produces a multiplicity of potential adhesins and toxins that may function as virulence factors. The outer membrane protein pertactin has been demonstrated to act as an adhesin. Extensive homology between the pertactin genes of *B. pertussis* and *B. bronchiseptica* (Li *et al.*, 1992), suggests that the proteins they encode may function similarly (Henderson and Nataro, 2001). Both active immunization using purified pertactin, and passive immunization with a pertactin-specific monoclonal antibody, have been shown to provide protection against mortality and to reduce turbinate pathology and lung colonization in mice and pigs challenged with *B. bronchiseptica* (Kobisch and Novotny, 1990).

Filamentous haemagglutinin (FHA) is an outer-membrane associated adhesin conserved within the genus *Bordetella*. FHA provides protection against *B. pertussis* infections in humans and is a component of acellular whooping cough vaccines (Pichichero *et al.*, 1997). Furthermore, FHA serves as a protective antigen in several animal models of infection with *B. bronchiseptica* and may serve as a protective antigen of canine bordetellosis (Leininger *et al.*, 1997). Polyclonal anti-*B. pertussis* FHA antiserum was used to identify an immunoreactive clone from the genomic DNA library of a canine *B. bronchiseptica* field isolate. The nucleotide and predicted amino acid sequences of the immunoreactive clone were compared to *fhaB* and FhaB from *B. pertussis*, revealing 94% identity at the nucleic acid level, and 86% identity at the protein level. A truncated fusion protein (FHAt) was prepared which included a conserved domain homologous to the immunodominant region in the FHA of *B. pertussis*. FHAt was shown to be safe and antigenic in rabbits. FHAt induced the formation of antibodies that inhibit the haemagglutination associated with full length *B. pertussis* FHA, and inhibit adherence of *B. bronchisepitca* to canine fibroblasts by as much as 65%. This information may have implications for the development of safe and efficacious subunit vaccines for the prevention of canine bordetellosis and may contribute to future acellular whooping cough vaccines (Keil *et al.*, 1999).

Parenterally and intranasally administered vaccines containing *B bronchiseptica* to protect puppies from infection with *B. bronchiseptica* have been reported. Vaccinated puppies with this vaccine may provide substantial protection

from clinical signs of respiratory tract disease associated with infection by this bacterium (Ellis *et al.*, 2001).

One approach to attenuating a bacterial pathogen is to knock out the gene encoding for a known virulence factor. An attenuated *B. bronchiseptica* (*aroA* mutant) expressing the non-toxic fragment C (FrgC) of tetanus toxin was used to immunise mice intranasally. Upon challenge with wild-type *B. bronchiseptica*, immunised mice rapidly reduced the numbers of *B. bronchiseptica* in their respiratory tract, although clearance was more pronounced in the lower than in the upper respiratory tract (Stevenson and Roberts, 2002).

1.2 Mast cells

Mast cells (MC) are mononuclear cells with distinctive cytoplasmic granules. They reside in connective tissue matrices and at epithelial surfaces. MC are considered as critical effector cells because they play important roles in the elicitation of anaphylactic reactions (i.e. when two IgE antibodies bind allergen). IgE antibody binds to high affinity receptors (FcεRI) on the mast cell surface. This cross-linking will lead to a cascade of reactions in mast cells which undergo a dramatic degranulation, releasing a variety of inflammatory mediators (e.g. histamine) and cytokines. The effect of these products will result in a variety of clinical symptoms such as asthma, high fever and chronic inflammation (type I hypersensitivity) (Yamaguchi *et al.*, 1997).

On the other hand, MC could represent a critical component of host defence in natural immunity. MC could contribute in the initiation of host defence mechanisms against many different species of microorganisms. This new concept of mast cells functions can be explained by their ability to contribute to defence mechanisms either by phagocytic capabilities, or by contributing to the acquired immune responses of the host cells as a result of their ability to produce cytokines which are considered as essentials elements for other cells of the immune response (Galli, 1993; Echtenacher *et al.*, 1996; Malaviya *et al.*, 1996a).

1.2.1 Mast cell biology

Mast cells are resident in tissues throughout the body particularly those that interface with the external environment (Metcalfe *et al.*, 1997). They are seen in particularly high concentrations in the submucosal tissues lying just beneath body surfaces, including those of the gastrointestinal and respiratory tract, and in connective tissues along blood vessels and especially in those layers called dermis that are located below the skin. It could be concluded from the distribution of mast cells throughout many different tissues that:

1- Their cell products are available to cells of the connective tissue, surface or glandular epithelial cells, nerve, vascular endothelial cells and vascular, respiratory, gastrointestinal or genitourinary smooth muscle cells.

2- This distribution places mast cells near to pathogens as well as near environmental antigens that come in contact with the skin or mucosal surfaces.

1.2.2 Mast cell progenitors and growth factors

MC originate from stem cell precursors (pluripotential haemopoietic), which depart from the bone marrow (BM) essentially as uncharacterised precursors and migrate into connective or mucosal tissues where they undergo differentiation and maturation (Kitamura *et al.*, 1978; Tsai *et al.*, 1991; Fodinger *et al.*, 1994). Then, MC proceed to synthesize their full complement of granules under the influence of many factors such as cytokines, acting on progenitors at early stages of development, including interleukin-3 (IL-3) (Small *et al.*, 1994), stem cell factor (SCF)(Macaldowie *et al.*, 1997a) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Saito *et al.*, 1996).

In the murine system, it has been elucidated that mast cells originate from hematopoietic stem cells *in vivo* (Kitamura *et al.*, 1981) or multipotential hematopoietic progenitors *in vitro* (Suda *et al.*, 1983). Cytokines such as SCF, IL-3, IL-4, Il-9 and IL-10 promote the development of immature mast cells into mature mast cells (Sawai *et al.*, 1999). In contrast with the murine mast cell system, *in vitro* development of human mast cells is different. In man, mast cell progenitors express $CD34^{+}$, and SCF has been shown to act as a major growth and differentiation factor (Mitsui *et al.*, 1993; Kirshenbaum *et al.*, 1999). In sheep, increased generation of multipotential eosinophil, macrophage and mast cells/basophil cells in bone marrow has been demonstrated (Haig *et al.*, 1995).

1.2.3 Mast cell heterogeneity

Mast cells can be divided into two different kinds that can be visualized under light microscopy by using Alcian blue staining (Roitt, Brostoff and Male, 1996):

1- Mast cells that are associated with mucosal tissue and called the mucosal mast cells (MMC) are located in the mucosa of the midgut and in the lung.
2- Mast cells that are associated with connective tissue (CTMC) are found around blood vessels in most tissues.

Mast cell mediators may be categorized into three groups; 1- granule-associated mediators such as histamine (Novak and Falus, 1997), polyanionic complex carbohydrates composed of repeating disaccharide units known as proteoglycans (heparin and chondroitin sulphate E)(Craig *et al.*, 1993) and proteases of the serine class (chymases, tryptases and carboxypeptidase)(Abe *et al.*, 2000).

Heterogeneity of mast cells could be explained by the fact that mast cells migrate from bone marrow to particular sites in the body as uncharacterized precursors and, under the influence of various growth factors present in a particular site, will undergo differentiation and maturation. Thus, the character and responsiveness of mast cells and their mediator content depends on these growth factors (Abraham *et al.*, 1997; Metcalfe *et al.*, 1997). For example, biochemical analysis of mast cell granule constituents showed that there are two two types of proteases, rat mast cell protease I (RMCP-I) and rat mast cell protease II (RMCP-II) (Woodbury and Neurath, 1980). In addition, it was demonstrated by using immunofluorescence technique that rat MMC and CTMC represent two approximately separate populations of mast cells containing RMCP-II and RMCP-I respectively (Gibson *et al.*, 1987). Furthermore, such observations of different mast cell population distinguished by their proteases have been observed in other species such as human (Irani *et al.*, 1989; Irani and Schwartz, 1989) and mice (Newlands *et al.*, 1993). In addition, in sheep, MC heterogeneity may also occur based on the distribution of a sheep MC granule protease (SMCP) (Sture *et al.*, 1995b). In this study, it was observed that the majority of intestinal MMC stained positive for SMCP whilst only a small percentage of dermal and hepatic mast cells contained the enzyme.

Recent studies demonstrated that human mast cells are a source of several pleiotropic cytokines including IL-4, IL-5, IL-6, IL-8 and TNF-α. But, not all mast cells contain all of these cytokines, suggesting that there is also functional heterogeneity with respect to cytokine content (Bradding *et al.*, 1995). Therefore, it could be concluded that heterogeneity of mast cells depends on many factors that affect their differentiation, proliferation, maturation and other characteristics.

Functional heterogeneity of mast cell sub-populations has been defined in both *in vitro* and *in vivo* derived mast cells from a number of species through their responses to chemical secretogogues. This effect was initially demonstrated *in vivo* in the rat by injecting the polycationic secretogogue Compound 48/80 which depleted peritoneal CTMC but not MMC of their mediators (Enerback, 1966). The neuropeptide substance-P has been used to distinguish MMC from CTMC in a variety of species. It was observed that substance-P activates human cutaneous CTMC but not intestinal MMC via a cell receptor dependent pathway (Regoli *et al.*, 1994). In contrast, calcium ionophore A21387 acts by non-specifically increasing intracellular

calcium levels in a variety of cell types activating calcium dependent cytoplasmic granule release mechanisms. Its effects are not entirely restricted to mast cells and has been shown to activate both CTMC and MMC populations present in laboratory animals (Pearce, 1986) and man (Cohan *et al.*, 1989).

Regarding ovine mast cells heterogeneity, it was reported that MC exhibit considerable structural, biochemical and functional heterogeneity dependent on the species and tissue location (Huntley, 1992). Further studies were carried out regarding sheep mast cells and their tissue distribution has been described (Sture *et al.*, 1995a). The presence or absence of the granule chymase, sheep mast cell proteinase (SMCP), was determined in trachea, bronchus, bronchial lymph node, lung, thymus, spleen, liver, flank skin, abomasum, duodenum, jejunum, ileum, colon and mesenteric lymph node by immunohistochemistry and by enzyme-linked immunosorbent assay using a polyclonal, affinity purified anti-SMCP antibody. It was confirmed by using reactivity to the anti-SMCP antibody that there are two populations of mast cells in sheep. Those in gastrointestinal tissues (analogous to the mucosal mast cell subset) and those present in skin (the putative ovine connective tissue mast cell subset) (Sture *et al.*, 1995b). Currently, studies carried out *in vitro* on ovine bone marrow mast cell grown in the presence of recombinant ovine interleukin-3 have shown that they are activated in the presence of calcium-ionophore A23187 (Huntley *et al.*, 1992). Moreover, ovine bone marrow mast cell in the presence of recombinant ovine interleukin-3 is also relatively refractory to stimulation with compound 48/80 or substance-P (Sture, 1996 PhD thesis). This, in combination with the fact that the majority of these cells contain SMCP (Huntley *et al.*, 1992) indicates that they may possess MMC phenotype.

1.2.4 The development of sheep mucosal mast cells *in vitro*

Mucosal mast cell hyperplasia is a T cell-dependent process (Miller, 1996; Malbec *et al.*, 2004). Both mice and humans with T-cell immunodeficiencies lack mast cells in intraepithelial compartment of their intestines, even though numbers of mast cells in the adjacent submucosal connective tissues are normal (Irani *et al.*, 1987). As in mice, several cytokines (IL-3, IL-4, IL-5, IL-6, and IL-9) support the survival of cultured human mast cells *in vitro* (Yanagida *et al.*, 1995), or synergistically support mast cell proliferation when provided in combination with

SCF, also known as the c-kit ligand because it binds to c-kit tyrosine kinase receptors (Bischoff *et al.*, 1999).

It was shown that mucosal mast cell development could be achieved in sheep by using recombinant ovine interleukin-3 (rOvIL-3) and recombinant ovine stem cell factor (rOvSCF) (Macaldowie *et al.*, 1997b). After approximately 2-3 weeks' growth in optimal concentrations of either rOvIL-3 alone or a combination of rOvIL-3 and rOvSCF, the majority of the cells produced in bone marrow culture were mast cells. The significant increase in the total numbers of cells and survival times of the cultures when both cytokines were present compared to either alone, indicated synergy between rOvIL-3 and rOvSCF on mast cell growth. Ovine and caprine cells cultured in rOvIL-3 alone produced a four-fold increase in cell numbers compared with medium only controls. The resulting cultures contained up to 52% mast cells by day 18 and had a lifespan of 3-4 weeks. In contrast, cells from both species grown in both rOvIL-3 and rOvSCF produced up to six times more cells than the equivalent rOvIL-3 stimulated cultures, contained up to 69% mast cells by day 21 and could be maintained for at least 6 weeks. Ovine cells grown in rOvIL-3 alone or rOvIL-3 and rOvSCF contained significantly more aryl-sulfatase and serine protease but similar amounts of beta-hexosaminidase compared with caprine cells during the second week of culture. There were no significant differences in the granule-associated mediator content of cells from either individual species grown in rOvIL-3 alone compared with those grown in rOvIL-3 and rOvSCF during the first 21 days of culture (Macaldowie *et al.*, 1997b).

1.2.5 Mast cell activation and mediator release

MC plays an important role in allergic reactions (Taube *et al.*, 2004). Interaction of IgE antibodies with high affinity (FcεRI) on mast cell surface, and aggregation of these FcεRI by the reaction of cell-bound IgE with specific antigens induces mast cells to secrete different biologically active performed or lipid mediators, and cytokines (Williams and Galli, 2000). Recent results further suggest that mast cells, by also expressing Fcγ receptor I, Fcγ receptor III, Toll-like receptor (TLR) types 2 and 4 are the antennae of the microenvironment that direct the immune response (Mekori and Metcalfe, 2000; Frossi *et al.*, 2004).

It was reported that activation of MCs and release of their content of granules occur due to two mechanisms, which may operate individually or in parallel. The first mechanism is a rapid process of anaphylactic degranulation and second mechanisms is a slow process of piecemeal degranulation (Dvorak, 1992). Finally, the activated and degranulated mast cells reconstitute their lost preformed mediators through the rapid onset of de novo synthesis, becoming ready to participate in a new process of activation and degranulation (Dvorak *et al.*, 1986; Dvorak, 1992). Generally, preformed mediators are associated with the mast cell cytoplasmic granules. On the other hand, newly-synthesised mediators are produced at the time of cell activation (Bradding, 1996).

Regarding sheep mast cells and their contents, sheep mast cell chymase was first isolated from mucosal mast cells of ovine origin (Huntley *et al.*, 1986) and antibodies raised against the enzyme demonstrated its immunohistochemical localisation to mast cells in the ovine gut as well as other tissues (Sture *et al.*, 1995b). Experiments involving isolated mucosal mast cells as well as *in vitro* derived ovine bone marrow mast cells have demonstrated that these cells also contain histamine, serotonin and dopamine (Huntley *et al.*, 1992) and in the case of the isolated cells are able to releases histamine on exposure of cells to larval antigens (Bendixsen *et al.*, 1995). Interaction of sheep mucosal mast cells with parasite antigen lead to release of mediators following cross-linking of surface bound antigen specific IgE (Jones *et al.*, 1992).

1.2.5.1 Coupling of different hypotheses in mast cell activation

Mast cell secretion is due to a cascade of reactions that end in the release of contents, which include different chemical mediators (e.g. histamine and cytokines), into the surrounding medium. Different mechanisms, immunological stimulation (IgE-dependent) and non-immunological (non-IgE dependent) provocation, under certain circumstances, cause mast cell activation:

1.2.5.1.1 The membrane stimulus

There are two possible ways to initiate mast cell activation, the IgE-dependent way and the IgE-independent way. The IgE-dependent way depends on interactions leading to cross-linking the high affinity IgE receptor (FcεRI) on mast cells and this

initiates cascades of biochemical events leading to degranulation, membrane ruffling and other physiological responses (Rivera *et al.*, 2002). Activation of mast cells through the IgE-independent way occurs via a receptor-independent pathway. For example, adenosine and calcium ionophore are able to induce significant release of tryptase and histamine from mast cells (Stassen *et al.*, 2002).

1.2.5.1.2 Calcium hypothesis

It has been reported that histamine secretion due to stimulation of mast cells requires the presence of extracellular calcium (Johansen, 1980). Furthermore, it was reported that histamine secretion from rat mast cells previously incubated with a chelating agent and stimulated by compound 48/80, is dependent on extracellular calcium. If calcium is absent from the incubation medium, secretion is prevented (Chabot *et al.*, 1981). A rise in intracellular calcium ion concentration might be the link between membrane stimulus and histamine secretion. The principle evidence for this hypothesis came from the experiments with the calcium ionophore A23187 which transports calcium across the mast cell membrane from extracellular to intracellular compartments and thereby induces histamine secretion (Reed and Lardy, 1972).

1.2.5.1.3 Phospholipid metabolism

A close linkage between activated phospholipid metabolism and mast cell activation was reported. It has been shown that inhibition of mast cell secretion is associated with parallel inhibition of the phospholipid metabolism that occurs during mast cell activation (Kennerly *et al.*, 1979b; Kennerly *et al.*, 1979a). Mast cell degranulation has been shown to be potentiated by phosphatidyl serine. The action of phosphatidyl serine was related to the role of calcium in cell permeability. Phosphatidyl serine increases the membrane permeability to calcium in cells stimulated by a cross-linking stimulus (Martin and Lagunoff, 1979).

1.2.5.1.4 Cyclic AMP

It was identified that adenosine 3',5'-monophosphate (cyclic AMP) is an intracellular second messenger of hepatic glycogenolysis (Sutherland and Rall, 1958 cited by Bourne, *et al.*, 1974). The discovery of cAMP triggered a series of investigations which demonstrated that cAMP regulates leukocyte functions (Bourne *et al.*, 1974). Regarding the importance of cAMP in secretory events in MC, it was

thus claimed that increase or decrease in levels of cAMP in mast cells either suppresses or enhances respectively the release of histamine (Kaliner and Austen, 1974). Furthermore, it was shown that secretory events in cells are generally accompanied by increased levels of cAMP. But, in mast cells, the pattern is reversed (Alm and Bloom, 1982). It has been suggested that the lowered levels of cAMP lead to an increase in membrane permeability towards calcium and that an influx of such ions triggers the release mechanisms. It has further been reported that high levels of cAMP inhibit histamine release by decreasing the permeability (Alm and Bloom, 1979). Stimulation of the mast cells is accompanied by falling levels of cAMP which ultimately results in increased membrane permeability to calcium. On the other hand, elevation on the cAMP level limits the permeability of the cell membrane to calcium, and histamine release dependent on extracellular calcium is inhibited (Foreman and Gomperts, 1975; Foreman *et al.*, 1975).

1.2.6 Importance of mast cells in immune response

The discovery that mast cells are a potential source of cytokines has suggested new ways in which mast-cell activation could lead to the development of late-phase reactions and influence other persistent inflammatory responses (Galli, 1993; Marshall *et al.*, 2003b). It is now believed that mast cells are more central to innate and adaptive immune responses. They are known to phagocytose particles (Sher *et al.*, 1979; Malaviya *et al.*, 1994), process antigens, produce cytokines and release vasoactive substances (Gordon *et al.*, 1990; Marshall *et al.*, 2003b).

1.2.6.1 Mast cells and phagocytic response

The possibility that mast cells are able to engulf bacteria was examined. Mixtures of mast cells and Fim^+ or Fim^- (*E. coli*) bacteria were incubated for 1h and then fixed and embedded and examined by EM of thin sections. It was observed that a high proportion (70%) of mast cells had at least one Fim^+ bacterium within distinct phagocytic vacuoles. In contrast, only 15% of mast cells exposed to Fim^- *E. coli,* showed engulfed bacteria (Malaviya *et al.*, 1994). By using a model system in which a well-characterized T cell epitope is expressed within bacteria as a fusion protein, it was demonstrated that mast cells are capable of processing bacterial antigens for

presentation through class I MHC molecules to T cell hybridomas after phagocytic uptake of live bacteria (Malaviya *et al.*, 1996b).

Cord blood-derived human mast cells (CBHMC) and their binding to various bacteria was also been examined. CBHMC bound the laboratory *E. coli* strain expressing recombinant type 1 fimbriae. The morphological aspects of the interaction between CBHMC and selected bacteria were examined by scanning and transmission electron microscopy and it was shown that mast cells employ protoplasmic protrusions on their surfaces to entrap bacteria. In addition, examination of cross-sections of mast cells after exposure to bacteria revealed a significant number of bacteria encased in vacuoles (Arock *et al.*, 1998).

Caveolae are pleomorphic membrane structures characterized biochemically by their buoyant density; resistance to nonionic detergents; and enrichment in cholesterol, glycolipids such as the glycosphingolipid GM1, and a specific protein, caveolin. Caveolae were detected in the microvilli and intracellular vesicles of cultured mouse bone marrow-derived mast cells (BMMCs). The involvement of caveolae in bacterial entry into BMMCs was indicated because caveolae-disrupting and -usurping agents specifically blocked *E. coli* entry, and markers of caveolae were actively recruited to sites of bacterial entry. The formation of bacteria-encapsulating caveolar chambers in BMMCs represents a distinct mechanism of microbial entry into phagocytes (Shin *et al.*, 2000).

The interaction of *B. pertussis* with mast cells has been investigated using transmission electron microscopy (TEM). Phagocytosis of *B. pertussis* by mast cells was described as follows; *B. pertussis* adhered to mice BMMCs and were often surrounded by cytoplasmic protrusions expressed on the cell surface, probably reflecting a mechanism for entrapment of *B. pertussis*. After contact, adherent bacteria were engulfed into invaginations of the mast cells. After 1 h, internalized bacteria were localized in membrane-bound intracytoplasmic vacuoles. In addition, BMMCs infected with virulent *B. pertussis* or a *B. pertussis* PT-deficient strain were equally able to present bacterial antigens to memory T cells isolated from the lungs of infected mice, as measured by the proliferation of the stimulated T cells. A dose-dependent increase in T-cell activation was observed when BMMCs were infected with a multiplicity of infection (MOI) of 1/100 compared with a MOI of 1/10. These data suggest that mast cells may play a role in the induction of immune responses

against *B. pertussis* through the release of cytokines, especially TNF-alpha (Mielcarek *et al.*, 2001).

1.2.6.2 Modulation of immune response by mast cell

An additional reason for attributing an important role for mast cells in various immune responses is the demonstration that mast cell mediators may have immunoregulatory or immunomodulatory effects(Marshall *et al.*, 2003b; Marshall and Jawdat, 2004). It was reported that cytokines proteins or glycoprotein molecules synthesized and secreted by mast cells have a broad spectrum of bioactivities (e.g. cell growth, repair, inflammation and the immune response) (Woolley and Tetlow, 2000; Marshall *et al.*, 2003a).

The recognition that non-Ig specific mechanisms are capable of causing mediators release from mast cells has increased recent interest in their role during the initiation and development of innate responses which may occur on initial exposure to parasites or bacteria (Mecheri and David, 1997). In this respect, innate mediator release from mast cells, including their ability to generate cytokines may have a direct effect on pathogens as well as being able to influence the development of subsequent acquired specific immune responses. This innate ability has been characterised most recently in experiments which demonstrate that the release of pre-formed mast cell-derived TNFα is critical *in vivo* for promoting the clearance of virulent strains of *K. pneumoniae* from the lungs and peritoneal cavities of mice (Malaviya *et al.*, 1996a).

It was observed that the coupling of Fim H-expressing bacteria with CD48 (glycosylphosphatidylinositol (GPI)-linked molecule) result in mast cell activation leading to the release of several proinflammatory mediators which can potentially play a role in determining the nature and intensity of the early inflammatory response to the infecting bacteria (Abraham and Malaviya, 2000).

Interaction of mast cells with *B. pertussis* was investigated (Mielcarek *et al.*, 2001). It was found that mast cells can take up the *B. pertussis*, present *B. pertussis* antigens to T cells, and induce the secretion of cytokines such as tumour necrosis factor alpha (TNF-α), interleukin 6 (IL-6), IL-10 and IL-4. In this regard, The ability of MC to release TNF-α and IL-6 following *in vitro* exposure to *B. pertussis* was investigated. It was found that, incubation with *B. pertussis*, MC released significant levels of TNF-α and IL-6 compared with BMMCs incubated with medium alone. To

determine whether PT produced by *B. pertussis* may modulate the ability of mast cells to release these cytokines, The levels of TNF-α and IL-6 released following exposure to wild-type *B. pertussis* (BPSM) was compared with the levels after incubation with PT-deficient strain (BPRA). The level of TNF-α released by MC after contact with BPRA was not significantly different from that observed following exposure to BPSM. In contrast, the level of IL-6 was consistently found to be significantly higher following exposure to BPRA bacteria than to the virulent BPSM strain. Mast cells express IL-4 and IL-10. The ability of *B. pertussis* to stimulate the release of these two cytokines using MC was investigated. Significant levels of IL-4 were secreted by MC following exposure to the bacteria. This effect was independent of the production of PT. In contrast, while exposure to BPSM did not induce any detectable IL-10 release by MC, an increased IL-10 production was observed following exposure to BPRA that was significantly different from the level of IL-10 released by BPSM-stimulated MC (Mielcarek *et al.*, 2001).

1.3 Bioluminescence-based assay for detection of mammalian cell-bacterial interaction

Recent progress in molecular biology has made available several biotechnological tools that take advantage of the high detectability and rapidity of biological processes (e.g. gene expression). Reporter gene technology represents one of the major recent achievements of molecular biology and has been used in the present project to study host-pathogen interactions.

1.3.1 Reporter genes

Reporter genes are DNA sequences that encode an easily detectable protein or enzyme (Alam and Cook, 1990). They can be artificially introduced into a cell to monitor gene expression, to obtain whole-cell biosensors or for cell-localization purposes. The principal advantage of reporter gene technology is based on their high sensitivity, reliability, convenience, and adaptability to large-scale measurements. The most common reporter genes used are; chloramphenicol acetyltransferase (CAT), β-galactosidase, luciferase (either firefly or bacterial), alkaline phosphatase and green fluorescent protein (GFP). Currently, bacterial luciferase genes (*lux*) as well as the eukaryotic *luc* and *ruc* genes from firefly and sea pansy represent the most widely used bioluminescence reporter genes (Lewis *et al.*, 1998). The following table (1.3) summarise of the advantages and disadvantages of the mostly used reporter genes (Naylor, 1999).

Table (1.3): Comparison of commonly used reporter genes, adapted from Naylor, 1999.

Reporter gene	Source	Measure by	advantages	Disadvantages
Chloramphenicol acetyltransferase (CAT)	Bacterial	Automated ELISA	- No endogenous activity	- Narrow linear range - Use of radioisotopes
β-Galactosidase	Bacterial	Colorimetric readouts	- Stable - No use of radioisotopes	Endogenous activity (mammalian cells)
Luciferase	Firefly	Luminometry	- High sensitivity	- Requires substrate (luciferin) - Presence of O_2 and ATP
Luciferase	Bacterial	Luminometry	- Heat-stable - analysing prokaryotic gene transcription	- Less sensitive than firefly - Not suitable for mammalian cells
Alkaline phosphatase	Human placental	- Colorimetric - Luminescence	- High sensitivity	Interference with compounds being screened
Green fluorescent protein (GFP)	Jellyfish	Immuno-fluorescence	- No substrate needed - No endogenous activity	- Post-translational modification - Low sensitivity (no signal)

1.3.1.1 Luciferase

Luciferase is a generic name for any enzyme that catalyzes a light-emitting reaction (Baldwin *et al.*, 1995). The measurable release of visible light by organisms containing luciferase is termed bioluminescence. Bioluminescent organisms, including bacteria, algae, dinoflagellates, fungi, jellyfish, clams, fish, insects, shrimp, and squid, are ubiquitous and can be found in aquatic as well as terrestrial environments with the vast majority occurring in the marine communities (Meighen, 1991). Among them, bacteria are the most abundant luminescent organisms and have been classified into three genera *Vibrio*, *Photobacterium*, and *Xenorhabdus*. The light-emitting (Lux) proteins of several of these bacterial species have been purified, and the ability to transfer the cDNA coding for Lux proteins into prokaryotic and eukaryotic organisms has resulted in their common use as reporters of gene expression (Daunert *et al.*, 2000).

1.3.1.1.1 Bacterial luciferase

Bacterial luciferase is found in bioluminescent bacterial species and catalyses oxidation of a reduced flavin mononucleotide ($FMNH_2$) and a long-chain aliphatic aldehyde, such as dodecanal, with molecular oxygen:

$$\mathbf{FMNH_2 + RCHO + O_2 \rightarrow FMN + RCOOH + H_2O + light\ (490\ nm)}$$

Since $FMNH_2$ production depends on a functional electron transport system, only viable cells will produce light. Extensively studied procaryotic luminescent systems are those from marine bacteria (e.g., *Vibrio fischeri*, *Vibrio harveyi*, and *Photobacterium phosphoreum*) (Francisco *et al.*, 1993).

1.3.1.1.2 Firefly luciferases

Firefly luciferases used from either the firefly (*Photinus pyralis*) or the click beetle (*Pyrophorus plagiophtalamus*). The firefly luciferase [EC 1.13.12.7] uses luciferin, a heterocyclic carboxylic acid, as a substrate whose conversion depends upon the presence of ATP. According to the enzymatic mechanism (White *et al.*, 1969), the beetle luciferases catalyze a sequence of reactions that convert firefly

luciferin into an electronically excited state oxyluciferin product which then emits light:

luciferin + ATP + O_2 → oxyluciferin + AMP + PPi + CO_2 + light (560 nm)

In this case, luciferase activity is also linked to cellular physiology and viability, as the reaction depends on the ATP concentration in the cell (Hastings, 1996).

1.3.1.2 Lux Genes Involved in Bioluminescence

1.3.1.2.1 Bacterial luciferase [E.C 1.14.14.3]

This is a heterodimeric enzyme whose subunits (40 kDa) and (37 kDa) are encoded by the *lux*A and *lux*B genes, respectively, which are both required for luminescence. In addition to the structural genes (*lux*AB), three other genes (*lux*C, D, and E) are found in all bacterial luminescence systems. These genes encode three proteins that associate to form a fatty acid reductase protein complex required for the synthesis and recycling of the aldehyde substrate (Meighen, 1991). The *lux* genes have now been transferred into a variety of different organisms to generate new luminescent species (e.g. *B. bronchiseptica*) (Forde *et al.*, 1998). Naturally dark bacteria containing the luxCDABE and luxAB genes, respectively, are luminescent or emit light on addition of aldehyde.

1.3.1.2.2 Eucaryotic luciferases

The most studied eucaryotic luminescence systems are those from the firefly and the click beetle, both possessing a luciferase encoded by the *luc* gene. In contrast to bacterial luciferases, the firefly enzyme is composed of a single polypeptide (62 kDa) of 550 amino acids that is active in a monomeric form. In fireflies, the luciferase reaction occurs in the peroxysomes of a light organ, but the reaction can also occur in bacteria and in cell free extracts, and requires only the enzyme, ATP, Mg^{2+}, O_2 and the substrate luciferin (Billard and DuBow, 1998).

1.3.1.3 Advantages of bioluminescence-based reporter systems

Several advantages of using a bioluminescence assay could be summarised as follows:

1- Reliable reporter assay. A number of luminescent genes have been isolated from diverse luminescent organisms, sequenced, and are now available as cloning vectors.

2- When fused to appropriate genes from a host organism, they will produce light under defined conditions, a signal that can be accurately measured and with great sensitivity, as well as quickly (real-time) and without perturbation of the sample (nondestructive).

3- *lux* or *luc* genes can be detected on the basis of unique DNA sequences by gene probing and PCR amplification, but the major advantage of luminescence-based systems is the ability to detect light emitted by marked organisms or by luciferase activity in cell-free extracts.

4- Luminescent colonies can be detected by eye, providing distinction from colonies of indigenous organisms. A number of methods have been developed to measure light emission. More sensitive techniques involving exposure of the sample to photographic or x-ray films, quantification using scintillation counting, or luminometry, are commonly used.

1.3.1.4 Biotechnological application of bioluminescence

1.3.1.4.1 Use of bioluminescent bacteria for determination of phagocytosis and bacterial survival

Using bioluminescence to study phagocytic activity depends on the fact that the level of the *in vivo* luminescence of luminous bacteria is directly proportional to the viability of the cells. Upon phagocytosis, the *in vivo* luminescence may decrease as a result of the intracellular killing of engulfed bacteria. Moreover, the bioluminescence assay being non-destructive to the experimental system, allows a continuous determination of the kinetics of the process (Barak *et al.*, 1983; Forde *et al.*, 1998).

Forde *et al.* (1998) developed an approach to evaluate survival of intracellular *B. bronchiseptica* within macrophage cells. The internalization and persistence of *B. bronchiseptica* was examined in a murine macrophage-like cell line and in murine

peritoneal phagocytes by using bioluminescence as a reporter of bacterial viability. In this work, A 7-kb EcoRI fragment bearing the intact *lux* operon (*lux*C, *lux*D, *lux*A, *lux*B, and *lux*E) from *P. luminescens* Hb was isolated from plasmid pT7-3. Ligation mixes of pUTmini-Tn5km and the 7-kb EcoRI *lux* fragment were used to transform the *E. coli* host strain by electroporation. Recombinant plasmid pUTmini-Tn5km*lux* was extracted from host *E. coli*, and used to transform *E. coli* donor strain SM10pir (which contain transfer vector) by electroporation. Lux+ (*E. coli)* transformants were isolated by CCD imaging. Exponential cultures of *B. bronchiseptica* 5376 and *E. coli* donor strain SM10pir (pUTmini-Tn5kmlux) were mixed for plate matings at 37°C on BG plates for 16 h. The resulting growth was resuspended in 100-µl aliquots were spread on BG selective plates (with rifampin and kanamycin) and incubated overnight at 37°C. Exconjugants of Lux strain of *B. bronchiseptica* (Kan^r Rif^r Amp^s) were picked randomly to gridded BG plates and examined by CCD imaging. Bioluminescence is shown to act as a convenient real-time technique for monitoring of intracellular survival of *B. bronchiseptica* in vitro and may provide a suitable means for examining the role of long-term intracellular survival of the bacterium in the host.

The relationship between protozoa and internalised bacteria is an area which has been investigated by employing a bioluminescent bacterial strain in coculture with the freshwater ciliate, *Tetrahymena pyriformis* (Nelson *et al.*, 2003). A bioluminescent bacterial strain was developed that enabled real-time monitoring of the uptake and survival of bioluminescent *E. coli* (O157) within the freshwater ciliate *Tetrahymena pyriformis*. Constitutively bioluminescent *E. coli* (O157) pLITE27 was cocultured with *T. pyriformis* in nutrient-deficient (Chalkley's) and in nutrient-rich (proteose peptone, yeast extract) media. Non-internalised bacteria were inactivated by addition of colistin, indicated by a decline in bioluminescence. Protozoa were subsequently lysed with Triton X-100 which lead to a further drop in bioluminescence, consistent with release of live internal bacteria from *T. pyriformis* into the colistin-containing environment. Bioluminescence measurements for non-lysed cultures indicated that internalised *E. coli* (O157) pLITE27 cells were only slowly digested by *T. pyriformis*, in both media, over the time period studied. The results suggest that bioluminescent bacteria are useful tools in the study of bacterial intra-protozoan survival.

1.3.1.4.2 Evaluation of antibiotics effect by bioluminescence assay of bacterial ATP

Studies of the persistent suppression of bacterial growth after short antimicrobial exposure is called postantibiotic effects (PAE). The *in vitro* postantibiotic effects (PAE) of aztreonam, ceftazidime, cefuroxime, imipenem, and piperacillin on *E. coli* were studied by a bioluminescence assay of bacterial ATP. The strain was exposed for 2 h to different concentrations of β-lactam antibiotics. The antibiotic activity was eliminated by 10(-4) dilutions, and regrowth of bacteria was monitored hourly by the bioluminescent assay of bacterial ATP. The length of PAE was dose dependent for ceftazidime (0.5 to 2.6 h), cefuroxime (0.4 to 2.6 h), and imipenem (0.3 to 4.5 h). The long PAE for these antibiotics at higher concentrations was associated with a potent initial killing and the presence of spheroplasts. Aztreonam and piperacillin produced a short, non-dose-dependent PAE (0.4 to 0.95 h). Short PAEs (below 1 h) were seen concomitantly with production of filaments, except in the case of imipenem, which only produced spheroplasts. The results of this work makes it possible to study PAE for β-lactam antibiotics on Gram-negative bacteria with bioluminescence (Hanberger *et al.*, 1990).

1.3.1.4.3 Use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity

The nucleotide adenosine triphosphate (ATP) plays a central role in energy exchanges in biological system. ATP is present in all metabolically active cells. Most ATP is found within living cells and links catabolic and anabolic processes. Cell injury or oxygen depletion results in a rapid decrease in cytoplasmic ATP. Therefore, measurement of ATP is important for study of living cells. This method has been used for ATP determination because of the sensitivity of the luciferin-luciferase bioluminescent assay (Higashi *et al.*, 1985 cited by Crouch, *et al* 1993). Adenosine triphosphate (ATP) bioluminescence was used to determine whether there was a linear relationship between cultured cell number and measured luminescence using the luciferin-luciferase reaction (Crouch *et al.*, 1993). These investigations have been carried out using peripheral blood mononuclear cells in addition to a number of cell lines including MOLT-4, HL-60, TF-1 and NFS-60 cells. The effect of granulocyte colony-stimulating factor (G-CSF) dependent proliferation on TF-1 cells was

investigated. The cell lines MOLT-4, HL-60, TF-1 and NFS-60 showed a strong correlation between thymidine uptake and ATP bioluminescence ($p > 0.00001$ for all cell types). Additionally the ATP method could detect the cytokine dependent proliferation on TF-1 and NFS-60 cells by granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) respectively. The tumour necrosis factor alpha (TNF)-induced cytotoxic effect on L-929 cells could also be accurately detected using this method. It would therefore appear to be possible to use ATP bioluminescence in the detection of cytokine activity in a number of different bioassays.

1.3.1.4.4 Investigation of quorum sensing by using bioluminescence assay using lux-based AHL on biolfim formation

Quorum sensing (QS) is a process by which bacteria regulate gene expression through the production and detection of extracellular signalling molecules known as autoinducers (Whitehead *et al.*, 2001). In Gram-negative bacteria, N-acyl homoserine lactones (AHLs) are a common diffusible signal, controlling the expression of characteristics associated with virulence and secondary metabolism (Swift *et al.*, 1996). AHL-mediated control of gene expression in quorum sensing occurs through the activity of proteins with homology to LuxI and LuxR, which control bioluminescence in the marine bacterium *Vibrio* (*Photobacterium*) *fischeri* (Chhabra *et al.*, 1993). To establish whether QS was occurring in *Aeromonas* biofilm, the presence of C4-HSL in biofilm samples was assayed using a bioluminescent AHL sensor strain *E. coli* strain JM109 (Lynch, 1999). It was shown that C4-HSL and the *ahyI* and *ahyR* genes are important in the differentiation of *Aeromonas* biofilm (Lynch *et al.*, 2002).

1.4 Aims of the project

In the light of recent findings about mast cell functions and their importance in the primary response of the immune system, the main aim of this study was to investigate their interaction with bacteria: their ability to recognize, engulf and kill bacteria and the effect of selected bacterial pathogens on the viability and degranulation of mast cells. The initial focus of interest was with the human pathogen *B. pertussis*, because of an apparent interaction with mast cells observed in a coughing rat model of pertussis, because of previous reports of the effects of *B. pertussis* and pertussis toxin on mast cells, and the possible links between pertussis and pertussis vaccination and allergic disorders. However, initial experiments failed to show that *B. pertussis* strains had any major effect of sheep bone marrow mast cells (sheep BMMC) that were available. Thus, attention was shifted to *B. bronchiseptica*, an important animal pathogen and possibly more relevant to study in conjunction with sheep BMMC. Other advantages of using this as the model organism were that it shares many of its virulence factors with *B. pertussis* but is less fastidious and easier to grow and also that a bioluminescent strain was available and thought to be highly suitable for monitoring bacterial-mast cell interactions.

Uptake of *Bordetella bronchiseptica* by sheep BMMC and other cell types will be studied by using a bioluminescent strain of *B. bronchiseptica* as a reporter strain. As well as to assess intracellular survival of ingested *B. bronchiseptica* (Lux 95). This bioluminescent strain has been used previously for studies on phagocytic uptake by, and intracellular survival in, mouse macrophage-like cells. Based on *in vitro* invasion assays, the kinetics of interaction between sheep BMMC and *B. bronchiseptica* will be followed.

B. bronchiseptica is an animal pathogen that can exist as virulent and avirulent forms that are governed by the Bvg virulence-regulatory system. This study will compare the effects of wild-type *B. bronchiseptica* and a Bvg-phase-locked mutant on viability and degranulation of mast cells and other types of mammalian cells, namely the RBL-2H3 cell line and J774A.1 mouse macrophage-like cell line. At the same time, cytotoxicity of *B. bronchiseptica* will be compared with that of other *Bordetella* species and other unrelated bacteria toward mast cells and other cell types.

Adenylate cyclase toxin (or CyaA) is a putative virulence factor that is presumed to have a central role in the pathogenesis of *B. bronchiseptica* and *B.*

pertussis infections. A main objective of this study was to compare the cytotoxic effect of different forms of purified recombinant ACT toward the mast cell population and, in addition, to specify the importance of either the enzymatic activity or pore-forming activity of adenylate cyclase toxin in its cytotoxicity towards sheep BMMC and other mammalian cells. Furthermore, the effect of other purified factors (PT, FHA and PRN) from *Bordetella* species on viability and degranulation of different mammalian cells will be investigated. Finally, the importance of adenylate cyclase toxin and pertussis toxin in modulation of mouse macrophage cells functions toward *B. bronchiseptica* infection will be studied based on the ability of mouse macrophage cells pre-treated by CyaA to take-up bacteria.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Bacteria and growth media

2.1.1 Gram negative bacteria

2.1.1.1 *Bordetella bronchiseptica*

Five different strains of *B. bronchiseptica* were used for assessing their effect on survival and degranulation of different mammalian cells. These strains are listed in table (2.1). All these strains were grown routinely on BG agar. Stocks were prepared as suspensions in 1% CAA-glycerol and aliquots were frozen at –70 °C and thawed for use.

Virulent-type *B. bronchiseptica* (BBC17) and avirulent-mutant type of *B. bronchiseptica* (Bvg^- phase) were kindly donated by Professor Mark Roberts (Department of Veterinary Pathology, University of Glasgow). Both strains of *B. bronchiseptica* were received as BG agar plate cultures and converted into –70°C CAA-glycerol suspension.

A novel bioluminescent *B. bronchiseptica* strain (Lux 95) was received from departmental stock (Fordc *et al.*, 1998) as CAA-glycerol suspension frozen at –70°C.

Two novel strains mutated in two-component systems (TCS), recently described, were created from *B. bronchiseptica* BBC17 by Dr. Martin Lynch (Infection and Immunity Division, University of Glasgow). They were obtained from our department stocks. Both strains, BagAS and BhoAS, were grown on BG agar and treated as previous strains.

Culture purity was checked by visual inspection and Gram stain. Virulence-related properties, adhesive and haemolytic effects of wild-type *B. bronchiseptica* BBC17 and other mutant strains were checked by methods such as conductimetry assay (for ACT activity), haemagglutination assay (with sheep red blood cells) and haemolysis of sheep RBC on sandwich BG agar.

For storage (if previous tests were satisfactory), a loopful of a heavy inoculum was suspended in 1.6 ml of sterile casamino acids (CAA)(Difco) and 0.4 ml sterile glycerol (BDH) and put into washed, sterilized 2ml polypropylene screw-cap vials (Elkay Lab products UK LTD) and stored at –70 °C.

2.1.1.2 *Bordetella pertussis*

Four different strains of *Bordetella pertussis* were received from departmental stocks as –70°C frozen suspensions and treated in same way as *B. bronchiseptica* (for

example, purity and virulence). Originally, they were kindly donated by Dr. Alison Weiss (University of Cincinnati, USA). These four strains are: 338 (wild strain), 347 (Bvg⁻ phase), 357 (defective in pertussis toxin production) and 348 (defective in ACT production). All these strains are listed in Table (2.1). *B. pertussis* strains were grown routinely on BG agar and were placed in a humidified box and incubated for 2-3 days at 37°C.

2.1.1.3 Growth of different *Bordetella* species from frozen stock cultures

Frozen stock suspensions in 1% CAA-glycerol were thawed at room temperature. One loopful of the suspension was streaked on to each of two to three BG plates which were incubated in a moistened box in a 37°C. The resultant growth was checked by Gram stain to confirm the presence of Gram-negative coccobacilli and absence of contaminants.

2.1.1.4 Other Gram-negative bacteria

Pasteurella multocida strains 85020 and JRMT12 and bioluminescent strain of *Escherichia coli* (DH5αpT7-3) were received as agar cultures from departmental stock. For stock, these strains were stored as CAA-glycerol suspensions at –70 °C. Culture purity was tested by visual inspection and Gram stain.

P. multocida was subcultured routinely on Brain-Heart Infusion (BHI) agar medium with the supplementation of 5% (v/v) defibrinated sheep blood. Liquid cultures were grown in the BHI broth medium in universal containers with shaking overnight on an orbital shaker at 150-200 rpm at 37°C.

The bioluminescent *E. coli* (DH5αpT7-3) strain was grown with 50 µg/ml ampicillin (Sigma) on LB agar or in 5ml LB broth media in Universal containers or dimpled flasks with shaking overnight on an orbital shaker (L.H. Engineering Co. LTD.) at 150-200 rpm at 37°C.

2.1.1.5 Construction of bioluminescent *E. coli*

The bioluminescent *E. coli lux* was strain DH5α containing plasmid pT7-3, which encodes ampicillin resistance and includes the *lux CDABE* genes from the naturally bioluminescent bacterium *Xenorhabdus luminescens*. In this plasmid, the *lux* genes are under the control of the phi-10 promoter for T7 RNA polymerase and are

transcribed at a low level by *E. coli* RNA polymerase (Szittner and Meighen, 1990). This *E. coli* strain was kindly provided by Professor E. Meighen (Department of Biochemistry, McGill University, Montreal, Quebec H3G 1Y6, Canada).

2.1.1.6 Construction of bioluminescent *B. bronchiseptica* (Lux 95)

This particular strain was constructed by Forde *et al.* (1998) in our laboratory by using a mini-Tn5 promoter probe carrying the *lux* operon (*luxC*, *luxD*, *luxA*, *luxB*, and *luxE*). Briefly, a 7-kb *Eco*RI fragment bearing the intact *lux* operon from *X. luminescens* was isolated from plasmid pT7-3 (see section 2.1.1.5) and blunt-ended with the Klenow fragment of DNA polymerase at 25°C for 15 min in the presence of 4 μM deoxynucleoside triphosphates. Plasmid pUTmini-Tn5km was linearized with *Not*I and blunt-ended. Ligation mixes of *Not*I-linearized, blunt-ended pUTmini-Tn5km and the 7-kb *Eco*RI *lux* fragment were used to transform the *E. coli* host strain CC118λ*pir* by electroporation, and transformants (Kan^r Amp^r) were screened for bioluminescence with a charge-coupled device (CCD) luminescence imager (Photonic Science). Recombinant plasmids in which the *lux* genes were oriented divergently with regard to the kanamycin resistance marker were identified by their lack of bioluminescence. Recombinant plasmid pUTmini-Tn5km*lux* was extracted from host cells with Qiagen Mini-Prep columns, and used to transform *E. coli* donor strain SM10λ*pir* by electroporation. Again Lux^+ transformants (Kan^r Amp^r) were isolated. *B. bronchiseptica* CBF1 (Rif^r mutant of strain 5376) and *E. coli* donor strain SM10λ*pir* (pUTmini-Tn5km*lux*) were resuspended in 10 mM $MgSO_4$. Plate matings were performed at 37°C on BG plates for 16 h at a recipient-to-donor ratio of 3:1. Growth was resuspended in 1 ml of 10 mM $MgSO_4$, and 100-μl aliquots were spread on BG selective plates (with rifampin and kanamycin) and incubated overnight at 37°C. Exconjugants (Kan^r Rif^r Amp^s) were picked randomly to gridded BG plates and examined by CCD imaging. The minitransposon pUTmini-Tn5km*lux* was used to generate a pool of 2,000 mutants of *B. bronchiseptica* CBF1(Forde *et al.*, 1998). From these, one strain, designated here as *B. bronchiseptica* (Lux 95) was chosen because it exhibited high light output under different growth conditions (i.e. constitutive expression of the *lux* genes)

Table 2.1: Different Gram-negative bacteria used in this project

Strain	Description	Growth media	Source/Remarks
B. bronchiseptica **BBC17**	Wild type (bvg^+ phase)	BG agar, LB agar, LB broth, SS and CL media.	Roberts, M. Dept. Vet. Path. University of Glasgow
B. bronchiseptica **BBC17**	Mutant type (Bvg^- phase)	BG agar, LB agar, LB broth, SS and CL media.	Roberts, M. Dept. Vet. Path. University of Glasgow
B. bronchiseptica **Lux 95**	Bioluminescent strain	BG agar, LB agar, LB broth, SS and CL media.	Department stock
B. bronchiseptica **BagAS**	Mutant of BBC17	BG agar, LB agar, LB broth, SS and CL media.	Department stock
B. bronchiseptica **BhoAS**	Mutant of BBC17	BG agar, LB agar, LB broth, SS and CL media.	Department stock
B. pertussis **338**	Antibiotic resistance derivative of Tohama (Bvg + phase)	BG agar, SS and CL media.	Weiss, A. Dept. Molecular Genetics, University of Cincinnati
B. pertussis **347**	Transposon mutant (Bvg^- phase) of *B. pertussis* 338	BG agar, SS and CL media.	Weiss, A. Dept. Molecular Genetics, University of Cincinnati
B. pertussis **357**	Transposon mutant (defective in pertussis toxin production) of *B. pertussis* 338	BG agar, SS and CL media.	Weiss, A. Dept. Molecular Genetics, University of Cincinnati
B. pertussis **348**	Transposon mutant (defective in ACT production) of *B. pertussis* 338	BG agar, SS and CL media.	Weiss, A. Dept. Molecular Genetics, University of Cincinnati
P. multocida **85020**	Wild strain	BHI agar and broth	Veterinary research institute, SriLanka
P. multocida **JRMT12**	Mutant strain (*aro*A)	BHI agar and broth	(Tabatabaei *et al.*, 2002)
E.coli **DH5αpT7-3**	Bioluminescent strain	LB agar	(Herrero *et al.*, 1990; Forde *et al.*, 1998)

Continued

Strain	Description	Growth media	Source/Remarks
B. pertussis 18323	Wild-type	BG ara, LB agar, LB broth, SS and CL media	Weiss, A. Dept. Molecular Genetics, University of Cincinnati
B. parapertussis	NCTC 5952	BG ara, LB agar, LB broth, SS and CL media	RSIL
B. avium	NCTC 12034	BG ara, LB agar, LB broth, SS and CL media	RSIL
B. holmesii	NCTC 13202	BG ara, LB agar, LB broth, SS and CL media	RSIL
B. trematum	NCTC 13203	BG ara, LB agar, LB broth, SS and CL media	RSIL
B. hinzii	NCTC 13200	BG ara, LB agar, LB broth, SS and CL media	RSIL

RSIL Respiratory and Systemic Infections Laboratory.

NCTC National Collection of type Cultures.

2.1.2 Gram-positive bacteria

These were received as plate cultures and were converted into –70 °C CAA-glycerol suspensions as done with previous strains, after growth in appropriate media as listed below.

2.1.2.1 *Staphylococcus aureus*

S. aureus strain 24 (Rodgers *et al.*, 2001) is an avian isolate and was received originally from Dr. J. J McCullagh (Veterinary Science Division of Agriculture for Northern Ireland). It was cultured on 4% (w/v) Tryptone soya agar (TSA) (Oxoid).

2.1.2.2 *Streptococcus pneumoniae*

Prof. Tim Mitchell, (Infection and Immunity Department, University of Glasgow) kindly provided two strains of *S. pneumoniae*, strain D39 (wild type) and strain D39 Δply (deficient in pneumolysin production) (Mitchell and Andrew, 1997). Both strains were grown on BHI agar with the supplementation of 5% (v/v) defibrinated sheep blood with incubation overnight at 37°C.

2.2 Preparation of growth media

2.2.1 Bordet-Gengou (BG) agar

For routine growth and maintenance of *Bordetella* species, BG agar medium was used. 36 g of Bordet-Gengou Agar Base (Difco, Becton, Dickinson and company, France) and 1% (v/v) glycerol were added to distilled water to give a final volume of 850ml in 1000ml screw cap bottle. The mixture was heated in a steamer to dissolve the agar completely and then sterilised by autoclaving at 121°C for 15 minutes. The agar was allowed to cool to 45-50 °C in a thermostatically-controlled water bath. Fresh sterile defibrinated horse blood (Tissue Culture Service Ltd, Buckingham, UK.) was warmed up to 37 °C and 150 ml (15% of total volume) of the blood was added to BG agar aseptically.

The mixture was rotated gently, avoiding the introduction of air bubbles, and was added aseptically into sterile petri dishes in a laminar flow cabinet. After cooling, the plates were stored at 4°C until required. Sandwich BG agar had a bottom layer of BG agar without blood (12 ml) and a top layer of BG with blood (12 ml). This aided the visualisation of haemolysis.

2.2.2 Luria Bertani (LB) agar

10 g of Tryptone powder (DUCHEFA, BIOCHEMIE, Netherlands) with 5g of yeast extract powder (DUCHEFA, BIOCHEMIE, Netherlands) and 5g of sodium chloride were dissolved into one litre of distilled water and mixed thoroughly, then heated with frequent agitation and boiled for 1 minute to completely dissolve the powder. For LB agar, 1.2% of Agar powder (Oxoid, CM3; 28g) was added. The mixture was heated to dissolve the agar and autoclaved at 121°C for 15 min. The medium was dispensed aseptically into sterile petri dishes in a laminar flow cabinet. The plates were stored at 4°C until required.

2.2.3 Cyclodextrin Liquid (CL) medium

CL-medium was prepared as described by Imaizumi *et al* (1983). 10.7g L-glutamic acid (Sigma), 2.5 g NaCl, 0.5 g KH_2PO_4, 0.1 g $MgCl_2.6H_2O$, 0.02 g $CaCl_2$, 0.2 g KCl, 6.25 g Tris (Sigma), 10 g casein hydrolysate (Difco), 0.24 g L-proline (Sigma) and 1 g dimethyl-β-cyclodextrin (J. Shimizu, Teijin Ltd, Tokyo, Japan) were dissolved in 1 litre of distilled water. The pH was adjusted to 7.4 with 1 N NaOH. The CL-medium base was sterilised at 121 °C for 15 minutes and stored at 4°C until required.

A supplement was prepared by dissolving 0.01 g $FeSO_4$, 0.04 g L-cysteine, 0.004 g niacin, 0.15 g glutathione and 0.4g ascorbic acid (all from Sigma) in 10 ml distilled water, which was then sterilised by passage through a membrane (0.45 µm pore size) filter (Sartorius). It was added to the CL-medium base immediately before use in a volume of 100 µl per 10 ml of medium. The supplement could be stored at –20°C, but was discarded after two months if not used.

2.2.4 Modified Stainer-Scholte (SS) medium

Stainer and Scholte (1971) described a simple chemically-defined medium for the production of phase I *B. pertussis*. SS medium was prepared as follows; 10.7g L-glutamic acid (Sigma), 2.5 g NaCl, 0.5 g KH_2PO_4, 0.1 g $MgCl_2.6H_2O$, 0.02 g $CaCl_2$, 0.2 g KCl, 6.25 g Tris (Sigma), 10 g casein hydrolysate (Difco) and 0.24 g L-proline (Sigma). The medium was sterilized at 121°C for 15 min and stored at 4°C until required.

A supplement was prepared by dissolving 0.01 g $FeSO_4$, 0.04 g L-cysteine, 0.004 g niacin, 0.15 g glutathione and 0.4 g ascorbic acid and 0.4 g ascorbic acid (all from Sigma) in 10ml distilled water, which was then sterilised by passage through a membrane (0.45 µm pore size) filter (Sartorius). It was added to the SS-medium base immediately before use in a volume of 50µl per 10 ml of medium. The supplement was stored at –20°C, and discarded after two months if not used.

2.2.5 Brain Heart Infusion (BHI) agar

37 g of Brain Heart Infusion powder (Oxoid) and 12 g of Agar powder were suspended into 1 litre of distilled water. The mixture was heated at 100°C to dissolve the powders and autoclaved at 121°C for 15 min. The agar was allowed to cool to 45-50°C in a thermostatically-controlled water bath. Fresh and sterile defibrinated horse blood (Tissue Culture Service Ltd, Buckingham, UK.) was warmed up to 37°C Media and was added slowly into mixture to a final concentration of 5%. The agar was mixed and dispensed aseptically into sterile petri dishes in a laminar flow cabinet. The plates were stored at 4°C until required.

2.2.6 Tryptone Soya Agar:

40 g of TSA powder and 12% of Agar powder (Oxoid, CM3; 28g) was added into one litre of H_2O. The mixture was heated and autoclaved at 121°C for 15 min. The medium was dispensed aseptically into sterile petri dishes in a laminar flow cabinet. The plates were stored at 4°C until required.

2.3 Virulence related properties of *B. bronchiseptica* strains

2.3.1 Determination of AC enzymatic activity by conductimetry assay

2.3.1.1 Conductimetry apparatus

The adenylate cyclase (AC) toxin (or CyaA) is a product of the Bvg $^+$ phase of certain *Bordetella* species. A conductimetry assay was used to detect the enzymatic activity due to CyaA which determined whether the strains used for other purpose were in the Bvg$^+$ phase or Bvg $^-$ phase. The assay measures a change in the electrical conductance of the reaction solution (see next section). The conductimetry equipment was developed originally by Mezna and Lawrence (1994) and consists of an eight glass-cell system. It has been modified recently for control from a computer, with automatic balancing and calibration. The enclosed water bath where the glass cells are suspended is allowed to heat to, and is maintained at, 37 °C and the stirrer inside maintains constant water temperature.

The sampling period is 1 sec/cell, cycling through the cells from cell 1 through to cell 8, with each cell containing a magnetic flea. This allows continuous mixing of the contents of each cell. The conductivity of incomplete assay mixture (2ml) is allowed to balance before the reactions are initiated by the addition of sample contains AC enzyme. Data processing of results includes blank subtraction (removes background noise) and on-screen line drawing to measure tangents of progress curves and total change in the electrical conductance of the reaction solution.

2.3.1.2 Conductimetry assay of AC enzymic activity

Lawrence *et al* (2002) described the conductimetry assay. Buffer containing 10 mM Bicine and 10 mM magnesium acetate (MgAc) was degassed by heating for 1-2 min to 90 °C and placed under vacuum pressure for 30 s. It was then allowed to cool to room temperature before the addition of ATP. A volume of 2 ml of buffer, 1 µl of 1 mg/ml calmodulin (CaM) (Sigma) and 1µl of 0.5 unit/µl of inorganic pyrophosphatase (PPiase) (Sigma) were added to each glass cell. Balancing of the cells was allowed to occur before addition of samples containing AC enzyme. After three readings, 2 µl of 43 ng/µl of purified recombinant ACT was added as a positive control (provided by Dr. B. Orr, Infection and Immunity Division, University of

Glasgow). The following reaction occurs in the cells where the buffer and reagents are present:

$$(ATP.Mg)^{2-} + Bicine^{-} \xrightarrow[Calmodulin]{AC\ enzyme} cAMP^{-} + (BicineH) + (PPiMg)^{2-} \xrightarrow{inorganic\ PPinse} 2Pi^{2-} + Mg^{2+}$$

This equation predicts no change in conductance unless the pyrophosphate product is cleaved to release the magnesium ion from chelation (Lawrence *et al* 2002). It is the release of the independently mobile ions, Pi^{2-} and Mg^{2+} that produces the change in conductance that is measured by the assay. Total conductance change and tangents to reaction curves were measured by an on-screen line drawing routine.

2.3.1.3 Detection of AC enzymic activity from *B. bronchiseptica*

The method developed for detection of AC enzymic activity from lysed cells of *Bordetella* species was described by Packard (PhD thesis, 2004).

Different *B. bronchsieptica* strains, wild-type *B. bronchiseptica* (Bvg^{+} phase), mutant strain (Bvg^{-} phase) and bioluminescence *B. bronchiseptica* (Lux 95) were grown on BG agar plates and incubated overnight at 37 ^{0}C. Cell suspensions of bacteria from the overnight growth were prepared at 2 x 10^{9} cfu/ml in saline, initially by comparison with an opacity rod, which was then later determined to be equivalent to OD_{600nm} = 0.46.

1 ml of cell suspension was transferred to a 1.5ml eppendorf tube (Sarstedt), and centrifuged at 13000 rpm for 10 min (Biofuge pico, Heraeus). The supernatant was discarded and the pellet was solubilized by adding 300µl of 10% v/v solution of Triton X-100 (a non ionic detergents) and mixing at 4 ^{0}C on a rotary shaker for 20 min. The conductimetry assay was carried out as described in 2.3.1.2, except that 100 µl of lysate was added to glass cells to start the reaction.

2.3.2 Haemagglutination assay

The filamentous haemagglutinin of *Bordetella* species is another product of the Bvg^{+} phase (Mattoo and Cherry, 2005). Thus, a haemagglutination assay could be used to determine the phase of the strain used in this study. Purified FHA was used as a positive control in this work, and was serially diluted five times. The procedure

involved the collection of different *B. bronchiseptica* strains from BG plates that had been incubated for 24h at 37 °C. Cell suspensions of bacteria were prepared to 2 x 10^9 cfu/ml in saline, which was determined to be equivalent to OD_{600nm} = 0.46. A 100 µl volume of cell suspension was added to individual wells in a 96-well u-shaped plate (Greiner, UK) and for the following five wells, five serial dilutions were carried out (100 µl as a final volume in each well). Thereafter, 100µl aliquots of 2% washed sheep red blood cells in saline were added to all wells. The plate was mixed for 15 min on a shaker at room temperature. The plate was then incubated at 37 °C for 1h and thereafter at 4 °C for 24 h. At the end of the experiment, haemagglutination at the bottom of each well was determined.

2.4 Mammalian cells and culture

2.4.1 Sheep bone marrow mast cells

2.4.1.1 Bone marrow collection and preparation

Dr. John Huntley (Moredun Research Institute, Edinburgh) generously provided sheep bone marrow cells. Preparation of bone marrow cells was carried out using a modification of the technique of Haig *et al.* (1988) for the collection of ovine sternal bone marrow. Suffolk-cross lambs, aged 6-12 months (the younger the animal the better, no older than 6 months if possible) were killed using captive bolt followed by exsanguination. Immediately post mortem, the sternum was removed and split longitudinally from the xiphoid cartilage to the manubrium with a surgical saw.

The marrow was extracted by squeezing the sternum in a vice and collecting the exudate from the cut surface with a sterile scalpel blade. The extracted marrow was resuspended in 20 ml of sterile Hanks balanced salt solution (HBSS) containing 5% foetal calf serum (FCS), 1% penicillin/streptomycin (P/S) and 20 U/ml heparin. The cell suspension was removed from below the fat layer, filtered through two thicknesses of sterile lens tissue to remove fat and debris and washed twice in fresh HBSS + 5% FCS + P/S + heparin by centrifugation at 200 x g in a Beckman Model TJ-6 centrifuge at 4 °C for 5 min before re-suspending each preparation in 24 ml of the same medium. 4 ml aliquots of the washed cell suspension were carefully overlaid onto 6 ml of lymphoprep™ (Bayer, GmBH) for centrifugation at 400 x g for 30 min at room temperature. The resulting inter-phase cells were harvested, pooled and diluted

in at least four volumes of HBSS + 5% FCS + P/S + heparin and washed three times as above, to remove any residual lymphoprep. After the final wash, the cells were resuspended in 20 ml of sterile Iscove's Modified Dulbecco's Media (IMDM, Gibco), containing 5 x 10^{-5} M mercaptoethanol (2-ME), 10 % FCS and P/S. A viable cell count was performed before adjusting the cell density to 2 or 3 x 10^5 cells/ml in IMDM and aliquoted into 75-225 cm^3 canted neck sterile tissue culture flasks (Corning) for different studies.

2.4.1.2 Preparation of rOvIL-3 and rOvSCF

Dr John Huntley (MRI) generously provided cell supernatants containing recombinant ovine interleukin-3 (rOvIL-3) and recombinant ovine stem cell factor (rOvSCF). These factors were prepared as described by McInnes *et al.* (1993) for the production of rOvIL-3. Stable expression of rOvIL-3 and rOvSCF was achieved in Chinese Hamster Ovary (CHO) cells using the Celltech pEE14 vector (Cockett *et al.*, 1990). The gene for rOvIL-3 (McInnes *et al.*, 1993) and the cDNA for the soluble form of rOvSCF (McInnes, unpublished, EMBL. NO. Z50743) were ligated into separate vectors adjacent to the human cytomegalovirus immediate early promoter sequence. The vector contained the glutamine synthetase (GS) gene as an amplifiable marker in CHO cells. Following transfection of ligated pEE14 IL-3/SCF plasmids into CHO cells, transfectants expressing elevated levels of GS were selected in the presence of 25-250 μM methionine sulphoxamine (Sigma). Expression of rOvIL-3 and rOvSCF mRNA was checked by Northern blotting, and recombinant protein expression by gel electrophoresis. Batches of supernatant were collected and assayed for biological activity in 24-well plate bone marrow culture.

2.4.1.3 *In vitro* growth of sheep bone marrow mast cells

All procedures of sheep bone marrow preparation and collection were carried out under aseptic conditions in a tissue culture hood (ICN flow Gelaire® Class II BSB48, ICN Pharmaceuticals LtD) using sterile media and equipment.

These procedures were carried out according to the method of Macaldowie, Huntley and Haig (1997). The recombinant growth factors, ovine interleukin-3 (rOvIL-3, Moredun batch no. 1H2 3/2/10/TF/1/C/9/2) and ovine stem cell factor (rOvSCF, Moredun batch no. 3/2/7/2/TF/2/A/3/12/3) were added in the appropriate

combination and concentrations for the current experiment. Then, the cells were incubated in an atmosphere of 5% CO_2 in air at 37 °C in a tissue culture incubator (ICN Flow automatic CO_2 incubator model 160, ICN Pharmaceuticals).

rOvIL-3 was added into bone marrow culture at 1:100 and rOvSCF were added at 1:50 (as final concentrations). For different studies, isolated bone marrow cells were set up at $1x10^6$ cells/ml in IMDM containing 10% FCS and 1% Penicillin / Streptomycin (P/S) in a 75 cm^3 sterile tissue culture flask. The initial culture volume 10ml was fixed during growth with observations of cell numbers during the growth.

Flask cultures were fed with both growth factors every 48h by adding the appropriate volume of fresh cytokine preparation to give the correct final dilution. Every seven days, the cells were centrifuged at 200 g for 5 min and resuspended in fresh IMDM + 10% FCS and 1% P/S. Throughout the culture period, the cells were monitored daily under a Leica DM 1L inverted microscope (Leica microscopy) and by microscopic examination of Leishman's cytosmear preparations.

2.4.1.4 Enumeration and staining of BMMC

Cytosmear preparations of sheep bone marrow mast cells were made using a Shandon Cytospin 2 cytocentrifuge (Shandon Southern Products LtD). Aliquots of 200µl of cell suspension containing $1x10^6$ cells/ml were centrifuged at 800 rpm for 5 min. The slides (cytosmears) were stained immediately with Leishman's stain.

2.4.1.5 Leishman's staining of cytosmears

Cytosmears slides were laid out on a staining rack and flooded with 1 ml Leishman's staining solution (BDH) for 2 min. 1 ml of tap water was then added and the mixture left for a further 5min. After rinsing thoroughly in tap water, the slides were air dried and mounted. Stained cells in the cytosmears were counted under a x40 objective lens by normal microscopy. A minimum of 400 cells were randomly counted per slide with the final results expressed as the percentage of mast cells relative to the total cell population present in the cytosmear.

2.4.1.6 Cell fixation for electron microscopy

Aliquots of 1.5ml of cell suspension containing 1 x 10^6 cells/ml were transferred to eppendorffs and centrifuged at 1200 rpm for 5 min in a microcentrifuge (Biofuge, Heraeus). The supernatant was removed and the pellet resuspended in 1 ml of freshly made up fixative containing 3% glutaraldehyde (Sigma) and 0.1M phosphate buffer. The cells were fixed for 24 h at 4°C before processing. The specimens were then rinsed in 0.1 M PBS for 3-10 min, and post-fixed in 2% osmium tetroxide (Sigma) in H_2O for 2h. The specimens were then dehydrated through an ethanol series (30, 50, 70, 90 and 100%) for 10 min each. The specimens were then infiltrated with white resin (London Resin Comp.), (50:50 resin/absolute alcohol overnight), with the caps on the vials.

The caps were then removed and the ethanol allowed to evaporate overnight. The resin was then replaced with fresh resin and allowed to infiltrate for another 12-24 h. The specimens were then embedded in Beter Equipment Electron Microscopy B.E.E.M.) capsules, and polymerised at 60 °C for 24 h. Sections were cut at 60 nanometeres on an L.K.B ultramicrotome, picked up on 100 mesh formvar coated grids, and stained with 2% methanolic uranyl acetate and lead citrate (5 min in each). The sections were visualized on a Zeiss 902 electron microscope at 80 KV. Images were captured digitally on an image slave software programme.

2.4.2 Cell lines and culture

2.4.2.1 Rat basophilic leukemia cell line (RBL-2H3)

RBL-2H3 (ATCC No. CRL-2256) is a tumour analogue of mucosal mast cells and widely used as a model for mucosal mast cells (Gentile and Skoner, 1996). The cell line was isolated and cloned in 1978 from Wistar rat basophilic cells that were maintained as tumours. These cells have high affinity IgE receptors (FcεRI). They can be activated to secrete histamine and other mediators by aggregation of these receptors or with calcium ionophore.

This cell line was received as frozen cells. All the following procedures were carried out under strict aseptic conditions in a tissue culture hood (ICN flow Gelaire® Class II BSB48, ICN Pharmaceuticals LtD) using sterile media and equipment. The cell line vial was thawed for 2 min by gentle agitation in a 37 °C water bath. Then, the

vial was decontaminated by dipping in 70 % ethanol. To remove the cryo protective agents, the cells were washed by transfer into a 30 ml Universal tube containing 15 ml of IMDM which contained 10 % FCS and 1% P/S. Then, the tube was centrifuged at 125 xg for 5 min.

The supernatant was discarded and cells were resuspended in 20 ml of fresh IMDM containing 10% FCS + and 1% P/S. A viable cell count was performed before adjusting the cell density to 1 x 10^6 cells/ml in IMDM and aliquoting into 75 cm^3 canted neck sterile tissue culture flasks (Corning) for different studies. The cell line was then incubated in an atmosphere of 5% CO_2 in air at 37 °C in a tissue culture incubator (ICN Flow automatic CO_2 incubator model 160, ICN Pharmaceuticals).

Within the first week, the culture medium was not changed and the morphology changes of the cells were observed by inverted microscope (Olympus, CK2). When cell proliferation started, IMDM medium was renewed each 48h, old medium was discard and replaced by fresh IMDM containing 10% FCS and 1% P/S. Throughout the culture time, the cells were monitored daily by using an inverted microscope (Olympus, CK2).

After two-three weeks of observation, cells were passaged in a fresh IMDM containing 10% FCS and 1% P/S. Attached RBL-2H3 cells were collected by scraping and centrifugation at 125 xg for 5 min and resuspended in a fresh IMDM containing 10% FCS and 1% P/S and viability was investigated by using trypan blue exclusion count. Then, the collected cells were passaged into four new 75 cm^3 canted neck sterile tissue culture flasks, each flask containing 20 ml of cell suspension.

For cell cultures which would be used for further experiments, it was observed that more than 10 passages could not be useful and an increase of cell death was observed which was estimated by trypan blue exclusion count. This meant that a new stock vial from the frozen RBL-2H3 cell line should be used.

After the first passage, RBL-2H3 cells were kept as stock in liquid nitrogen for future use. For storage, after collection by centrifugation, 1ml of cell freezing medium -DMSO serum free (Sigma, C6295) was added to each cell pellet and transferred into sterilized 2-ml volume polypropylene plastic vials with O-ring screw-caps and labelled by writing the name of cell and date of collection. The vial was frozen in -70° C for 3-4 h and stored in liquid nitrogen for future use.

2.4.2.2 Macrophage cell lines

Three types of macrophage cell lines have been used in this study.

Table (2.2) Macrophage cell lines and sources

No.	Description	Source
P338D1	Mouse lymphoid macrophage	ECACC 85011439 (Departmental Stock)
RAW.264.7	Mouse monocyte macrophage	ECACC 91062702 (Departmental Stock)
J774A.1	Mouse monocyte macrophage	ECACC 91051511 (Departmental Stock)

All three cell lines were maintained as cell suspensions in 1ml of freezing medium -DMSO serum free (Sigma) and stored in liquid nitrogen. At the time of use, cells were washed twice with IMDM medium by centrifugation at 1200 rpm for 5 min. and resuspended in a fresh IMDM containing 10% FCS and 1% P/S and aliquoting into 75 cm^3 canted neck sterile tissue culture flasks (Corning) for different studies. Within the first week, the culture medium was not changed and proliferation was observed by inverted microscope (Olympus, CK2). When cell proliferation started, IMDM medium was renewed each 48h, the old medium was discarded and replaced by fresh IMDM containing 10 % FCS and 1% P/S.

After two to three weeks of observation, cells were passaged in a fresh IMDM containing 10% FCS and 1% P/S by using the method of Westrop *et al.* (1994). Attached macrophage cells were collected by shaking the flask to detach the cells and centrifugation at 125 xg for 5 min. Cells were resuspended in a fresh IMDM containing 10% FCS and 1% P/S and viability was investigated by trypan blue exclusion count. Then, the collected cells were passaged into four new 75 cm^3 canted neck sterile tissue culture flasks, each flask containing 20 ml of cell suspension.

2.5 Automated luminometry method

2.5.1 Equipment

The luminometer (Anthos lucy 1) is a bench-top luminescence photometer (Figure 2.1a) intended for the measurement of all types of luminescence (Anthos Labtec Instruments Ges.m.b.H, AUSTRIA, www.anthos-labtec.com). The sample holder was a 96-well fluorescence plate, black, with clear flat-bottom wells (Sigma-Aldrich) (Figure 2.1b). The luminometer was run on Stingray multi-use software (www.dazdaq.com), which controls the instrument and asseses bioluminescence emission data (Figure 2.1c). Addition of reagents was done manually in the measuring plate. The cover of the sample holder was closed except when applying reagents, so as to avoid change of incubation temperature or quick evaporation of buffer. The sample volume in each well ranged from 200-250 µl to avoid any flooding from one well to another well that could cause interference. The sample changing (moving the cursor) and luminescence measurement was done automatically. The light emitted from each well (sample) was detected and was converted into an electrical signal which was then amplified, recorded, saved and displayed on the computer screen in the form of digital data and graph output against time. The temperature in the luminometer was adjusted to 37 °C, and the time interval between each reading could be controlled and ranged from 0.1 sec to 900 sec (15 min).

Figure 2.1a: The luminometer (Anthos lucy 1) (Anthos Labtec Instruments Ges.m.b.H, AUSTRIA, www.anthos-labtec.com). **A)** Plate carrier which is suitable for 96-well plate, tranpsort in x/y direction. A certain position on the plate carrier is searched (hole) during transport. **B)** Control-board, containing keys for control of different functions. **C)** The luminometer was run on Stingray multi-use software, which is able to record, save and display on the computer screen.

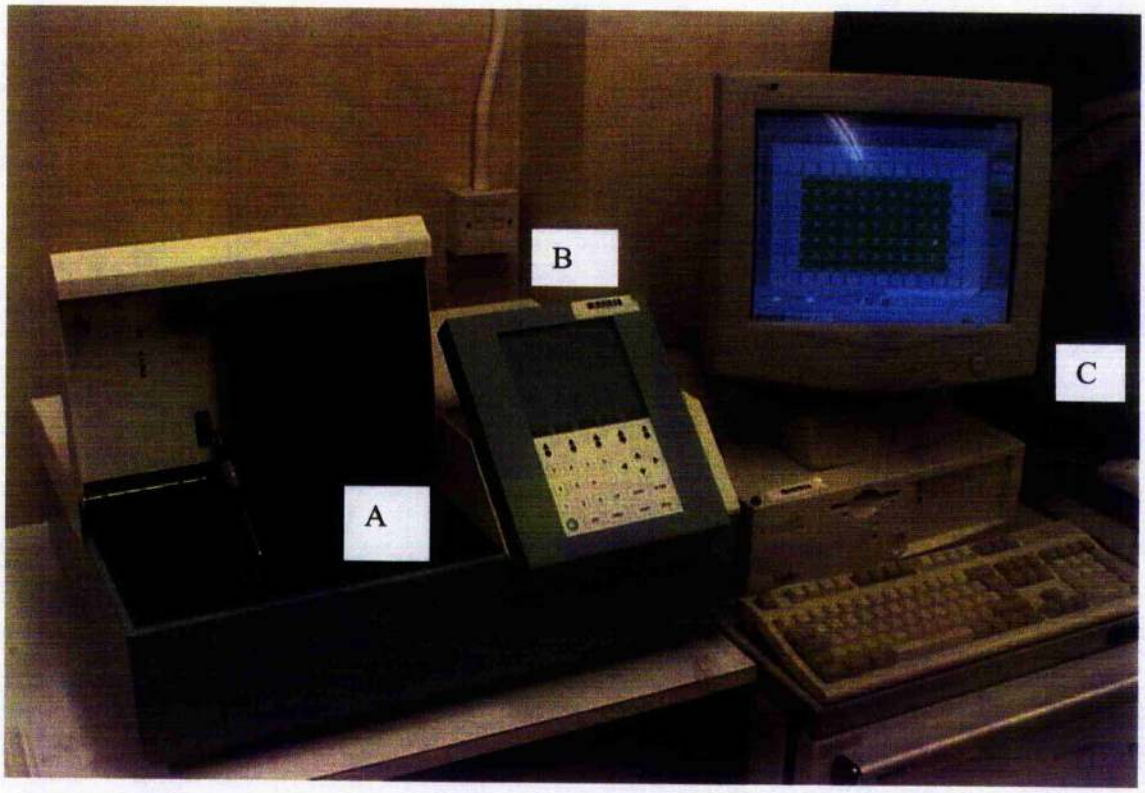
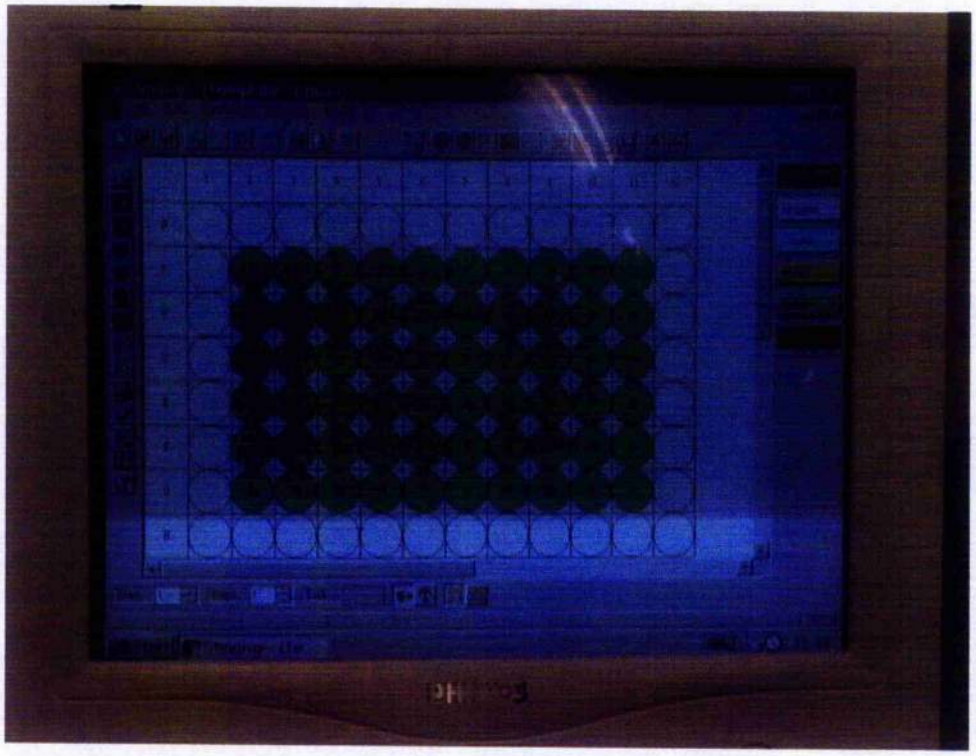

A
B
C

Figure 2.1b: 96-well Fluorescence plate, black, with clear flat-bottom wells (Sigma-Aldrich).

Figure 2.1c: The luminometer was run on Stingray multi-use software, which is able to record, save and display on the computer screen. Green circles indicate the wells which contain sample.



2.6 Phagocytosis of different strains of *B. bronchiseptica*

Interaction of different strains of *B. bronchiseptica* and sheep BMMC (or RBL-2H3 or macrophage cell lines) was done based on the method described by Ford, Parton and Coote (1998) with some modifications. Sheep BMMCs (or RBL-2H3 cell line or mouse-macrophage cells) routinely maintained in IMDM medium containing 10% FCS and 1% P/S, were collected in a 30 ml Universal tube. Then, the collected cells were centrifuged at 1200 rpm for 5 min, the supernatant was discarded and replaced with fresh IMDM medium containing 10% FCS. This step was repeated twice to remove antibiotics from the surrounding medium and the cell pellet was then resuspended in fresh IMDM medium containing 10 % FCS only. Viability of cells was confirmed by trypan blue exclusion count and cell number was adjusted to 1 x 10^6 cells/ml.

Infection of sheep BMMC (or RBL-2H3 or mouse macrophage cells) with different bacterial strains was carried out at a ratio of 1:500 (one mammalian cell, 500 bacterial cells) in 96-well plate in triplicate. The 96-well plate was then centrifuged at low speed, 150 rpm for 5 min, to accelerate interaction between the two cell types. Incubation times were varied from 1h to 3h at 37 °C in 5% CO_2.

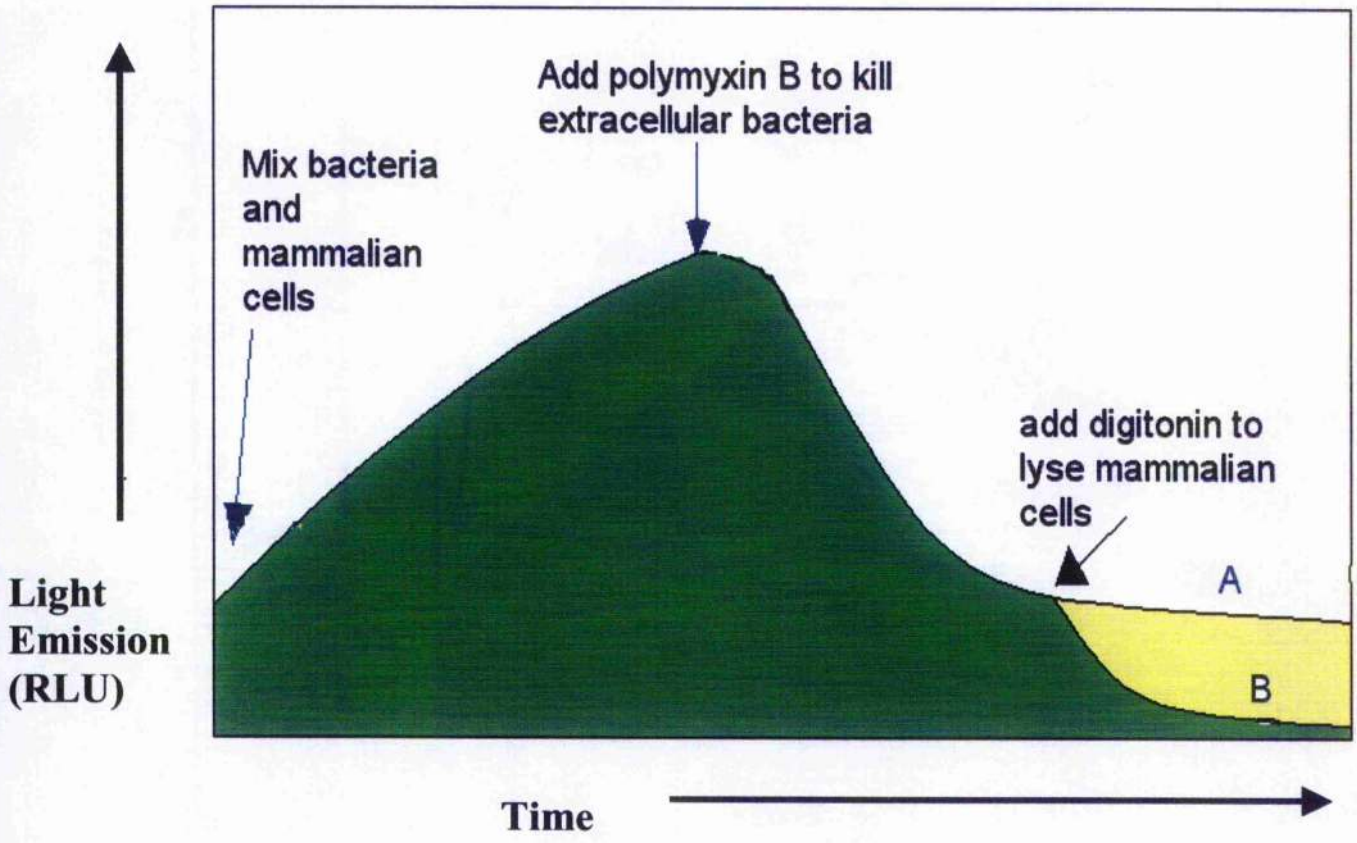
2.6.1 Automated luminometry of bioluminescent bacteria

Sheep BMMC or other cell lines were infected with the bioluminescent stain of *B. bronchiseptica* (*lux* 95) (or bioluminescent *E. coli*) at an infection ratio of 1:500 and incubated for 1 – 3 h as described above (section2.6). Then, to some wells, 50 µg/ml (final concentration) of polymyxin B was added to kill extracellular bacteria. Preliminary experiments had shown that this concentration of polymyxin B killed >99.99% of 5 x10^9 cells/ml of *B. bronchiseptica* (Lux 95) (over the period of 1h). After a further incubation period of 1 h, 50 µg/ml (as a final concentration) of digitonin was added into some wells (to some of which polymyxin B had been added) to lyse the sheep BMMC (or other mammalian cells) and to expose any intracellular *B. bronchiseptica* (Lux 95) to the polymyxin B.

Killing or survival intracellulary of *B. bronchiseptica* (Lux 95) was assessed by comparison of the light emission from digitonin and polymyxin B treated, and polymyxin B only treated wells (see figure 2.2).

Figure 2.2: Invasion assay

Survival of *B. bronchiseptica* (*lux* 95) was assessed by comparison of the light emission from polymyxin B-only treated wells (A) and digitonin and polymyxin B treated wells (B).


Add polymyxin B to kill extracellular bacteria
Mix bacteria and mammalian cells
add digitonin to lyse mammalian cells
A
B
Light Emission (RLU)
Time

2.6.2 Intracellular survival of different *B. bronchiseptica* strains

Intracellular *B. bronchiseptica* (Lux 95) within sheep BMMC was determined by luminometry. Furthermore, intracellular survival of the bioluminescent strain of *B. bronchiseptica* (Lux 95) was compared with intracellular survival of the virulent strain of *B. bronchiseptica* (Bvg $^+$ phase), and the avirulent stain of *B. bronchiseptica* (Bvg $^-$ phase) within either sheep BMMC or J774A.1 macrophage cells.

Bacterial suspensions prepared from growth for 24h on BG agar of different strains of *B. bronchiseptica* (Lux 95, Bvg$^+$ phase or bvg $^-$ phase) were made in IMDM medium containing 10% FCS only and bacterial number was adjusted at OD_{600nm} = 0.46. Sheep BMMC (or other mammalian cells) were adjusted to $1x10^6$ cells/ml in IMDM medium containing 10% FCS only. Infection of sheep BMMC (or other mammalian cells) with different strains *B. bronchiseptica* was carried out at MOI 1:500 in a total volume of 6 ml. Each infection was carried out in duplicate in a $25cm^2$ cell culture flask with vent cap and incubated at 37°C in 5% CO_2. After incubation for 3h, culture flasks which contained sheep BMMC and bacterial suspensions were shaken to detach sheep BMMC (or other mammalian cells) and collected in a 30 ml Universal tube. Cell suspensions were centrifuged at 2000 rpm for 5 min in IMDM medium three times, old supernatants were discarded and antibiotic free IMDM medium containing 10% FCS was added.

The washing step was repeated 3 times. This was done to remove most of the extracellular bacteria because bacteria attached to the cell surface of mammalian cells could not be easily removed by washing. Due to this, sheep BMMC (or other mammalian cells) were resuspended in fresh IMDM medium containing 10% FCS and 1µg/ml polymyxin B (as a final concentration) and incubated at 37°C in 5% CO_2.

Finally, at the end of each incubation time (16 h, 24 h and 36 h), extracellular polymyxin B was removed by extensive washing by centrifugation at 2000 rpm for 5 min and resuspeneded in fresh IMDM. At this point, viability of sheep BMMC (or other mammalian cells) was estimated by using trypan blue exclusion count. Intracellular bacteria within sheep BMMC (or mammalian cells) were released by adding 100 µg/ml of digitonin as a final concentration. Then, 100 µl of cell suspension was diluted 1:100 with PBS, another 100 ul (from the first dilution) diluted 1:100 with PBS. Finally, 100 µl of the second dilution was inoculated on a BG

agar plate, which was incubated at 37°C for 48h. Colonies were counted and the number of viable bacteria per ml of the original suspension was estimated.

2.7 Methods for assessing cytotoxicity of different bacterial strains

Sheep BMMC (or other cell lines) were mixed with different strains of *B. bronchiseptica* (or other related or unrelated bacteria) and loss of mammalian cells viability was estimated over time. A variety of assays were applied.

2.7.1 Trypan blue exclusion count

Cell numbers and viability were confirmed by diluting a sample of mammalian cell suspensions with an equal volume of 0.1% trypan blue (Sigma). Then 10µl from the mixture was pipetted into an improved Neubauer haematocytometer cell counting chamber (Hawksley, England). Light microscopy used to assess cell viability. Viable cells were seen as bright cells whereas dead cells stained blue.

2.7.2 CellTiter-Glo™ Luminescent cell viability assay (Promega)

The luciferin-luciferase-based ATP bioluminescence assay was designed for determining adenosine triphosphate (ATP) present in metabolically-active cells (Crouch *et al* 1993). The amount of ATP present was estimated by adding homogeneous reagent (CellTiter-Glo™ Reagent) into a mammalian cell suspension. This results in cell lysis and generation of a luminescent signal which is proportional to the number of metabolically-active cells. The luminescent signal was detected by using an Anthos lucy 1 luminometer (see 2.5.1). 100 µl of mammalian cell-bacterial suspension in IMDM medium was mixed with an equal volume of homogenous reagent in black, 96-well, flat- bottom microtitre plates in triplicate. All contents were mixed at 300-500 rpm for at least 2 minutes on an orbital shaker to induce cell lysis, and left for 10 minutes to stabilize the luminescence signal. Bioluminescence emission was measured within 3 minutes. Non-infected sheep BMMCs were used as controls for ATP levels, and were compared with those of infected cells.

2.7.3 MTT Dye reduction assay

Cytotoxic activity of different strains of *B. bronchiseptica* and their extracts was determined by the MTT assay (Hussain *et al* 1993) using the cell titre assay (Promega) kit. This assay is based on the reduction of a yellow tetrazolium dye (3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl tetrazolium bromide, MTT) into insoluble purple formazan by dehydrogenases in active mitochondria of living cells.

Sheep BMMC (or other mammalian cells) were maintained in IMDM medium and adjusted to $1x10^6$ cells/ml. Infection of sheep BMMC (or other mammalian cells) with different strains of *B. bronchiseptica* was carried out at infection ratio of 1:500. 100µl of cell suspension was added into 96-well, flat-bottom microtitre plates (Nunclon) in triplicate. The plate was incubated at 37 °C in 5% CO_2 for 3h before addition of 15 µl/well of MTT dye (Promega) and incubation continued at 37 °C in 5% CO_2 for another 4h. The solubilization / stop solution (100 µl/well) (Promega) was then added and incubation continued at 37 °C in the humidified box overnight.

An ELISA reader (*rosys anthos* 2001) was used to measure the optical denisty at a wavelength of 540nm. The percentage of cell death was calculated by applying the following formula:

$$\% \text{ of cell death} = \left(\frac{\text{Sample (OD 540 nm)} - \text{Positive (OD 540nm)}}{\text{Negative (OD 540nm)} - \text{Positive (OD 540nm)}} - 1 \right) \times 100$$

Sample: test sample (mammalian cells infected with *B. bronchiseptica*)
Positive: lysed sample of sheep BMMC (or other mammalin cells), Sheep BMMCs were lysed by the method of rapid freeze-thawing at –70°C.
Negative: IMDM alone.

2.7.4 Estimation of caspase 3/7 activity

The cysteine aspartic acid-specific protease (caspase) family plays important roles in apoptosis in mammalian cells. Caspase-3 and caspase-7 are members of this family, which can be detected by using the Apo-ONE® Homogenous Caspase-3/7 assay (Promega) kit. The caspase–3/7 assay kit consists of a profluorescent substrate

rhodamine110, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide)(Z-DEVD-R110) which is diluted 1:100 with optimized bifunctional cell lysis buffer, prior to addition to mammalian cells. Upon sequential cleavage and removal of the DEVD peptides by caspase-3/7 activity, the rhodamine 110 leaving group becomes intensely fluorescent. The amount of fluorescent product generated is proportional to the amount of caspase-3/7 cleavage activity present in the sample and can be detected by fluorimetry in a luminescence spectrometer LS55 reader (Perkin Elmer).

Different concentrations of purified factors were used to stimulate $5x10^5$ cells/ml of mammalian cell suspension in 100µl IMDM medium supplemented with 10% FCS dispensed in triplicate in black, 96-well plates and mixed with 100µl of homogenous Caspase –3/7 reagent. The plate was gently mixed using a plate shaker at 300-500 rpm for different times (30 min – 4 h) at room temperature. The relative fluorescence light units (RFLU) of the products were measured at a wavelength of OD_{485nm}. The following formula was used to calculate caspase activity:

Caspase activity = Test sample – Negative control

Test sample: sheep BMMC (or other mammalian cells) treated with different concentrations of purified factors.
Negative sample: sheep BMMC (or other mammalian cells) incubated with IMDM medium only.

2.8 Degranulation assay for sheep BMMC (or other mammalian cells)

2.8.1 β-hexosaminidase Assay

Activity of the granule-associated acid hydrolase, β-hexosaminidase, was measured by hydrolysis of the substrate p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma). It was reported that 1 unit of enzyme cleaves 1 µmole of substrate per hour at 37 °C (Schwartz, Austen and Wasserman 1979). Degranulation of sheep BMMC (or other mammalian cells) was estimated after treatment with different bacterial strains, different purified factors of *Bordetella* species, or treatment with chemical

stimulators such as calcium ionophore or substance-P in a total volume of 200µl which was added in triplicate in 96-well microtitre plates and the mixture plate was centrifuged (Multifuge 3 s-R, Hereaus) at 150 rpm for 5 min to accelerate interaction between mammalian cells, different bacteria, or factors.

After a certain period of treatment, 100µl of mixture supernatant (from each well) was transferred into clean wells. The 25 µl from mixed supernatant was mixed with 50 µl of aliquots of 5 mM substrate diluted in citrate buffer pH 4.5 (Appendix) in a 96-well microtitre ELISA plate (M129A, Dynatech Laboratories Ltd). The last step was repeated in triplicate. The mixture was incubated at 37°C for 45 min then the reaction terminated with the addition of 100 µl of ice-cold glycine-NaOH buffer pH 10.7 (Appendix). The resulting colour change was read at $OD_{405\ nm}$ on an Anthos Labtech 2001 reader with the mean OD result being calculated by using the following formula:

% of β-hexosaminidase release =

$$\left(\frac{\text{sample supernatant (OD 405nm)} - \text{spontaneous release supernatant (OD 405nm)}}{\text{total cell extract (OD 405 nm)}} \right) (100)$$

Sample supernatant: The optical density of test sample supernatant.

Spontaneous release supernatant: The optical density of the supernatant of the negative control (cell incubated with IMDM only).

Total cell extract: The optical density due to the total available activity of β-hexosaminidase from mammalian cells, obtained by freezing and thawing (3 times) to release all β-hexosaminidase.

2.9 Effect of cell extracts of sheep BMMC on *B. bronchiseptica* (Lux 95)

The effect of viable sheep BMMC (or other viable cell lines) was compared with the effect of cell extracts of sheep BMMC (or other cell line contents) on bioluminescence emission of *B. bronchiseptica* (*lux* 95). Different cell numbers of

sheep BMMC (or other cell lines) were adjusted and maintained in LMDM medium containing 10% FCS only. The method of rapid freeze-thawing at –70°C was used to lyse cell membranes and release cell contents. This step was repeated three times. The resulting extract was centrifuged at 4000 rpm for five minutes and the supernatant was removed into a sterilized universal tube. *B. bronchiseptica* (Lux 95) was grown and adjusted and maintained in IMDM. 100µl of *B. bronchiseptica* (Lux 95) suspension was mixed with 100 µl of sheep BMMC contents. The luminescence out-put was measured as described previously.

2.10 Effect of different *Bordetella* extracts on sheep BMMC

Four different purified factors of *Bordetella* species were used to investigate their effects on sheep BMMC or other cell lines (RBL-2H3 or J774A.1).

2.10.1 Recombinant adenylate cyclase toxin (or Cya A)

The following toxin was produced in the Infection and Immunity Department, IBLS, University of Glasgow and was kindly provided from departmental stock. Three different forms of ACT were used, these forms being:

Table (2.3) different forms of rACT.

ACT	Description	Concentration (µg/ml)	Producer strain	Activity	
				AC	Hly
44/188	Purified	2020	*E. coli*	-	-
44/54	Purified	4000	*E. coli*	+	+
44/54/188	Purified	2551	*E. coli*	-	+

AC: acylated and enzymatically active, **Hly:** haemolytic activity and pore-forming

2.10.2 Pertussis toxin (PT), filamentous haemagglutinin (FHA) and pertactin (PRN)

These purified *B. pertussis* proteins were generously provided Dr D. Xing (National Biological Standards Boards, NBSB) as freeze-dried preparations. The amount in each vial was 20 µg protein. Each of the extracts was dissolved in 5 ml of

saline to a protein concentration of 4 µg/ml and immediately stored in small volumes at –20 °C.

2.11 DNA extraction

The effect of different forms of rACT (44/54 and 44/54/188) and PT on genomic DNA structure of sheep BMMC (or cell lines) was investigated. 1 x 10^6 cells/ml of sheep BMMC stimulated with different concentrations of different forms of rACT or PT was incubated for 24 h at 37 °C and in 5% CO_2. At the end of incubation time, cells were collected and genomic DNA was purified.

2.11.1 Genomic DNA of sheep BMMC (or other mammalian cells)

For isolation of genomic DNA from sheep BMMC, RBL-2H3 or J774A.1, Promega's Wizard® genomic DNA purification kit was used. 10 ml of suspension of mammalian cells treated with different purified virulence factors were centrifuged at 4000 rpm for 5 min. Supernatant was discarded and the pellet was resuspended in 1 ml of fresh IMDM medium and collected in 1.5 ml eppendorf tubes by centrifugation at 13000 rpm for 2 min. To the pellet was added 600 µl of nuclei lysis solution (Promega) and the mixture was pipetted to completely mix and the sample was incubated at 37 °C for 15 min and then cooled at room temperature. 3 µl of RNase solution was then added and, after through mixing, the sample was incubated at 37 °C for 30 min and then cooled at room temperature. After cooling, 200 µl of protein precipitation solution was added, the mixture vortexed and incubated on ice for 5 min. The sample was centrifuged at 13000 rpm for 3 min and the supernatant was carefully transferred to an eppendorf tube containing 600 µl of isopropanol and mixed thoroughly. The sample was centrifuged at 13000 rpm for 2 min and to the pellet was added 600 µl of 70% ethanol. The sample was then mixed and centrifuged for 2 min at 13000 rpm. The supernatant was removed and the pellet was air dried to completely remove traces of ethanol. The DNA pellet was rehydrated by adding 100 µl of rehydration solution (Promega) for 1 h at 65 °C, or occasionally overnight at 4 °C. The DNA was aliquoted and stored at -20 °C for further steps.

2.12 Agarose gel electrophoresis

2.12.1 Sample preparation

The sample DNA (5-30 µl) was mixed with 6 x DNA loading buffer (Appendix) in a 5:1 (vol/vol) ratio prior to loading into the wells. Molecular weight markers (1 Kb ladder, Gibco BRL, UK) were utilised according to manufacturer's instructions.

2.12.2 Gel preparation

Pre-weighed agarose (type II-A medium EEO, Sigma) was suspended in 0.5 x Tris-Borate-EDTA (TBE) buffer (appendix) and heated until the agarose solution became clear (completely dissolved). The solution was allowed to cool to the extent that it was still warm and ethidium bromide (Bio-Rad, UK) was added to a final concentration of 0.5 µg/ml. A gel tray was prepared by taping the edges with adhesive tape and the gel was cast to the desired thickness. Upon setting, the gel was immersed in 0.5 x TBE buffers containing ethidium bromide (0.5 µg/ml) in a horizontal submarine electrophoresis tank (E-C apparatus Corporation, USA). A power pack (model SL3655, Scotlab, UK) was used to provide a constant voltage corresponding to 1-5 volt/cm. Electrophoresis was carried out until the marker dye in the loading buffer migrated an appropriate distance.

2.12.3 Visualisation of DNA

The ethidium bromide-stained DNA was visualised using a UV transilluminator (model TM-40, UVP Inc, California, USA). Images were stored electronically as appropriate using the Gel Documentation System Image Store 5000, version 7.2 (Ultra Violet Products, Cambridge, UK), as well as being printed using a video graphic printer (model UP-860, Sony). Electronic images were edited using Adobe Photoshop 3.0 and images labelled with Microsoft Power point 4.0.

CHAPTER THREE: RESULTS

Section One: Differentiation of sheep bone marrow mast cells (Sheep BMMC)

3.1.1 Differentiation of sheep bone marrow mast cells *in vitro*

Bone marrow cell cultures were grown in 25 cm^2 tissue culture flasks in a total of 10 ml volume of IMDM medium containing 10% FCS, 50µl of rOvSCF suspension and 100µl of rOvIL-3 suspension. Feeding with both rOvSCF and rOvIL-3 started from day zero and was done every 48 h (see Material and Methods).

Total viable cell numbers were estimated by trypan blue exclusion and mast cell numbers were estimated by cytosmear preparation and staining cells by Leishman's stain (Figure 3.1.1a). In most stem cell cultures, the initial viable cell numbers were approximately $1x10^6$ cells/ml and it was observed that total viable cell numbers and number of viable mast cells differentiated from primary stem cells were highest at day 12. Subsequently, there was a gradual decrease in both total cell numbers and mast cell numbers.

The percentage of mast cells and other cell types were estimated. However, the percentage of mast cells in cell cultures increased with time compared with other cell types in the culture (Figure 3.1.1b).

During the first week of culture, contamination of stem cells with red blood cells (RBCs) was evident, but their numbers declined during the first week. Also, contamination with other cell types like fibroblast cells and macrophages was observed.

It was difficult to differentiate mast cells from other cells in bone marrow culture by Leishman's stain during the first 10 to 12 days. Some cells with empty granules or vacuoles were observed, so it was difficult to determine whether these were mast cells or not. Therefore, all observations were made after 12 days to enable identification and differentiation of mast cell from other cells

Figure 3.1.1a Differentiation of sheep bone marrow mast cells *in vitro*

Total cell numbers and mast cell numbers for sheep bone marrow cell culture fed with rOvIL-3 and rOvSCF. Total cell numbers were estimated by trypan blue exclusion and mast cell numbers by Leishmain's stain. Within the first 12 days it was difficult to differentiate between mast cells and other cells by Leishman's stain (mean of 6 observations ± SEM).

Figure 3.1.1b Percentage of mast cells in sheep bone marrow cell cultures with time

The graph shows that the percentage of mast cells in the population increased, in comparison with other cell types, in sheep bone marrow cell cultures fed with rOvIL-3 and rOvSCF (mean of 6 observations ± SEM).

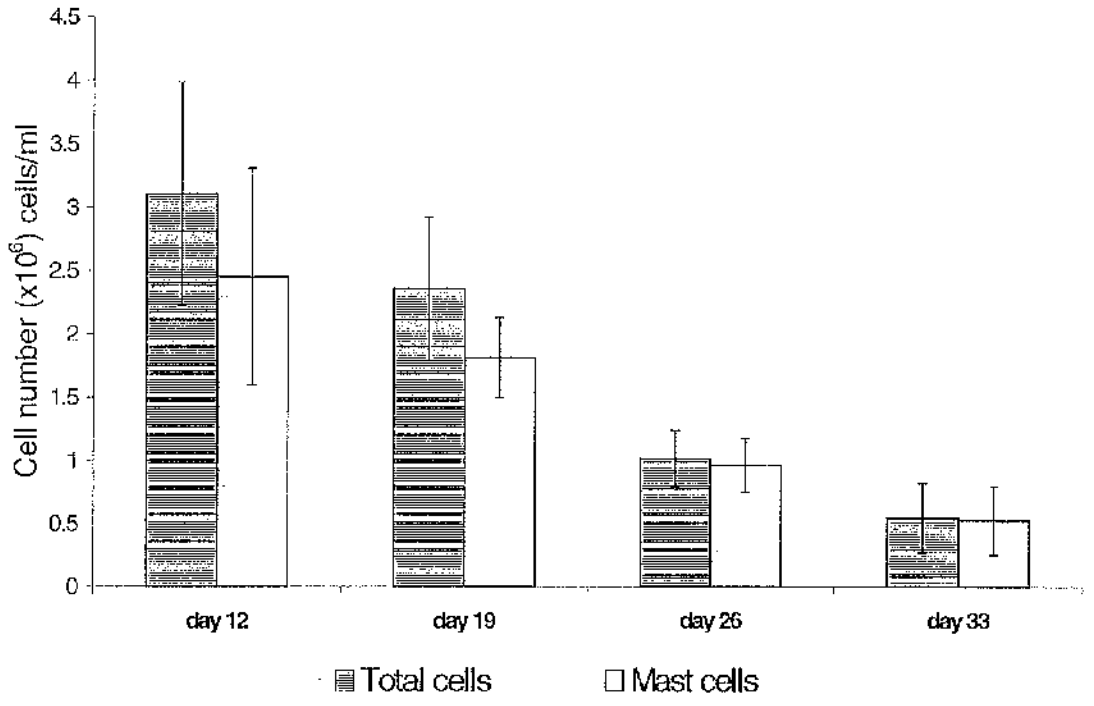


Figure 3.1.1a

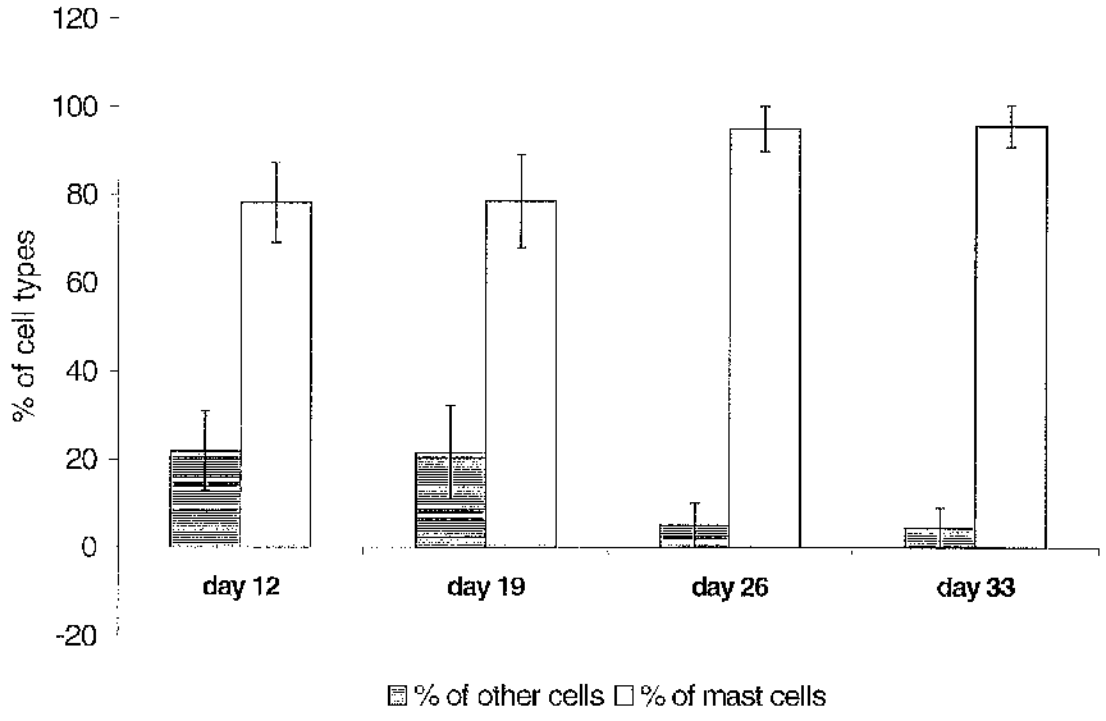


Figure 3.1.1b

3.1.2 Microscopic study of mast cell differentiation

Leishman's stain was used to stain mast cell contents (Figure 3.1.2a). Light microscopy (original magnification x 600) was used to differentiate and count mast cell and other cells. As can be seen at day 30, most of the cells had the appearance of granulated mast cells. Some of these cells contained highly condensed granules. A small proportion of other cell types (macrophages and neutrophils) could also be observed.

Further microscopic studies were carried out by transmission electron microscopy (TEM). This method helped to observe mast cell differentiation. Mast cells differentiated from sheep bone marrow cells from different culture ages were examined by TEM. The images showed that there were differences in morphology, especially in their contents (in different cell cultures). The intracellular contents of mast cells differed and depended on the age of culture. Figure 3.1.2b shows a single mast cell from cell culture at 7 days. As can be seen, this mast cell has many pseudopodia and contains mainly empty cytoplasmic vacuoles. Figure 3.1.2c, shows a mast cell from culture at 15 days. It could be observed that the mast cell is again surrounded by pseudopodia but the proportion of empty cytoplasmic vacuoles was less than the previous figure and some intracytoplasmic dense granules were evident. Figure 3.1.2d shows a mast cell from culture at 29 days. Again, pseudopodia were evident on the mast cell surface but the number of dense granules was increased compared with the previous two figures (3.1.2b and 3.1.2c). These figures (3.1.2b,c, d) were typical of mast cells at these different ages of culture.

Figure 3.1.2a A cytosmear specimen of mast cell culture

A cytosmear specimen of mast cell culture at 30-day stained by Leishman's stain. Both mature (A) and immature (B) mast cells were observed (⟶) with other cell types (C) (⟶) (X1000)

Figure 3.1.2b Section of sheep bone marrow mast cell (TEM)

Thin section of sheep bone marrow mast cell from 7-day culture obtained by TEM. The cell is surrounded by pseudopodia (A) and contain mainly empty cytoplasmic vacuoles (B). Scale bar = 10 μm

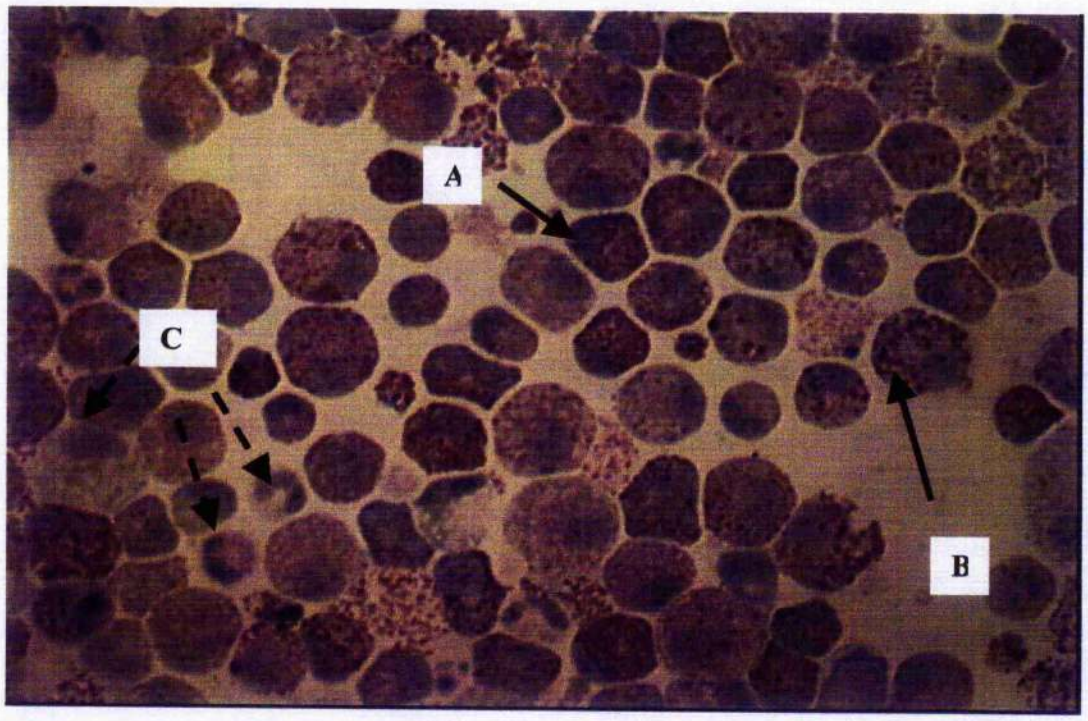


Figure 3.1.2a

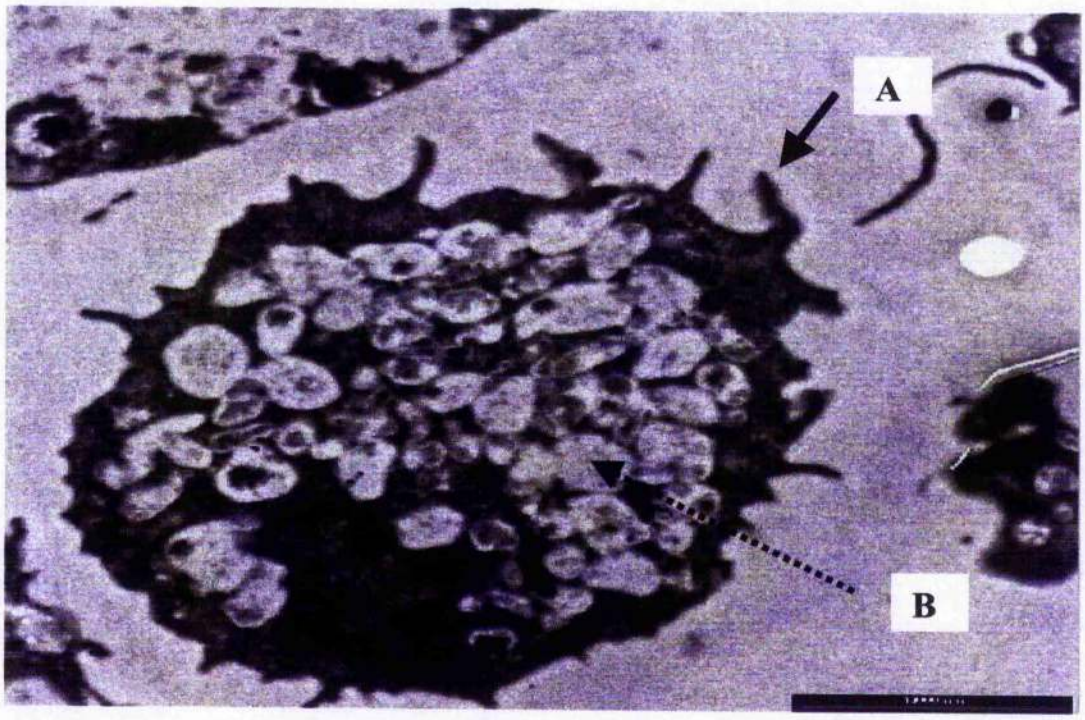


Figure 3.1.2b

Figure 3.1.2c TEM of sheep BMMC (15 day)

TEM of sheep bone marrow mast cell from 15-day culture. Scale bar = 10μm

Figure 3.1.2d TEM of sheep BMMC (29 day)

TEM of sheep bone marrow mast cell from 29-day culture. Scale bar = 10μm

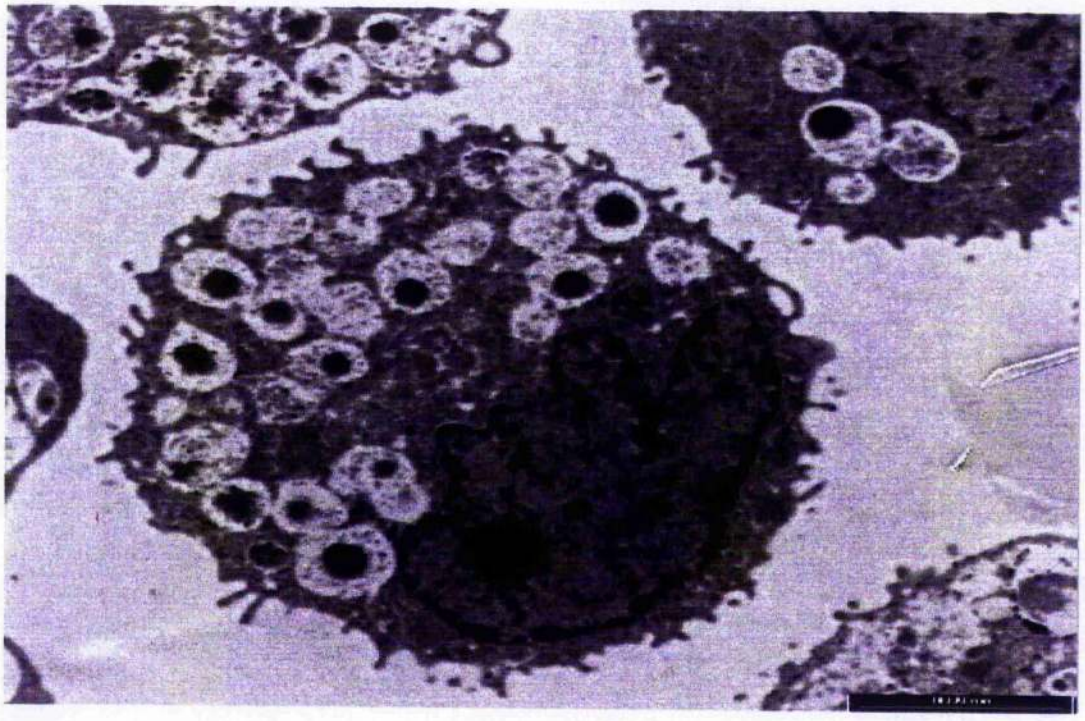


Figure 3.1.2c

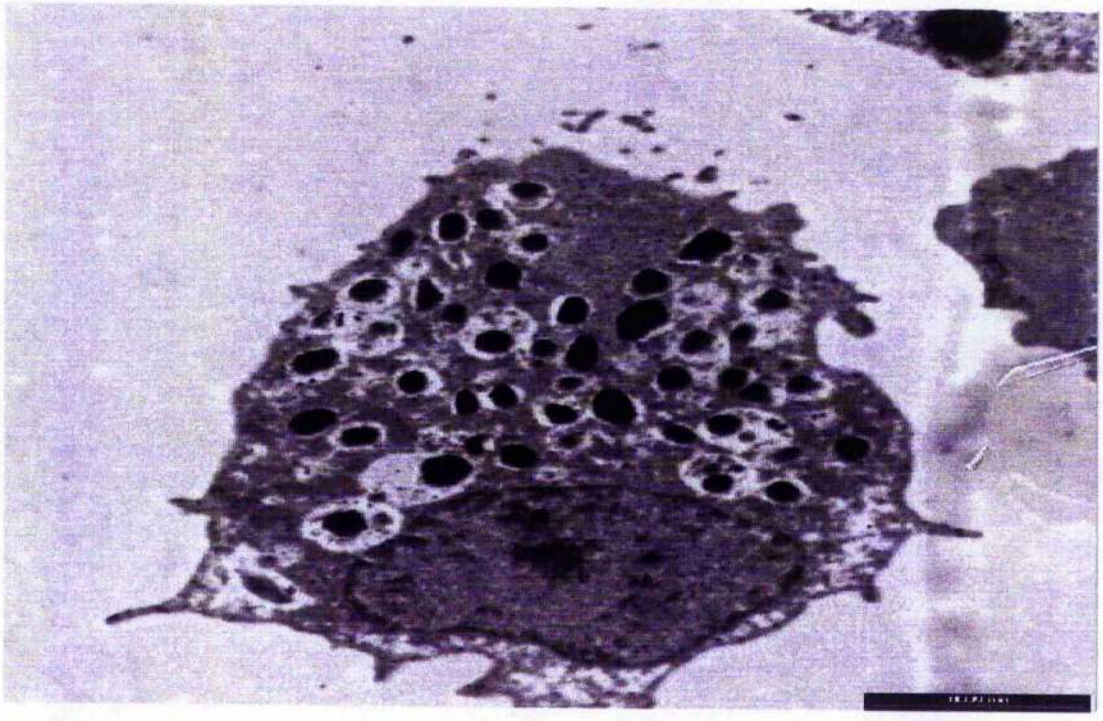


Figure 3.1.2d

3.1.3 Characterisation of sheep BMMC

The ability of either calcium ionophore A23187 (carboxylic acid antibiotic) or substance-P (neuropeptide) to stimulate bone marrow mast cells at different ages of culture was tested. β-hexosaminidase was used as a marker for degranulation of differentiated mast cells from sheep bone marrow in cultures supplemented with both rOvIL-3 and rOvSCF. It is known that there are two mast cell types, connective tissue mast cells and mucosal mast cells. It has been reported that substance-P is not able to stimulate mucosal mast cells and release their contents (Stewart *et al.*, 1996). Therefore, the sensitivity of mast cells differentiated from sheep bone marrow to different concentrations of both substance–P or calcium ionophore was estimated.

Sheep bone marrow mast cells from two different cultures (at 18 days or 27 days) were used. Viability and number of mast cells were estimated by the trypan blue exclusion count and adjusted to 10^6 cells/ml. Both cell suspensions were challenged for 30 min with different concentrations (10^{-4} M, 10^{-6} M or 10^{-8} M) of calcium ionophore or substance-P. Control mast cells were treated with IMDM medium alone, to determine spontaneous β-hexosaminidase release. Other mast cells were frozen at -70°C and thawed three times, to release their total content of β-hexosaminidase.

As can be seen from Fig. 3.1.3a, 10^{-4}M or 10^{-6}M of calcium ionophore A23187 caused significant release (15-25% of total) of β-hexosaminidase from the two ages of sheep BMMC culture compared with the effect of 10^{-8}M of calcium ionophore. Substance-P at any concentration failed to release a significant amount of β-hexosaminidase from any of these cultures. This indicates that mast cells differentiated from sheep bone marrow are relatively refractory to stimulation due to incubation with different concentrations of substance-P. This, in combination with the fact that the majority of their cell contents were released due to the effect of calcium ionophore, indicates that they may possess a mucosal mast cell phenotype.

β-hexosaminidase release from sheep BMMC was further examined after treatment with 10^{-6}M of calcium ionophore. This step was done to investigate the timing of β-hexosaminidase release due to stimulation with calcium ionophore. As can be seen from Fig. 3.1.3b, there was a gradual increase in β-hexosaminidase release up to 30 min but after that, and apparently lower release of β-hexosaminidase was observed.

Figure 3.1.3a Effect of substance-P or calcium ionophore on β-hexosaminidase release from sheep BMMC

Sheep BMMC from 18 day and 27 day cultures were incubated with different concentrations of either calcium ionophore (cal-ion) or substance-P (sub-P). β-hexosaminidase release, as a percentage of cell total content was calculated after incubation for 30 min (mean of 9 observations ± SEM).

1) 10^{-4}M on sheep BMMC (18 days)
2) 10^{-6}M on sheep BMMC (18 days)
3) 10^{-8}M on sheep BMMC (18 days)
4) 10^{-4}M on sheep BMMC (27 days)
5) 10^{-6}M on sheep BMMC (27 days)
6) 10^{-8}M on sheep BMMC (27 days)

Figure 3.1.3b Effect of time of incubation with calcium ionophore on β-hexosaminidase release from sheep BMMC (20 day culture)

Sheep BMMC was treated with 10^{-6}M of calcium ionophore for different times (5 min, 15 min, 30 min, 1 h and 3 h). β-hexosaminidase release, as a percentage of cell total content was estimated at the end of each treatment (mean of 9 observations ± SEM).

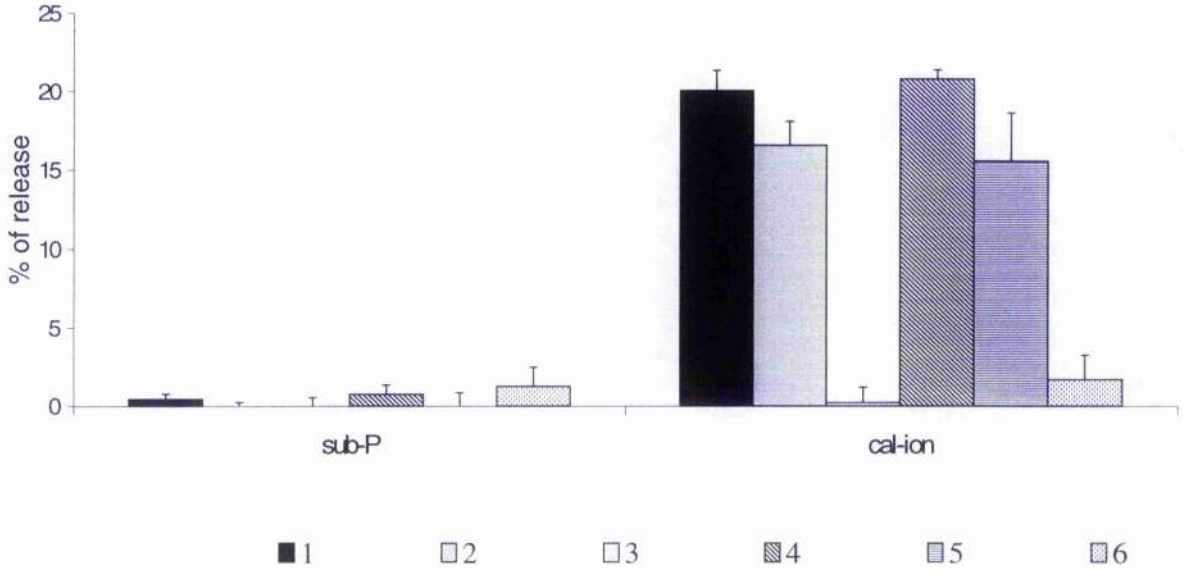


Figure 3.1.3a

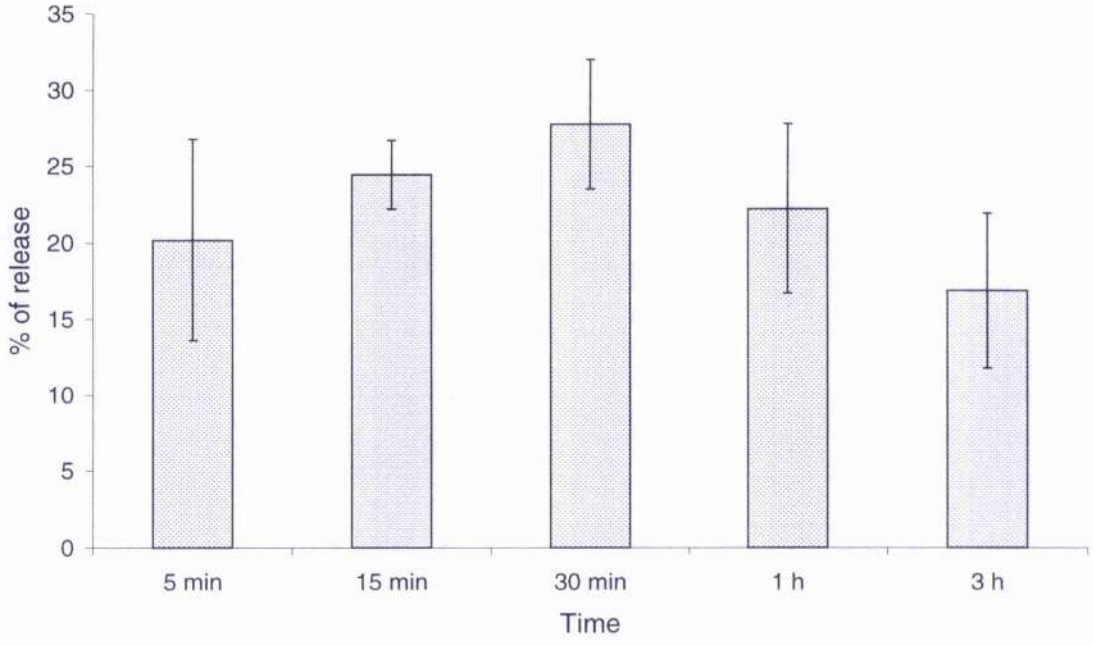


Figure 3.1.3b

3.1.4 Growth and maintenance of RBL-2H3 cells

RBL-2H3 cells (ATCC CRL-2256) were maintained in IMDM medium containing 10% heat inactivated FCS and 1% penicillin/streptomycin. Stock samples from liquid nitrogen required three weeks to recover and proliferate. The subsequent growth rate of RBL-2H3 cells was high (i.e. formation of a monolayer of RBL-2H3 within 48 h). Therefore, it was important to change the IMDM medium every 48 h.

The morphology of RBL-2H3 cells was studied by both light microscopy and transmission electron microscopy (TEM). The growth rate of RBL-2H3 cells in tissue culture flask was investigated by light microscopy. As can be seen from figures 3.1.4a the appearance of these cells differed from that of mast cells, RBL-2H3 cells have longer pseudopodia (which could help these cells to attach to a substrate surface of a culture flask) that could be observed by light microscopy (Ohshiro *et al.*, 2000). In contrast, by TEM the appearance of RBL-2H3 cells (Fig. 3.1.4b) resembled that of the sheep BMMC. They both have similar surface pseudopodia and cytoplasmic vacuoles were observed with granules of different densities.

Figure 3.1.4a RBL-2H3 cell culture

RBL-2H3 cells show long pseudopodia that could be observed by light microscopy (X1000).

Figure 3.1.4b TEM of an RBL-2H3 cell

TEM of a single RBL-2H3 cell. Scale bar = 10μm.

Figure 3.1.4a

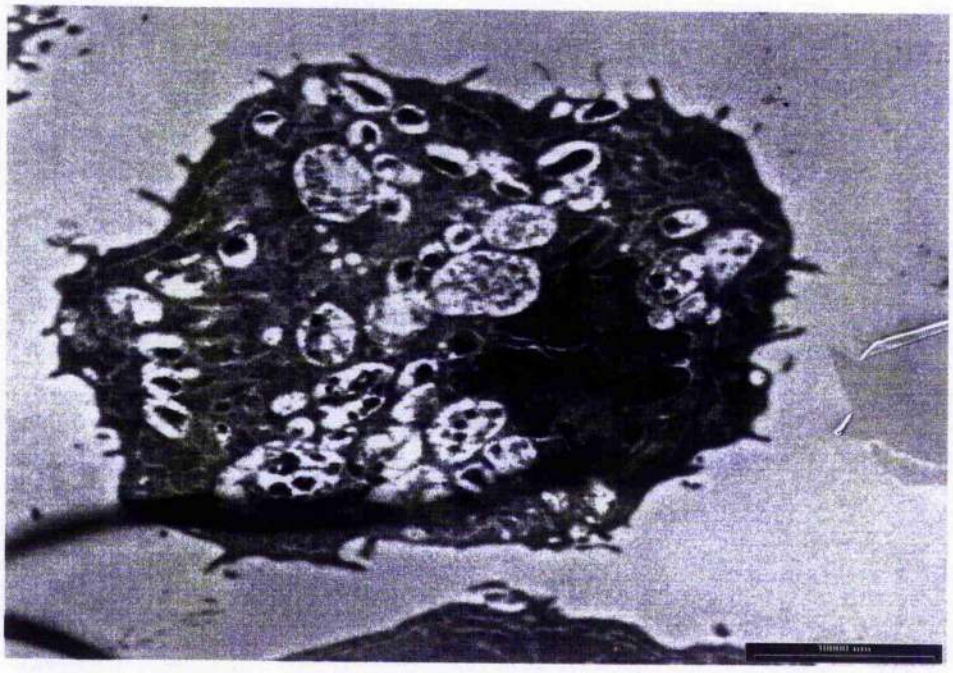


Figure 3.1.4b

3.1.4.1 Interaction of RBL-2H3 cells with different chemical stimulators

The RBL-2H3 cell line is a rat leukemia line that provides a model for the study of mucosal mast cell function. So, a comparison of the effects of calcium ionophore and substance-P on stimulation of sheep BMMC and RBL-2H3 cell was performed. Figure 3.1.5 shows β-hexosaminidase release from either sheep BMMC or RBL-2H3 cells after incubation for 30 min with 10^{-4}M or 10^{-6}M of calcium ionophore which was significantly (p-value < 0.001) higher than that caused by compared to 10^{-8}M. But, as before, (section 3.1.3) substance-P failed to stimulate sheep BMMC to release a significant amount of β-hexosaminidase. In contrast, there was an apparently low β-hexosaminidase release from RBL-2H3 cells due to interaction with 10^{-4}M and 10^{-6}M of substance-P compared with the effect of similar concentrations of calcium ionophore (p-value < 0.0001).

From these observations (section 3.1.3 and section 3.1.4.1), it could be concluded that the effect of calcium ionophore A23187 on degranulation of sheep BMMC and RBL-2H3 cells was similar, and mast cells differentiated from sheep bone marrow cells had similar characteristics to those of a mucosal mast cell-type.

Figure 3.1.5 Effect of different concentrations of substance-P and calcium ionophore on β-hexosaminidase release from sheep BMMC and RBL-2H3 cells

$1x10^6$ cells/ml of sheep BMMC or RBL-2H3 cells were incubated with different concentrations (10^{-4}, 10^{-6} and 10^{-8} M) of calcium ionophore or substance-P for 30 min. Substance-P failed to release β-hexosaminidase from sheep BMMC but with RBL-2H3 cells there was a slight β-hexosaminidase release. A similar concentration of calcium ionophore caused a significant β-hexosaminidase release from both sheep BMMC and RBL-2H3 cells (mean of 9 observations ± SEM).

1) Substance-P 10^{-4} M
2) Substance-P 10^{-6} M
3) Substance-P 10^{-8} M
4) Calcium ionophore 10^{-4} M
5) Calcium ionophore 10^{-6} M
6) Calcium ionophore 10^{-8} M

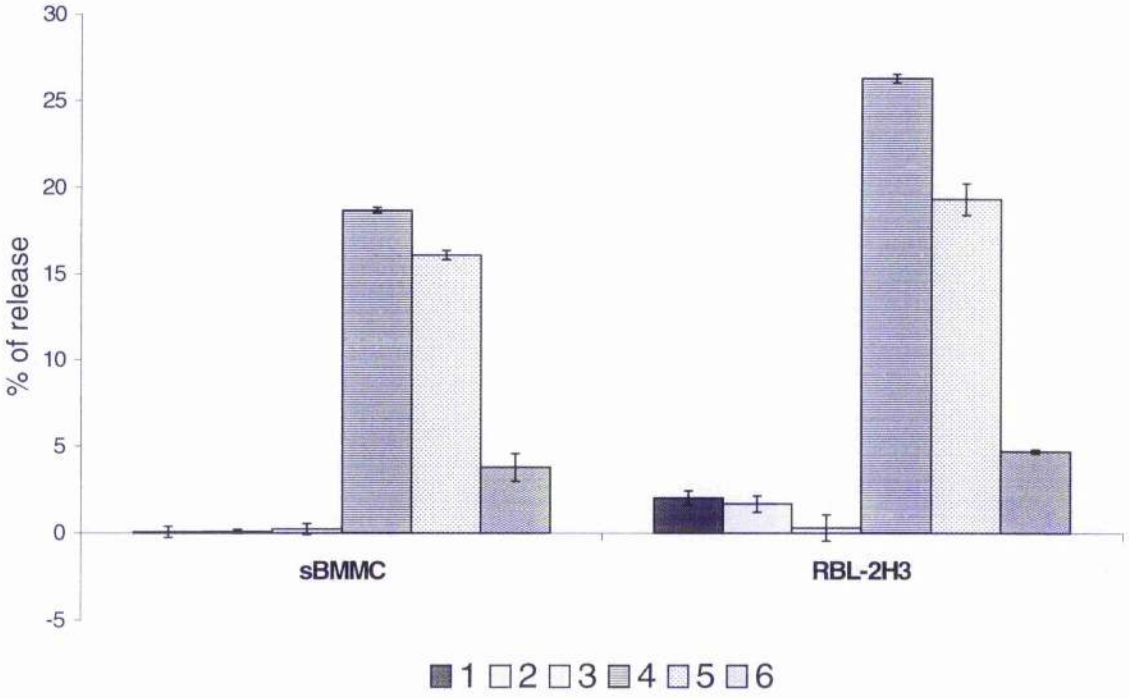


Figure 3.1.5

Section Two: Application of bioluminescent bacteria as an in vitro reporter of the interaction of *B. bronchiseptica* and sheep BMMC

3.2.1 Characterisation of *B. bronchiseptica* (Lux 95)

At the start of the present study, an attempt was made to determine whether the bioluminescent strain of *B. bronchiseptica* (Lux 95) had the typical virulent phenotype of *B. bronchiseptica* BBC17 (Bvg $^+$ phase) or not. In addition, an avirulent (Bvg $^-$ phase) mutant strain of *B. bronchiseptica* (BBC17) was used as a control strain.

B. bronchiseptica (Lux 95) was compared with the virulent strain of *B. bronchiseptica* (Bvg $^+$ phase) and avirulent strain of *B. bronchsieptica* (Bvg $^-$ phase) for a number of virulence-related properties.

3.2.1.1 Colony morphology

All three strains of *B. bronchiseptica*: the virulent strain (Bvg $^+$ phase), the avirulent strain (Bvg $^-$ phase) and the bioluminescent strain (Lux 95) were grown on BG agar plates and allowed to grow for 48 h in humid conditions at 37°C. Colonies on the plate were then observed with the naked eye and variation between colonies was recorded. After 24 h, it was observed that colonies of the avirulent strain (Bvg$^-$ phase) were visible. But both the virulent strain (Bvg $^+$ phase) and the bioluminescent strain (Lux 95) needed more than 24 h to form colonies. It was observed that the avirulent strain (Bvg $^-$ phase) colonies were large and flat compared with those of the virulent strain (Bvg $^+$ phase) and of the bioluminescent strain (Lux 95) which could be characterised as small and domed.

3.2.1.2 Haemolysis effect

BG sandwich agar plates were used to study the haemolytic effect of *B. bronchiseptica* (Lux 95) and compared with the haemolytic effect of both the virulent (Bvg $^+$ phase) and the avirulent (Bvg $^-$ phase) strains. It was observed visually that there was clear zone of haemolysis surrounding the colonies of the virulent strain (Bvg $^+$ phase) after growth for 48h. No zone of haemolysis surrounded the colonies of either the avirulent strain (Bvg $^-$ phase) or the bioluminescent strain (Lux 95).

3.2.1.3 Haemagglutination assay

The haemagglutination assay was carried out to identify surface adhesion molecules of the bioluminescent strain (Lux 95) compared with the virulent strain (Bvg $^{+}$ phase) and the avirulent (Bvg $^{-}$ phase) strains. Purified filamentous haemagglutinin (FHA) was used as a positive control for haemagglutination and was received from a departmental stock. It was used at a net concentration of 45 µg/ml.

Growth of *B. bronchiseptica* (Bvg $^{+}$, Bvg $^{-}$ and Lux 95) on BG agar for 24h was collected in phosphate buffered saline (PBS). Cell numbers were adjusted at OD_{600nm}= 0.46 equivalent ($2x10^{9}$ cfu/ml) and diluted to $5x10^{8}$ cfu/ml in PBS.

There was a weak haemagglutination effect due to $5x10^{8}$ cfu/ml of *B. bronchiseptica* (Bvg $^{+}$ phase), but this effect was not observed with greater dilution of the bacterial suspension. In contrast, no haemaggultination effect was observed for either mutant strain of *B. bronchiseptica* (Bvg $^{-}$ or Lux 95) at $5x10^{8}$ cfu/ml. Haemagglutination of different strains *B. bronchiseptica* was compared with the effect of purified FHA (positive control) which gave a clear haemagglutination effect and IMDM medium (negative control) which gave no haemagglutination.

3.2.1.4 Determination of AC enzymatic activity in *B. bronchiseptica* (Lux 95)

Adenylate cyclase (AC) enzymatic activity of *B. bronchiseptica* (Lux 95) was estimated by a conductimetry assay (Chapter two; Materials and Methods) and compared with AC enzymatic activity of the virulent *B. bronchiseptica* (Bvg $^+$ phase) or avirulent *B. bronchiseptica* (Bvg $^-$ phase). Growth of *B. bronchiseptica* (Bvg $^+$, Bvg$^-$ and Lux 95) on BG agar for 24h was collected in PBS and washed twice by centrifugation at 4000 rpm for 5 min. Cell numbers were adjusted to $OD_{600nm} = 0.46$ (equivalent to $2x10^9$ cfu/ml) and diluted to $5x10^8$ cfu/ml in PBS. 1% of Triton X100 was added to lyse the bacterial cells and released their contents.

Figure 3.2.1 shows that the AC enzymatic activity of *B. bronchiseptica* (Lux 95) was low and similar to AC enzymatic activity of the avirulent strain of *B. bronchiseptica* (Bvg $^-$ phase), compared with the high enzymatic activity of the virulent strain of *B. bronchiseptica* (Bvg $^+$ phase). These observations suggested that the contents of AC toxin in the bioluminescent strain of *B. bronchiseptica* (Lux 95) grown on BG agar is weak.

Furthermore, it is known that adenylate cyclase toxin, either as a soluble protein or when associated with whole bacteria, is a bi-functional molecule and, in addition to AC enzymic activity, is also able to lyse erythrocytes. The conductimetry assay is one way of characterization of ACT activity but it is also possible to estimate ACT activity in whole bacteria by observing haemolysis on sandwich BG agar (section 2.1.2). Thus, these were two estimates of ACT activity.

In conclusion, the findings with both the virulent strain of *B. bronchiseptica* (Bvg $^+$ phase) and avirulent *B. bronchiseptica* (Bvg $^-$ phase) agree with the known properties of these two strains. Furthermore, the bioluminescent strain of *B. bronchiseptica* (Lux 95) has a colony morphology like that of the virulent *B. bronchiseptica* (Bvg $^+$ phase) but otherwise resembles the avirulent *B. bronchiseptica* (Bvg $^-$ phase). Thus, the bioluminescent *B. bronchiseptica* strain appears to be an intermediate phenotypic phase of *B. bronchiseptica* rather than a wild type (virulent Bvg $^+$ phase) strain.

Figure 3.2.1 Comparison of the AC enzymatic activity in bioluminescent *B. bronchiseptica* (*Lux* 95) with AC activity in virulent and avirulent *B. bronchiseptica* by conductimetry assay

This graph shows a comparison between the AC enzymatic activity of *B. bronchiseptica* (Lux 95) that of *B. bronchiseptica* (Bvg^{+}) and (Bvg^{-}) grown on BG agar. **A** represent the scale of conductivity of the reaction mixture (Y-axis) and **B** represents the scale of time (sec) (X-axis). AC enzymatic activity of both *B. bronchiseptica* (Lux 95) and *B. bronchiseptica* (Bvg^{-} phase) was low compared with the high AC enzymatic activity of *B. bronchiseptica* (Bvg^{+} phase).

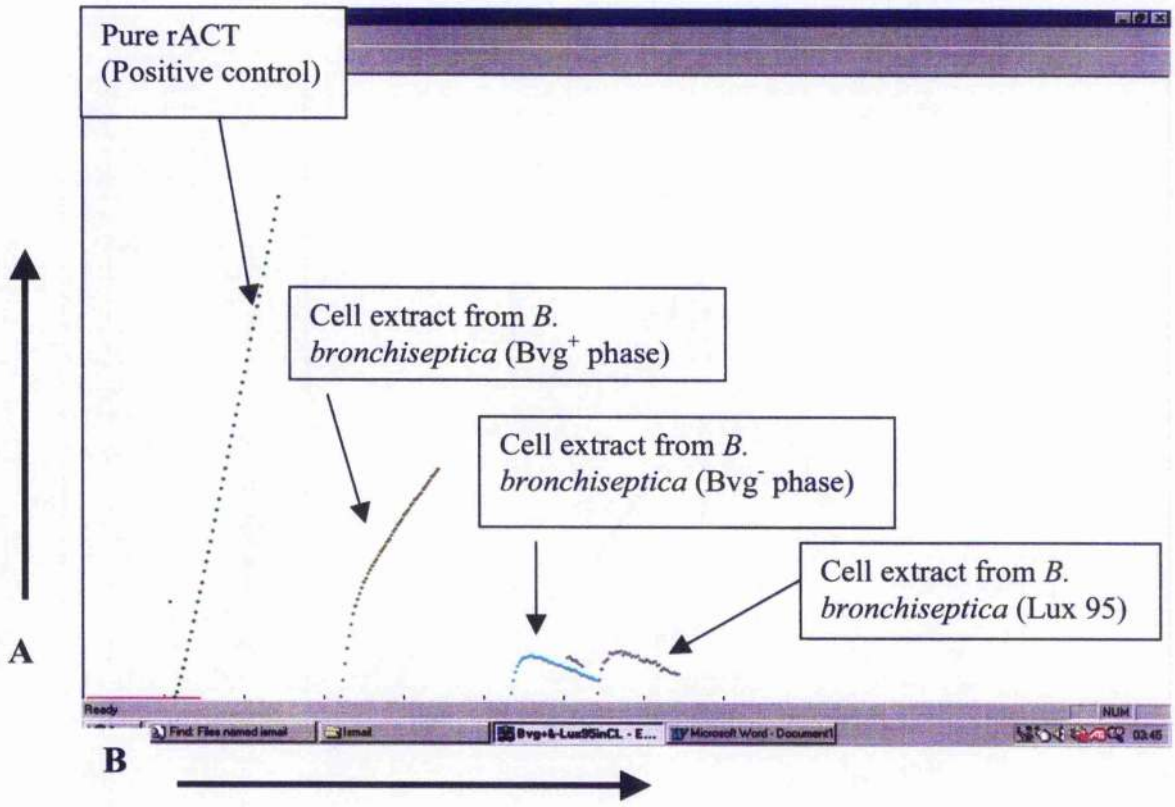

Pure rACT
(Positive control)
Cell extract from *B. bronchiseptica* (Bvg$^+$ phase)
Cell extract from *B. bronchiseptica* (Bvg$^-$ phase)
Cell extract from *B. bronchiseptica* (Lux 95)
A
B

Figure 3.2.1

3.2.2 Bioluminescence activity of *B. bronchiseptica* (Lux 95)

3.2.2.1 Bioluminescence activity in different media

In order to study the interaction of *B. bronchiseptica* and mammalian cells, it was important to show that *B. bronchsieptica* (Lux 95) would survive, and its bioluminescence activity would be maintained, in the IMDM medium used routinely for culture of mammalian cells. It was assumed that the light output of live bacteria would be directly proportional to the viability of the bacteria cells (Nelson *et al.*, 2003). In addition, *B. bronchiseptica* can survive and even grow in natural waters and in PBS (Porter *et al.*, 1991). Therefore, bioluminescence activity of *B. bronchiseptica* (Lux 95) in IMDM was compared with that in PBS. The effect of adding heat-inactivated FCS to the media was also investigated. Growth of *B. bronchiseptica* (Lux 95) for 24h on BG agar was collected in IMDM medium containing 10% FCS, IMDM medium only, PBS containing 10% FCS and PBS only. Bacterial cell numbers were adjusted to $5x10^8$ cfu/ml in the same medium or buffer. Bacterial suspension (200µl) was then added in triplicate into 96-well plates and incubated at 37°C in the luminometer. Bioluminescence output as relative light units (RLU), of the bacterial cell suspension was measured with time.

Figure (3.2.2a) shows that, within the first 0 – 6 h of incubation, there was only low bioluminescence activity of the Lux 95 suspension either in IMDM medium or in PBS. After 6 h, there was an increase in the bioluminescence activity of Lux 95 suspension in IMDM medium, especially if the IMDM medium containing 10% heat-inactivated FCS. Bioluminescence activity in PBS only remained low. But, if PBS contained 10% heat-inactivated FCS, bioluminescence activity increased. From these observations, it could be concluded that IMDM medium containing 10% FCS helped *B. bronchiseptica* (Lux 95) to be more active in its bioluminescence emission presumably as a result of metabolic activity, growth and increase in cell numbers. Figure 3.2.2b shows bioluminescence activity from different numbers of *B. bronchiseptica* (Lux 95) suspended in IMDM medium containing 10% FCS and measured immediately. There was a linear correlation between bioluminescence output (RLU) and cell numbers (cfu/ml). With an increase in cell numbers, there was increased bioluminescence activity.

Figure 3.2.2a Bioluminescence output of *B. bronchiseptica* (Lux 95) in different media

B. bronchiseptica (Lux 95) was maintained in either IMDM medium or PBS in the presence or absence of 10% FCS. Bioluminescence output was measured and expressed as relative light units (RLU) (mean of 9 observations ± SD).

Figure 3.2.2b Bioluminescence output from different numbers *B. bronchiseptica* (Lux 95)

Different numbers of *B. bronchiseptica* (Lux 95) were prepared in IMDM medium and 10% FCS and bioluminescence output (RLU) was measured (means of 3 observations)

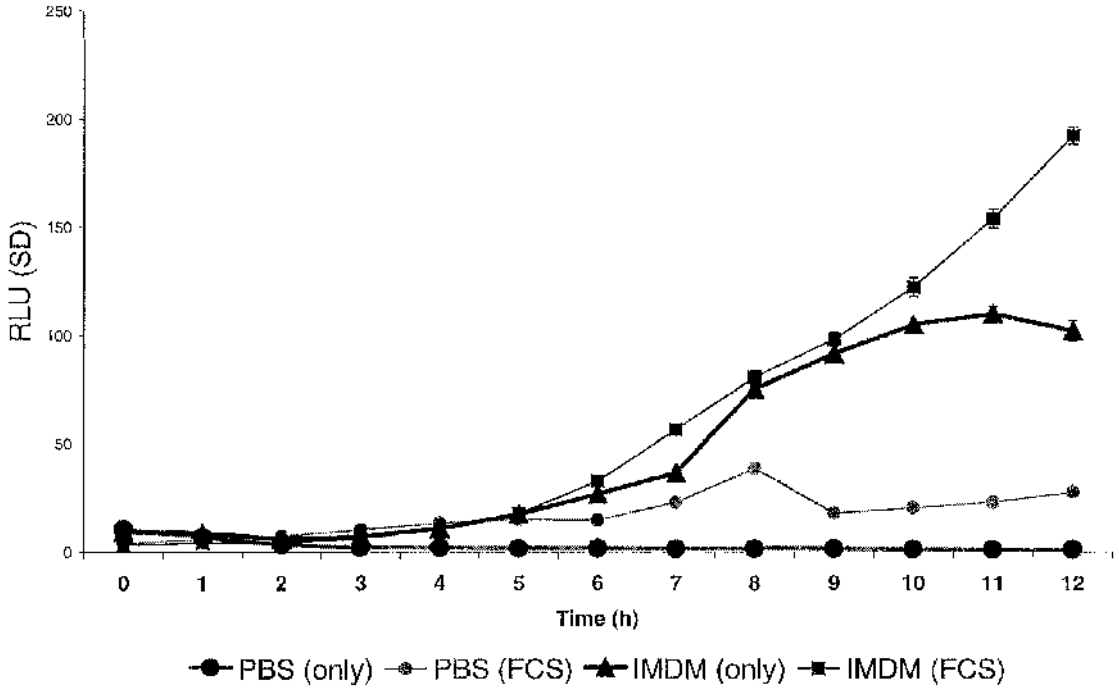


Figure 3.2.2a

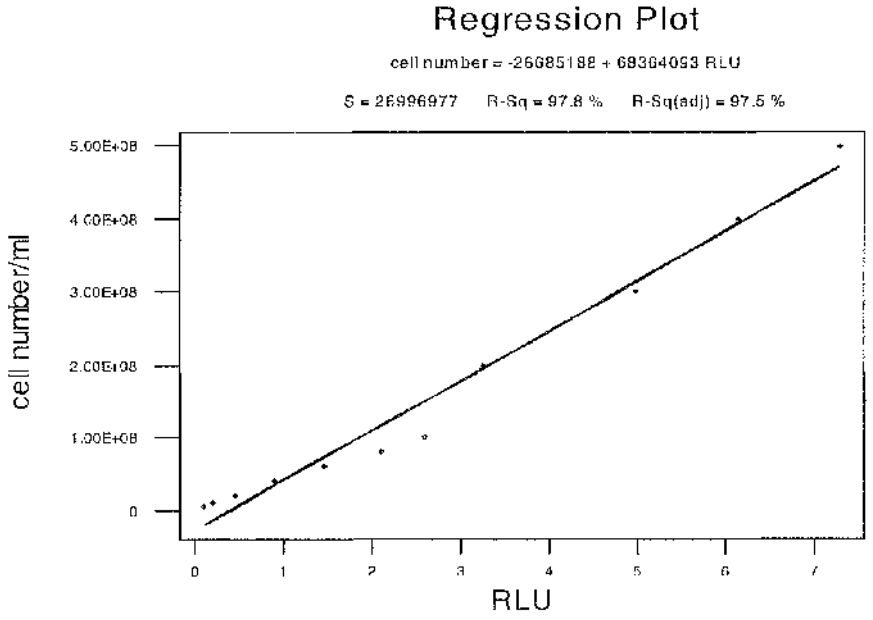


Figure 3.2.2b

3.2.2.1.1 Bioluminescence activity of *B. bronchiseptica* (Lux 95) in a short-run experiment (20 h)

Bioluminescence activity in bacteria expressing *lux* genes is assumed to depend on metabolic activity. Therefore, it was important to investigate whether an increase in the bioluminescence activity of a *B. bronchiseptica* (Lux 95) culture correlated with an increase in cell numbers as determined by optical density (OD). Bacterial numbers of *B. bronchiseptica* (Lux 95) were adjusted to $5x10^8$ cfu/ml in IMDM medium containing 10% FCS. Bacterial suspension (200µl) was then added in triplicate into 96 -well plates and incubated at 37°C in the luminometer.

Figure 3.2.3a shows the overall measurement of both RLU and OD_{492nm} estimated in a short-run (20 h) experiment. There was a parallel increase in both RLU and OD of *B. bronchiseptica* (Lux 95) culture over the first 10 h of incubation. From 10 – 20 h, there was a continued steady increase in RLU but only a gradual increase in OD and lack of correlation between the two curves.

The correlation between bioluminescence activity (RLU) of *B. bronchiseptica* (Lux 95) and optical density OD_{492nm} was estimated by a regression plot (Figure 3.2.3d). The following data shows output in the Session window of MINITAB that accompanied the graphical plot in Figure 3.2.3b.

Table 3.2.1: Analysis of variance of the regression between RLU and OD (short-run)

Regression Analysis: RLU versus OD

Predictor	**Coef**	**SE Coef**	**T**	**P**	
Constant	-45.084	5.500	-8.20	0.000	
OD	132.606	7.685	17.26	0.000	
Analysis of Variance					
Source	**DF**	**SS**	**MS**	**F**	**P**
Regression	1	14559	14559	297.75	0.000
Residual Error	19	929	49		
Total	20	15489			

DF: Degree of freedom, **SS:** sums of squares, **MS:** mean square, **F:** variance ratio, **T:** Student's *t*, **P:** null hypothesis, **S:** the error standard deviation, **R-Sq:** % of the variability (**S** and **R-Sq** are shown in Fig. 3.2.3b).

Figure 3.2.3b shows the regression diagram between RLU and OD surrounded by additional dotted and dashed lines. The inner, dotted, lines are the 95% confidence bands of the regression itself. These are narrowest at the centre, indicating that the position of the regression line is known with greatest certainty in the middle region and with some wider uncertainty as the dotted lines flare out slightly at the ends.

With reference to figure 3.2.3b and table 3.2.1, and for 95% confidence bands and 95% prediction bands and p-value = 0.000 (< 0.001) which is very highly significant, it could be said that, over a short-run (20h) experiment, there was a good linear correlation between bioluminescence activity (RLU) and optical density (OD) of the *B. bronchiseptica* (Lux 95). These observations suggested that bioluminescence output would be an easy and accurate measure of viable cell numbers.

Figure 3.2.3a Correlation between bioluminescence output and optical density of *B. bronchiseptica* (Lux 95)

$5x10^8$ cfu/ml of *B. bronchiseptica* (Lux 95) were inoculated into IMDM medium containing 10% heat-inactivated FCS. Bioluminescence output (RLU) was measured in parallel with optical density (OD_{492nm}) (means of 9 observations ± SD).

Figure 3.2.3b Regression plot of bioluminescence output and optical density

Regression of bioluminescence output (RLU) of *B. bronchiseptica* (Lux 95) and optical density (OD_{492nm}) in IMDM medium containing 10% FCS. With 95% confidence bands (dotted lines) for the regression and 95% prediction bands (dashed line).

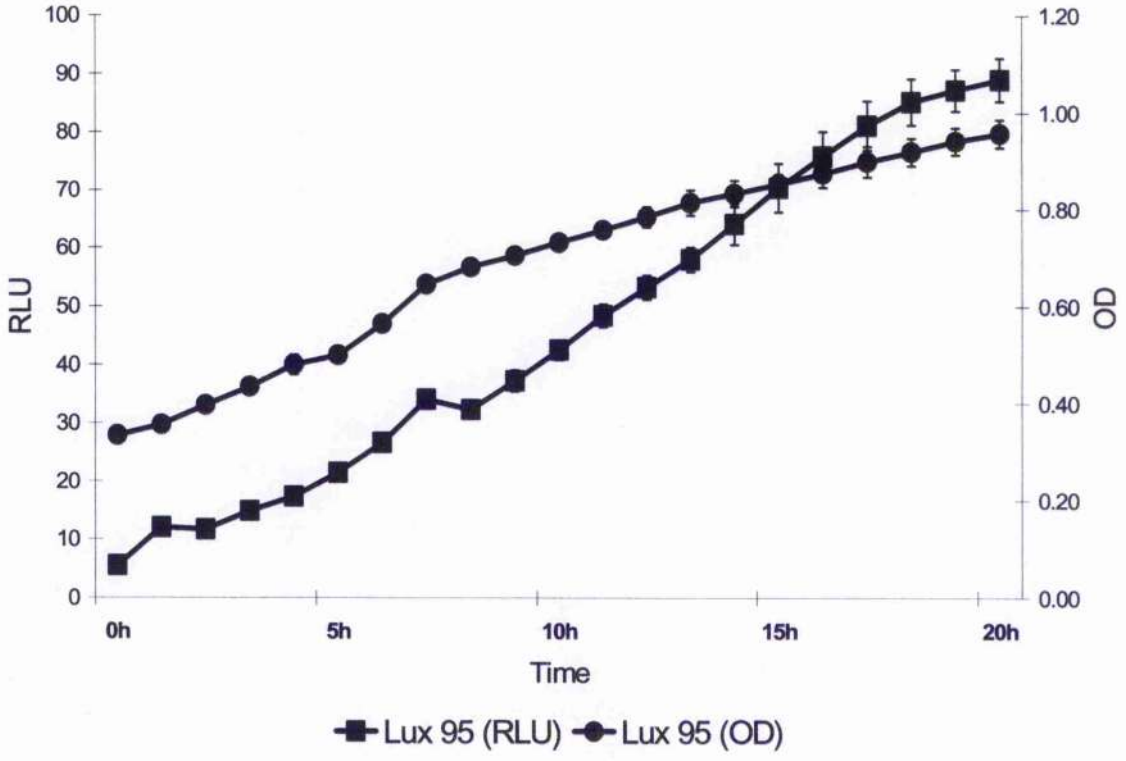


Figure 3.2.3a

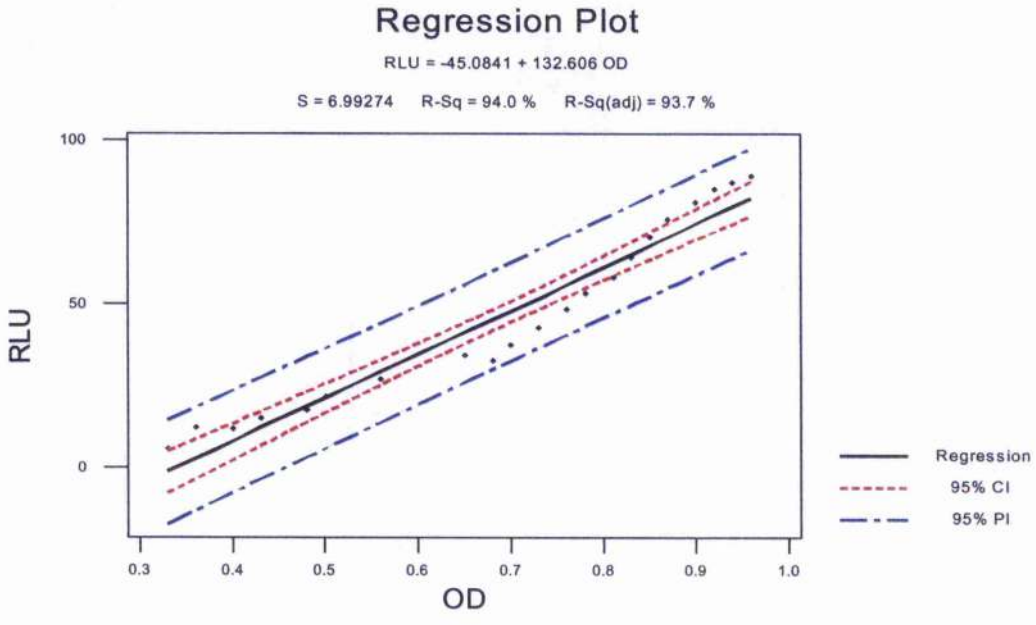


Figure 3.2.3b

3.2.2.1.2 Bioluminescence activity of *B. bronchiseptica* (Lux 95) in a long-run experiment (>20 h)

Bioluminescence activity and optical density of *B. bronchiseptica* (Lux 95) were monitored in a long-run experiment over 40 h. As can be seen from 3.2.3c there was a steady increase in both RLU and OD within the first 20 h (1199min). But, after 20 h, it was observed that there was a sharp decline of bioluminescence output of *B. bronchiseptica* (Lux 95) and there was a slowing down of the rate of increase in OD. The correlation between bioluminescence activity (RLU) of *B. bronchiseptica* (Lux 95) and optical density (OD_{492nm}) was estimated by regression plot (Figure 3.2.3d). The following data shows output in the Session window of MINITAB that accompanied the graphical plot in Figure 3.2.3d.

Table 3.2.2: Analysis of variance of the regression between RLU and OD (long-run)

Regression Analysis: RLU versus OD

Predictor	**Coef**	**SE Coef**	**T**	**P**
Constant	113.25	46.86	2.42	0.024
OD	-35.09	34.17	-1.03	0.315

Analysis of Variance

Source	**DF**	**SS**	**MS**	**F**	**P**
Regression	1	2877	2877	1.05	0.315
Residual Error	24	65483	2728		
Total	25	68361			

DF: Degree of freedom, **SS:** sums of squares, **MS:** mean square, **F:** variance ratio, **T:** Student's *t*, **P:** null hypothesis, **S:** the error standard deviation. (**S** is shown in Fig. 3.2.3d)

At the end of the 40 h incubation period in the luminometer, viability of *B. bronchiseptica* (Lux 95) was determined by viable counting on BG agar plates. A total of three samples was taken from each incubation well (n = 3) which had contained $5x10^8$ cfu/ml of *B. bronchiseptica* (Lux 95) at the start of experiment. These counts showed that there were not less than $1x10^8$ cfu/ml in the 40h culture. From these observations it appears that, with increase in incubation time up to 40 h at 37°C in the luminometer, bioluminescence output (RLU) reduces in *B. bronchiseptica* (Lux 95) culture but there is not a parallel reduction in bacterial viability. So, it appears that, in older cultures (> 20 h) of *B. bronchiseptica* (Lux 95), the linear relationship between RLU and viable cell numbers breaks down.

Figure 3.2.3c Bioluminescence output and optical density of *B. bronchiseptica* (Lux 95) in a long-run experiment

$5x10^8$ cfu/ml of *B. bronchiseptica* (Lux 95) were incubated in IMDM medium containing 10% heat-inactivated FCS. Bioluminescence output (RLU) was measured along with optical density (OD) (means of 9 observations ± SEM). At the end of the run, viability of B. *bronchiseptica* (Lux 95) was determined by plating on BG agar.

Figure 3.2.3d Regression plot of bioluminescence output and optical density

Regression of bioluminescence output of *B. bronchiseptica* (Lux 95) and optical density in IMDM medium containing 10% FCS. With 95% confidence bands (dotted lines) for the regression and 95% prediction bands (dashed line).

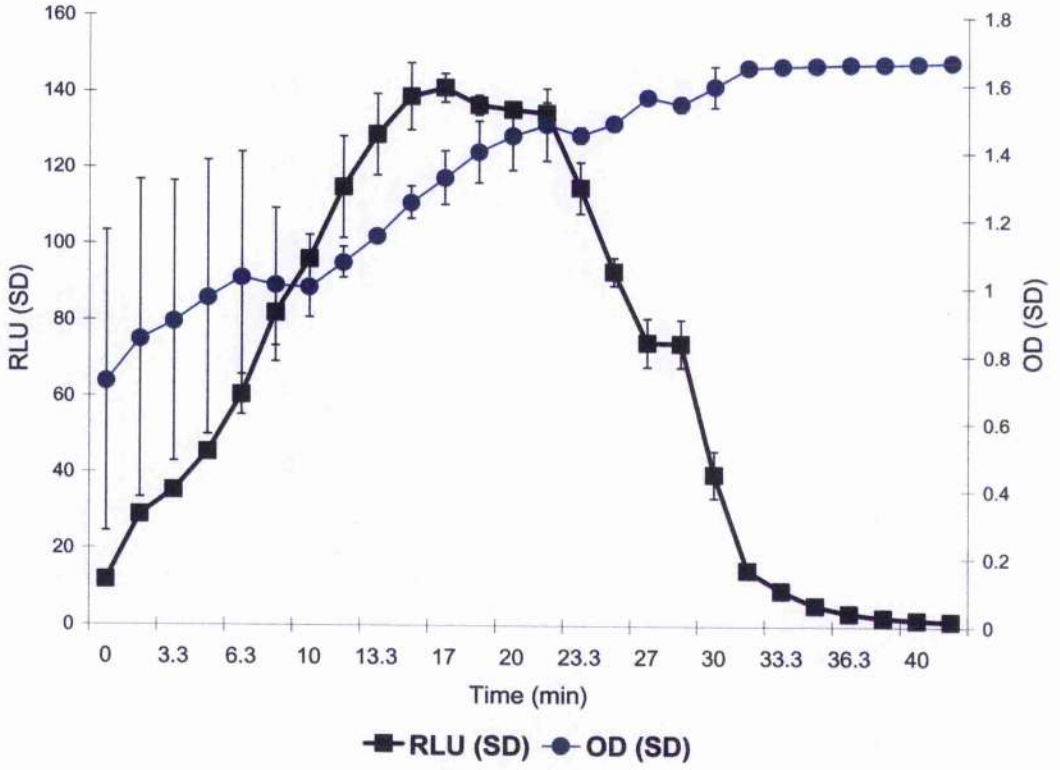


Figure 3.2.3c

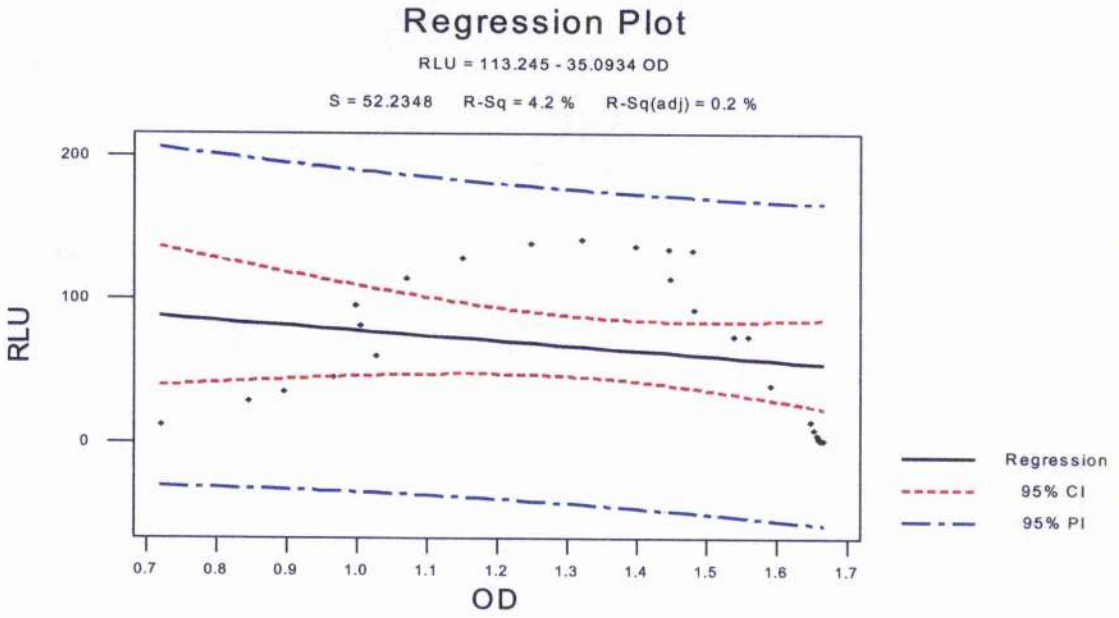


Figure 3.2.3d

3.2.2.2 Effect of polymyxin B on bioluminescence activity

Polymyxin B is used to kill extracellular bacteria in studies of bacterial uptake/phagocytosis by mammalian cells. 10^8 cfu/ml (lower number) and $5x10^8$ cfu/ml (higher number) of *B. bronchiseptica* (Lux 95) were prepared in IMDM medium containing 10% FCS and 200 µl volumes were incubated in 96-well plates at 37°C in the luminometer. Bioluminescence output was monitored. After incubation for 3 h, 50 µg/ml of polymyxin B was added to some wells and other wells served as control wells which contained bacteria in IMDM medium only. As can be seen from Fig. 3.2.4, there was a sharp drop in bioluminescence output from low and high numbers of *B. bronchiseptica* (Lux 95) due to the addition of polymyxin B, compared with a gradual increase in bioluminescence output from *B. bronchiseptica* (Lux 95) in control wells. After incubation for 8 h in the luminometer, viability of *B. bronchiseptica* (Lux 95) was determined by viable counting on BG agar plates. In wells originally containing 10^8 cfu/ml and polymyxin B, it was observed that 50 µg/ml polymyxin B killed > 99.99% of 10^8 cfu/ml *B. bronchiseptica* (Lux 95). Whereas, without polymyxin B, the viable count was more than $1x10^8$ cfu/ml (number of observations = 6).

Figure 3.2.4 Effect of polymyxin B on bioluminescence output from *B. bronchiseptica* (Lux 95)

Bioluminescence output was monitored from $1x10^8$ and $5x10^8$ cfu/ml of *B. bronchiseptica* (Lux 95) in IMDM medium containing 10% FCS. After 3 h incubation, 50 μg/ml polymyxin B was added to some wells and other wells served as control wells (mean of 9 observations ± SD).

a) *B. bronchiseptica* $1x10^8$ cfu/ml in IMDM medium alone.

b) *B. bronchiseptica* $1x10^8$ cfu/ml and polymyxin B.

c) *B. bronchiseptica* $5x10^8$ cfu/ml in IMDM medium alone.

d) *B. bronchiseptica* $5x10^8$ cfu/ml and polymyxin B.

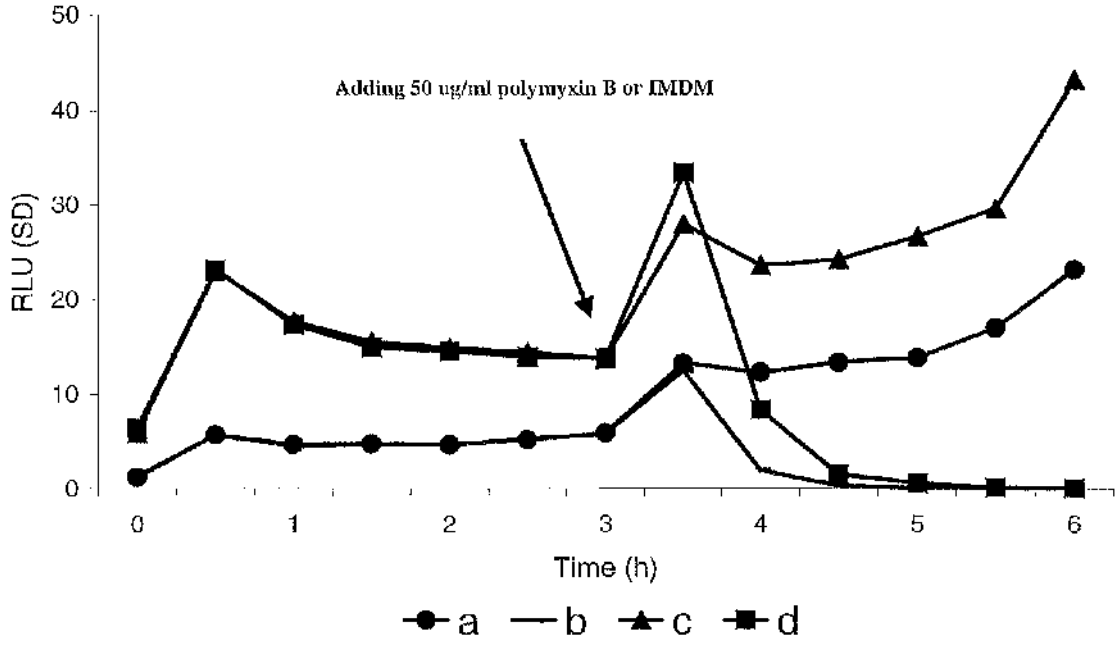

Adding 50 ug/ml polymyxin B or IMDM
RLU (SD)
50
40
30
20
10
0
0
1
2
3
4
5
6
Time (h)
a
b
c
d

Figure 3.2.4

3.2.2.3 Effect of digitonin on bioluminescence activity

Digitonin is a cholesterol-complexing agent soluble in water and dissolved by heating to 95 - 98°C. Pre-treatment of mammalian cells with digitonin results in rapid lysis of the cell membrane and release of intracellular contents. In these studies, it was used to disrupt the cell membrane of sheep BMMC and other mammalian cell lines, to determine whether bacteria are intracellular and for viable counts purpose (see section 2.6.2, Materials and Methods). When digitonin was added to the mammalian cell/bacteria mixtures with polymyxin B, it allowed any intracellular bacteria to be released and killed by polymyxin B. The following study was planned to determine if digitonin itself had any effect on viability and bioluminescence activity of *B. bronchiseptica* (Lux 95).

B. bronchiseptica (Lux 95) was grown on BG agar for 24h, then $5x10^8$ cfu/ml was prepared in IMDM medium containing 10% FCS. 200µl of the bacterial suspension was then added in triplicate to 96 -well plates and incubated at 37°C in the luminometer. Bioluminescence output was measured and, after incubation for 1h in the luminometer, 50 µg/ml of digitonin and/or polymyxin B were added.

Figure 3.2.5 shows the mean of the bioluminescence output from *B. bronchiseptica* (Lux 95) under different conditions. As can be seen, there was a steady increase in bioluminescence output from *B. bronchiseptica* (Lux 95) in IMDM medium only. A similar increase in bioluminescence output from *B. bronchiseptica* (Lux 95) was observed when 50 µg/ml digitonin was present. However, bioluminescence output from *B. bronchiseptica* (Lux 95) declined due to the presence of 50 µg/ml polymyxin. A similar observation of decline in bioluminescence output was observed when both digitonin and polymyxin B were present. These observations suggested that this concentration of digitonin had no effect on viability of *B. bronchiseptica* (Lux 95) and could be used in invasion assays.

Figure 3.2.5 Comparison of the effect of digitonin and polymyxin B on light output from *B. bronchiseptica* (Lux 95)

$5x10^8$ cfu/ml of *B. bronchiseptica* (Lux 95) were maintained in IMDM medium containing 10% FCS. Bioluminescence output was measured at 37°C in a luminometer. After 60min, 50µg/ml digitonin and polymyxin B were added to some wells and IMDM medium was added to other wells (controls). Data are shown in relative light units (RLU) (means of 9 observations, error bars are omitted when SD of the mean do not exceed the dimensions of the symbols)

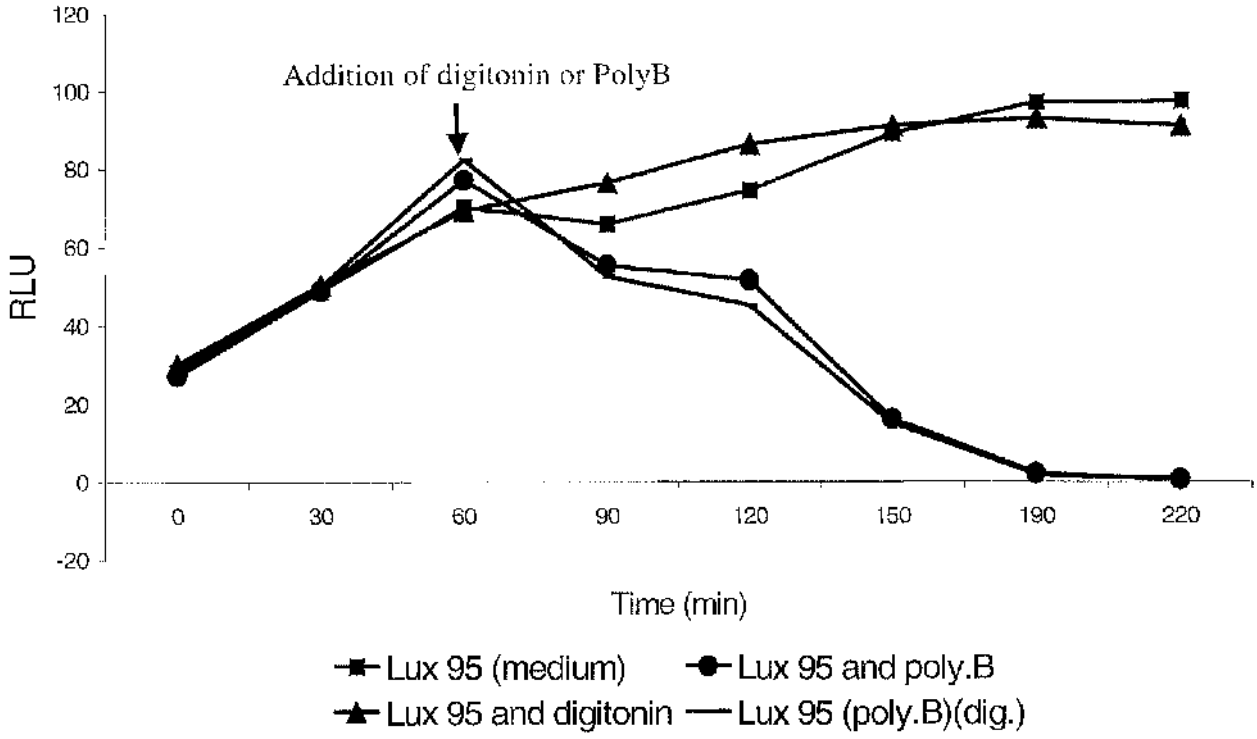

120
100
80
60
40
20
0
-20
RLU
Addition of digitonin or PolyB
0
30
60
90
120
150
190
220
Time (min)
Lux 95 (medium)
Lux 95 and poly.B
Lux 95 and digitonin
Lux 95 (poly.B)(dig.)

Figure 3.2.5

3.2.2.4 Bioluminescent *E. coli*

A bioluminescent strain of *E. coli*, Hb101pT7-3, was used in the study to compare its uptake and intracellular survival in mammalian cells with that of *B. bronchiseptica* (Lux 95). Its bioluminescence emission when maintained in IMDM medium containing 10% FCS was monitored. The *E. coli* was grown routinely in 5 ml LB broth with 50 µg/ml of ampicillin. Bacteria were harvested from LB broth, and suspended to $5x10^8$ cfu/ml in IMDM containing 10% FCS. 200 µl of cell suspension was added in triplicate in 96-well plates and incubated at 37°C in the luminometer. Figure 3.2.6 shows the relationship between RLU and OD over 20 h at 37°C in the luminometer. There was a gradual increase in OD (up to 10 h), and then there was slower increase in OD (up to 20 h). Bioluminescence output shows a slow rise initially (up to 5h) then a more rapid increase (up to 10 h), and then there was a sharp decline in RLU down to baseline. At the end of the 20 h incubation time, viability of the *E. coli* was determined by viable counting on LB agar plates. A total of three samples was taken from each incubation well (n = 3) which contained $5x10^8$ cfu/ml of *E. coli* at the start of experiment. These counts showed that there were > $1x10^8$ cfu/ml in the 20 h culture. From these observations it appears that, with increase in incubation time up to 20h at 37°C in the luminometer, bioluminescence output RLU declines in the *E. coli* culture, but there is not a parallel reduction in bacterial viability. So, it appears that with *E. coli*, the linear relationship between RLU and viability breaks down.

Thus, the correlation between bioluminescence output and optical density or viability of the bioluminescent *E. coli* is not as good as with *B. bronchiseptica* (Lux 95) even with a short incubation (up to 20 h). With a longer incubation time, presumably when the culture reaches stationary phase, the bacteria rapidly cease to emit light even though they remain viable. Such older cultures would therefore not be useful for invasion assays. This particular construct of *E. coli* is possibly less useful than *B. bronchiseptica* (Lux 95).

Figure 3.2.6 Correlation between bioluminescence output and optical density of bioluminescent *E. coli*

$5x10^8$ cfu/ml of bioluminescent *E. coli* (Hb101pT7-3) was inoculated into IMDM medium containing 10% of heat-inactivated FCS. Bioluminescence output was measured in parallel with optical density (OD). Data are means of 9 observations ± SEM.

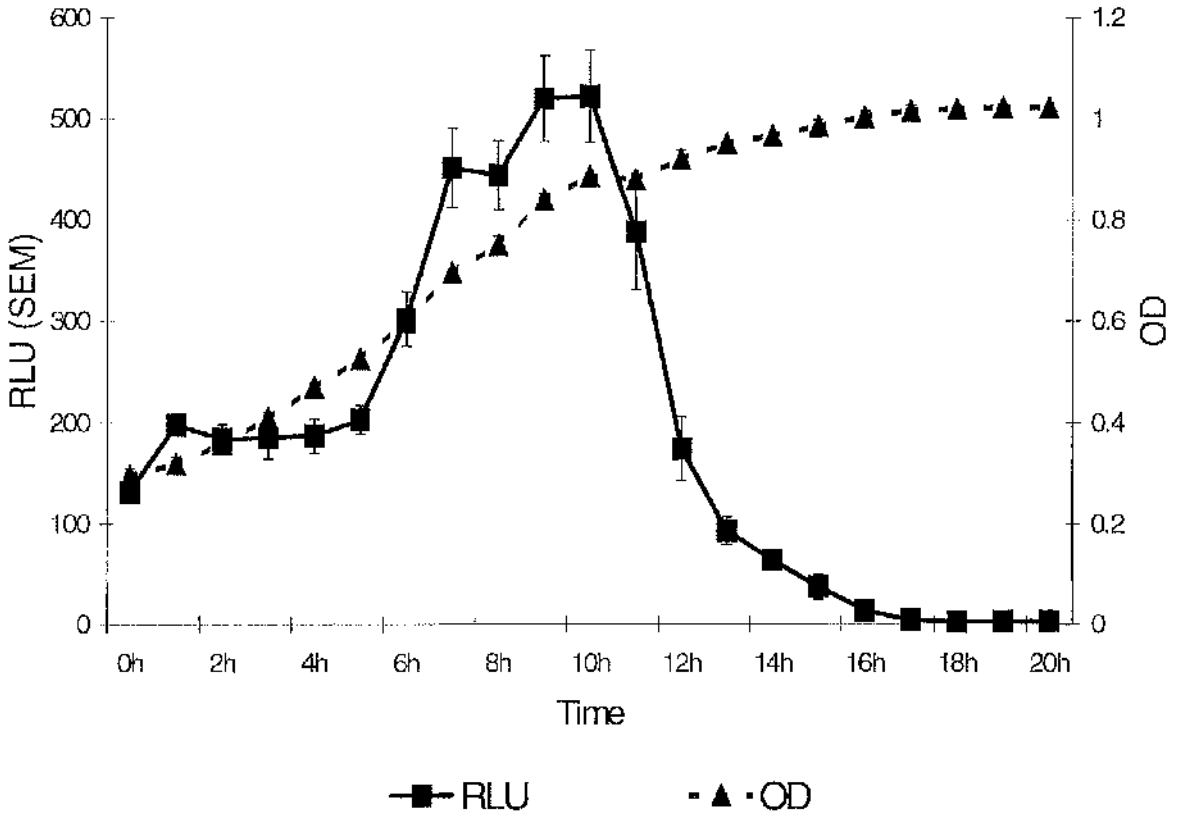

600
500
400
300
200
100
0
RLU (SEM)
1.2
1
0.8
0.6
0.4
0.2
0
OD
0h
2h
4h
6h
8h
10h
12h
14h
16h
18h
20h
Time
RLU
OD

Figure 3.2.6

3.2.3 Detection of intracellular *B. bronchiseptica* (Lux 95) within sheep BMMC and P388D1 murine macrophage cell line

3.2.3.1 Luminometry method

Sheep BMMC (25 day culture) and murine macrophage-like cell line P388D1 were maintained in IMDM medium and adjusted to $1x10^6$ cells/ml by using trypan blue exclusion. Infection of sheep BMMC or P388D1 by *B. bronchiseptica* (Lux 95) were carried out at on infection ratio of 1:500. Then, volumes of 200 µl of cell-bacteria suspension were seeded in triplicate in 96-well micro-plates. To enhance bacterial uptake by sheep BMMC and P388D1 cells, the microtitre plates were centrifuged at low speed of 250 rpm for 10 min and then incubated at 37°C in the luminometer. After incubation for 4 h at 37°C, extracellular bacteria were killed by adding polymyxin B sulfate (Sigma) to a final concentration of 50 µg/ml and incubation was continued. After 1 h, 50 µg/ml digitonin was added to some wells to lyse mammalian cells and to expose any intracellular bacteria to the polymyxin in the suspending medium.

Figure 3.2.7a1 (with P388D1 macrophage cell line) and figure 3.2.7 a2 (with sheep BMMC) show the overall light emissions at the infection ratio of 1:500. Figure 3.2.7b (macrophage cell P388D1) and Fig. 3.2.7c (sheep BMMC) show light output in more detail after adding digitonin, using an expand scale for the Y-axis. In the two figures (3.2.7b and 3.2.7c), line A, represents the effect of polymyxin B on light emission of *B. bronchiseptica* (Lux 95) in IMDM medium only. A reduction in light output was observed and this had already reached baseline level at 390 min. In contrast, the difference in light output represented by line B (*B. bronchiseptica* (Lux 95) in presence of mammalian cells and polymyxin only) and line C (*B. bronchiseptica* (Lux 95) in presence of mammalian cells and both polymyxin B and digitonin) shows the light emission from that is presumably from intracellular *B. bronchiseptica* (Lux 95) within either the sheep BMMC or the murine macrophage cells. With both cell types, in the absence of digitonin, internalized bacteria continued to emit a low level of light throughout the assay, demonstrating that some had retained viability and that polymyxin did not accumulate within these mammalian cells. However, addition of digitonin led to exposure of intracellular bacteria to the polymyxin in the suspending medium, and bacterial killing was shown by a rapid decrease in light output to reach the baseline level.

Figure 3.2.7a1 Interaction of *B. bronchiseptica* (Lux 95) with P388D1 macrophage cells

$1x10^6$ cells/ml of P388D1 macrophage cell line maintained in IMDM medium containing 10% FCS were infected with *B. bronchiseptica* (Lux 95) at an infection ratio of 1:500 and incubated for 4 h. 50 µg/ml of polymyxin B was added to kill extracellular *B. bronchiseptica* (Lux 95) and after 1h, 50 µg/ml digitonin was added to some wells containing polymyxin B. The overall bioluminescence output was monitored (mean of 6 observations ± SD).

Figure 3.2.7a2 Interaction of *B. bronchiseptica* (Lux 95) with sheep BMMC

$1x10^6$ cells/ml of sheep BMMC maintained in IMDM medium containing 10% FCS were infected with *B. bronchiseptica* (Lux 95) at an infection ratio of 1:500 and incubated for 4 h. 50 µg/ml of polymyxin B was added to kill extracellular *B. bronchiseptica* (Lux 95) and after 1h, 50 µg/ml digitonin was added to some wells containing polymyxin B. The overall bioluminescence output was monitored (mean of 6 observations ± SD).

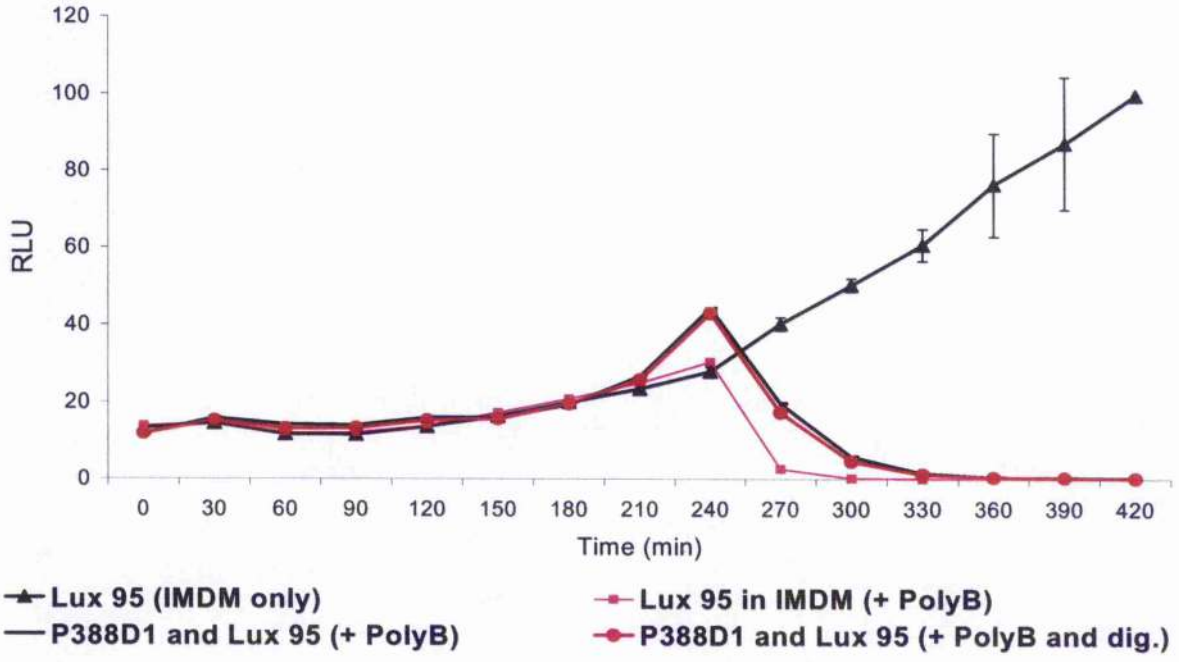


Figure 3.2.7 a1

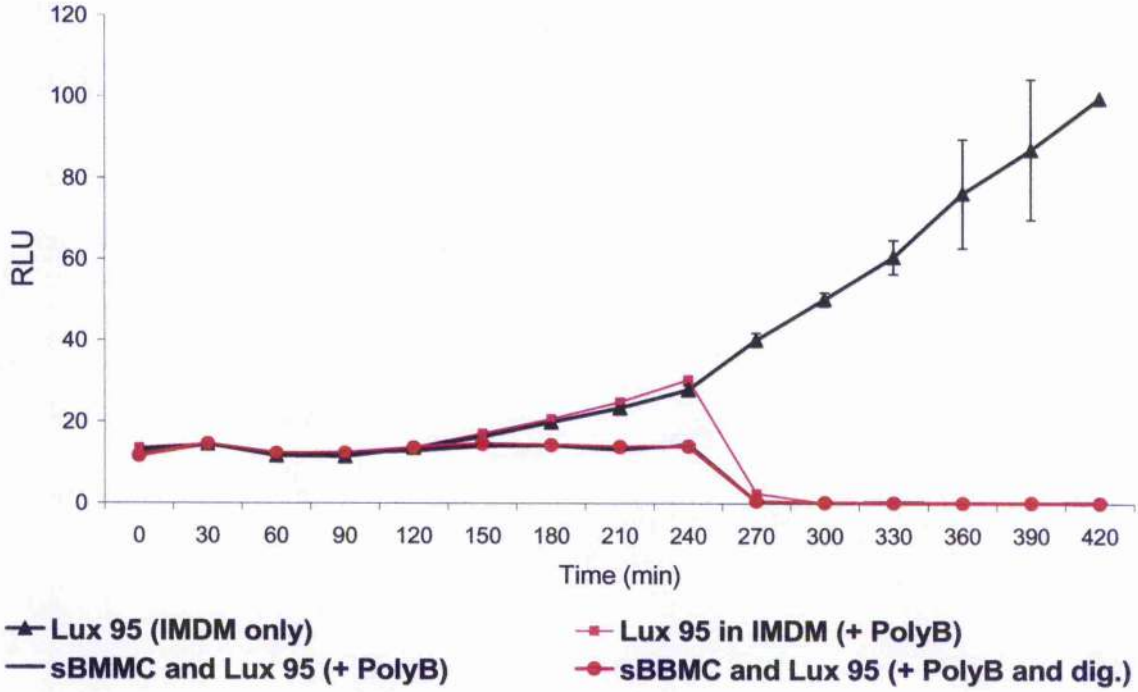


Figure 3.2.7 a2

Figure 3.2.7b Intracellular *B. bronchiseptica* (Lux 95) within macrophage cell P388D1

This graph shows enlargement of the previous graph (2.8a) after addition of digitonin. The light emission from intracellular *B. bronchiseptica* (Lux 95) in macrophage cells treated with of polymyxin B only, can be compared with light output from cells treated with both polymyxin B and digitonin. These data represent the mean of 6 observations. The range of the error bars was between 0.01 and 0.1, therefore the bars were omitted to avoid exceed with other lines.

A) *B. bronchiseptica* (Lux 95) in IMDM medium containing 50 µg/ml of polymyxin B

B) P388D1 cell line and *B. bronchiseptica* (Lux 95) and 50 µg/ml of polymyxin B.

C) P388D1 cell line and *B. bronchiseptica* (Lux 95) and 50 µg/ml of polymyxin B and 50 µg/ml of digitonin.

Figure 3.2.7c Intracellular *B. bronchiseptica* (Lux 95) within sheep BMMC

This graph shows enlargement of the previous graph (2.8a) after addition of digitonin. The light emission from intracellular *B. bronchiseptica* (Lux 95) in macrophage cells treated with of polymyxin B only, can be compared with light output from cells treated with both polymyxin B and digitonin. These data represent the mean of 6 observations. The range of the error bars was between 0.01 and 0.1, therefore the bars were omitted to avoid exceed with other lines.

A) *B. bronchiseptica* (Lux 95) in IMDM medium containing 50 µg/ml of polymyxin B

B) Sheep BMMC and *B. bronchiseptica* (Lux 95) and 50 µg/ml of polymyxin B.

C) Sheep BMMC and *B. bronchiseptica* (Lux 95) and 50 µg/ml of polymyxin B and 50 µg/ml of digitonin.

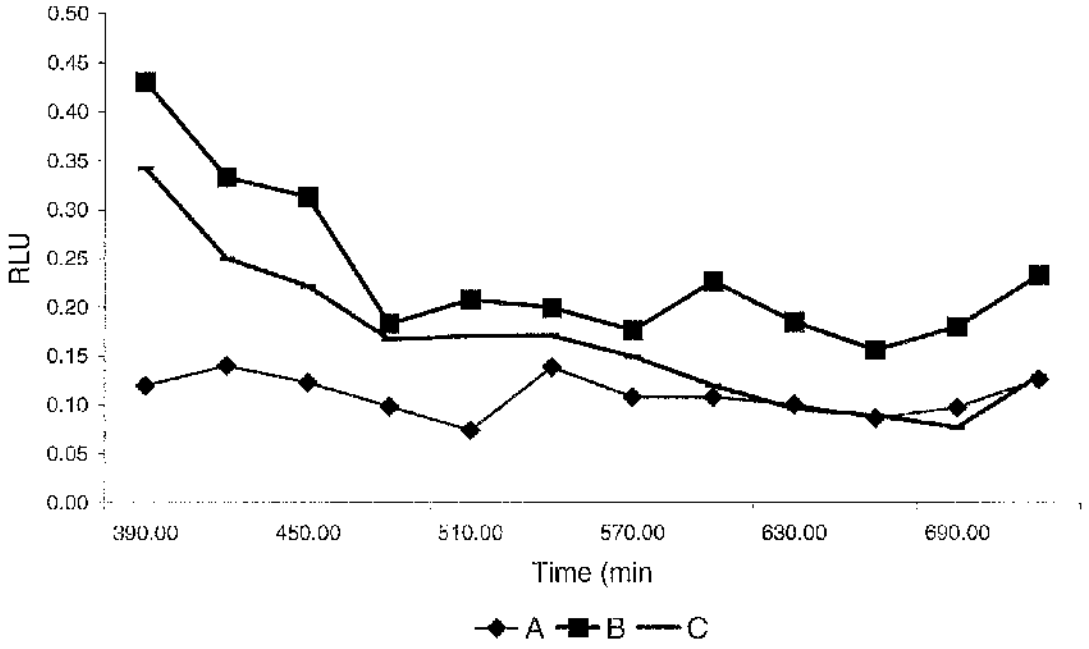


Figure 3.2.7b

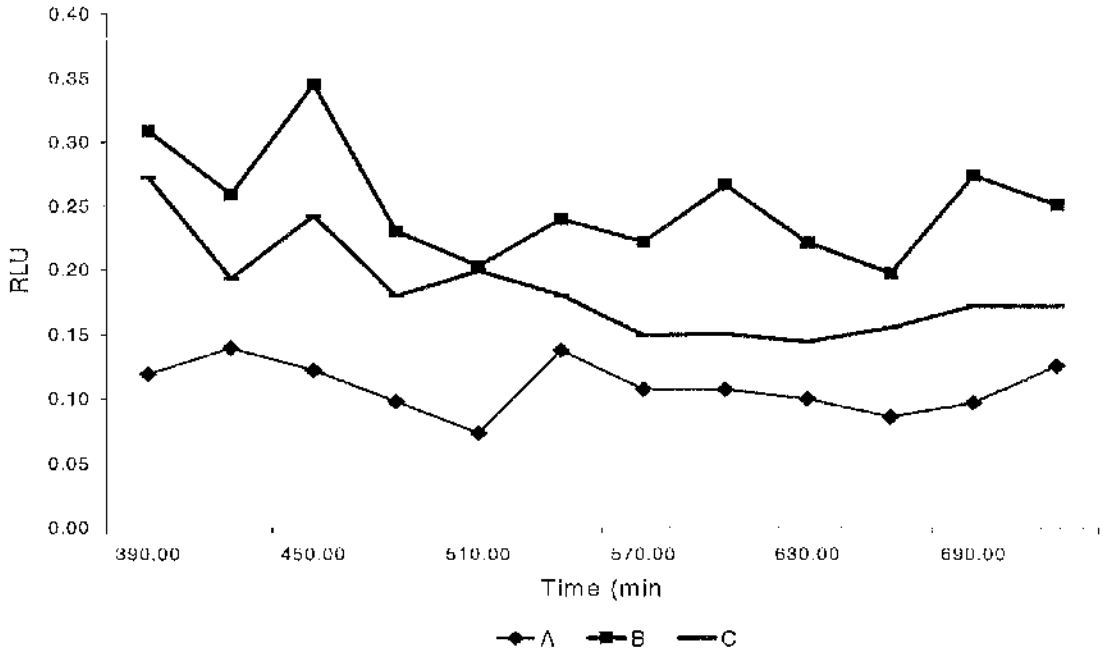


Figure 3.2.7c

3.2.3.2 Microscopic study

The presence of intracellular *B. bronchiseptica* (Lux 95) within sheep BMMC was investigated by using either light microscopy or electron microscopy.

3.2.3.2.1 Light microscopy

At 4 h postinfection of sheep BMMC infected at MOI of 1:500, cytosmear samples of infected culture were stained by Leishman's dye. As can be seen from figure 3.2.8, it was difficult to differentiate between bacterial cells and cytoplasmic granules within sheep BMMC.

3.2.3.2.2 Transmission electron microscopy

Transmission electron microscopy was used to investigate samples of sheep BMMC (Figure 3.2.9a and Figure 3.2.9b) and p388D1 macrophage cell line (Figure 3.2.9c) infected at an MOI 1:500. Within sheep BMMC, bacteria were seen to be localized in the phagosomal vacuoles (Figure 3.2.9a and 3.2.9b) of the infected mast cells examined. The bacteria were present in low numbers in the phagosomes, usually a single bacterium was observed within each vacuole.

In contrast, transmission electron microscopy (TEM) of samples of P388D1 cultured macrophages infected at an MOI of 1:500 at 4h postinfection showed more bacteria localized within vacuoles (Figure 3.2.9c) and in around 90% of the infected cells examined. The bacteria were present in low numbers in the phagosomes, usually singly, although in some cells several bacteria could be observed within the same vacuole as shown in figure 3.2.9c. No bacteria were observed free in the cytoplasm in the macrophage cells.

Figure 3.2.8 Cytosmear of sheep BMMC infected with *B. bronchiseptica* (Lux 95).

At 4 h postinfection of sheep BMMC infected at MOI of 1:500, cytosmear samples of infected culture were stained by Leishman's dye. The sheep BMMC contained highly condensed granules by Leishman's stain (original magnification X600).

Figure 3.2.9a TEM of single sheep bone marrow mast cell infected with *B. bronchiseptica* (Lux 95). Arrows indicate bacteria in phagosomal vacuoles. Scale bar = 10μm .

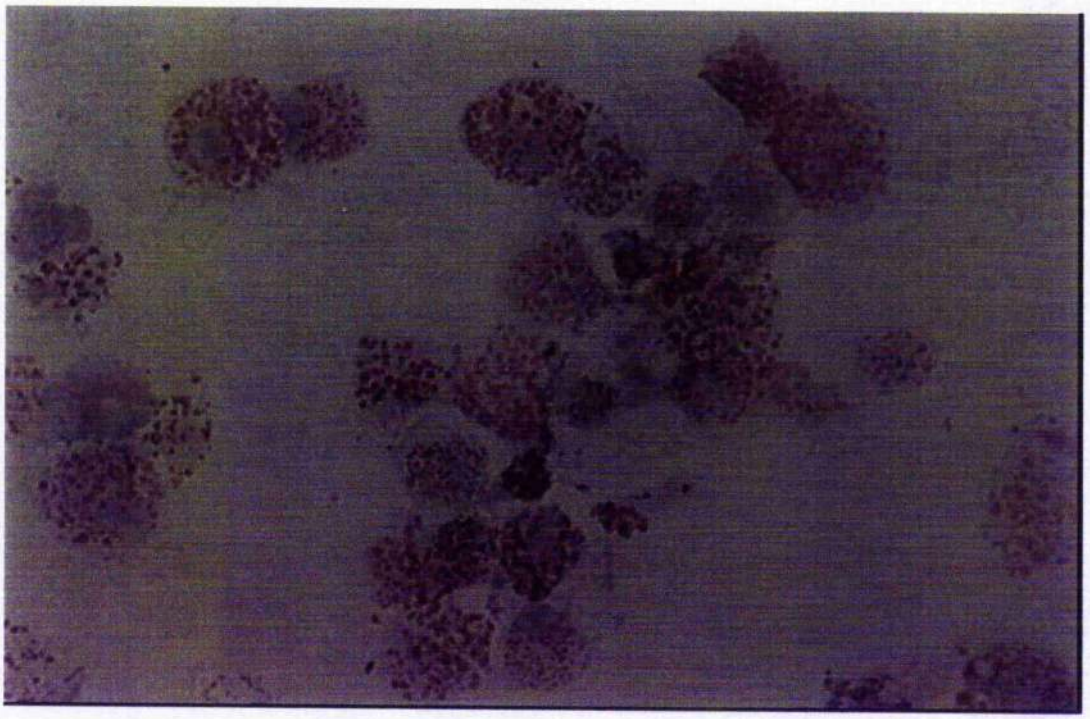


Figure 3.2.8

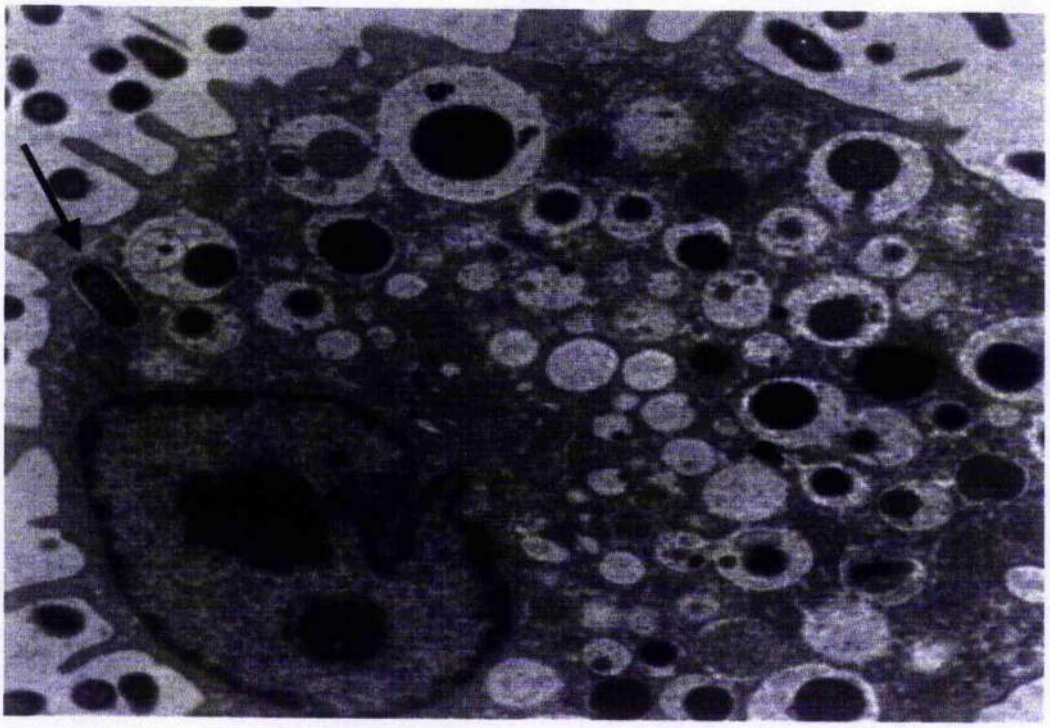


Figure 3.2.9a

Figure 3.2.9b TEM of single sheep bone marrow mast cell infected with *B. bronchiseptica* (Lux 95). Arrows indicate bacteria in phagosomal vacuoles. Scale bar = 10 μm.

Figure 3.2.9c TEM of single P388D1 macrophage cell infected with *B. bronchiseptica* (Lux 95). Arrows indicate bacteria in phagosomal vacuoles. Scale bar = 10 μm.

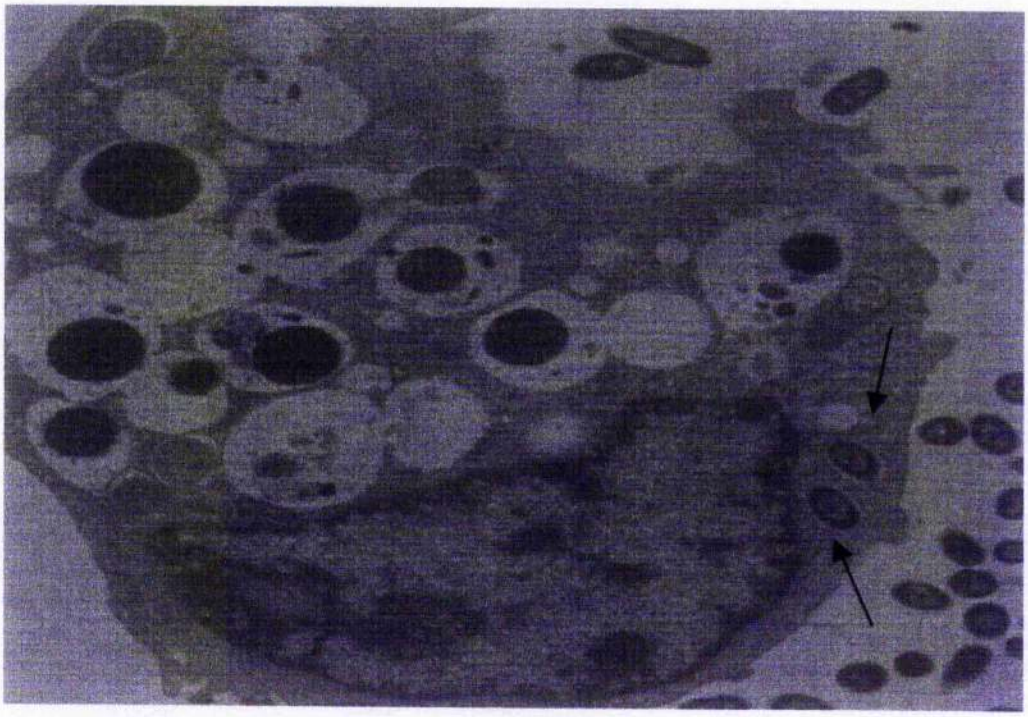


Figure 3.2.9b

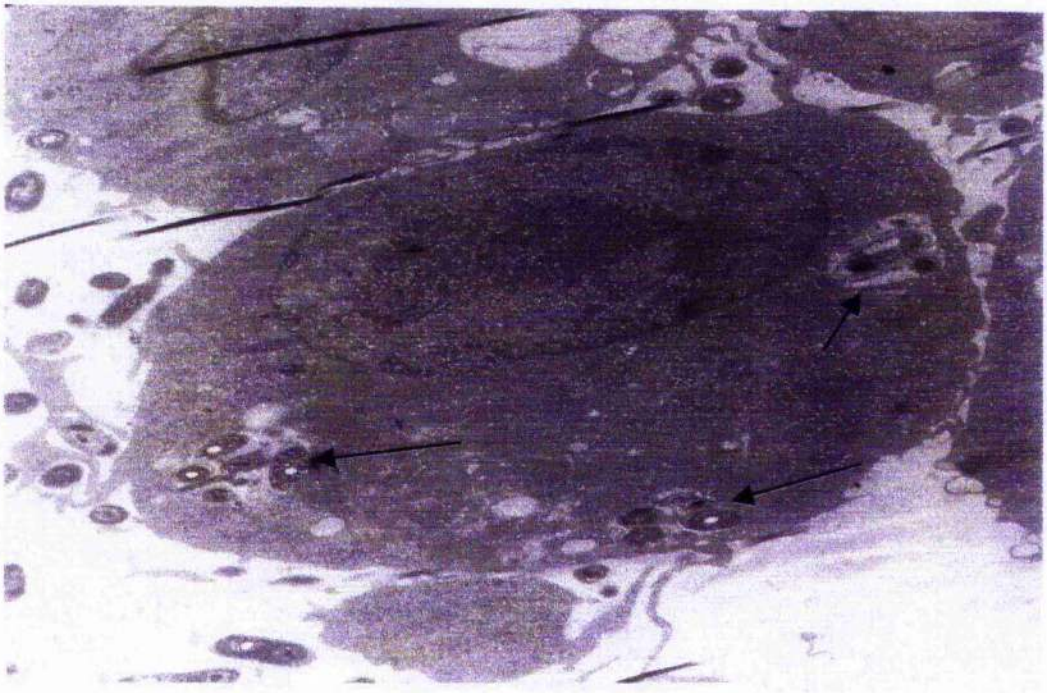


Figure 3.2.9c

3.2.4 Detection of intracellular *B. bronchiseptica* (Lux 95) within sheep BMMC depends on age of culture

It was observed that differentiation of the sheep bone marrow mast cells depends on the age of culture (section 3.1.2). Cells from the earliest age of culture (i.e. within the first two weeks) were devoid of cytoplasmic granules, but the number of dense granules within the cytoplasmic vacuoles subsequently increased with the increasing of age culture. The aim of the following work was to investigate the ability of different ages of sheep BMMC to take-up bacteria.

The presence of intracellular *B. bronchiseptica* (Lux 95) within sheep BMMC was investigated with different ages of cell culture. From cell cultures at day 14 and at day 29 (mast cells more than 90% of total population in both cultures) from the same batch of sheep bone marrow, cell numbers were adjusted to $1x10^6$ cells/ml using trypan blue exclusion. Infection of sheep BMMC with *B. bronchiseptica* (Lux 95) was carried out at MOI 1:500 (one mast cell to 500 bacterial cells) and bioluminescence output was monitored in the luminometer. To confirm intracellular *B. bronchiseptica* (Lux 95) within sheep BMMC, 50 µg/ml polymyxin B was added at 4 h post-infection. A decline in bioluminescence output was observed (data not shown) due to killing of extracellular *B. bronchiseptica* (Lux 95). After a further 1 h, 50 µg/ml digitonin was added to selected wells to expose intracellular *B. bronchiseptica* (Lux 95) to the polymyxin B. Bioluminescence output of *B. bronchiseptica* (Lux 95) with sheep BMMC (14 day culture) (Fig. 3.2.10a) and 29 day culture (Fig. 3.2.10b) was compared. With the 29-day culture, there was a much higher bioluminescence output with polymyxin B alone (assumed to be from intracellular bacteria) than with polymyxin B and digitonin. This difference was not observed with the 14-day culture. Therefore, bioluminescence output from intracellular *B. bronchiseptica* (Lux 95) demonstrated that only the mast cells which, by microscopy, appeared to be mature (at 29 days) seemed to be capable of take-up of the bacteria. An alternative but less likely explanation is that both ages of sheep BMMC were able to take-up the bacteria but that they only survived intracellulary in the mature mast cells.

The above observation was confirmed by a repeat experiment but with a new batch of sheep BMMC.

For statistical analysis of the differences in bioluminescence output from intracellular *B. bonchiseptica* (Lux 95) incubated with sheep BMMC from 14-day and 29-day cultures with polymyxin B alone, the areas under the curves (Fig. 3.2.10a and 3.2.10b) could be determined. Trendlines (Excel 98) may be used to analyze the problems of area under curves, such analysis is also called regression analysis. For this purpose, polynomial equation (calculates the least squares fit through points) may be used and the following equation may be applied:

In the case of the 29-day culture:

$y = 2E\text{-}08x^6 - 2E\text{-}06x^5 + 8E\text{-}05x^4 - 0.0018x^3 + 0.0215x^2 - 0.1285x + 0.4015$

$R^2 = 0.9663$ (constant of polynomial equation)

(6,5...etc, refer to R^2 value)

In the case of the 14-day culture:

$y = 2E\text{-}08x^6 - 3E\text{-}06x^5 + 0.0001x^4 - 0.0024x^3 + 0.0266x^2 - 0.1476x + 0.3196$

$R^2 = 0.9766$ (constant of polynomial equation)

(6,5...etc, refer to R^2 value)

Then, integration of the following equation may be used for calculating the areas under the different curves:

$$\text{Area (under curve)} = \int_{a1}^{a2} y\,dx$$

a 1 = lower time (e.g. at time 200 min)

a 2 = higher time (e.g. at time 900 min)

y = curve equation (polynomial equation)

dx = a number raised to a power (value of R^2).

In order to do this, a number of replicate observations would be required.

Figure 3.2.10a Effect of sheep BMMC (14-day culture) on uptake of *B. bronchiseptica* (Lux 95)

$1x10^6$ cells/ml of sheep BMMC (14-day culture) was infected with *B. bronchiseptica* (Lux 95) at MOI 1:500. After 4h incubation, 50 μg/ml polymyxin B was added to kill extracellular *B. bronchiseptica* (Lux 95) and 1 h later 50 μg/ml digitonin was added to selected wells to lyse sheep BMMC and release intracellular *B. bronchiseptica* (Lux 95). This graph shows light output (RLU) after adding polymyxin B and digitonin. These data represent the mean of 9 observations. The range of the error bars was between 0.01 and 0.1, therefore the bars were omitted to avoid exceed with other lines.

Figure 3.2.10b Effect of sheep BMMC (29-day culture) on uptake of *B. bronchiseptica* (Lux 95)

$1x10^6$ cell/ml of sheep BMMC (29-day culture) was infected with *B. bronchiseptica* (Lux 95) at MOI 1:500. After 4h incubation, 50 μg/ml polymyxin B was added to kill extracellular *B. bronchiseptica* (Lux 95) and 1 h later, 50 μg/ml digitonin was added to selected wells to lyse sheep BMMC and release intracellular *B. bronchiseptica* (Lux 95). This graph shows light output (RLU) after adding polymyxin B and digitonin. These data represent the mean of 6 observations. The range of the error bars was between 0.01 and 0.1, therefore the bars were omitted to avoid exceed with other lines.

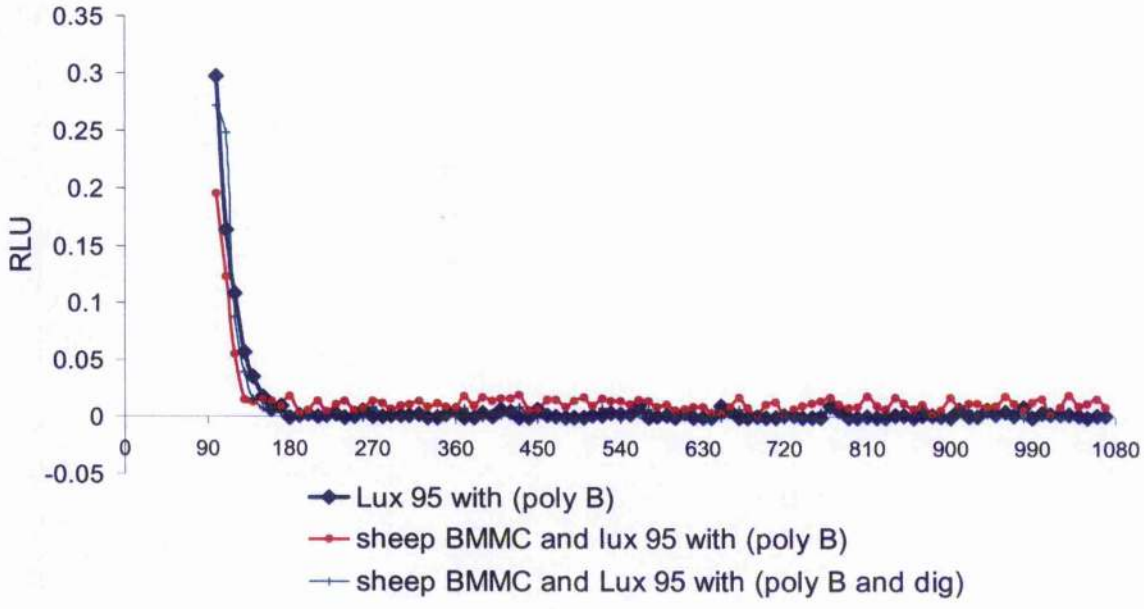


Figure 3.2.10a

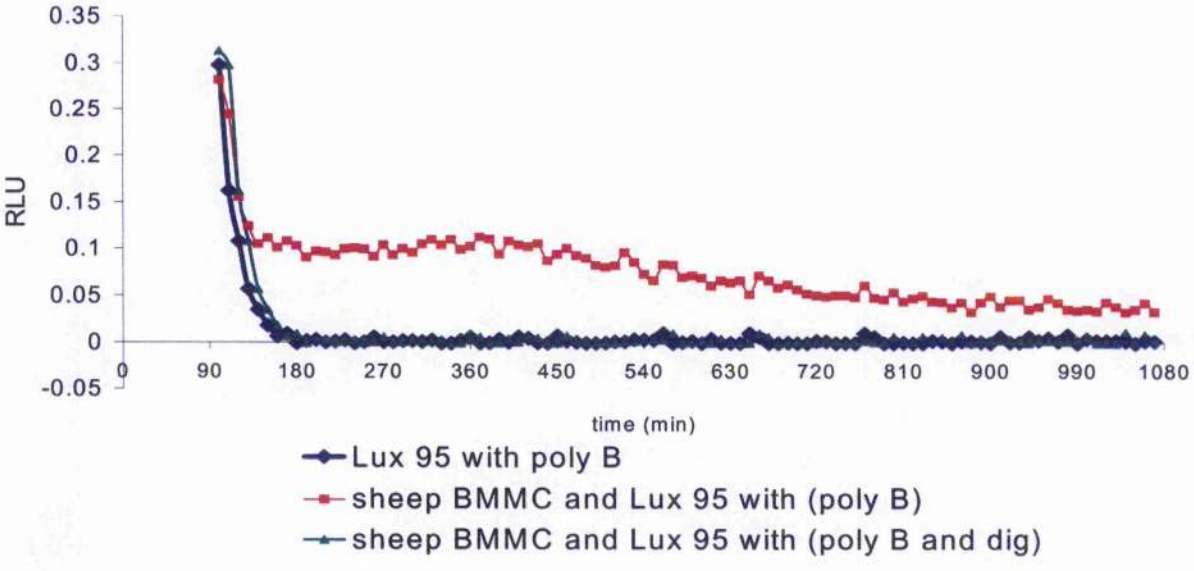


Figure 3.2.10b

3.2.5 Effect of sheep BMMC culture fluid on *B. bronchiseptica* (Lux 95)

The following experiments were designed to investigate that the possibility of sheep BMMC releases any substances during normal growth into the surrounding medium which could be antimicrobial. Sheep BMMC (30-day culture) were maintained routinely in IMDM medium containing 10% FCS, 50 µl of rOvSCF and 100 µl of rOv IL-3. 1% of P/S suspension was usually added into IMDM medium to avoid any contamination. For these experiments, sheep BMMC was grown in antibiotic-free medium to avoid any possibility that antibiotics had an effect on viability of *B. bronchiseptica* (Lux 95). Three days before the experiment, fresh antibiotic-free IMDM medium containing 10% FCS and growth factors was used to replace the old IMDM culture medium and cells were incubated at 37°C under 5% CO_2. Then, after 3 days, sheep BMMCs were collected in fresh IMDM medium containing 10% FCS and cell numbers were adjusted to $1x10^6$ cells/ml. Sheep BMMC culture fluid (3 days old) was collected in sterilised 30 ml universal tubes and kept in aseptic conditions at 37°C. *B. bronchiseptica* (Lux 95) grown for 24 h on BG agar were collected and cell numbers were adjusted to $1x10^8$ cfu/ml or $5x10^8$ cfu/ml in two different media: IMDM antibiotic-free medium containing 10% FCS only and three day old sheep BMMC culture fluid. Figure 3.2.11 shows the bioluminescence output from either 10^8cfu/ml or $5x10^8$ cfu/ml under three different conditions. First, in IMDM antibiotic-free medium containing 10% FCS only. Second, in three day old sheep BMMC culture fluid. Finally, mixed with $1x10^6$ cells/ml sheep BMMC (in fresh IMDM medium). Each mixture was done in triplicate in 96-well plates and incubated at 37 °C in the luminometer.

There was a gradual increase in bioluminescence output of *B. bronchiseptica* (Lux 95) (10^8 cfu/ml and $5x10^8$ cfu/ml) in IMDM medium containing 10% FCS only and from *B. bronchiseptica* (Lux 95) maintained in sheep BMMC culture fluid. However, there was a decline in bioluminescence output from both concentrations of *B. bronchiseptica* (Lux 95) due to incubation with $1x10^6$ cells/ml of sheep BMMC. These observations suggest that mast cells had not released any substances during normal growth which were antimicrobial. It also suggests that the mast cells themselves were causing the decline in the bioluminescence output from *B. bronchiseptica* (Lux 95). This possible mechanism was further investigated in the next experiment.

Figure 3.2.11 Effect of sheep BMMC and growth culture fluid on bioluminescence of *B. bronchiseptica* (Lux 95)

Bioluminescence output from 10^8 cfu/ml or $5x10^8$ cfu/ml of *B. bronchiseptica* (Lux 95) was monitored in three different conditions: IMDM medium containing 10% FCS, sheep BMMC culture fluid and $1x10^6$ cells/ml of sheep BMMC). The data shown are the means of 3 observations ± SD.

a) 10^8cfu/ml of *B. bronchiseptica* (Lux 95) in IMDM medium containing 10% FCS only
b) 10^8cfu/ml of *B. bronchiseptica* (Lux 95) in sheep BMMC culture fluid (three days old).
c) 10^8cfu/ml of *B. bronchiseptica* (Lux 95) co-cultured with $1x10^6$ cell/ml of sheep BMMC.
d) $5x10^8$cfu/ml of *B. bronchiseptica* (Lux 95) in IMDM medium containing 10% FCS only
e) $5x10^8$cfu/ml of *B. bronchiseptica* (Lux 95) in sheep BMMC culture fluid (three days old).
f) $5x10^8$cfu/ml of *B. bronchiseptica* (Lux 95) co-cultured with $1x10^6$ cells/ml of sheep BMMC.

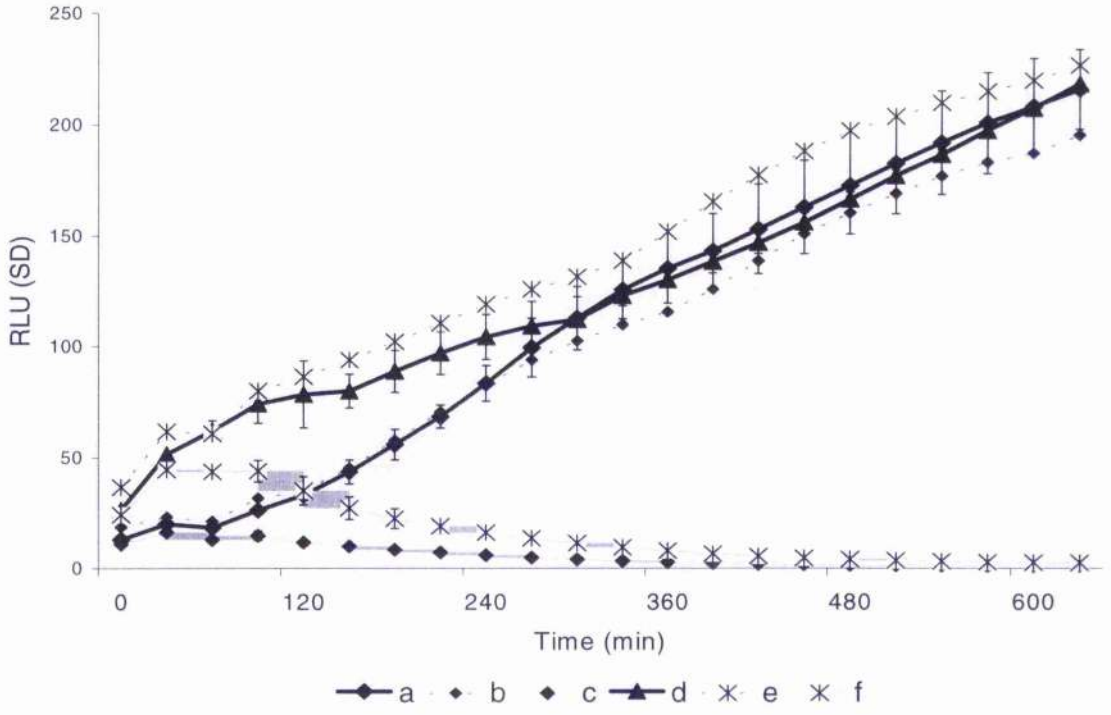


Figure 3.2.11

3.2.6 Effect of sheep BMMC contents on *B. bronchiseptica* (Lux 95)

Sheep BMMC (32 day culture) was adjusted to $1x10^6$ cells/ml in fresh IMDM medium containing 10% FCS and lysed by the freeze/thaw method at –70°C. The cell contents of the lysed BMMC suspension was used to study the effect on bioluminescence output from *B. bronchiseptica* (Lux 95) and compared with the effect of viable sheep BMMC. Co-culture of *B. bronchiseptica* (Lux 95) with sheep BMMC was carried at MOI of 1:100 (one sheep BMMC to 100 *B. bronchiseptica*) or with the contents of an equivalent number of lysed cells. 200µl of each mixture was added in triplicate in 96-well plates and incubated at 37°C in the luminometer.

The bioluminescence output of *B. bronchiseptica* (Lux 95) in IMDM medium only was compared with the effect of either the contents of lysed sheep BMMC or viable sheep BMMC. All showed a gradual increase in bioluminescence, but the increases due to the effect of either lysed or viable sheep BMMC were lower than the increase in bioluminescence output from *B. bronchiseptica* (Lux 95) in IMDM medium only (Fig. 3.2.12a). These effects on bioluminescence output were compared with the sharp decline in bioluminescence output from *B. bronchiseptica* (Lux 95) killed due to addition of 50 µg/ml polymyxin B. At the same time, optical density (OD) of suspension of each mixture was measured. There was a gradual increase in OD of all cell suspensions, but a slight decline in OD of the *B. bronchiseptica* (Lux 95) when polymyxin was added (Fig. 3.2.12b).

The above observations suggest that there are very similar effects of both viable sheep BMMC and the contents of lysed sheep BMMC on bioluminescence emission (RLU) from *B. bronchiseptica* (Lux 95). However, such effects were not observed on the optical density of the suspension containing both *B. bronchiseptica* (Lux 95) and sheep BMMC in IMDM medium. Thus, the bioluminescence method is more sensitive than the OD method for measuring changes in the *B. bronchiseptica* culture. Also, these observations suggest that further investigation should be done by estimating viable bacteria when mixed with viable sheep BMMC or contents of lysed cells at different times of incubation.

Figure 3.2.12a Effect of sheep BMMC contents on bioluminescence output from *B. bronchiseptica* (Lux 95)

The effect of cell contents of $1x10^6$ cells/ml of lysed sheep BMMC on *B. bronchiseptica* (Lux 95) was compared with the effect of either viable sheep BMMC or IMDM medium containing 10% FCS only. These effects were compared with the effect of 50 µg/ml polymyxin B. The data are means of three observations ± SD.

a) 10^8 cfu/ml of *B. bronchiseptica* (Lux 95) in IMDM medium containing 10% FCS.

b) Effect of 50 µg/ml polymyxin B on 10^8 cfu/ml of *B. bronchiseptica* (Lux 95).

c) Effect of $1x10^6$ cells/ml of viable sheep BMMC on *B. bronchiseptica* (Lux 95).

d) Effect of lysed sheep BMMC contents on *B. bronchiseptica* (Lux 95)

Figure 3.2.12b Effect of sheep BMMC contents on optical density of *B. bronchiseptica* (Lux 95)

The effect of cell contents of $1x10^6$ cells/ml of lysed sheep BMMC on *B. bronchiseptica* (Lux 95) was compared with the effect of either viable sheep BMMC or IMDM medium containing 10% FCS only. These effects were compared with the effect of 50µg/ml polymyxin B on *B. bronchiseptica* (Lux 95). The data are means of three observations.

a) 10^8 cfu/ml of *B. bronchiseptica* (Lux 95) in IMDM medium containing 10% FCS.

b) Effect of 50 µg/ml polymyxin B on 10^8 cfu/ml of *B. bronchiseptica* (Lux 95).

c) Effect of $1x10^6$ cells/ml of viable sheep BMMC on *B. bronchiseptica* (Lux 95).

d) Effect of lysed sheep BMMC contents on *B. bronchiseptica* (Lux 95)

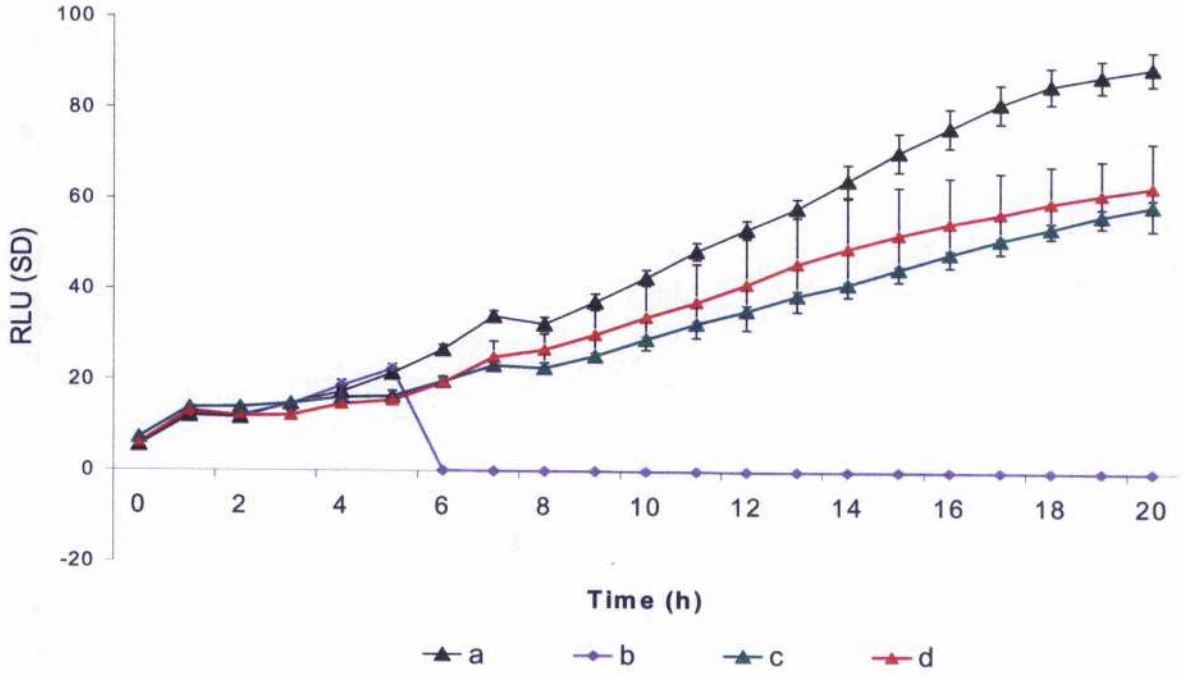


Figure 3.2.12a

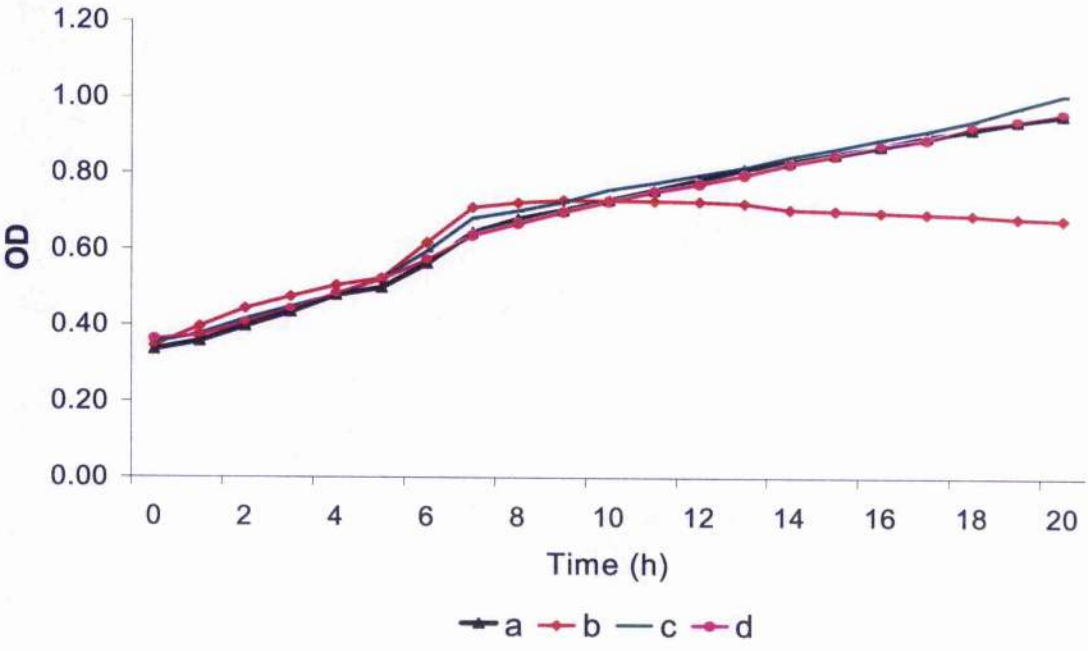


Figure 3.2.12b

3.2.7 Comparing the effect of sheep BMMC on *B. bronchiseptica* (Lux 95) with the effect of RBL-2H3 cells and J774A.1 macrophage cells

The following work aimed to investigate the effect of different mammalian cells on bioluminescence output from *B. bronchiseptica* (Lux 95) and compare it with bioluminescence output of *B. bronchiseptica* in IMDM medium only.

3.2.7.1 Overall effect of different mammalian cells on bioluminescence output of *B. bronchiseptica* (Lux 95)

All three cell types: sheep BMMC (from 25 day culture), RBL-2H3 cells and J774A.1 macrophage cells were adjusted to $1x10^6$ cells/ml in IMDM medium containing 10% FCS only and treated as before to remove antibiotics. All three cell types were infected with *B. bronchiseptica* (Lux 95) at MOI 1:100 (one mammalian cells to 100 bacterial cells). A total of 200 µl of mixture was added in triplicate in 96-well micro-plates and incubated at 37°C in the luminometer. Bioluminescence output from *B. bronchiseptica* (Lux 95) in IMDM medium containing 10% FCS only was compared with bioluminescence output from *B. bronchiseptica* (Lux 95) co-cultured with the different cell types (Figure 3.2.13).

During the first 24 h (1440 min), there was a gradual increase in bioluminescence output (RLU) of *B. bronchiseptica* (Lux 95) mixed with different mammalian cell types and of *B. bronchiseptica* (Lux 95) in control wells, but at different rates. With sheep BMMC, bioluminescence output (RLU) of *B. bronchiseptica* (Lux 95) was slower than bioluminescence output from the control culture, as in previous observations (section 2.3 and section 2.7). On the other hand, bioluminescence output from *B. bronchiseptica* (Lux 95) co-cultured with J774A.1 cells was similar to bioluminescence output from the control. Bioluminescence output with the RBL-2H3 cell line was higher than the control, which could mean that RBL-2H3 cells could stimulate growth or metabolic activity of *B. bronchiseptica* (Lux 95) in some way. From 24 h - 48 h (2880 min) there was a gradual decline in bioluminescence output from all cell suspensions even from the control cultures of *B. bronchiseptica* (Lux 95) in IMDM medium alone. In addition, with RBL-2H3 cells, the decline in light output was slower than with the other cell types or the control culture. These observations suggested that sheep BMMC have greater antibacterial activity than the two cell lines.

Figure 3.2.13 Effect of different mammalian cells on bioluminescence output of *B. bronchiseptica* (Lux 95)

$1x10^6$ cells/ml of different cell types were infected with *B. bronchiseptica* (Lux 95) at MOI 1:100. Bioluminescence output was monitored at 37°C in the luminometer. These data are the means of 3 observations ± SD. Error bars are omitted when standard errors of the mean do not exceed the dimension of the symbols.

a) 10^8 cfu/ml of *B. bronchiseptica* (Lux 95) in IMDM medium containing 10% FCS only.

b) Effect of sheep BMMC (25-day culture) on bioluminescence output of *B. bronchiseptica* (Lux 95).

c) Effect of sheep J774A.1 macrophage cell line on bioluminescence output of *B. bronchiseptica* (Lux 95).

d) Effect of RBL-2H3 cell line on bioluminescence output of *B. bronchiseptica* (Lux 95).

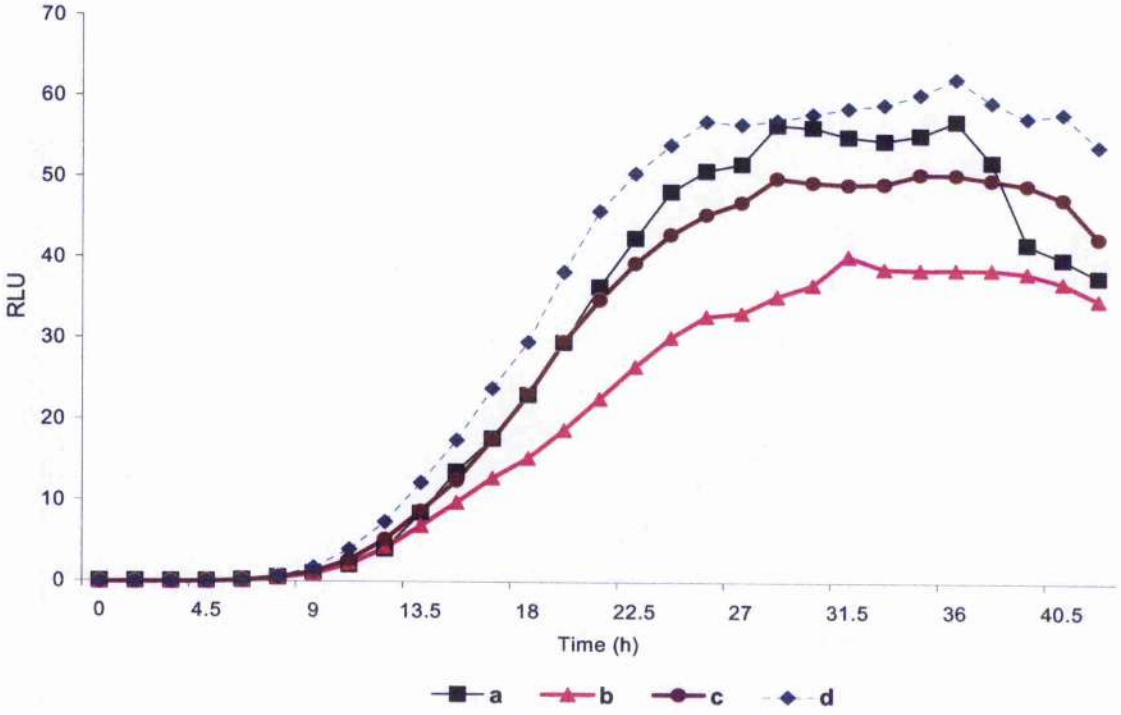


Figure 3.2.13

3.2.7.2 Comparing the internalization of *B. bronchiseptica* (Lux 95) by RBL-2H3 cells and sheep BMMC

3.2.7.2.1 Luminometery assay

The ability of the RBL-2H3 cells was compared with the ability of sheep BMMC (from 29-day culture) for uptake of *B. bronchiseptica* (Lux 95). Both cell types were adjusted to $1x10^6$ cells/ml in IMDM medium containing 10% FCS and infected with *B. bronchiseptica* (Lux 95) at MOI 1:500. (one mammalian cell to 500 *B. bronchiseptica*). 200 µl volumes of the mixture were added in triplicate in 96-well plates. Contact between mammalian cells and *B. bronchiseptica* (Lux 96) was accelerated by centrifuging the micro-well plate at 250 rpm for 10 min. Then, the plate was incubated at 37°C and in 5% CO_2 for 4 h. Polymyxin B 50 µg/ml was then added to kill extracellular *B. bronchiseptica* (Lux 95) and the micro-well plate was incubated at 37°C in 5% CO_2 for a further 1 h. 50 µg/ml digitonin was then added to some wells. Then, plates were incubated in 37°C in the luminometer and bioluminescence output was monitored (Figure 3.2.14a).

The effect of polymyxin B on *B. bronchiseptica* (Lux 95) in the absence of mammalian cells was used as a control for all other conditions. In the presence of mammalian cells (i.e. RBL-2H3 cell line or sheep BMMC) and 50 µg/ml polymyxin B, light output in the presence of sheep BMMC was higher than light output in the presence of RBL-2H3 cells which was similar to the control measurement. However, in the presence of 50 µg/ml digitonin, all RLU measurements were similar for light output of the control. From the above observations, it appeared either there was no evidence of internalization of *B. bronchiseptica* (Lux 95) by the RBL-2H3 cells, or that any internalized bacteria had been killed. Further investigation was done by TEM and viable counting.

3.2.7.2.2 TEM

Transmission electron microscopy (TEM) of sections of RBL-2H3 cells that had been mixed with *B. bronchiseptica* (Lux 95) at an MOI of 1:500 at 4 h postinfection did not show any bacteria localized within vacuoles of RBL-2H3 cells. Representative cells are shown in figure 3.2.14b. Extracellular bacteria were clearly

visible (arrows). With sheep BMMC, internalized *B. bronchiseptica* (Lux 95) were clearly visible, as described in section (3.2.4.2.2).

3.2.7.2.3 Viable count

RBL-2H3 cells were infected with *B. bronchiseptica* (Lux 95) at MOI 1:500 and incubated for 4 h at 37°C in 5% CO_2. Then, extracellular *B. bronchiseptica* (Lux 95) were removed by washing three times. Each time, the cell suspension was centrifuged at 2000 rpm for 5 min and fresh IMDM medium was added. At the third wash, the RBL-2H3 cell pellet was resuspended in a fresh IMDM medium containing 50 µg/ml polymyxin B as a final concentration and incubated for 1 h under the same conditions to kill any remaining extracellular *B. bronchiseptica* (Lux 95). To remove polymyxin B from the medium, the cell suspension was centrifuged twice at a speed of 2000 rpm for 5 min and fresh IMDM medium was added. 50 µg/ml digitonin was then added into the cell suspensions and incubated for 1h under the previous conditions. Then, 100 µl of cell suspension from each sample were diluted 1:10 in PBS and 100 µl of dilution were placed on BG agar. After incubation for 24 h at 37°C in humid conditions, no colonies were observed. These observations supported the previous results in that there was no evidence of uptake of *B. bronchiseptica* by the RBL-2H3 cells.

Figure 3.2.14a Comparison of internalization of *B. bronchiseptica* (Lux 95) by RBL-2H3 cells and sheep BMMC

This graph shows enlargement scale after addition of polymyxin B and digitonin. The light emission from intracellular *B. bronchiseptica* (Lux 95) in sheep BMMC and RBL-2H3 cells treated with of polymyxin B only, can be compared with light output from cells treated with both polymyxin B and digitonin. These data represent the mean of 6 observations. The range of the error bars was between 0.01 and 0.1, therefore the bars were omitted to avoid exceed with other lines.

A) *B. bronchiseptica* (Lux 95) in IMDM medium containing 50 µg/ml of polymyxin B

B) Sheep BMMC and *B. bronchiseptica* (Lux 95) and 50 µg/ml of polymyxin B.

C) Sheep BMMC and *B. bronchiseptica* (Lux 95) and 50 µg/ml of polymyxin B and 50 µg/ml of digitonin

D) RBL-2H3 cells and *B. bronchiseptica* (Lux 95) and 50 µg/ml of polymyxin B.

E) RBL-2H3 cells and *B. bronchiseptica* (Lux 95) and 50 µg/ml of polymyxin B and 50 µg/ml of digitonin.

Figure 3.2.14b TEM of RBL-2H3 cells infected with *B. bronchiseptica* (Lux 95)

TEM did not show any intracellular *B. bronchiseptica* (Lux 95) within RBL-2H3 cells, only extracellular bacteria (arrows) are seen, scale bar =10µm.

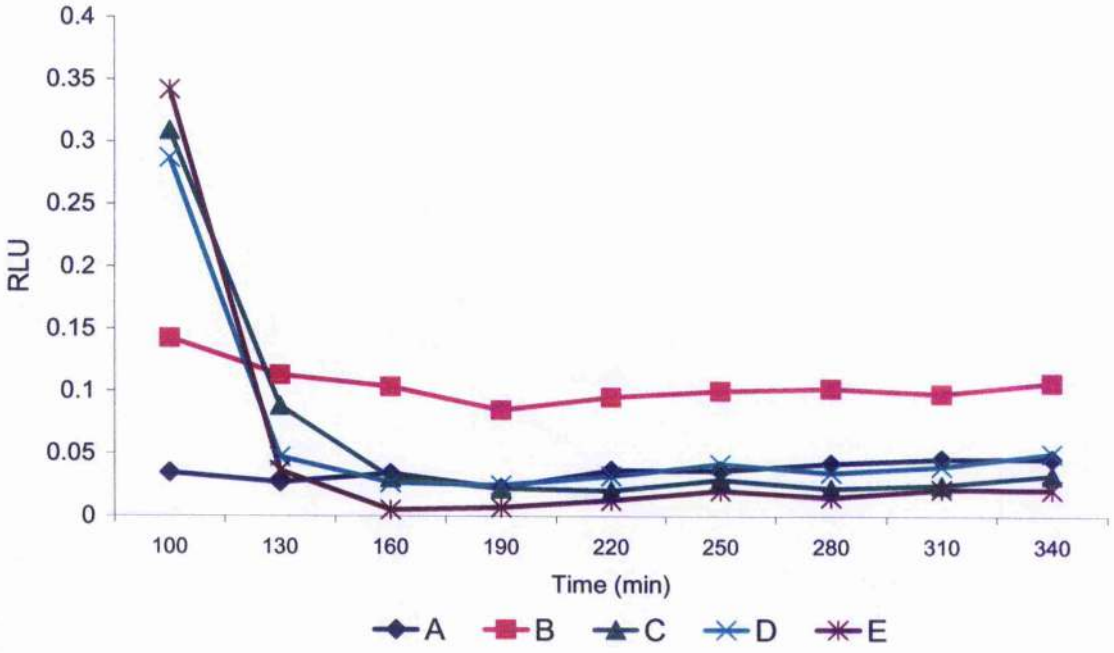


Figure 3.2.14a

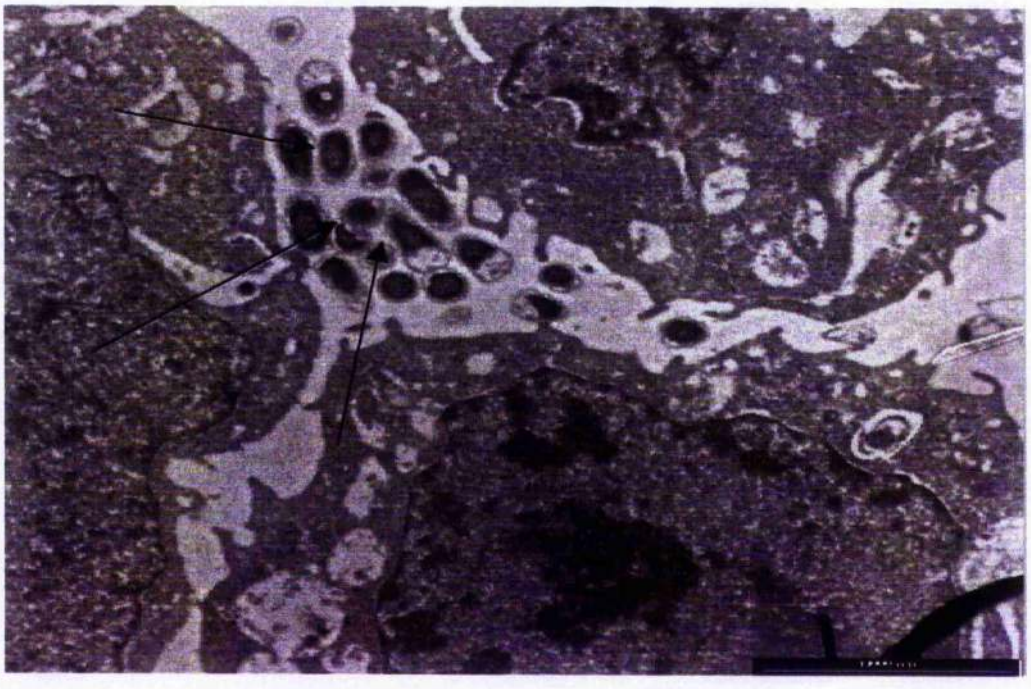


Figure 3.2.14b

3.2.8 Internalization of bioluminescent *E. coli* by sheep BMMC

$1x10^6$ cells/ml of sheep BMMC (28-day culture) were maintained in IMDM medium containing 10% FCS. Cell numbers and viability were confirmed by trypan blue exclusion counts. Infection of sheep BMMC with the *E. coli* strain (Hb101pT7-3) was carried out at MOI 1:500 (one sheep BMMC to 500 bacterial cells). Volume of 200 µl of cell-bacteria suspension were added in triplicate in 96-well plates then incubated at 37°C in the luminometer and bioluminescence output was monitored. At 4 h postinfection, 50µg/ml polymyxin B sulphate (Sigma) was added to some wells to kill extracellular bacteria. After 1 h, 50 µg/ml of digitonin was added to some wells to lyse sheep BMMC.

Figure 3.2.15a shows the overall light emission for the experiment. There was a gradual increase in bioluminescence output from *E. coli* maintained in IMDM medium only (over the first 10h of incubation). Then, there was a gradual decline in bioluminescence output. With sheep BMMC, there was an increase and decline in bioluminescence output similar to those in the control containing bacteria in IMDM medium only. But, the light outputof *E. coli* mixed with sheep BMMC was less than the light output of *E. coli* mixed with IMDM medium only. This obersvtion was similar to the light output of *B. bronchiseptica* (lux 95) mixed with sheep BMMC. A sharp decline in bioluminescence output from *E. coli* was observed due to addition of 50µg/ml polymyxin.

As can be seen from Fig 3.2.15b, the effect of polymyxin B on the light output from *E. coli* in absence of sheep BMMC was used as a control for light output measurement. In the presence of sheep BMMC and polymyxin B, light output measurement was higher than light output measurement in presence of sheep BMMC and digitonin. These differences could suggest that the light output in absence of digitonin could be due to intracellular viable bioluminescent *E. coli* (Fig. 3.2.15b). Unlike *B. bronchiseptica* (Lux 95) (Fig. 3.2.7c) there is a gradual decline in light output from assumed intracellular *E. coli*. This observations suggested that decline in light output of intracellular *E. coli* was due to either killing of intracellular *E. coli* by sheep BMMC or due to their lowering of light output as the culture age (see Fig. 3.2.6).

When digitonin was added, the intracellular *E. coli* were exposed to extracellular polymyxin B and bioluminescence output rapidaly declined to base level, even though there is not a good correlation between light output from bioluminescent *E. coli* and viable cell numbers in older cultures (section 3.2.2.4). The bioluminescence assay still provided evidence for uptake and survival for some time of *E. coli* in sheep BMMC.

Figure 3.2.15a Overall effect of sheep BMMC on light output from bioluminescent *E. coli*

$1x10^6$ cells/ml of sheep BMMC maintained in IMDM medium containing 10% FCS were infected with *E. coli* (Hb101pT7-3) at an infection ratio of 1:500 and incubated for 4 h. Polymyxin B was added to kill extracellular *E. coli* (Hb101pT7-3) and after a further 1 h, digitonin was added to selected wells containing polymyxin B. The overall bioluminescence output (RLU) was monitored (mean of 6 observations ± SD).

A) *E. coli* maintained in IMDM only.

B) *E. coli* maintained in IMDM medium containing sheep BMMC.

C) The effect of polymyxin B on light output from *E. coli*.

D) The effect of polymyxin on light output from *E. coli* maintained in IMDM medium with sheep BMMC

E) The effect of digitonin and polymyxin on light output from *E. coli* maintained in IMDM medium with sheep BMMC.

Figure 3.2.15b Evidence for intracellular *E. coli* (DHα5pT7-3) within sheep BMMC

This graph shows an enlargement of the selected samples (Y-axis) for the previous graph (2.16a). The light emission in samples which containing polymyxin B only were compared with light emission in samples containing polymyxin B and digitonin. These differences could suggest that the light output in absence of digitonin could be due to intracellular viable bioluminescent *E. coli* (mean of 6 observations, the range of error bars was between 0.01 and 0.1, therefore error bars were omitted to avoid overlap with other lines).

A) *E. coli* in IMDM medium containing 50 μg/ml of polymyxin B.

B) Sheep BMMC and *E. coli* and 50 μg/ml of polymyxin B.

C) Sheep BMMC and *E. coli* and 50 μg/ml of polymyxin B and 50 μg/ml of digitonin.

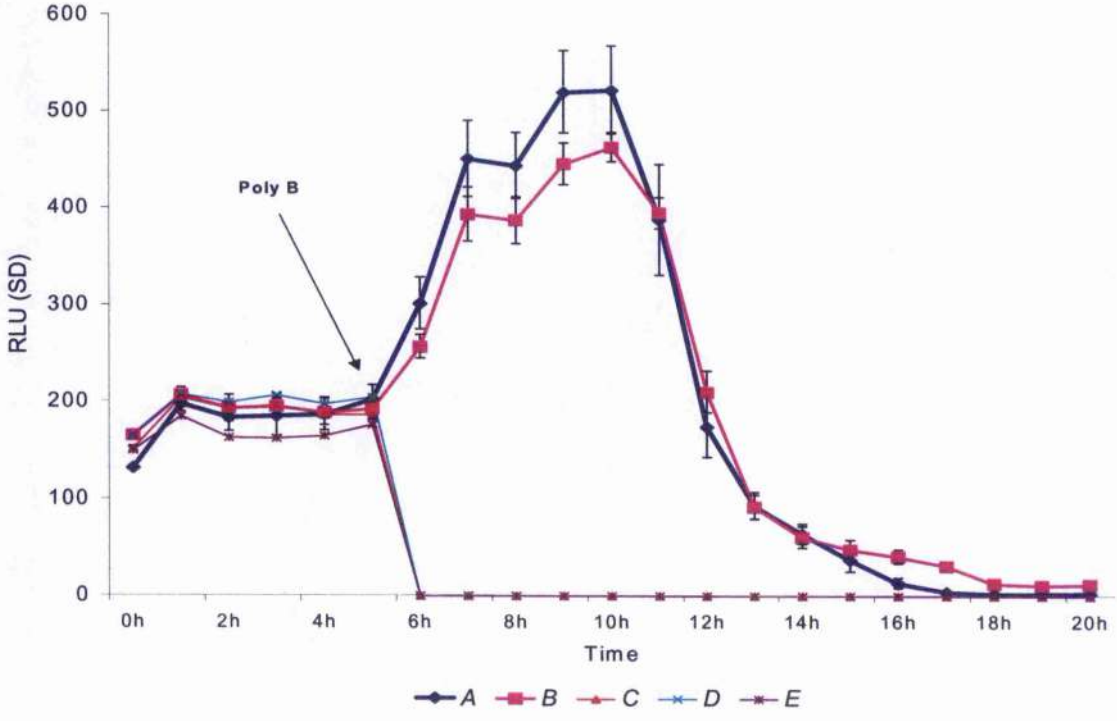


Figure 3.2.15a

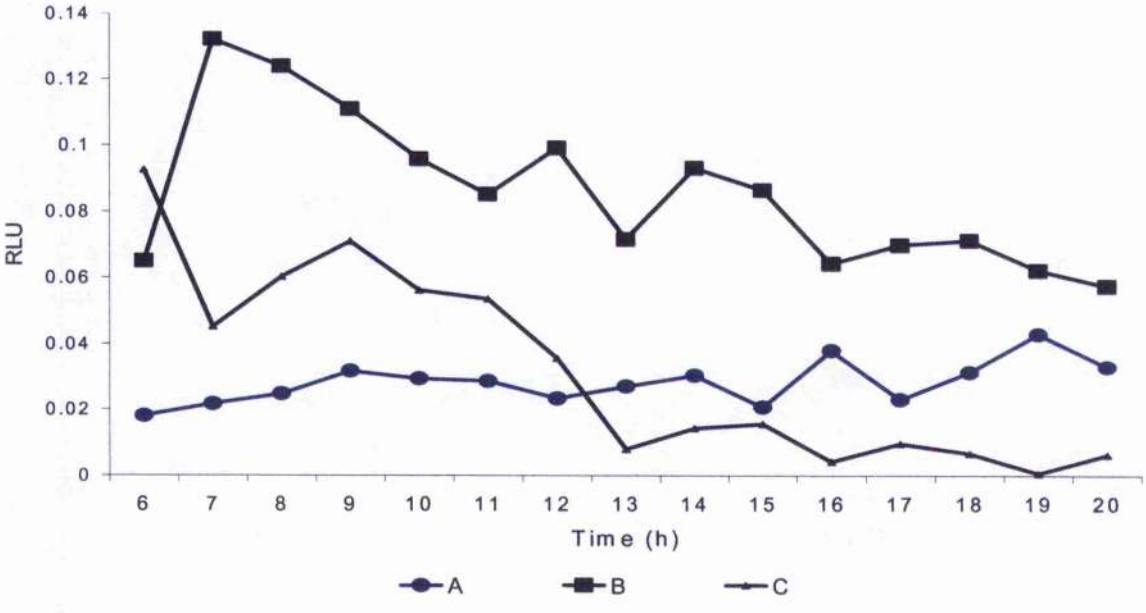


Figure 3.2.15b

Section Three: Interaction of *B. bronchiseptica* and other bacteria with sheep BMMC and other cell types

3.3.1 Effect of sheep BMMC and murine macrophage cell line (P388D1) on biouminescence output from *B. bronchiseptica* (Lux 95)

Bioluminescence output from *B. bronchiseptica* (Lux 95) was used as a reporter for the effect of either sheep BMMC or murine macrophage cell line (P388D1) on the bacteria. Two different infection ratios were used: 1:500 (one mammalian cell to 500 bacterial cells) or 1:2500 (one mammalian cell to 2500 bacterial cells).

Viability of sheep BMMCs from a 25-day culture was investigated using trypan blue exclusion and cell numbers were adjusted to $1x10^6$ cell/ml in antibiotic-free IMDM medium containing 10% FCS. Viability and cell number of murine macrophage cells P388D1 were also adjusted to $1x10^6$ cell/ml in antibiotic-free IMDM medium containing 10% FCS. *B. bronchiseptica* (Lux 95) grown for 24 h on BG agar were collected. A suspension of pelleted bacteria was made in IMDM medium and cell numbers were adjusted to either $5x10^8$ cfu/ml or $25x10^8$ cfu/ml. 100 µl of sheep BMMC suspension (or P388D1) was mixed with 100µl of bacterial suspension (total volume 200 µl) and was added in triplicate in 96-well plates and incubated at 37°C in the luminometer for 12 h. Bioluminescence output from the mixture of mammalian cell and bacteria was compared with bioluminescence output from control wells which contain *B. bronchiseptica* (Lux 95) in IMDM medium only. Figure 3.3.1a and Figure 3.3.1b show a comparison of the effect of sheep BMMC and P388D1 macrophage cell line on bioluminescence output from *B. bronchiseptica* (Lux 95) at both infection ratios.

There was a gradual increase in bioluminescence output with time from the control well which contained *B. bronchiseptica* (Lux 95) in IMDM medium only. With sheep BMMC at either lower and higher infection ratios, there was only a slow increase in bioluminescence output from *B. bronchiseptica* (Lux 95). In contrast, there was increase in bioluminescence output from *B. bronchiseptica* (Lux 95) co-cultured with P388D1 macrophage cells compared with bioluminescence output in control wells. Thus, two different effects were observed. Co-culture of *B. bronchiseptica* (Lux 95) with sheep BMMC appeared to reduce the growth rate of *B. bronchiseptica* (Lux 95) in that there was a slow rate of increase in bioluminescence output. But, in the case of co-culture of *B. bronchiseptica* (Lux 95) with P388D1 macrophage cells there was an increase in bioluminescence output suggesting that there was enhancement of growth rate.

Figure 3.3.1a Comparison of effects of sheep BMMC and P388D1 cells on *B. bronchiseptica* (Lux 95) (Low infection ratio)

$5x10^8$ cfu/ml of *B. bronchiseptica* (Lux 95) were co-cultured with either sheep BMMC or P388D1 macrophage cell line at an infection ratio of 500:1 (500 bacteria to one mammalian cell) in IMDM medium containing 10% FCS and incubated at 37°C in the luminometer for 12h (mean of 9 observations ± SD).

a) Control wells containing $5x10^8$ cfu/ml *B. bronchiseptica* (Lux 95) in IMDM medium only.
b) $5x10^8$ cfu/ml *B. bronchiseptica* (Lux 95) were co-cultured with $1x10^6$ cells/ml of P388D1 macrophage cells
c) $5x10^8$ cfu/ml *B. bronchiseptica* (Lux 95) were co-cultured with $1x10^6$ cells/ml of sheep BMMC.

Figure 3.3.1b Comparison of effects of sheep BMMC and P388D1 cells on *B. bronchiseptica* (Lux 95)(High infection ratio)

$25x10^8$ cfu/ml of *B. bronchiseptica* (Lux 95) were co-cultured with either sheep BMMC or P388D1 macrophage cell line at infection ratio 2500:1 (2500 bacteria to one mammalian cell) in IMDM medium containing 10% FCS and incubated at 37°C in luminometer for 12 h (mean of 9 observations ± SD).

d) Control wells containing $25x10^8$ cfu/ml *B. bronchiseptica* (Lux 95) in IMDM medium only.
e) $25x10^8$ cfu/ml *B. bronchiseptica* (Lux 95) were co-cultured with $1x10^6$ cells/ml of P388D1 macrophage cell.
f) $25x10^8$ cfu/ml *B. bronchiseptica* (Lux 95) were co-cultured with $1x10^6$ cells/ml of sheep BMMC.

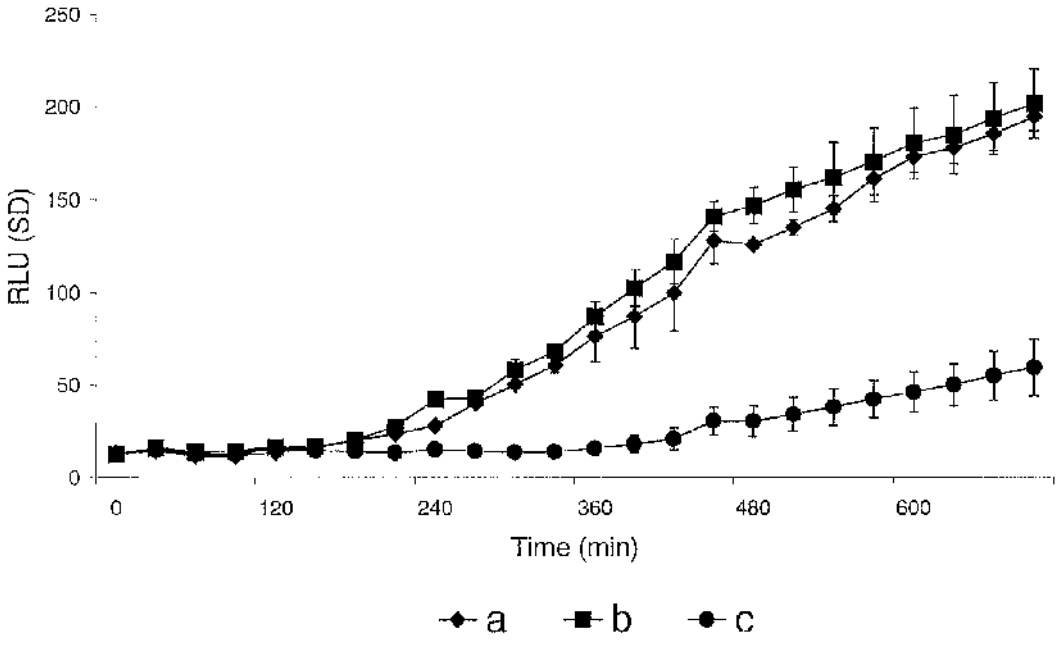


Figure 3.3.1a

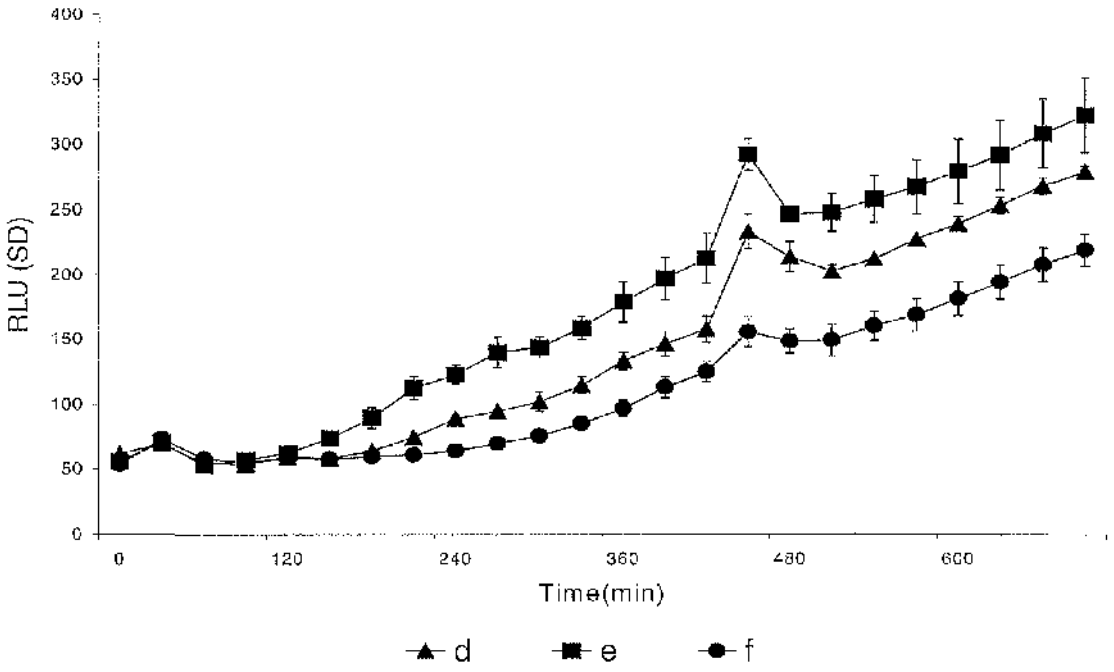


Figure 3.3.1b

3.3.2 Interaction of different *B. bronchiseptica* strains with sheep BMMC and J774A.1 macrophage cells

In the previous experiments, interaction of sheep BMMC with *B. bronchiseptica* was studied by using a bioluminescent strain of *B. bronchiseptica* (Lux 95). Intracellular *B. bronchiseptica* (Lux 95) were observed within sheep BMMC at 4 h post-infection (section 3.2.3). As there was some doubt about the virulence mode of *B. bronchiseptica* (Lux 95) from preliminary characterisation tests (section 3.2.1), intracellular survival was compared with that of the virulent strain of *B. bronchiseptica* (Bvg $^+$ phase) and an avirulent strain of *B. bronchiseptica* (Bvg $^-$ phase) within sheep BMMC and the J774A.1 macrophage cells.

Bacterial suspensions of different strains of *B. bronchiseptica* were prepared from 24 h BG agar plates. Bacterial numbers were adjusted to $5x10^8$ cfu/ml. Sheep BMMC (from 35-day culture) and J774A.1 macrophage cells were adjusted to $1x10^6$ cells/ml in IMDM medium containing 10% FCS only. Infection of both cell types with different strains of *B. bronchiseptica* was carried out at MOI 1:500. Each infection was carried out in duplicate in $25cm^2$ cell culture flasks with vent caps and incubated at 37°C in 5% CO_2. After incubation for 4 h, extracellular *B. bronchiseptica* were removed by centrifugation of the cell suspensions at 2000 rpm for 5 min, three times. Each time, fresh IMDM was added and the old IMDM medium was discarded. Pellets were resuspended in fresh IMDM medium containing 10% FCS and 1 µg/ml polymyxin B was added as a final concentration at zero time, and incubation continued at 37°C in 5% CO_2. At different times (12, 20 and 32 h) after zero time, extracellular polymyxin B was removed by extensive washing by centrifugation of the cell suspensions at 2000 rpm for 5 min and resuspension in fresh IMDM. This step was repeated twice. Then, the viability of sheep BMMC or J774A.1 cells was estimated by using trypan blue exclusion count. Intracellular bacteria within sheep BMMC or J744A.1 cells were released by adding 100 µg/ml of digitonin as a final concentration and dilution in PBS was carried out and plated on BG agar plate for viable counting.

3.3.2.1 Comparison of survival of sheep BMMC and J774A.1 cells after infection with different *B. bronchiseptica* strains

The survival of sheep BMMC or J774A.1 after infection with different strains of *B. bronchiseptica* was measured at different incubation times (0 h, 12 h, 20 h and 32 h)(Fig. 3.3.2a). All cultures showed some loss of viability with time. Untreated cultures of both cell types showed around 20% loss of viability over 32 h. The most dramatic reduction in survival of both sheep BMMC and J774A.1 cells was caused by the *B. bronchiseptica* (Bvg^+ phase). At zero time (4 h after infecting mammalian cells with bacteria), there was 60 % survival of sheep BMMC and around 30 % survival of J774A.1. Furthermore, more reduction in percentage survival of both cell types due to the effect of *B. bronchiseptica* (Bvg^I phase) was observed at 12 h incubation. Then, little further reduction was observed for both cell types at 20 h and 32 h incubation. In contrast, the avirulent strain of *B. bronchiseptica* (Bvg^- phase) allowed around 85 % survival of sheep BMMC and similar percentage of survival of J774A.1 cells at zero time. At 32 h, around 70% survival of sheep BMMC and 30% survival of J774A.1 cells were observed due to the effect of *B. bronchiseptica* (Bvg^- phase). In contrast, the percentage survival of sheep BMMC due to the effect of *B. bronchiseptica* (Lux 95) was higher than the percentage survival of sheep BMMC incubated with *B. bronchiseptica* (Bvg^+ phase), but lower than the percentage survival of sheep BMMC due to the effect of *B. bronchiseptica* (Bvg^- phase) at all incubation times. For example, there was around 75 % survival of sheep BMMC at zero time. At 32 h, around 60% survival of sheep BMMC was observed. At all incubation times, there was a statistically significant difference in percentage survival of sheep BMMC infected with bioluminescent *B. bronchiseptica* (Lux 95) strain when compared with sheep BMMC infected with the virulent *B. bronchiseptica* (Bvg^+ phase) (Student *t*-test, p-value ≤ 0.001). Also, there was a significant difference between survival of sheep BMMC with avirulent *B. bronchiseptica* (Bvg^- phase) and with *B. bronchiseptica* (Lux 95) (p-value < 0.05).

In contrast, the percentage survival of J774A.1 at zero time, 12 h and 32 h due to the effect of *B. bronchiseptica* (Lux 95) were similar to the effect of *B. bronchiseptica* (Bvg^- phase) which were higher than the percentage survival of J774A.1 cells due to the effect of *B. bronchiseptica* (Bvg^I phase). In general, the

effects of the different strains of *B. bronchiseptica* on survival of J774A.1 macrophage cells (Fig. 3.3.2b) were similar to the pattern of reduction due to same strains on viability of sheep BMMC.

Therefore, the above observations, suggested that the cytotoxicity of the bioluminescent *B. bronchiseptica* (Lux 95) was intermediate between that of the Bvg^+ phase and Bvg^- phase toward sheep BMMC which confirms the previous observations (section 3.2.1).

Figure 3.3.2a Survival of sheep BMMC due to infection with different strains of *B. bronchiseptica*

Sheep BMMC were infected with different strains of *B. bronchiseptica* (Lux 95), (Bvg $^{+}$ phase) and (Bvg $^{-}$ phase) at MOI 1:500. At different incubation times (0 h, 12 h, 20 h and 32 h) survival of sheep BMMC was measured by trypan blue exclusion count (mean of 4 observations ± SEM).

Figure 3.3.2b Survival of J774A.1 macrophage cell line due to infection with different strains of *B. bronchiseptica*

J774A.1 macrophage cell lines were infected with different strains of *B. bronchiseptica* (Lux 95), (Bvg $^{+}$ phase) and (Bvg $^{-}$ phase) at MOI 1:500. At different incubation times (0 h, 12 h, 20 h and 32 h) survival of J774A.1 was measured by trypan blue exclusion count (mean of 4 observations ± SEM).

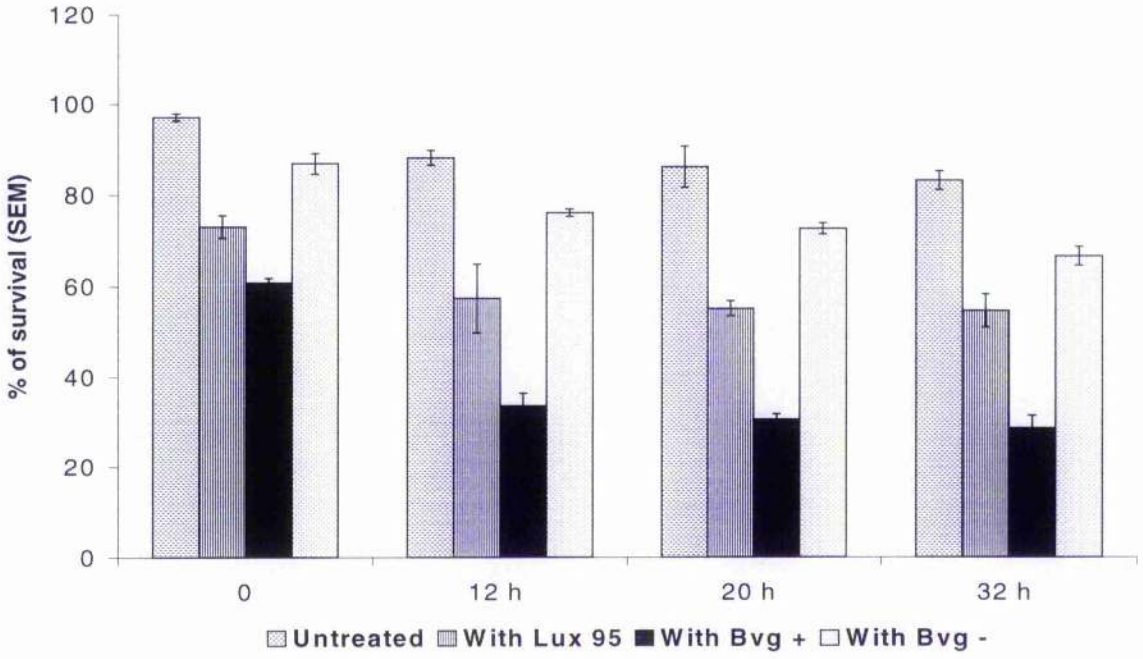


Figure 3.3.2a

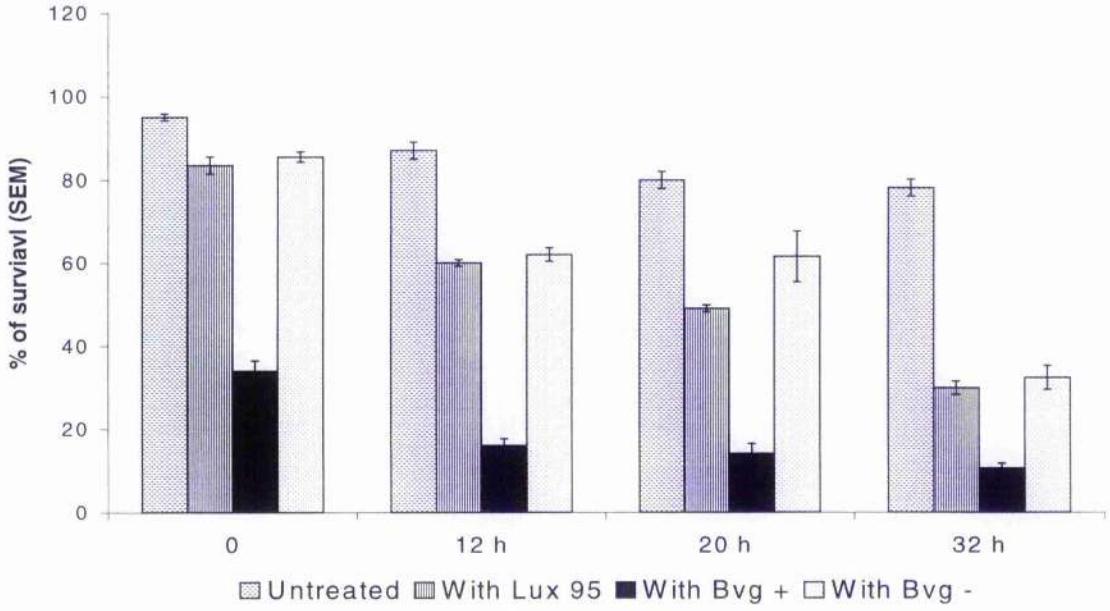


Figure 3.3.2b

3.3.2.2 Survival of different *B. bronchiseptica* strains within sheep BMMC or J774A.1 macrophage cells

Viable counts of the different *B. bronchiseptica* strains (Lux 95), (Bvg^+ phase) and (Bvg^- phase) were taken at different incubation times with the sheep BMMC and J774A.1 cells (section 3.3.2.1). This step was done to confirm the previous suggestion that *B. bronchiseptica* (Lux 95) is at intermediate phase between the Bvg^+ and Bvg^- phase observations.

Figure 3.3.3a shows that, after 12 h, the avirulent *B. bronchiseptica* (Bvg^- phase) showed a significantly higher survival rate than the virulent *B. bronchiseptica* (Bvg^+ phase) (p-value < 0.001) within sheep BMMC or J774 macrophage cells (Figure 3.3.4a). But with increasing incubation time, survival of B. *bronchiseptica* (Bvg^- phase) decreased within sheep BMMC (Fig. 3.3.3a) or J774A.1 cells (figure 3.3.4a). A similar significant survival of *B. bronchiseptica* (Lux 95) was observed within both cell types, but within sheep BMMC at 20 h was lower than survival of *B. bronchiseptica* (Lux 95) within J774A.1 cells. The numbers of viable intracellular *B. bronchiseptica* (Lux 95) or the avirulent strain (Bvg^- phase) within sheep BMMC or J774A.1 cells had declined by 20 h and 32 h when compared with numbers of intracellular survivors at 12 h. The last observation suggests that both strains of *B. bronchiseptica* (Bvg^- phase) and (Lux 95) were eventually killed by the sheep BMMC and J774A.1 cells (Fig. 3.3.3a and Fig. 3.3.4a) or that these strains eventually killed the sheep BMMC or J774A.1 cells and were then exposed to the extracellular 1 μg/ml polymyxin B. Another possibility is that polymyxin B eventually leaks into phagosoms of the mammalian cells to kill the intracellular bacteria.

In contrast, the number of viable intracellular *B. bronchiseptica* (Bvg^+ phase) was low compared with other strains at all incubation times. These results indicate that the virulent strain of *B. bronchiseptica* (Bvg^+) reduced the viability of the sheep BMMC or J774A.1 cells as shown in Fig. 3.3.3b and Fig. 3.3.4b and in turn any intracellular Bvg^+ could be then killed by extracellular polymyxin B. With reference to the original infection number of different *B. bronchiseptica* strains ($5x10^8$ cfu/ml), the percentage of intracellular *B. bronchiseptica* within either sheep BMMC or J774A.1 cell line was calculated at different incubation times. The percentages of

different strains of intracellular *B. bronchiseptica* declined with incubation time (Fig. 3.3.3b and Fig. 3.3.4b).

The above observations suggested that *B. bronchiseptica* (Lux 95) may be as intermediate phase of *B. bronchiseptica*. Furthermore, the numbers of intracellular *B. bronchiseptica* (Bvg ⁻ phase) and (Lux 95) within J774A.1 cells was higher than number within sheep BMMC which suggest that uptake of bacteria by sheep BMMC is less than the uptake ability of the professional phagocytic J774A.1 cells.

Figure 3.3.3a Long-term intracellular survival of different strains of *B. bronchiseptica* within sheep BMMC

$1x10^6$ cells/ml of sheep BMMC were mixed with different strains of *B. bronchiseptica* at MOI 1:500 and incubated for 4 h. Extracellular bacteria were removed by washing and the cell suspension was incubated with 1 μg/ml of polymyxin. The time scale refers to the time point of washing and addition of polymyxin B to the cell suspension. At different times, the number of viable intracellular bacteria was determined by viable counting (means of 4 observations ± SEM).

Figure 3.3.3b Percentage of intracellular B. *bronchiseptica* (Lux 95), (Bvg^+) phase and (Bvg^-) phase within sheep BMMC

Sheep BMMC were infected with different *B. bronchiseptica* strains at MOI 1:500 and incubated for 4h. The time scale of viability of intracellular bacteria of different strains refers to the time point of washing and addition of polymyxin B to the cell suspension. Intracellular *B. bronchiseptica* at different incubation times calculated as a percentage of the original bacterial number.

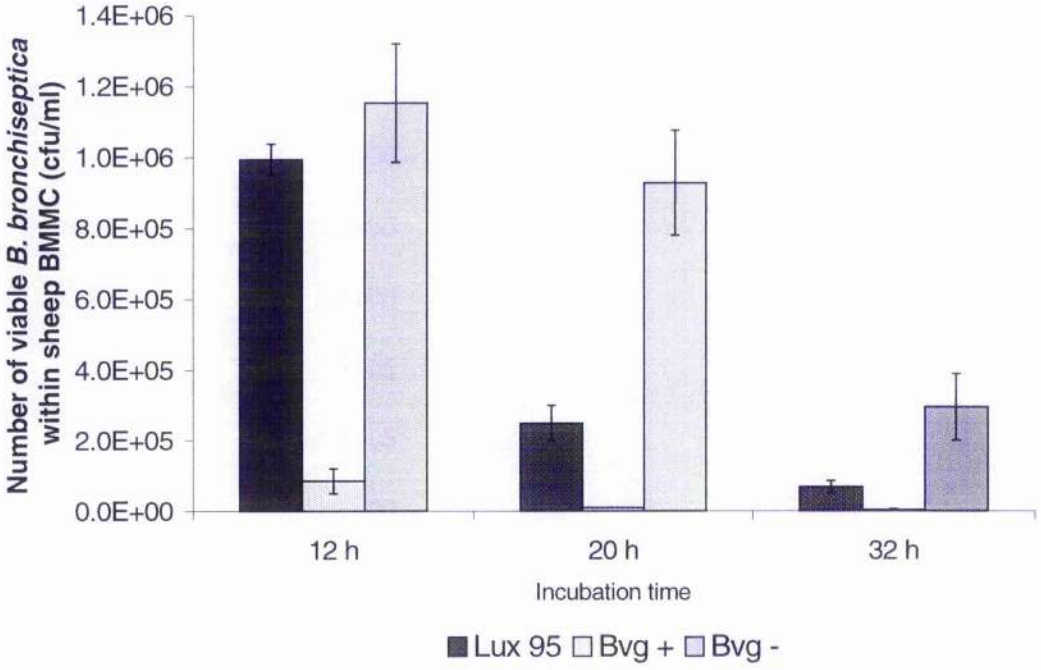


Figure 3.3.3a

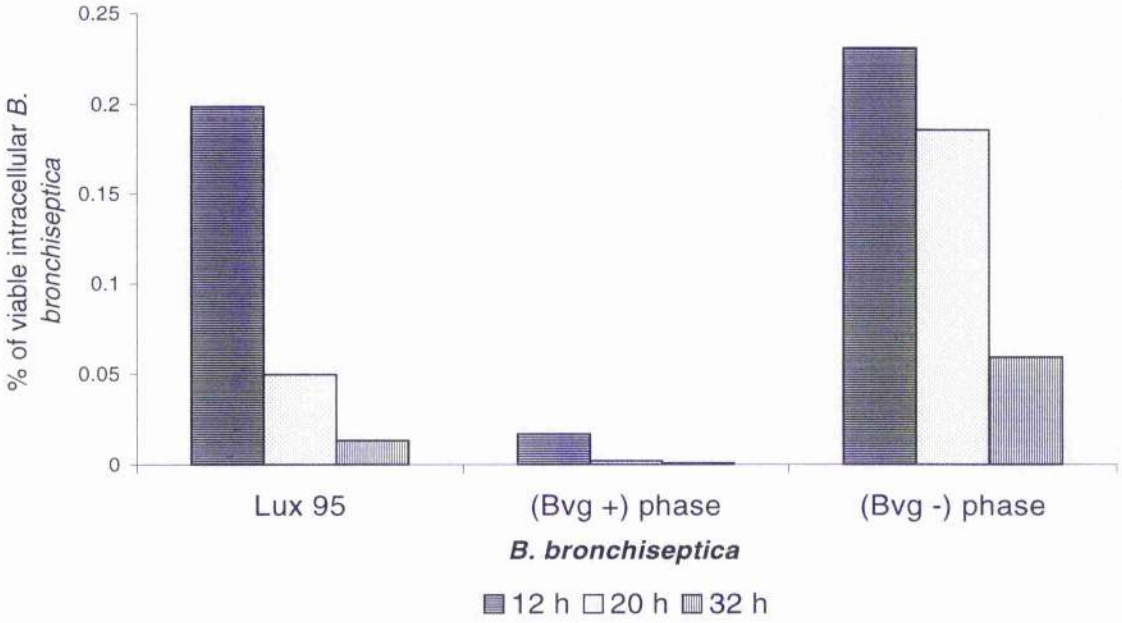


Figure 3.3.3b

Figure 3.3.4a Long-term intracellular survival of different strains of *B. bronchiseptica* within J774A.1 cells

$1x10^6$ cells/ml of J774A.1 were mixed with different strains of *B. bronchiseptica* at MOI 1:500 and incubated for 4 h. Extracellular bacteria were removed by washing and the cell suspension was incubated with 1 μg/ml of polymyxin. The time scale refers to the time point of washing and addition of polymyxin B to the cell suspension. At different times, the number of viable intracellular bacteria was determined by viable counting (means of 4 observations ± SEM).

Figure 3.3.4b Percentage of intracellular B. *bronchiseptica* (Lux 95), (Bvg^+) phase and (Bvg^-) phase within J774A.1 cells

J774A.1 were infected with different *B. bronchiseptica* strains at MOI 1:500 and incubated for 4 h. The time scale of viability of intracellular bacteria of different strains was refers to the time point of washing and addition of polymyxin B to the cell suspension. Intracellular *B. bronchiseptica* at different incubation times calculated as a percentage of the original bacterial number.

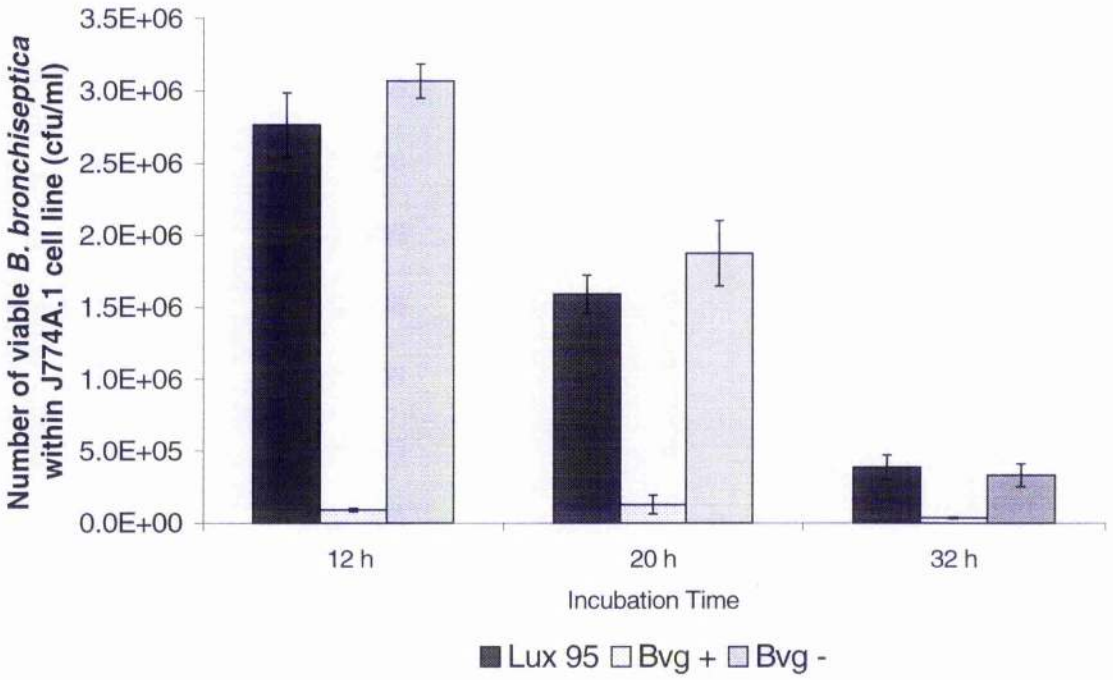


Figure 3.3.4a

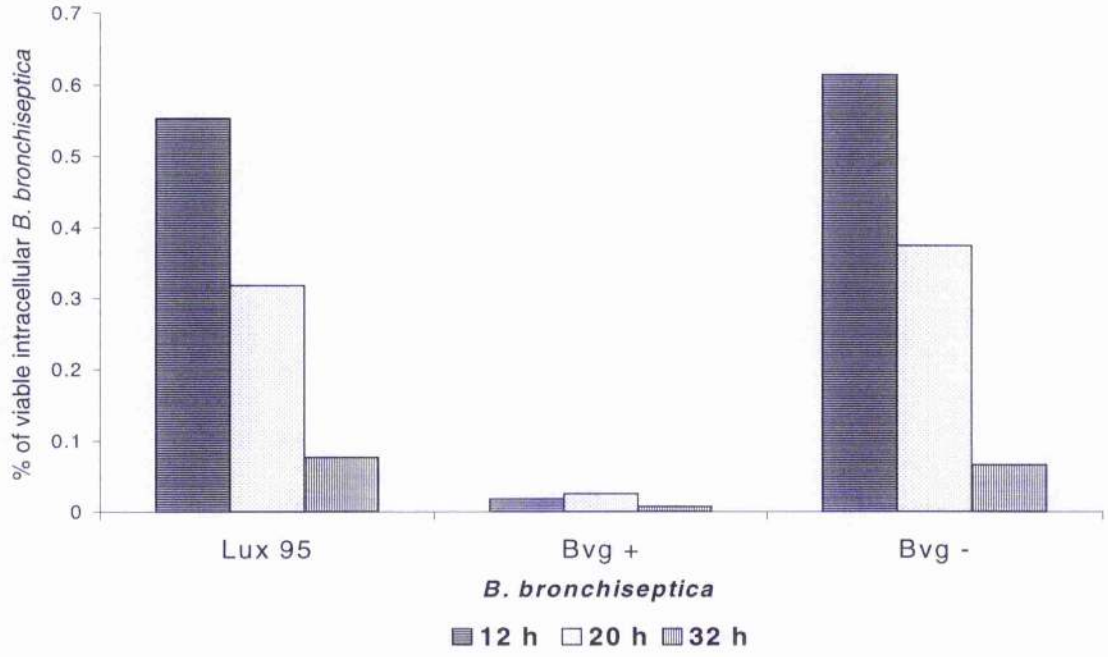


Figure 3.3.4b

3.3.3 ATP-bioluminescent assay and bioluminescence output strain from *B. bronchiseptica* (Lux 95)

The nucleotide adenosine triphosphate (ATP) plays a central role in energy exchange in biological systems. Many methods have been used for ATP determination, but now the most widely used, because of its sensitivity is the luciferin-luciferase bioluminescence assay which provides an assessment of viable cell numbers. The aim of this section was to further study the cytotoxic effect of different strains of *B. bronchiseptica* and to compare it with that of other related bacteria (i.e. different *Bordetella* species) and unrelated bacteria (i.e. Gram-positive bacteria). Therefore, different bacterial species were used and their effect on viability and degranulation of different mammalian cells was investigated. Viability of mammalian cells was assessed based on quantitation of the ATP present (by using the cellTiter-Glo™ luminescent cell viability assay), which signals the presence of metabolically-active cells by light output (RLU).

A preliminary study was set-up to examine the correlation between metabolic activities of different numbers of viable mammalian cells and light output (RLU). RBL-2H3 cells were used as a model for the following study. Initially, different numbers of RBL-2H3 cells were adjusted in IMDM medium and the metabolic activities of these different cell numbers were estimated in IMDM medium only. Figure 3.3.5a showed that there was a linear relationship between cell numbers and ATP levels (RLU). As the ATP-bioluminescent assay was to be used for estimation of metabolic activity of different mammalian cells in the presence of bacteria, therefore it was important to investigate the level of metabolic activity of bacterial cells (e.g. different strains of *B. bronchiseptica*) in the same assay. This would show whether there was any interference due to the ATP content of the bacterial cells themselves in the final readings. Figure 3.3.5b shows a comparison of the ATP levels of different mammalian cells and different strains of *B. bronchiseptica.*

As can be seen, there were different levels of ATP detected in the different mammalian and bacterial cells by using the cellTiter-Glo™ luminescent cell viability assay. But, in the case of *B. bronchiseptica* (Bvg^+ phase) and its mutants (Bvg^- or

Lux 95) at a cell concentration $5x10^8$ cfu/ml, the ATP-bioluminescent assay showed only very low light output from the bacterial cells compared with the different mammalian cells at $1x10^6$ cells/ml (p-value < 0.0001). These observations suggested that the ATP contents of the different strains of *B. bronchiseptica* would not interfere with measurment of ATP contents (and viability) of the different mammalian cells. In addition, the above results suggested that the bioluminescence output of *B. bronchiseptica* (Lux 95) did not interfere with the bioluminescence output (RLU) measured using the cellTiter-Glo™ luminescent cell viability assay at the same wavelength.

Figure 3.3.5a The correlation between ATP measurement and mammalian viable cell numbers

The figure shows the correlation between bioluminescence output (RLU) detected by the ATP-bioluminescent assay and different viable cell numbers of RBL-2H3 cell line (mean of 9 observations and ± SEM).

Figure 3.3.5b ATP contents of different mammalian cells and different strains of *B. bronchiseptica*.

ATP contents of $1x10^6$ cells/ml of different mammalian cells compared with ATP contents of different strains of *B. bronchiseptica* ($5x10^8$ cfu/ml) by using the the ATP-bioluminescent assay (mean of 9 observations ± SEM).

MC: sheep BMMC

RBL-2H3 cell line

J774A.1 macrophage cell line

Bvg $^+$: virulent type of *B. bronchiseptica*

Bvg $^-$: avirulent type of *B. bronchiseptica*

Lux 95: bioluminescent strain of *B. bronchiseptica*

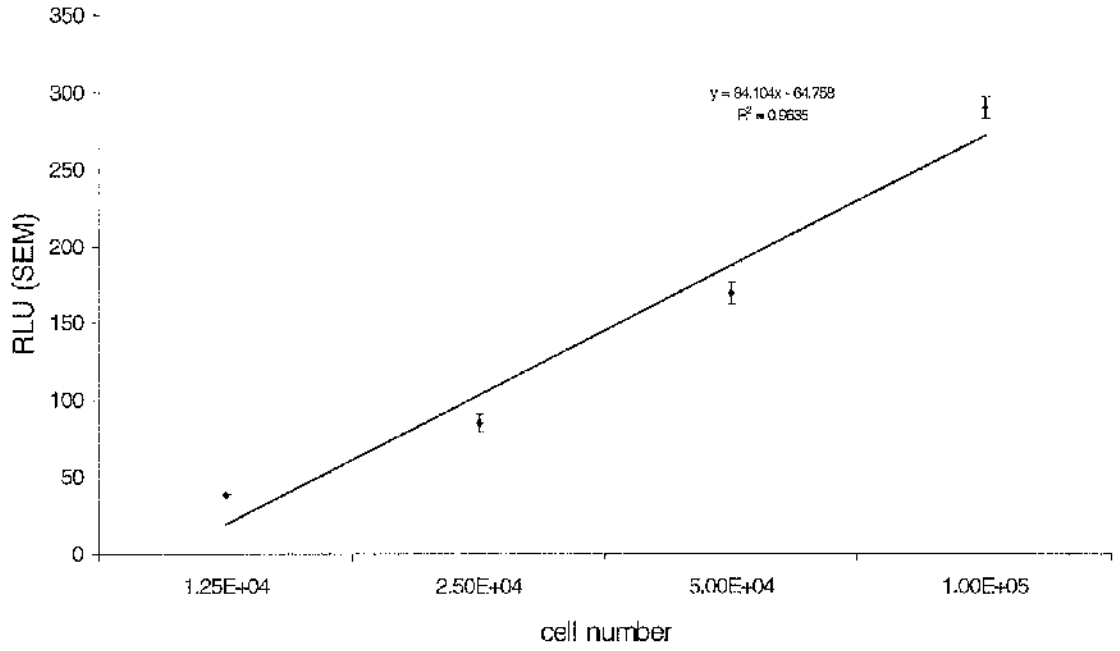


Figure 3.3.5a

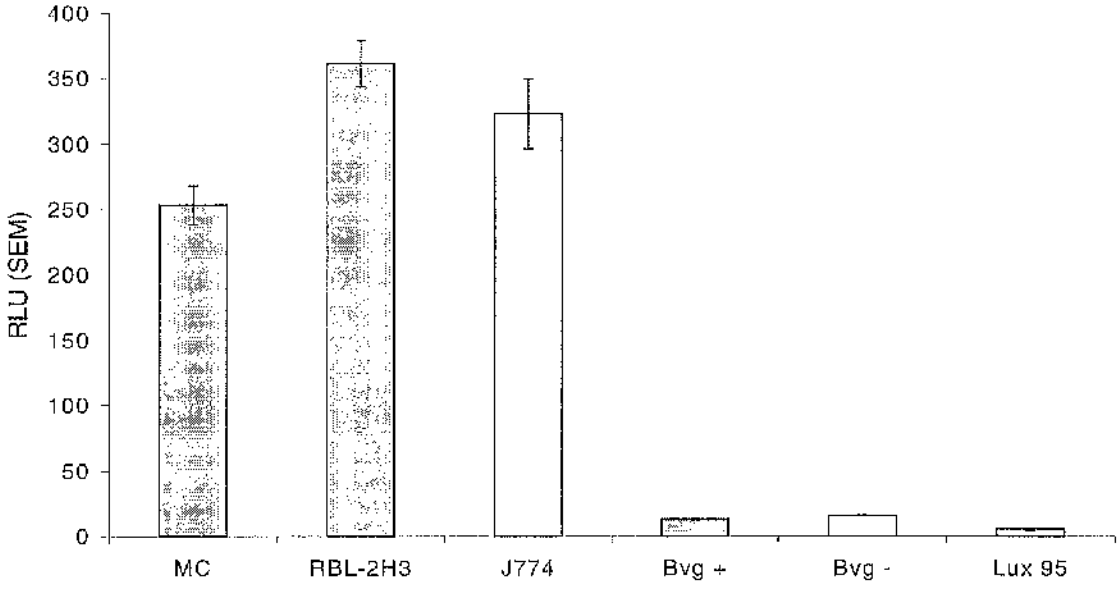


Figure 3.3.5b

3.3.4 Importance of growth medium on cytotoxic activity of *B. bronchiseptica*

3.3.4.1 Effect on cytotoxicity of *B. bronchiseptica*

To determine whether different growth media had an effect on the cytotoxicity of the virulent strain of *B. bronchiseptica*, cultures grown for 24 h on BG agar or in CL broth, on LB agar or other media were collected in IMDM medium containing 10% FCS. Cell numbers were adjusted to $OD_{600nm} = 0.46$ (equivalent to $2x10^9$ cfu/ml) and diluted to $5x10^8$ cfu/ml. RBL-2H3 cells were adjusted to $1x10^6$ cells/ml in antibiotic-free IMDM and mixed with *B. bronchiseptica* at an infection ratio of 1:500 (one RBL-2H3 cell to 500 bacterial cells). 200 µl volumes of the mixture were added in triplicate in 96-well plates and incubated for 1 h at 37°C in 5% CO_2. Viability of the RBL-2H3 cells was estimated by the ATP bioluminescence assay and the percentage of survival was estimated. Previous, degranulation of RBL-2H3 cells after incubation with 10^{-6} M of calcium ionophore showed that β-hexosaminidase release was highest after incubation for 3 h (data not shown). Therefore, estimation of β-hexosaminidase release was used as a marker for RBL-2H3 cell degranulation after incubation for 3h with different stimulators.

As can be seen from Figure 3.3.6a, the percentage of survival of the RBL-2H3 cells infected with the virulent strain of *B. bronchiseptica* (Bvg^+ phase) grown on BG agar was low compared with the percentage of survival of RBL-2H3 cells infected with virulent strain of *B. bronchiseptica* (Bvg^+ phase) grown in CL broth or on LB agar (p-value ≤ 0.001). Figure 3.3.6b showed that there was statistically significant β-hexosaminidase release from RBL-2H3 cells due to interaction with *B. bronchiseptica* (Bvg^-) grown on BG agar compared with β-hexosaminindase release from RBL-2H3 cells due to the effect of *B. bronchiseptica* (Bvg^+) grown on the other media including BG agra, CL broth, SS broth, LB agar or LB broth (p-value < 0.001). 10^{-6} M of calcium ionophore stimulated β-hexosaminidase release from RBL-2H3 cells, but 10^{-6} M of substance-P did not show significant β-hexosaminidase release from RBL-2H3 cells, as shown previously.

Previously, it has been show that the medium cultur is an important factor in modifying *Bordetella* virulence (Wardlaw *et al.*, 1976). Expression of all of the cytotoxic factors was reduced under phenotype-modulating growth conditions (Coote,

2001) and in phase variants, demonstrating that their transcription was found to be affected by the growth conditions, with maximal expression being observed using BG agar. Therefore, these results suggest that BG agar is the preferred medium to grow *B. bronchiseptica* to obtain a cytotoxic effect.

Figure 3.3.6a Effect of different growth media on cytotoxicity of *B. bronchiseptica* (Bvg $^{+}$) for RBL-2h3 cells.

This figure shows the cytotoxicity of virulent strain of *B. bronchiseptica* (Bvg $^{+}$) grown on BG agar, in CL broth or on LB agar. RBL-2H3 cells were infected at a ratio of 1:500 and incubated for 1 h. The percentage of survival of the RBL-2H3 was estimated by comparing the viability of RBL-2H3 cells treated in IMDM only with the viability of RBL-2H3 cells infected with *B. bronchisetica* grown in different growth media (mean of 9 observations ± SEM). Viability of RBL-2H3 cells was estimated by the ATP-bioluminescence assay.

Figure 3.3.6b Effect of cytotoxicity of *B. bronchiseptica* (Bvg $^{+}$) on degranulation of RBL-2H3 cells.

The graph shows the percentage of β-hexosaminidase release from RBL-2H3 cells due to infection with *B. bronchiseptica* (Bvg^{+}) grown on different media. Calcium ionophore and substance-P (10^{-4}M) were used as controls. The results shown are the percentage of β-hexosaminidase release estimated after incubation for 3 h (mean of 9 observations ± SEM).

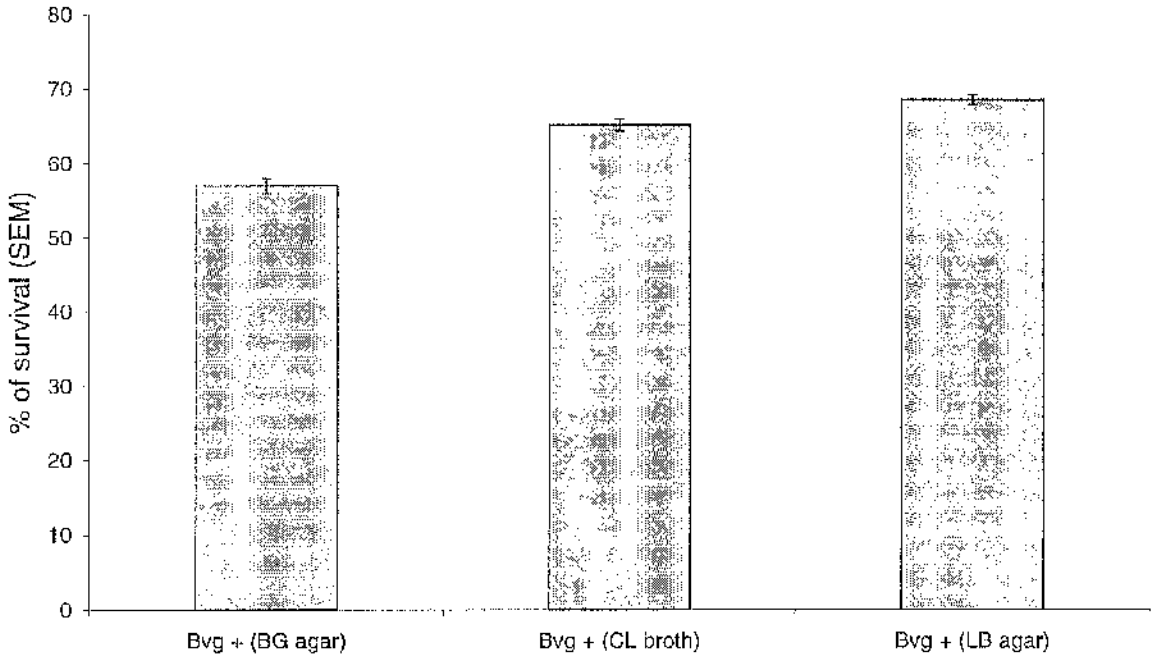


Figure 3.3.6a

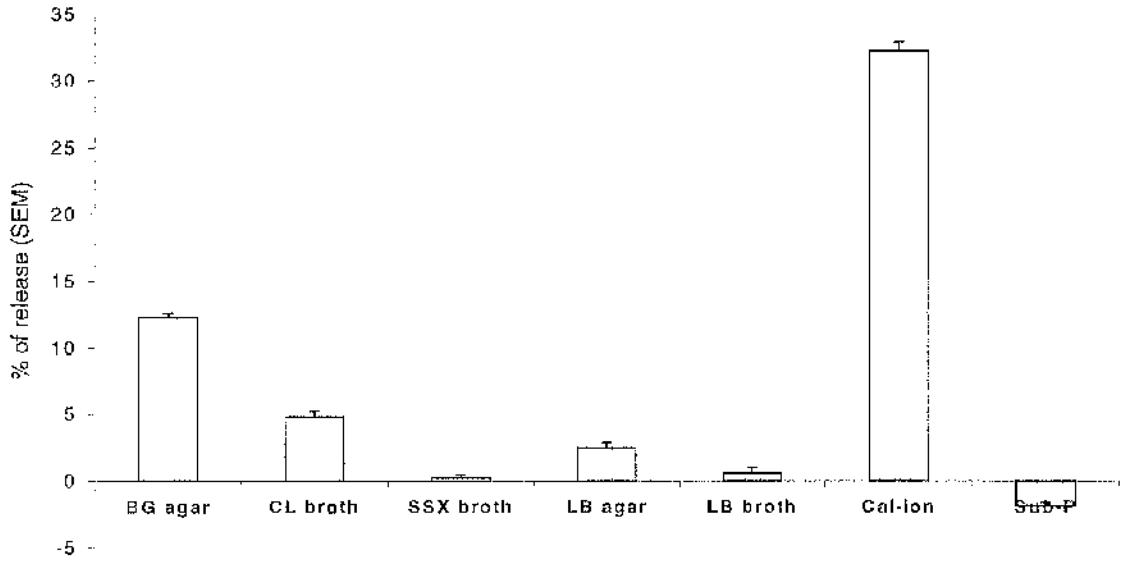


Figure 3.3.6b

3.3.4.2 Effect of different media on enzymatic activity of adenylate cyclase toxin (ACT)

The previous observations suggested that growth media on BG agar gave greater cytotoxicity of the virulent strain of *B. bronchiseptica* towards RBL-2H3 cells than other growth media. Therefore, it was important to investigate the importance of growth medium on expression of different cytotoxic factors in *B. bronchiseptica* (Bvg^+). For example, adenylate cyclase toxin (or CyaA) is a known Bvg-regulated virulence factor in *B. bronchiseptica*, and it is considered to be one of the most important virulence factors. Therefore, *B. bronchiseptica* (Bvg^+) was grown on different media (BG agar, LB agar and CL broth) for 24 h at 37°C and the resulting growth collected in PBS. Then, bacterial numbers were adjusted to OD_{600nm}=0.46 (equivalent to $2x10^9$ cfu/ml). 1 % of Triton X100 (Sigma) was added to lyse the bacterial cells. After 30 min, the mixture was centrifuged at 13000 rpm for 5 min and then the supernatant was placed in an ice bath. AC enzymic activity was detected in the supernatant by conductimetry assay (see Materials and Methods).

Figure 3.3.7 shows that the AC enzymic activity of the virulent strain of *B. bronchiseptica* (Bvg^-) cells grown on BG agar was higher than the enzymic activity of *B. bronchiseptica* (Bvg^+) grown on LB agar or in CL broth. These observations again suggest that BG agar could maximise the cytotoxicity of *B. bronchiseptica* (Bvg^+) phase compared with the effect of other media. Therefore, in all future work, BG agar was used as the growth medium for *B. bronchiseptica*.

Figure 3.3.7 Adenylate cyclase enzymatic activity from lysed *B. bronchispetica* (Bvg$^+$ phase) grown on different media.

B. bronchiseptica (Bvg $^+$ phase) grown on different media were collected and bacterial numbers were adjusted to $2x10^9$ cfu/ml. 1 % of Triton X100 was added to lyse the bacterial cells. AC activity was detected in the supernatant by conductimetry assay. This figure shows that the enzymic activity cells grown on BG agar was higher than when grown on LB agar or in CL broth. **A)** indicates an increase conductivity and **B)** is the time scale (sec).

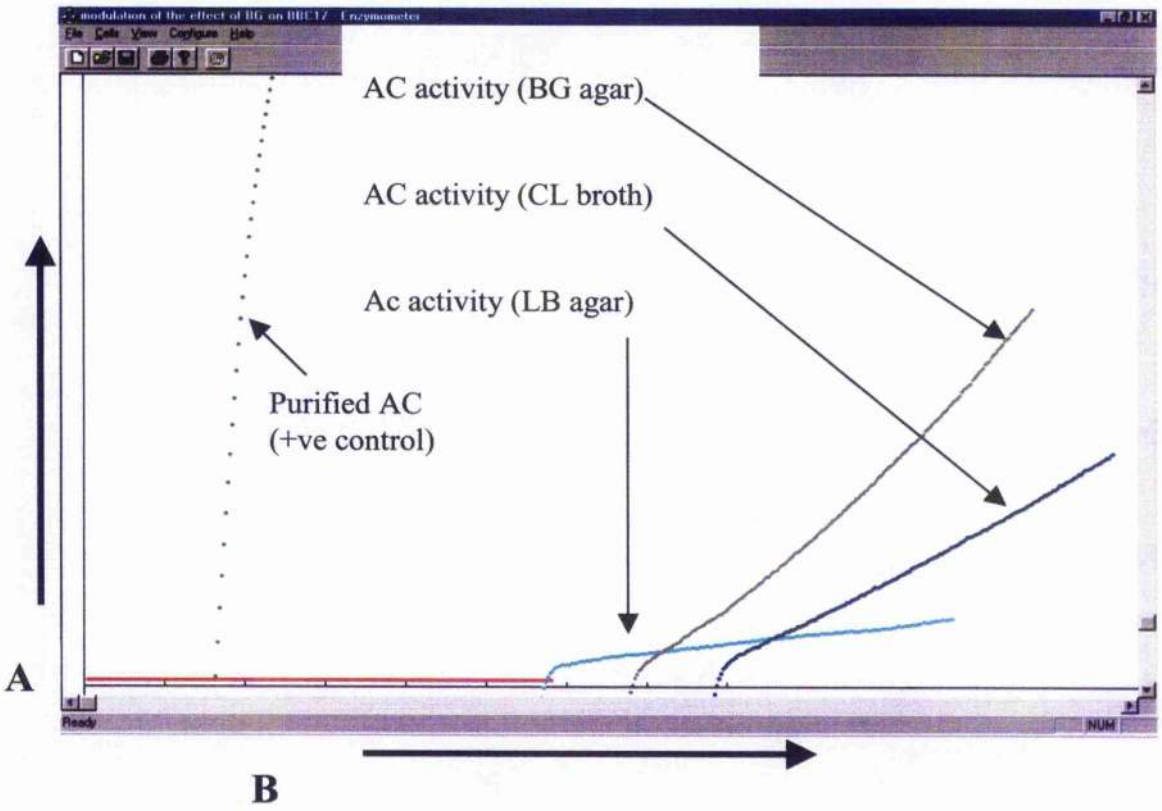

AC activity (BG agar)
AC activity (CL broth)
Ac activity (LB agar)
Purified AC
(+ve control)
A
B

Figure 3.3.7

3.3.5 Importance of Bvg mode on cytotoxicity of *B. bronchiseptica*

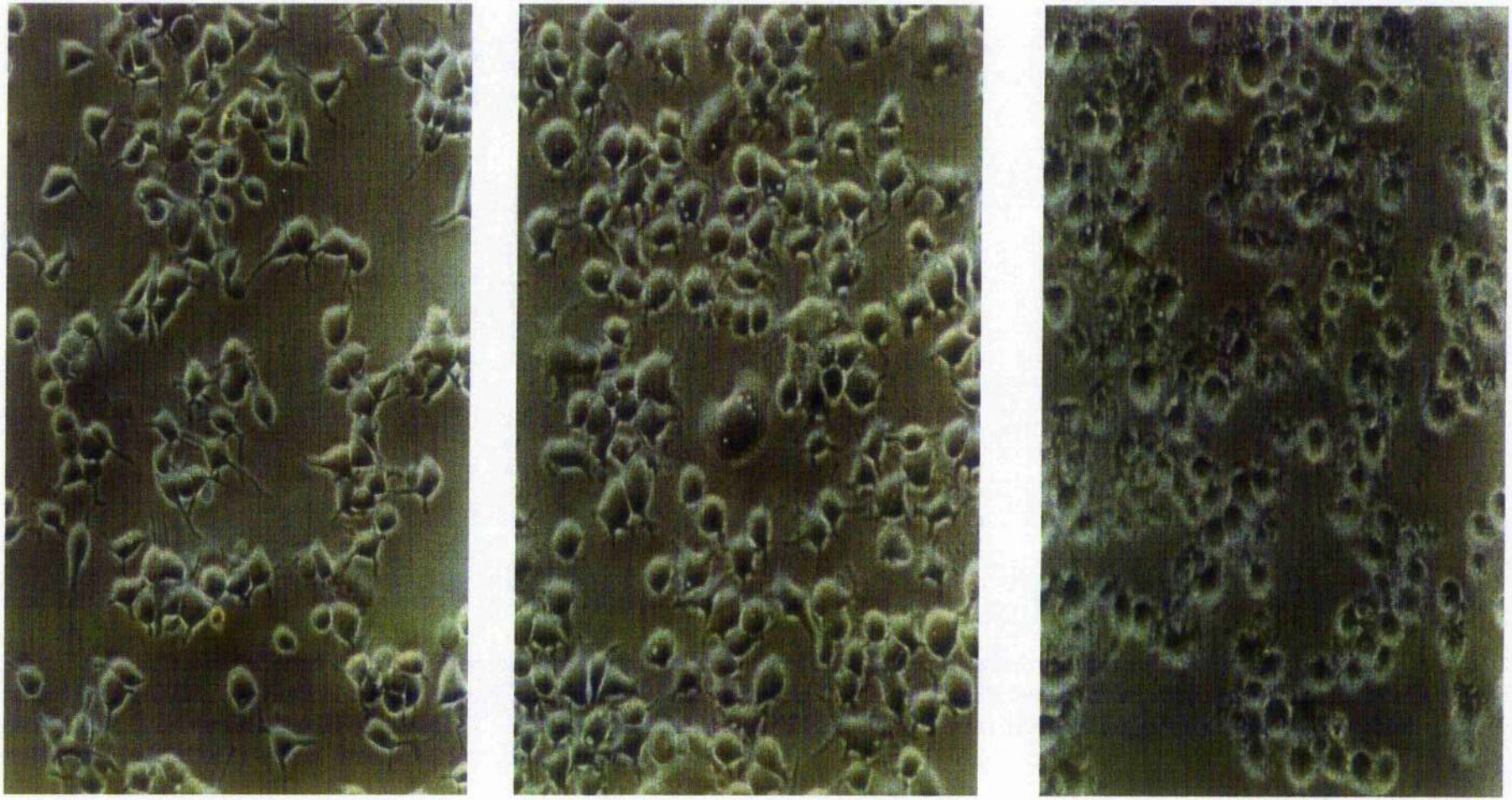
Bvg-regulated factors are known to play a role in the virulence of *B. bronchiseptica*. Here, cytotoxicity of *B. bronchiseptica* towards different mammalian cells was investigated with regard to the Bvg mode of the bacteria. Two different strains of *B. bronchiseptica* were used, the virulent strain (Bvg $^+$) and an avirulent strain (Bvg$^-$) to investigate their effects on the morphology, viability and degranulation of different mammalian cell types (sheep BMMC, RBL-2H3 cells and J774A.1 cells).

3.3.5.1 Microscopic study of interaction of different strains of *B. bronchiseptica* with RBL-2H3 cells

RBL-2H3 cells were infected with either *B. bronchiseptica* (Bvg $^+$) or *B. bronchiseptica* (Bvg $^-$). Both strains were grown on BG agar and the infection carried out at MOI 1:500 (one RBL-2H3 cell to 500 bacterial cells). The infection was carried out in tissue culture flasks (25 cm) and incubated for 3 h at 37°C in 5% CO_2. The cell suspension was then examined by using an inverted microscope (OLYMPUS CK2).

Figure 3.3.4a shows the effect of *B. bronchiseptica* on the morphology of RBL-2H3 cells after incubation for 3 h. Figure 3.3.8a.A. shows the normal appearance of RBL-2H3 cells in IMDM medium only, with many pseudopodia that help RBL-2H3 cells to attach to the surface of the tissue culture flask. This appearance was similar to the appearance of RBL-2H3 cells when infected with *B. bronchiseptica* (Bvg $^-$) strain (Fig. 3.3.8a.B) but the latter showed more vacuoles which were not apparent in RBL-2H3 cells maintained in IMDM only. However, the normal morphological appearance was completely changed due to infection with *B. bronchiseptica* (Bvg$^+$) (Fig. 3.3.8a.C). For example, they had lost most of their pseudopodia and also they appeared to be smaller with rounding up of cells which could suggest that they had lost their contents due to treatment with *B. bronchiseptica* (Bvg $^+$) mode.

Therefore, it was shown that infection of RBL-2H3 cells with *B. bronchiseptica* (Bvg $^+$) leads to dramatic changes in their morphological appearances, but such changes were not observed with *B. bronchiseptica* (Bvg $^-$) mode.



A B C

Figure 3.3.8a Effect of different strains of *B. bronchiseptica* on morphology of RBL-2H3 cells after incubation for 3 h by using light microscopy (X1000).. (**A**) Normal morphology of RBL-2H3 cells maintained in IMDM medium only. (**B**) More vacuoles in RBL-2H3 cells infected with *B. bronchiseptica* (Bvg^- phase). (**C**) Smaller with rounding up of RBL-2H3 cells which could suggest that they had lost their contents due to treatment with *B. bronchiseptica* (Bvg^+) mode.

3.3.5.2 Effect of incubation time on the cytotoxic effect of *B. bronchispetica* (Bvg^+ and Bvg^-) toward RBL-2H3 cells

The cytotoxic effect of the different modes of *B. bronchiseptica* on the viability of RBL-2H3 cells was estimated at different incubation times (1 h, 3 h and 6 h). The numbers of RBL-2H3 cells was adjusted to $1x10^6$ cells/ml and infected with different strains of *B. bronchiseptica* (Bvg^+ or Bvg^-) at an infection ratio of 1:500. The infection was carried out at 37°C in 5% CO_2 condition and incubated for different times. At the end of each incubation time, the viability of RBL-2H3 cells was estimated by trypan blue exclusion and the percentage of survival was estimated compared with the control which contain RBL-2H3 cells in IMDM medium only (Fig. 3.3.8b). It could be seen that *B. bronchiseptica* (Bvg^+) reduced the survival of RBL-2H3 cells more than *B. bronchiseptica* (Bvg^- phase) (p-value ≤ 0.001) at all three different incubation times. Also, it was observed that the effect of *B. bronchiseptica* (Bvg^+ phase) on survival of RBL-2H3 cell was rapid and had occurred within 1 h. The effect of *B. bronchiseptica* (Bvg^- phase) on RBL-2H3 cells increased with increasing incubation times. There was around 90% survival of RBL-2H3 cells at 1 h but at 6 h the percentage of survival of RB-2H3 cells was reduced to 75%.

Figure 3.3.8b Effect of different strains of *B. bronchiseptica* (Bvg $^{+}$ and Bvg $^{-}$) on viability of RBL-2H3 cells.

The graph shows the percentage of survival of RBL-2H3 cells infected with different strains of *B. bronchiseptica* (Bvg $^{+}$ or Bvg $^{-}$) strains at MOI 1:500 and incubated for different times. The viability of RBL-2H3 cells was estimated by trypan blue exclusion (mean of 6 observations ± SEM).

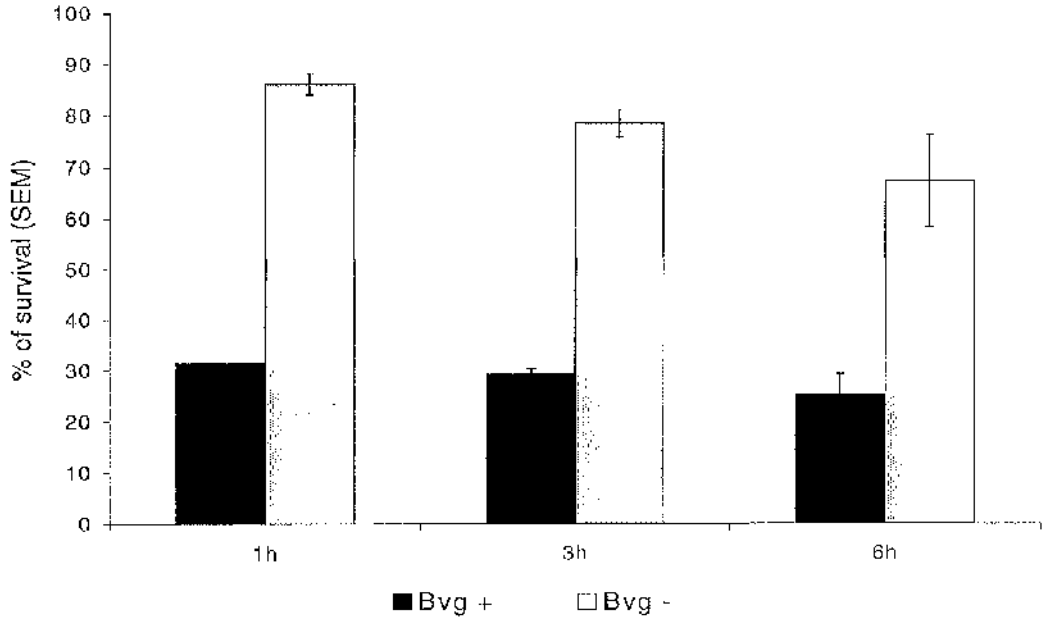


Figure 3.3.8b

3.3.5.3 Effect of *B. bronchiseptica* (Bvg^+ or Bvg^-) on viability of different mammalian cell types (ATP-bioluminescent assay)

The previous results (section 3.3.5.2) were confirmed by estimating the effect of different strains of *B. bronchiseptica* on the ATP content of different mammalian cells. $1x10^6$ cells/ml of different mammalian cells (sheep BMMC, RBL-2H3 cells and J774A.1 cells) maintined in IMDM containing 10 % FCS were infected with either *B. bronchiseptica* (Bvg^+) or (Bvg^-) strains at an MOI of 1:500. 200 µl volume of mixtures were added in triplicate in 96-well plates and incubated for 3 h at 37°C in 5% CO_2. The percentage of survival of all mammalian cells types were estimated by the ATP assay. Figure 3.3.8c shows the percentage of survival of different mammalian cells. As can be seen, there was a significant reduction in survival of different mammalian cells types after infection with *B. bronchiseptica* (Bvg^+) compared with *B. bronchiseptica* (Bvg^-) (p-value ≤ 0.001). For example, there was 60% survival of sheep BMMC after incubation with *B. bronchiseptica* (Bvg^+) and 95% survival after incubation with *B. bronchiseptica* (Bvg^- phase). Similar results were observed with RBL-2H3 cells and J774A.1 cells, except that *B. bronchiseptica* (Bvg^+ phase) showed a greater killing effect with J774A.1 cell types than with the other cell types (sheep BMMC or RBL-2H3 cells) (p-value ≤ 0.0001).

The above observations, confirm that the *B. bronchiseptica* (Bvg^+ phase) is more cytotoxic toward different mammalian cells than the Bvg^- phase, presumably due to one or more virulence factors produced by the virulent strain.

Figure 3.3.8c The effect of *B. bronchiseptica* (Bvg^+ and Bvg^-) on viability of different mammalian cell types.

The graph shows the effect of different strains of *B. bronchiseptica* (Bvg^+ and Bvg^-) on the viability of different mammalian cell types (sheep BMMC, RBL-2H3 and J774A.1 cells) at an infection ratio of 1:500 and incubated for 3 h. The results shown are the percentage of survival of different cell types compared with the control group for each mammalian cell type maintained in IMDM only. The viability of different mammalian cells determined by using by using the ATP-bioluminescent assay (mean of 9 observations ± SEM).

Bvg +: *B. bronchiseptica* virulent strain.

Bvg -: *B. bronchiseptica* avirulent strain (mutant strain).

MC: sheep BMMC

RBL-2H3 cell line

J774A.1 macrophage cell line.

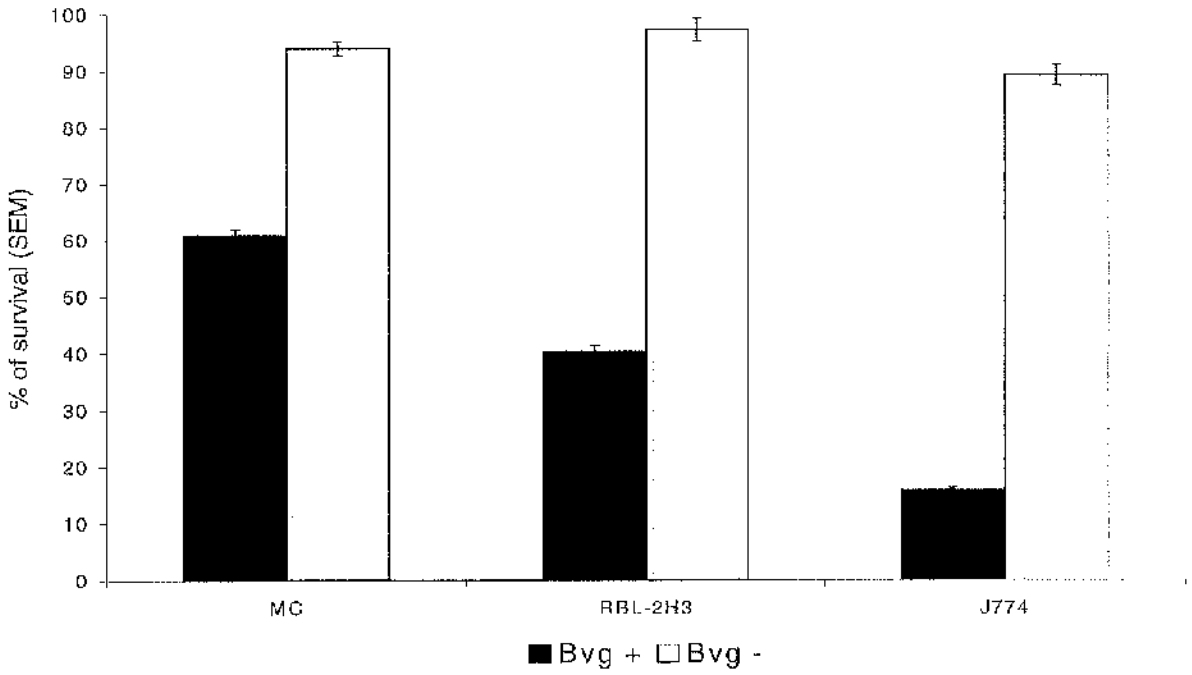

100
90
80
70
60
50
40
30
20
10
0
% of survival (SEM)
MC
RBL-2H3
J774
Bvg +
Bvg -

Figure 3.3.8c

3.3.6 Cytotoxicity of two novel mutant strains of *B. bronchiseptica* (Bag and Bho) on the viability of different mammalian cells

Dr. M. Lynch (Infection and Immunity Division, University of Glasgow) created two novel mutant strains, Bag and Bho, from a virulent strain of *B. bronchiseptica* (BBC17). He used comparative genomics to identify new putative two-component systems and then created mutants in two of these by using sequence probes (data not published). Furthermore, virulence experiments of the two new strains in a mouse model suggested that both new strains were attenuated their ability to colonise the mouse respiratory tract (personal communication). Cytotoxicity of both new strains on the viability of different mammalian cell types was investigated *in vitro* and compared with *B. bronchiseptica* (Bvg $^+$) and (Bvg $^-$) strains. $1x10^6$ cells/ml of different mammalian cells maintained in IMDM medium containing 10% FCS were infected with different strains of *B. bronchiseptica* (Bvg $^+$, Bvg $^-$, Lux 95, Bag and Bho) grown on BG agar for 24 h. Infection was carried out at MOI 1:500 and cells were incubated for 3 h at 37°C in 5% CO_2.

The ATP-bioluminescent assay was used to estimate viability of different mammalian cells. Therefore, the ATP contents of different strains of *B. bronchiseptica* were estimated by the ATP-bioluminescent assay to avoid any interference with the assay of viability of different mammalian cells. Figure 3.3.9a shows the ATP content (RLU) of different strains of *B. bronchiseptica* compared with the ATP content (RLU) of different mammalian cells. Statistically, there was a highly significant difference between the ATP content of the *B. bronchiseptica* strains and the mammalian cell types (p-value < 0.0001). Thus, the ATP content of the *B. bronchiseptica* strains would not interfere with this cytotoxicity assay for mammalian cells.

The percentage of survival of different mammalian cells (sheep BMMC, RBL-2H3 cells and J774A.1 cells) was estimated after incubation for 3 h with different strains of *B. bronchiseptica* (Fig. 3.3.9b). As can be seen, there was a significant decrease in survival of different mammalian cells due to incubation with *B. bronchiseptica* (Bvg $^+$) compared with other strains of *B. bronchiseptica*.

Cytotoxicity of the new mutant strains of *B. bronchiseptica* (Bag and Bho) was different towards the different mammalian cells. They caused a significant reduction in survival of J774A.1 cells, some reduction with sheep BMMC and had little effect on survival for RBL-2H3 cells. Furthermore, these results suggest that the new mutant strains (Bag and Bho) have a higher cytotoxic effect compared with the *B. bronchiseptica* (Bvg^- and Lux 95), but lower than that of *B. bronchiseptica* (Bvg^+). Thus, cytotoxicity of *B. bronchiseptica* (Bag) and (Bho) may be intermediate between that of the Bvg^+ mode and Bvg^-. 10^{-6}M of calcium ionophore was used as a control to compare with the effect of different strains of *B. bronchiseptica*.

Furthermore, β-hexosaminidase release was estimated from sheep BMMC and RBL-2H3 cells due to incubation with different strains of *B. bronchiseptica*. Figure 3.3.9c shows that the percentage of β-hexosaminidase release from both cell types were significantly high due to incubation with *B. bronchiseptica* (Bvg^+) mode compared with the effect of other strains of *B. bronchiseptica*. There was a significant β-hexosaminidase release from both cell types due to the effect of the new mutant *B. bronchiseptica* strains (Bag and Bho) comapred with the effect of *B. bronchiseptica* (Bvg^-) mode, but it was lower than β-hexosaminidase release from both mammalian cells due to the effect of *B. bronchiseptica* (Bvg^+). Interestingly, *B. bronchiseptica* (Bho) strain showed higher β-hexosaminidase release from both cell types compared with effect of *B. bronchiseptica* (Bag) mode (p-value > 0.05).

The above observations showed that the new mutant strains of *B. bronchiseptica* (Bag and Bho) able to stimulate sheep BMMC and RBL-2H3 cell types to release its contents, but this effect was intermediate between those of the Bvg^+ and Bvg^- strains.

Figure 3.3.9a Comparison of ATP-content of different strains of *B. bronchiseptica* with that of different mammalian cells

ATP contents of $1x10^6$ cells/ml of different mammalian cells was compared with the ATP contents of different strains of *B. bronchiseptica* ($5x10^8$ cfu/ml) by using the ATP-bioluminescent assay (cellTiter-Glo™ luminescent cell viability assay (mean of 9 observations ± SEM).

Sheep BMMC: sheep bone marrow mast cells

J774A.1 macrophage cells

Bvg^+ : virulent type of *B. bronchiseptica*

Bvg^- : avirulent type of *B. bronchiseptica*

Lux 95: bioluminescent strain of *B. bronchiseptica*

Bag: mutant type of *B. bronchiseptica*

Bho: mutant type of *B. bronchiseptica*

Figure 3.3.9b Effect of different strains of *B. bronchiseptica* on viability of different mammalian cells

$1x10^6$ cells/ml of different mammalian cells were treated with different strains of *B. bronchiseptica* at infection ratio of 1:500 and incubated for 3 h. Survival of treated mammalian cells were estimated by the ATP-bioluminescent assay (CellTiter-Glo™ luminescent cell viability) (mean of 9 observations ± SEM).

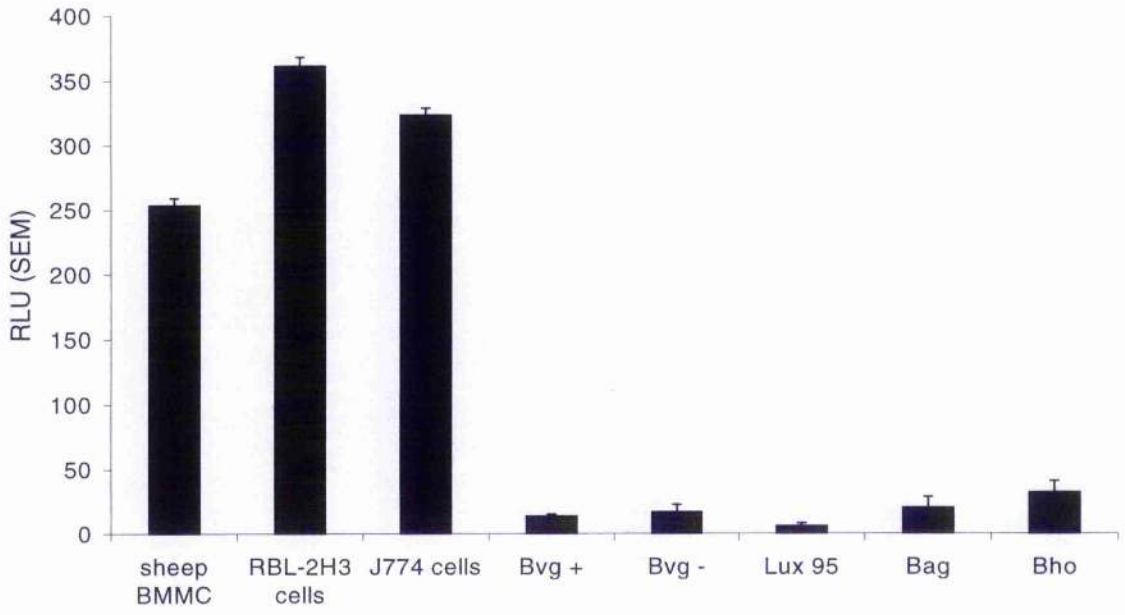


Figure 3.3.9a

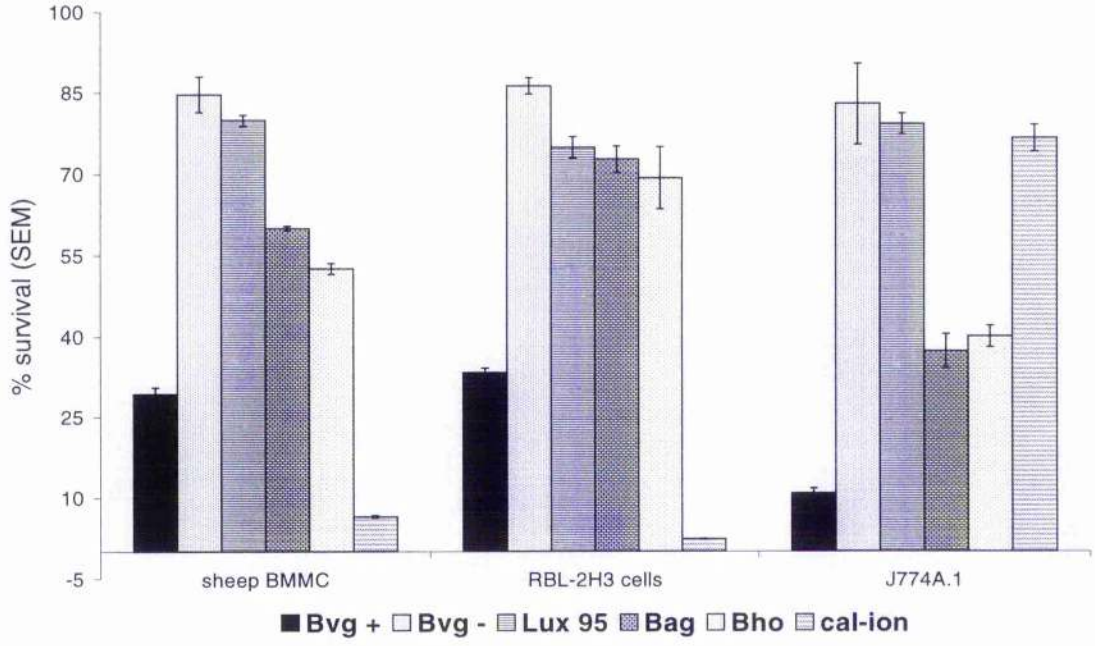


Figure 3.3.9b

Figure 3.3.9c Effect of different strains of *B. bronchiseptica* on degranulation of sheep BMMC and RBL-2H3 cells

$1x10^6$ cells/ml of sheep BMMC and RBL-2H3 cells were treated with different strains of *B. bronchiseptica* at an infection ratio of 1:500 and incubated for 3 h. β-hexosaminidase release was estimated and 10^{-6}M of calcium ionophore was used as a control (mean of 9 observations ± SEM).

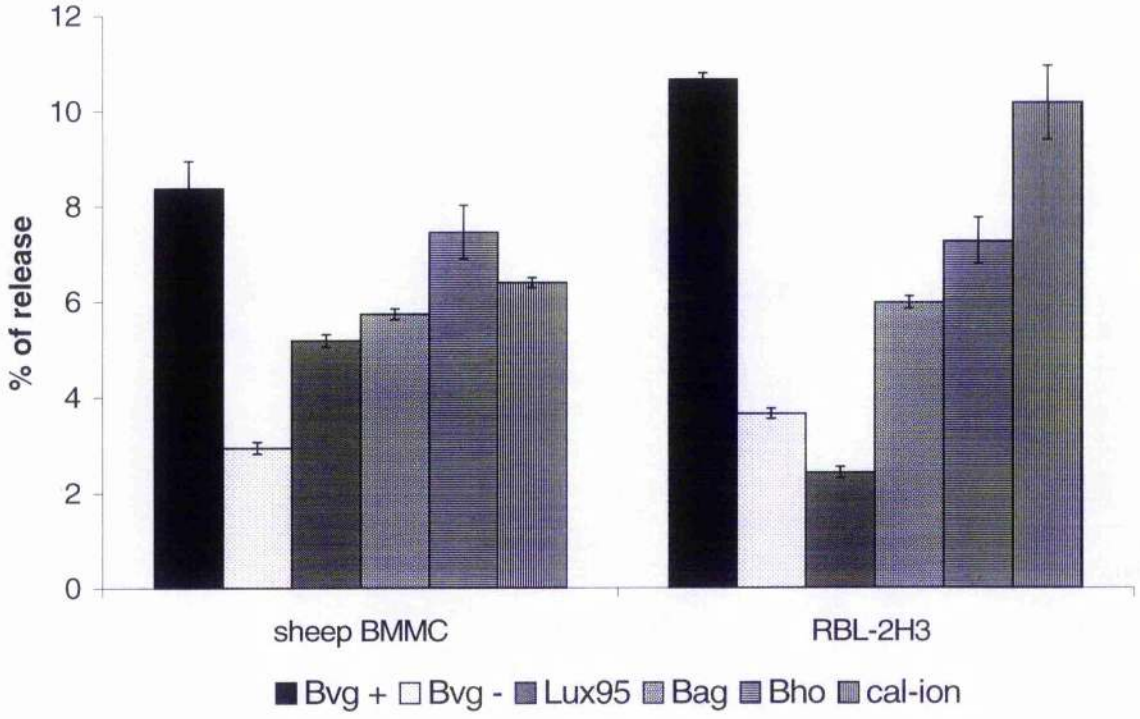

12
10
8
6
4
2
0
% of release
sheep BMMC
RBL-2H3
Bvg +
Bvg -
Lux95
Bag
Bho
cal-ion

Figure 3.3.9c

3.3.7 Influence of temperature on cytotoxicity of *B. bronchiseptica*

The previous results (Fig. 3.3.8c) presented viability data for RBL-2H3 cells mixed with the virulent strain of *B. bronchiseptica* based on quantitation of the ATP levels by using the cellTiter-Glo™ luminescent cell viability assay. This assay signals the presence of metabolically active cells by light output (RLU). In further work, a tetrazolium salt reduction (MTT) assay was used in parallel with the ATP bioluminescence assay for measuring cell viability (see Materials and Methods). The following study aimed to determine the effect of temperature on cytotoxicity of different strains of *B. bronchiseptica*. *B. bronchiseptica* (Bvg $^+$) or (Bvg $^-$) cells grown for 24 h on BG agar were collected and bacterial numbers adjusted to $5x10^8$ cfu/ml in IMDM medium. One sample was heated to 56°C for 10 min. The second sample was heated to 100°C for 10 min and the third sample was kept at room temperature. Then the contents of each were mixed with 10^6 cells/ml of RBL-2H3 cells maintained in IMDM medium containing 10% FCS, equivalent to an infection ratio of 1:500 (one mammalian cells to 500 bacterial cells). 200µl volumes of mixture were added in triplicate in 96 -well plates and incubated for 3h at 37°C under 5% CO_2. Survival of both RBL-2H3 cells and J774A.1 cells was estimated by both ATP-bioluminescence and MTT assays.

3.3.7.1 RBL-2H3 cells

Figure 3.3.10a shows survival of RBL-2H3 cells by ATP-bioluminescence assay after incubation with different samples of either *B. bronchiseptica* (Bvg $^+$ or Bvg $^-$) treated with different temperatures and figure. 3.3.10b shows survival of RBL-2H3 cells, measured by the MTT assay. Both assays (ATP-bioluminescence and MTT) sugggest that there was a statistically significant reduction in the percentage survival of RBL-2H3 cells due to infection with the untreated samples of *B. bronchiseptica* (Bvg $^+$) compared with other samples of *B. bronchiseptica* which were treated at different temperatures (p-value < 0.0001). The increase in survival of RBL-2H3 cells when mixed with *B. bronchiseptica* (Bvg $^+$) strain treated at 56°C suggested that cytotoxic factors were not totally destroyed at 56°C for 10 min.

Interestingly, there was a statistically significant differences in survival of RBL-2H3 cells due to the effect of 10^{-6}M of calcium ionophore. Less than 10 % survival of RBL-2H3 cells was observed according to the ATP-bioluminescent assay compared with 70 % survival by the MTT assay. Calcium ionophore presumably causes rapid depletion of ATP levels but has little effect on the dehydrogenase enzymes that are measured in the MTT assay.

Similar results were observed with J774A.1 macrophage cells (data not shown). Viability of J774A.1 macrophage cells treated with *B. bronchiseptica* suspension that had been treated to 56°C or 100°C for 10 min was higher than the viability of infected J774A.1 cells treated with *B. bronchiseptica* suspension kept at room temperature (p-value < 0.001). Degranulation of RBL-2H3 cells due to interaction with these *B. bronchiseptica* suspensions was also investigated and, as can be seen from figure. 3.3.10c, the ability of *B. bronchiseptica* (Bvg^{-}) to cause β-hexosaminidase release was also abolished at either 56°C or 100°C for 10 min (p-value ≤ 0.001).

The above observations demonstrated that the cytotoxic factor(s) of *B. bronchiseptica* are heat-labile.

Figure 3.3.10a The effect of temperature on cytotoxic effect of *B. bronchiseptica* (Bvg $^{+}$).

The graph shows the percentage survival of RBL-2H3 cells mixed with *B. bronchiseptica* suspensions treated at different temperatures (not-treated (NT), 56°C or 100°C (boil) for 10 min). Viability of the RBL-2H3 cells was estimated by the ATP-bioluminescence assay after incubation for 3 h (mean of 9 observations ± SEM).

Figure 3.3.10b The effect of temperature on cytotoxic effect of *B. bronchiseptica* (Bvg $^{+}$).

The graph shows the percentage survival of RBL-2H3 cells mixed with *B. bronchiseptica* suspensions treated at different temperatures (not-treated (NT), 56°C or 100°C (boil) for 10 min). Viability of the RBL-2H3 cells was estimated by the MTT assay after incubation for 3 h (mean of 9 observations ± SEM).

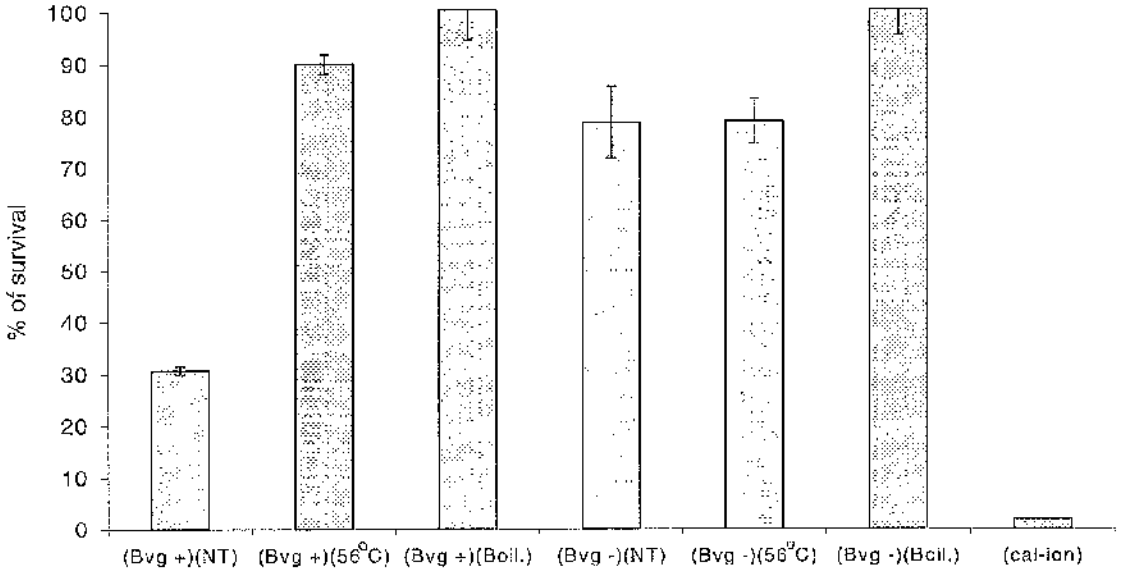


Figure 3.3.10a

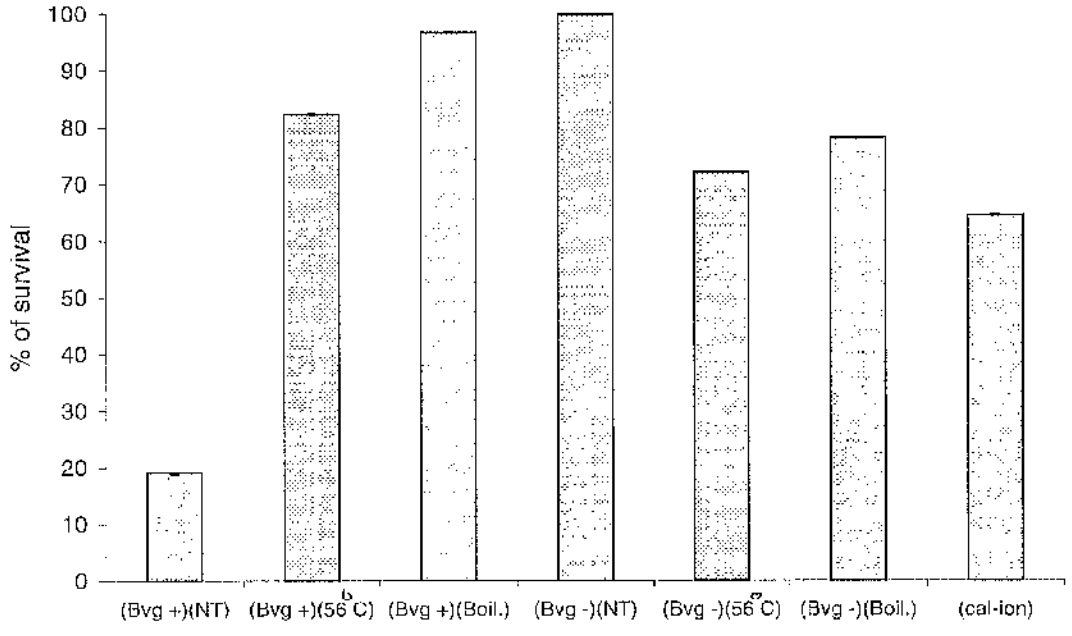


Figure 3.3.10b

Figure 3.3.10c Degranulation of RBL-2H3 cells treated with *B. bronchiseptica* (Bvg^+) suspensions heated at different temperatures.

The graph shows percentage of β-hexosaminidase release (mean of 9 observations ± SEM) from RBL-2H3 cells treated with *B. bronchiseptica* suspensions that had been heated at different temperatures (not-treated (NT), 56°C or 100°) and incubated for 3 h.

Bvg + (NT): *B. bronchiseptica* (Bvg^+) suspension kept at room temperature.

Bvg + (56°C): *B. bronchiseptica* (Bvg^+) suspension heated at 56°C for 10min.

Bvg + (Boil.): *B. bronchiseptica* (Bvg^+) suspension heated at 100°C for 10min.

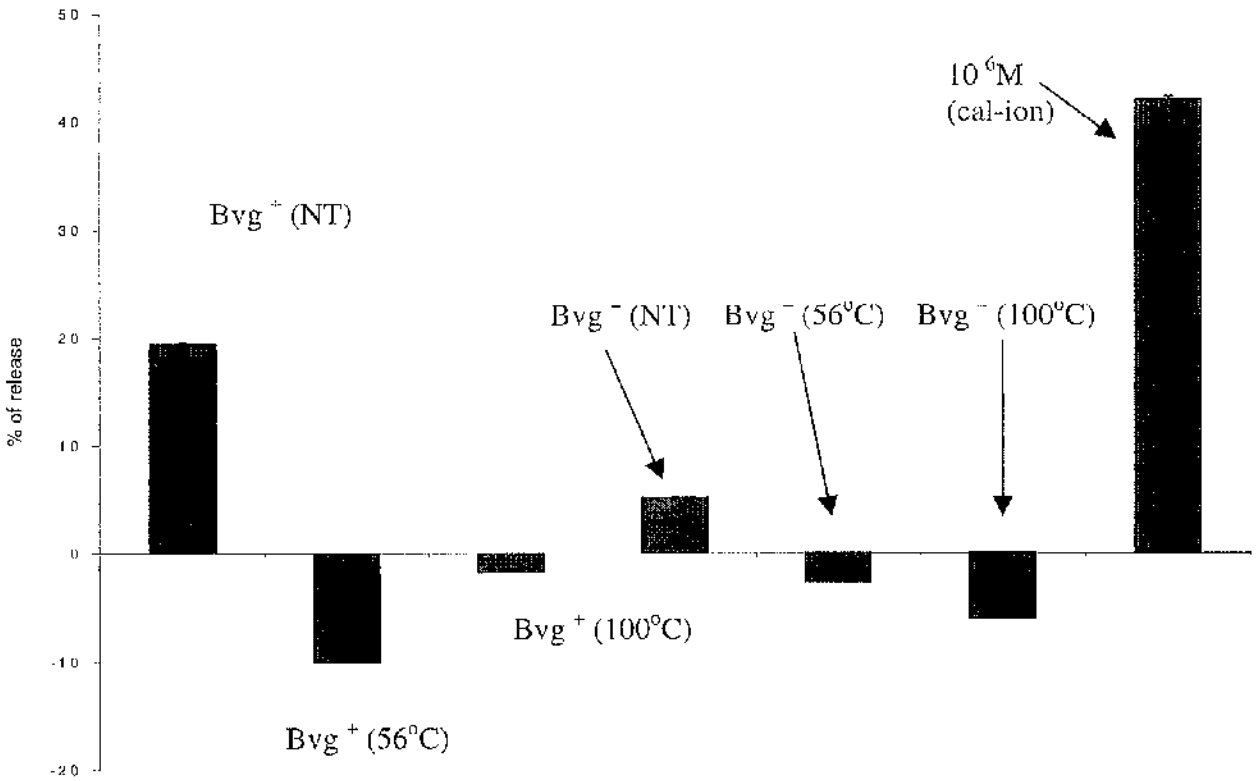


Figure 3.3.10c

3.3.8 Comparison of the effect of different strains of *Bordetella pertussis* on viability of RBL-2H3 cells

The following strains of *B. pertussis* were grown on BG agar and cell numbers were adjusted to $5x10^8$ cfu/ml in IMDM medium containing 1% of FCS:

1) *B. pertussis* 338 (virulent strain)
2) *B. pertussis* 347 (avirulent strain)
3) *B. pertussis* 357 (pertussis toxin-deficient strain)
4) *B. pertussis* 348 (ACT-deficient strain)

10^6 cells/ml of RBL-2H3 cells were maintained in IMDM medium containing 10% FCS and infected with different strains of *B. pertussis* at an infection ratio of 1:500. 200 µl volumes of mixtures was added in triplicate in 96-well plates and incubated for 3 h at 37°C in 5% CO_2. At the end of 3 h incubation, the percentage of survival of infected cells was estimated by the ATP-bioluminescent assay. It was important in the beginning to investigate any interference between the ATP content of RBL-2H3 cells with that of the *B. pertussis* strains. Figure 3.3.11a shows the ATP content of suspension of the different strains of *B. pertussis* compared with that of RBL-2H3 cells estimated by the ATP-bioluminescent assay. This figure shows that the ATP-bioluminescent assay would be a useful method to estimate the percentage of survival of RBL-2H3 cells treated with *B. pertussis* because there was little (non-significant, p-value > 0.001) contribution to light output due to the ATP content of the *B. pertussis*.

Surprisingly, there was no effect of any strain of *B. pertussis* on the survival of RBL-2H3 cells. Figure 3.3.11b shows the effect of different *B. pertussis* strains on survival of RBL-2H3 after incubation for 3 h compared with the effect of 10^{-6}M of calcium ionophore. Furthermore, β-hexosaminidase release was estimated after incubation for 3 h. Figure 3.3.11c shows no siginficant effect of any strain of *B. pertussis* on degranulation of RBL-2H3 cells compared with the effect of 10^{-6}M calcium ionophore.

The above observations suggest that to show any cytotoxic effect of different strains of *B. pertussis*, a higher infection ratio or longer incubation could be required.

Figure 3.3.11a Comparison of ATP content of different strains of *B. pertussis* with that of RBL-2H3 cells

$1x10^6$ cells/ml of RBL-2H3 cells and different strains of *B. pertussis* ($5x10^8$ cfu/ml) were maintained in IMDM medium containing 10% FCS. Their ATP contents were compared by using ATP-bioluminescent method and expressed in RLU (mean of 9 observations ± SEM).

Figure 3.3.11b Effect of different strains of *B. pertussis* on viability of RBL-2H3 cells

$1x10^6$ cells/ml of RBL-2H3 cells were infected with different strains of *B. pertussis* ($5x10^8$ cfu/ml) at an infection ratio of 1:500. Survival of RBL-2H3 after 3 h incubation was estimated by the ATP-bioluminescent assay (mean of 9 observations ± SEM). 10^{-6}M of calcium ionophore was used as a control.

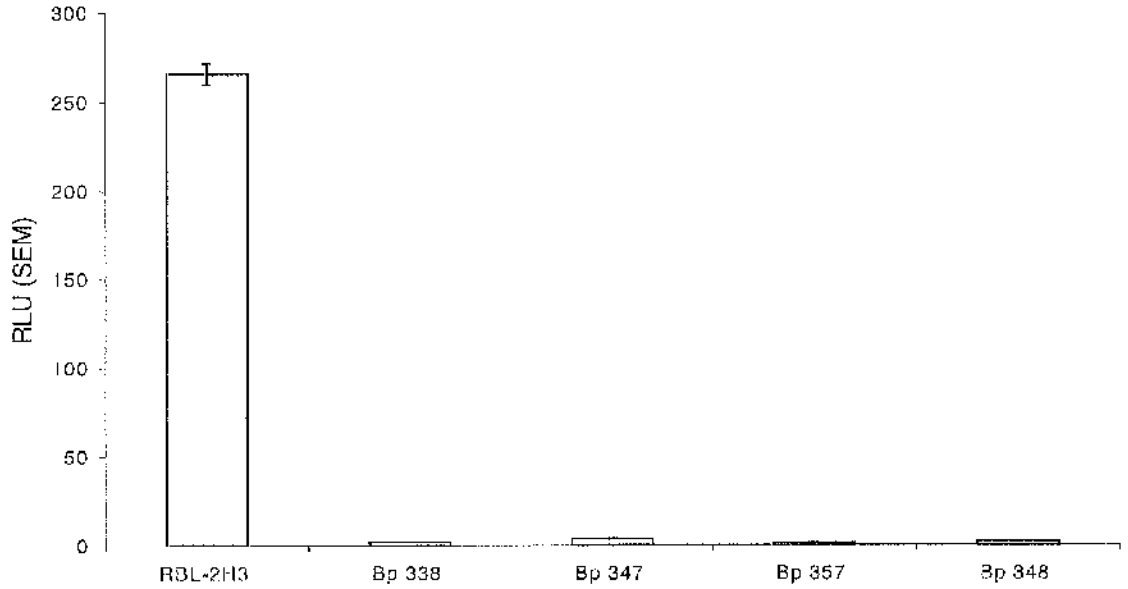


Figure 3.3.11a

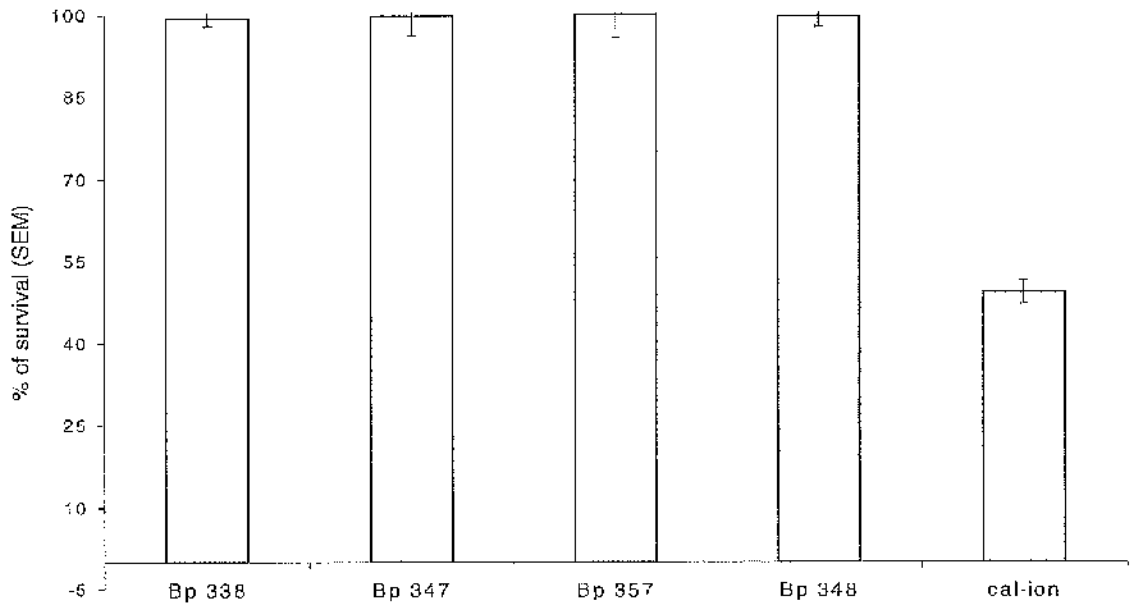


Figure 3.3.11b

Figure 3.3.11c Effect of different strains of *B. pertussis* on degranulation of RBL-2H3 cells

$1x10^6$ cells/ml of RBL-2H3 cells were infected with different strains of *B. pertussis* ($5x10^8$ cfu/ml) at an infection ratio of 1:500 and incubated for 3 h. β-hexosaminidase release was estimated after incubation for 3 h (mean of 9 observations ± SEM). 10^{-6}M of calcium ionophore was used as control.

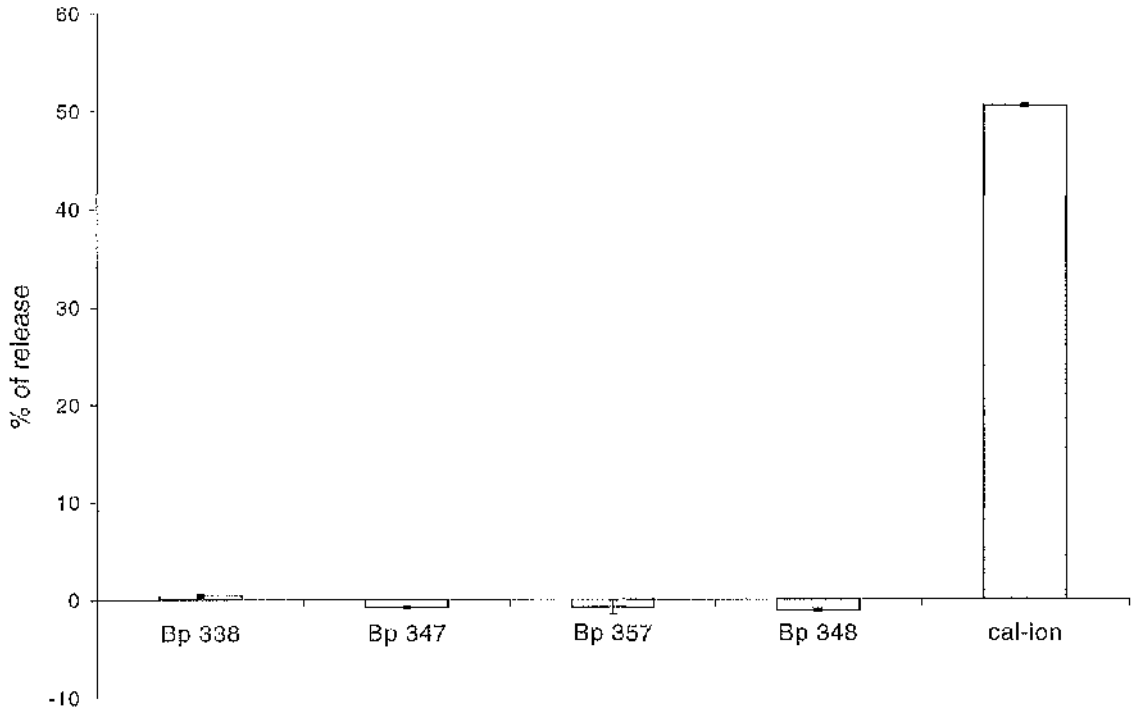


Figure 3.3.11c

3.3.9 Comparison of the cytotoxic effect of *B. bronchiseptica* with that other *Bordetella species*

Different species of *Bordetella* were grown on BG agar, and bacterial numbers were adjusted to $5x10^8$ cfu/ml in IMDM medium containing 1% of FCS. $1x10^6$ cells/ml of RBL-2H3 or J774A.1 cells were maintained in IMDM medium containing 10% FCS and mixed with different *Bordetella* species at an infection ratio of 1:500. 200 µl volumes of mixture were added in triplicate in 96-well plates and incubated for 3 h at 37°C in 5% CO_2. Survival of both infected cell types was estimated by the ATP-bioluminescent assay.

The percentage of survival of RBL-2H3 or J774A.1 cells after infection with different *Bordetella* species was estimated (Fig. 3.3.12). There was a statistically significant reduction (p-value ≤ 0.001) in survival of both RBL-2H3 and J774 A.1 cells due to interaction with *B. bronchiseptica* (Bvg $^+$) compared with other *Bordetella* species. Furthermore, the J774A.1 cells showed slightly lower survival than RBL-2H3 cells, but none of these species had any great effect on both cell types, unlike *B. bronchiseptica* (Bvg $^+$).

The effect of the different *Bordetella* species on survival of the two cell types was different. *B. bronchiseptica* (Bvg $^-$) showed a low effect, less than 10% reduction in survival of RBL-2H3 cells but, with J774A.1 cells, there was around 15% reduction. *B. bronchiseptica* (Lux 95) reduced survival of RBL-2H3 cells by 15% but with J774A.1 cells there was 30 % reduction in the percentage of survival. In contrast, *B. pertussis* (18323) showed 15% reduction in survival of RBL-2H3 cells and 25% reduction of survival of J774A.1 cells which were similar to the effect of *B. bronchiseptica* (Lux 95). Less cytotoxic effect of *B. pertussis* 338 toward RBL-2H3 cells and around 10% reduction in survival of J774A.1 cells. There was no significant difference between the cytotoxic effects of *B. parapertussis* and *B. avium* toward the percentage of survival of RBL-2H3 cells and J774A.1 cells. In contrast, a less cytotoxic effect towards RBL-2H3 cells and J774A.1 cells was observed with *B. holmesii*.

B. trematum reduced the percentage of survival of RBL-2H3 cells as compared with the effect of other species of *Bordetella* but was less than the cytotoxic effect of *B. bronchiseptica* (Bvg^+ phase). There was around 10% reduction in survival of RBL-2H3 and J774A.1 cells due to incubation with *B. hinzii*.

The above observations, suggested that *B. bronchiseptica* (Bvg^+) mode showed the highst cytotoxic effect against both RBL-2H3 cells or J774A.1 cells compared with other *Bordetella* species. With the other *Bordetella* species (i.e. *B. pertussis* 338, *B. pertussis* 18323, *B. parapertussis*, *B. avium*, *B. holmesii*, *B. tretamutum* and *B. hinzii*) higher infection ratios and longer incubation times could be used to show any killing effect on these mammalian cells.

Figure 3.3.12 Effect of different *Bordetella* species on viability of RBL-2H3 and J774A.1 cells (ATP-bioluminescent assay)

The graph shows the percentage survival (mean of 9 observations ± SEM) of RBL-2H3 cells and J774A.1 cells treated with different *Bordetella* species grown on BG agar. Viability was estimated by the ATP-bioluminescent method after incubation for 3 h at infection ratio of 1:500.

B.b (+): *B. bronchiseptica* virulent (Bvg^+) strain.

B.b (-): *B. bronchiseptica* avirulent (Bvg^-)strain

Lux 95: *B. bronchiseptica* (bioluminescent strain)

Bp: *B. pertussis*, 338 and 18323 strains.

B.pp: *B. parapertussis*

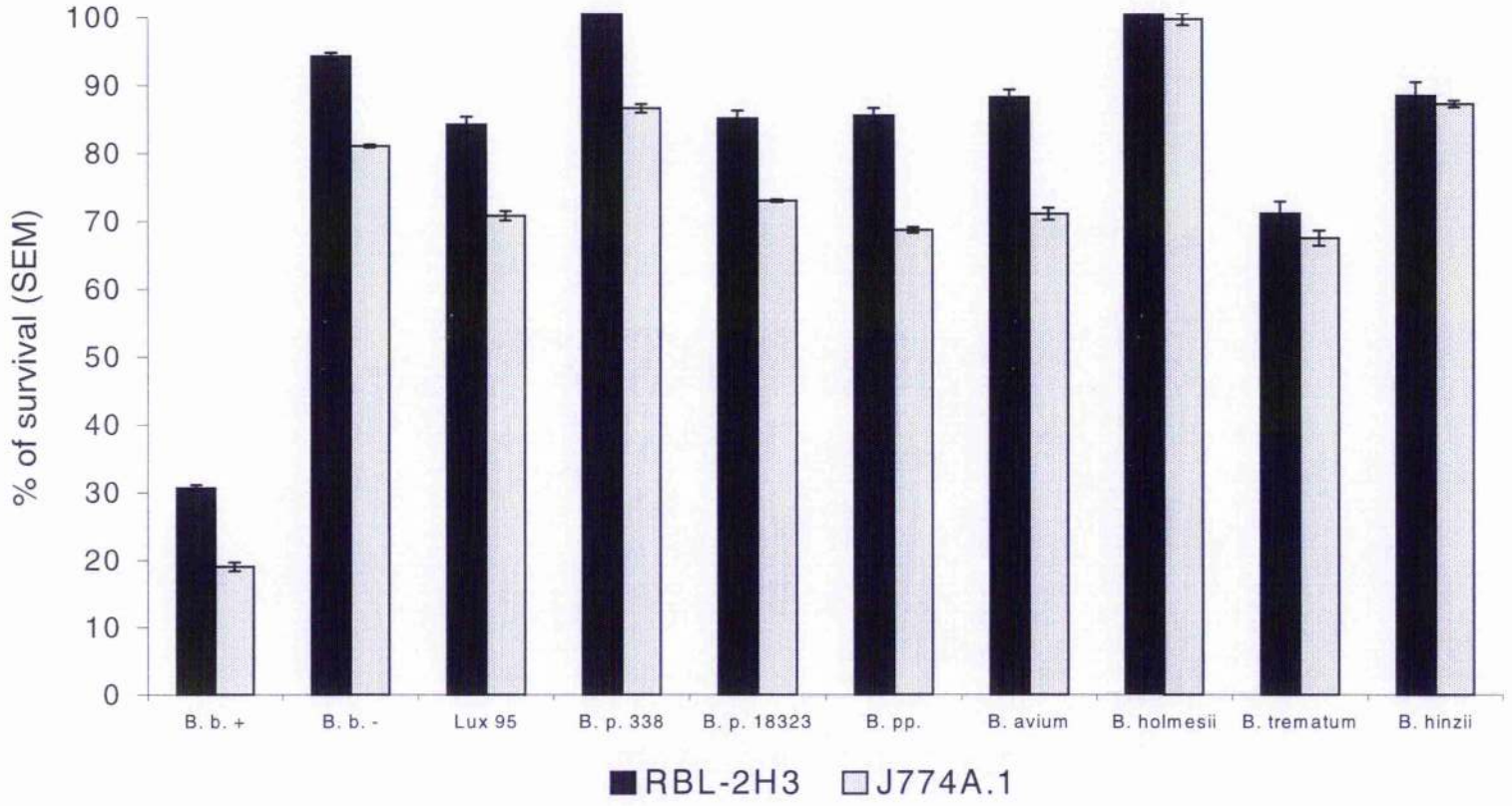

100
90
80
70
60
50
40
30
20
10
0
% of survival (SEM)
B. b. +
B. b. -
Lux 95
B. p. 338
B. p. 18323
B. pp.
B. avium
B. holmesii
B. trematum
B. hinzii
RBL-2H3
J774A.1

Figure 3.3.12

3.3.9.1 β-hexosaminidase release

All *Bordetella* species were grown on BG agar. Infection of RBL-2H3 cells was carried out at MOI 1:500 (one RBL-2H3 cell to 500 bacterial cells). 200 µl volumes of mixture were added in triplicate in 96-well plates and incubated at 37°C in 5% CO_2 for 3 h. At the end of incubation, β-hexosaminidase release was estimated.

Figure 3.3.13 shows the effect of the different *Bordetella* species on degranulation of the RBL-3H3 cells, it is the difference between hexosaminidase release caused by different *Bordetella* species. A statistically significant release of β-hexosaminidase was detected from RBL-2H3 cells due to interaction with *B. bronchiseptica* (Bvg^{I}) compared with either *B. bronchiseptica* (Bvg^{-}) or *B. bronchiseptica* (Lux 95) (P-value < 0.005). *B. trematum* and *B. hinzii* species showed a significant β-hexosaminidase release from RBL-2H3 cells compared with other species and similar to that caused by *B. bronchiseptica* (Bvg^{+}) (P-value < 0.005). *B. holmesii* showed very low β-hexosaminidase release compared with the effect of the *B. bronchiseptica* (Bvg^{-}). Both strains of *B. pertussis* (338 and 18323) showed β-hexosaminidase from RBL-2H3 cells, although lower than the effect of *B. bronchiseptica* (Bvg^{+}). The effect of *B. parapertussis* was similar to that of *B. pertussis* 18323. Also, *B. avium* showan effect similar to that of *B. bronchiseptica* (Bvg^{-})

These observations showed that degranulation effects differ from one species to another, which could suggest that more work is needed to investigate different factors (e.g. Type three secretion system (TTSS)) which could cause such different effects between different species, especially with *B. trematum* and *B. hinzii,* because both species showed an interesting effect on degranulation of RBL-2H3 cells but, unlike *B. bronchiseptica* (Bvg^{+}) having a major cytotoxic effect on these cells.

Figure 3.3.13 Effect of different *Bordetella* species on β-hexosaminidase release from RBL-2H3 cells

This graph shows β-hexosaminidase release from $1x10^6$ cells/ml of RBL-2H3 cells due to the effect of different *Bordetella* species at MOI 1:500 and 3 h incubation (mean of 9 observations ± SEM).

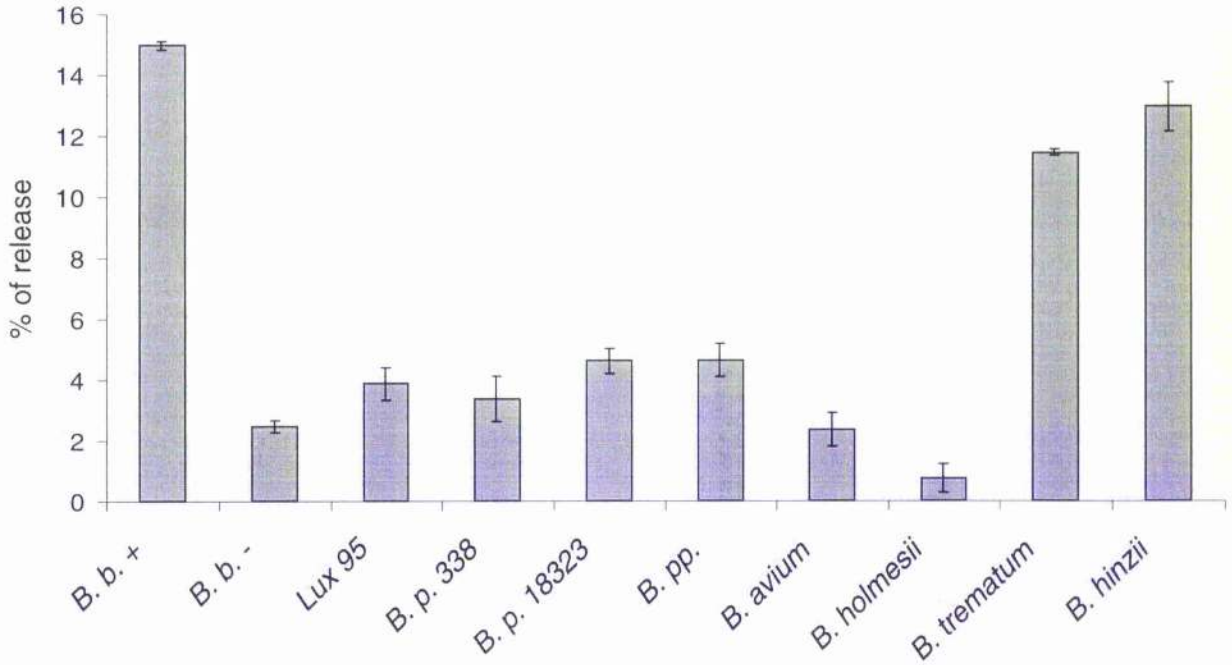

16
14
12
10
8
6
4
2
0
% of release
B. b. +
B. b. -
Lux 95
B. p. 338
B. p. 18323
B. pp.
B. avium
B. holmesii
B. trematum
B. hinzii

Figure 3.3.13

3.3.10 Comparison of the cytotoxicity of *B. bronchiseptica* with that of unrelated bacteria

The effect of *B. bronchiseptica* (Bvg^+ phase) on the viability of different mammalian cells was compared with the effect of different species of unrelated bacteria which were available in the laboratory. The ATP-bioluminescent assay was used and, as a preliminary step, it was important to avoid any interference between the light signals from the ATP content of the bacterial cells, therefore the ATP content of different bacterial strains was compared with the ATP contents of different mammalian cells. $1x10^6$ cells/ml of different mammalian cells were maintained in IMDM medium containing 10% FCS. Also, different bacterial cells grown on different growth media were collected and adjusted to $5x10^8$ cfu/ml in IMDM medium containing 10% FCS. Figure 3.3.14a shows the bioluminescence output (RLU), which reflects the ATP levels of the different mammalian and bacterial cell suspensions. The graph shows the mean of RLU from $1x10^6$ cells/ml of sheep BMMC, RBL-2H3 cells and J774A.1 cells and from $5x10^8$ cfu/ml of the different bacteria after incubation for 3 h in IMDM medium containing 10% FCS.

There was a statistically significant differences in light output from different mammalian cells compared with the light output of *B. bronchiseptica* (Bvg^+) and most of the other bacterial species and these therefore would be unlikely to interfere with the estimate of viability of the mammalian cells in this particular assay. But, there was statistically significant increase in light output of *Staphylococcus aureus* (strain 24) compared with the other bacterial species (p-value ≤ 0.001), equal to more than 50% of the RLU from sheep BMMC. This finding could suggest that the ATP-bioluminescence assay may not be useful with *S. aureus* (strain 24). It may be that *Staphylococcus aureus* (strain 24) has more rapid multiplication rate compared to the other bacteria in IMDM medium.

Figure 3.3.14a ATP content of different bacteria compared with that of different mammalian cells

$1x10^6$ cells/ml of different mammalian cells and $5x10^8$ cfu/ml of different bacteria were maintained in IMDM medium containing 10% FCS. The ATP content of different cell types was estimated by the ATP-bioluminescent assay (mean of 9 observations ± SEM).

MC: sheep bone marrow mast cells

B. bronchiseptica: wild-type strain

S. pneumoniae: wild-type strain (D39)

S. pneumoniae: pneumolysin-deficient strain (Ply^-)

S. aureus: strain 24

P. multocida: strain 85020

P. multocida: mutant strain (JRMT/2)

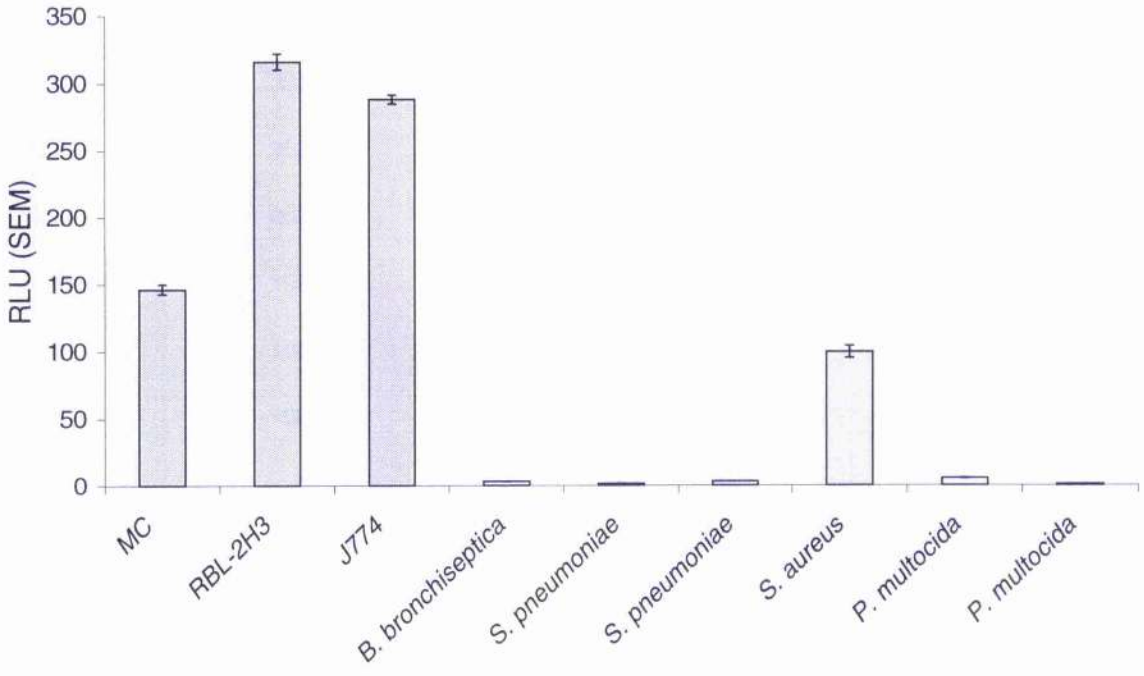


Figure 3.3.14a

3.3.10.1 Effect of different bacterial species on viability of sheep BMMC and other cell types

The effect of *B. bronchiseptica* (Bvg^+) on the viability of different mammalian cells was compared with the effect of different unrelated bacteria. Different mammalian cells were maintained in an antibiotic-free IMDM medium containing 10% FCS and cell numbers were adjusted to 10^6 cells/ml. These cells were treated with different bacterial species (grown in the appropriate growth media) at an infection ratio of 1:500 and 200 µl volumes of total mixture were added in triplicate in 96-well plates and incubated for 3 h at 37°C in 5% CO_2. At the end of the incubation time, the ATP levels (RLU) were determined by the ATP bioluminescent assay and the percentage survival of different mammalian cells was estimated. The effect of *B. bronchiseptica* (Bvg^+) on the viability of different mammalian cells was compared with the effect of virulent strains of *Streptococcus pneumoniae* (D39 wild-type) and *P. multocida* (85020). In addition, avirulent strains of *Streptococcus pneumoniae* (D39), which is pneumolysin-deficient (Ply^-) and *P. multocida* (JRMT/2) which is an avirulent *aroA* mutant, were used to compare their effect on survival of different mammalian cells.

As can be seen from figure 3.3.14b the virulent strains of *Str. pneumoniae* (D39) showed a highly cytotoxic effect towards all mammalian cell types compared with *B. bronchiseptica* (Bvg^+) and both strains caused a statistically significant (p-value ≤ 0.05) reduction in the viability of all three mammalian cells compared with the cytotoxic effect of the virulent *P. multocida* (85020) and other bacterial strains. Neither the virulent strain of *P. multocida* (85020) nor their avirulent mutant strain of *P. multocida* (JRMT) showed any significant reduction in the percentage survival of the three mammalian cells compared with the effect of *B. bronchiseptica* (Bvg^+).

The above observations suggest that pneumolysin (potent cytotoxic toxin) was the factor responsible for the dramatic reduction in the percentage survival of the three mammalian cells after incubation with *Str. pneumoniae* (D39) because the pneumolysin-deficient *Str. pneumoniae* strain did not show such a significant reduction in the percentage survival of different mammalian cells. In contrast, both virulent and mutant strain of *P. multocida* did not show any reduction in the percentage survival of mammalian cells. Neither strain is known to produce cytotoxic toxins.

Figure 3.3.14b: Effect of different bacteria on viability of sheep BMMC, RBL-2H3 cells or J774A.1 cells.

$1x10^6$ cells/ml of different mammalian cell types were infected with $5x10^8$ cfu/ml of different bacteria. The ATP content of sheep BMMC, RBL-2H3 and J774A.1 cells was estimated by the ATP-bioluminescent method (RLU) after incubation for 3 h and the percentage of survival of the infected mammalian cells was estimated (mean of 9 observations ± SEM). The effect on survival of different mammalian cells was compared with the effect of 10^{-6} M of calcium ionophore, which was used as a control.

A) *B. bronchiseptica* (Bvg^+)

B) *S. pneumoniae* (D39 wild-type)

C) *S. pneumoniae* (Ply^- mutant type)

D) *P. multocida* (85020)

E) *P. multocida* (JRMT/2)

F) Calcium ionophore

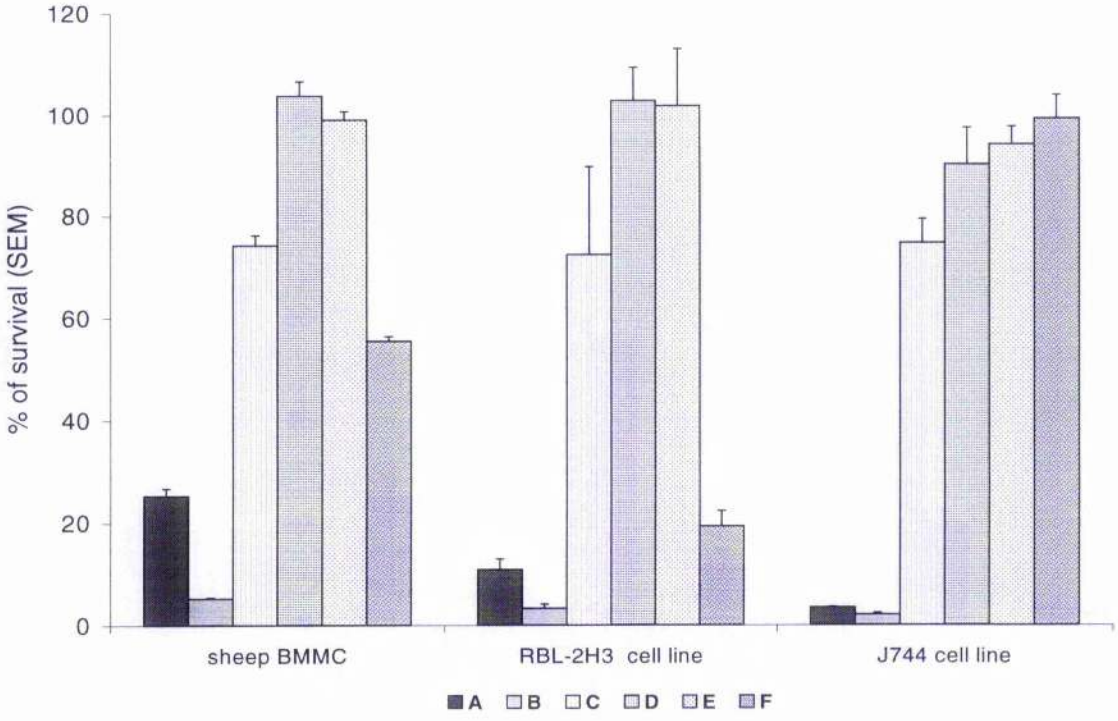


Figure 3.3.14b

3.3.10.2 Effect of different bacteria on degranulation of sheep BMMC and RBL-2H3 cells

The effect of previous bacterial strains on degranulation of sheep BMMC was investigated by estimating β-hexosaminidase release from sheep BMMC and RBL-2H3 cells treated with different bacterial strains at infection ratio of 1:500 and incubated for 3 h.

Previous observations (Fig. 3.3.9c and Fig 3.3.10c) had shown that *B. bronchiseptica* (Bvg^+ phase) caused significant β-hexosaminidase release from both sheep BMMC and RBL-2H3 cells after incubation for 3 h. But, as can seen from Fig 3.3.14c, in this particular study that there was only around 6% of the total β-hexosaminidase released from sheep BMMC with this strain. This could be due to use of a different batch of sheep bone marrow (see discussion). In contrast, there was higher β-hexosaminidase release due to the effect of both *Str. pneumoniae* strains compared with the effect of *B. bronchiseptica* (Bvg^+ phase). In fact, *Str. pneumoniae* has a gene for β-hexosaminidase production (www.tigr.net) and thus is unsuitable for use in this particular release assay. It would be necessary to use an assay to determine other release factors (e.g. histamine) to measure the effect of these bacterial strains on degranulation of sheep BMMC or RBL-2H3 cells. Neither strain of *P. multocida* stimulated β-hexosaminidase release from either sheep BMMC or RBL-2H3 cells.

Figure 3.3.14c Effect of different bacteria on β-hexosaminidase release

$1x10^6$ cells/ml of sheep BMMC or RBL-2H3 cells were treated with $5x10^8$ cfu/ml of different bacteria. β-hexosaminidase release from sheep BMMC or RBL-2H3 cells was estimated after incubation for 3 h (mean of 9 observations ± SEM). 10^{-6} M of calcium ionophore, which was used as a control.

A) *B. bronchiseptica* (Bvg^+)

B) *S. pneumoniae* (D39 wild-type)

C) *S. pneumoniae* (Ply^- mutant type)

D) *P. multocida* (85020)

E) *P. multocida* (JRMT/2)

F) Calcium ionophore

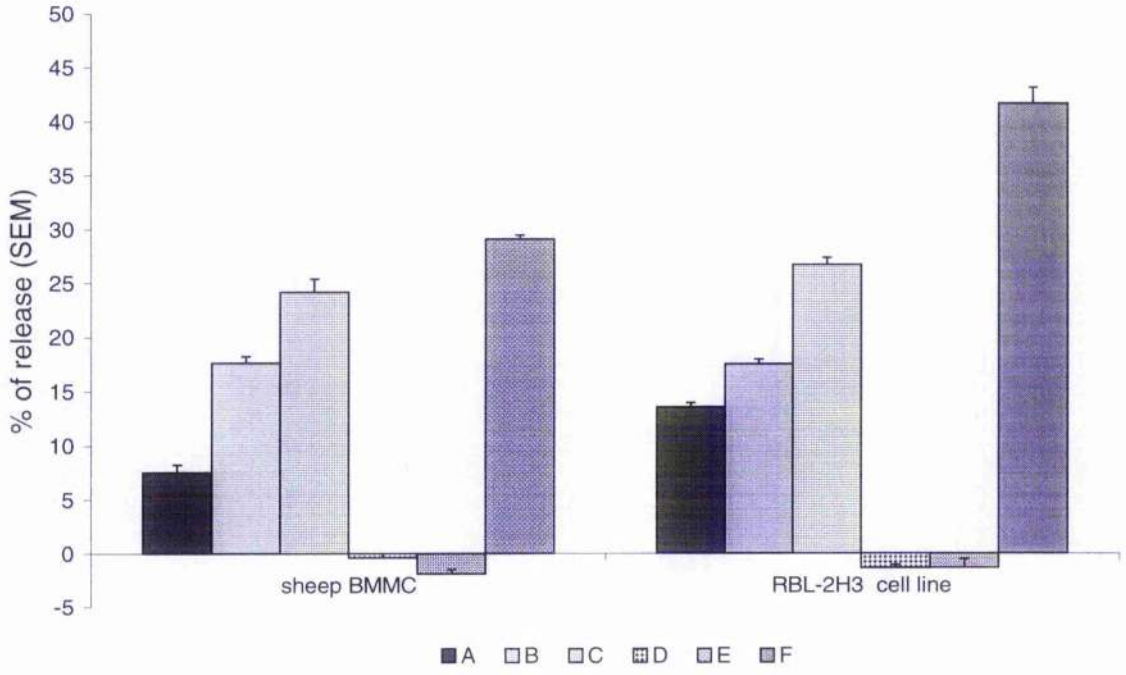


Figure 3.3.14c

Section Four: Interaction of different purified factors of *Bordetella* species with sheep BMMC and other cell types

3.4.1 Interaction of purified factors from *Bordetella* with sheep BMMC and other cell types

Previous observations (section 3 of Results) had shown that the virulent strain of *B. bronchiseptica* (Bvg$^+$ phase) has a significant cytotoxic effect on viability and degranulation of different mammalian cells. Therefore, the next aim was to investigate the effect of different *Bordetella* extracts on viability and degranulation of different mammalian cells. Different purified factors of *Bordetella* species were used:

1- Adenylate cyclase toxin (or CyaA), an anti-phagocytic factor produced by certain *Bordetella*, including *B. bronchiseptica* and *B. pertussis*.
2- Pertussis toxin (PT), produced by *B. pertussis* only. A component of all acellular pertussis vaccine (in toxoided form).
3- Pertactin (PRN), an adhesin in *Bordetella*, including *B. bronchiseptica* and *B. pertussis* and a component of some acellular pertussis vaccines.
4- Filamentous haemagglutinin (FHA), an adhesin in *Bordetella* including *B. bronchiseptica* and *B. pertussis* and a component of some acellular pertussis vaccines.

The effect of these factors on different mammalian cells were studied in different assays: viability (ATP-bioluminescent assay, MTT assay and trypan blue exclusion), degranulation (β-hexosamindase release assays) and apoptosis (Caspase 3/7 activity and DNA fragmentation). Furthermore, it has been reported that adenylate cyclase toxin can modulate immune responses and inhibit phagocytosis (Weingart and Weiss, 2000; Boyd *et al.*, 2005). Therefore, it was important to study the effect of *B. bronchiseptica* (Bvg$^+$ mode) in J774A.1 macrophage cells pre-treated with different concentrations of ACT.

3.4.1.1 Interaction of recombinant adenylate cyclase toxin with sheep BMMC and other cell types

The cytotoxic effect of purified recombinant ACT from *E. coli* against sheep BMMC and other cell types was studied. Purification of recombinant ACT was done in 8M Urea/Tris buffer and steps were taken to reduce the amount of other factors such as lipopolysaccharide of *E. coli* in the purified preparation. Therefore, as a preliminary study, it was important to investigate the effect of different factors

(Urea/Tris buffer and LPS) on the interaction of ACT with sheep BMMC and other cell types.

3.4.1.1.1 Effect of Urea/Tris buffer on viability of sheep BMMC

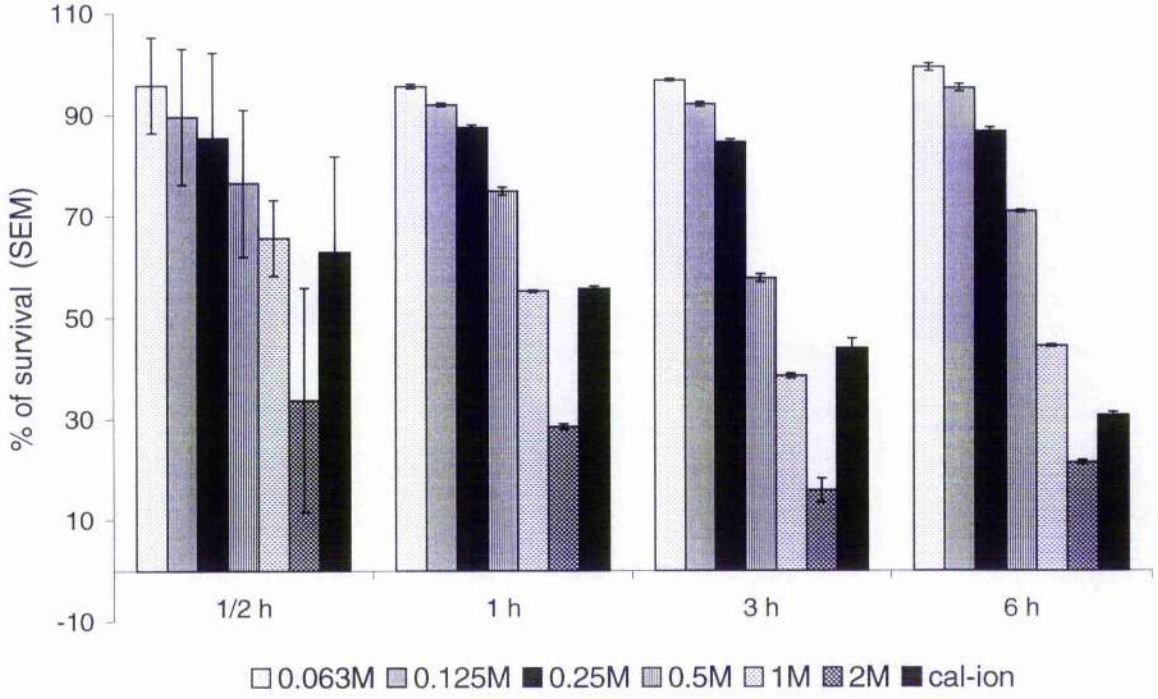
The cytotoxic effect of Urea/Tris buffer on sheep BMMC was studied. Sheep BMMC were adjusted to $1x10^6$ cells/ml in IMDM medium only (as a control for total survival) and IMDM medium containing different concentrations of Urea/Tris buffer in IMDM medium: 2 M, 1 M, 0.5 M, 0.25 M, 0.125 M and 0.063 M as a final Urea concentration. A total volume of (200 µl) of mixture was added in triplicate to 96-well plates and incubated for different times (30 min, 1 h, 3 h and 6 h) at 37°C in 5% CO_2 Viability of sheep BMMC was estimated by the ATP-bioluminescence assay and percentage survival was determined by comparison with controls of sheep BMMC in IMDM medium alone.

Figure 3.4.1a shows that statistically, there no significant effect on the percentage survival of sheep BMMC due to incubation with 0.063M of urea/Tris buffer at different incubation times, compared to cells in IMDM medium alone. IMDM medium containing 0.05-2 M Urea/Tris buffer showed a significant reduction in viability of sheep BMMC at all incubation times (p-value ≤ 0.001) compared with cells in IMDM medium containing 0.063 M Urea/Tris buffer. In contrast, 0.0125 M and 0.25 M of Urea/Tris buffer showed no significant effect compared with the effect of 0.063 M of Urea/Tris buffer.

The above results suggested that high concentrations of urea/Tris buffer in ACT preparation may have an effect on viability of different mammalian cells. Therefore, it was important to use Urea buffer in concentration equal to the content of urea/Tris buffer in the ACT preparation as controls to avoid any interference of the urea/Tris buffer in determining the effect of ACT on mammalian cells.

Figure 3.4.1a Effect of different concentrations of urea/Tris buffer on sheep BMMC viability

10^6 cells/ml of sheep BMMC were maintained in IMDM medium containing different concentrations of urea/Tris buffer. Percentage survival of sheep BMMC was estimated by the ATP-bioluminescent assay. 10^{-6}M of calcium ionophore (cal-ion) was used as control (mean of 9 observations ± SEM).


110
90
70
50
30
10
-10
% of survival (SEM)
1/2 h
1 h
3 h
6 h
0.063M
0.125M
0.25M
0.5M
1M
2M
cal-ion

3.4.1.1.2 Effect of lipopolysaccharide on cytotoxicity of rACT (44/54) for sheep BMMC

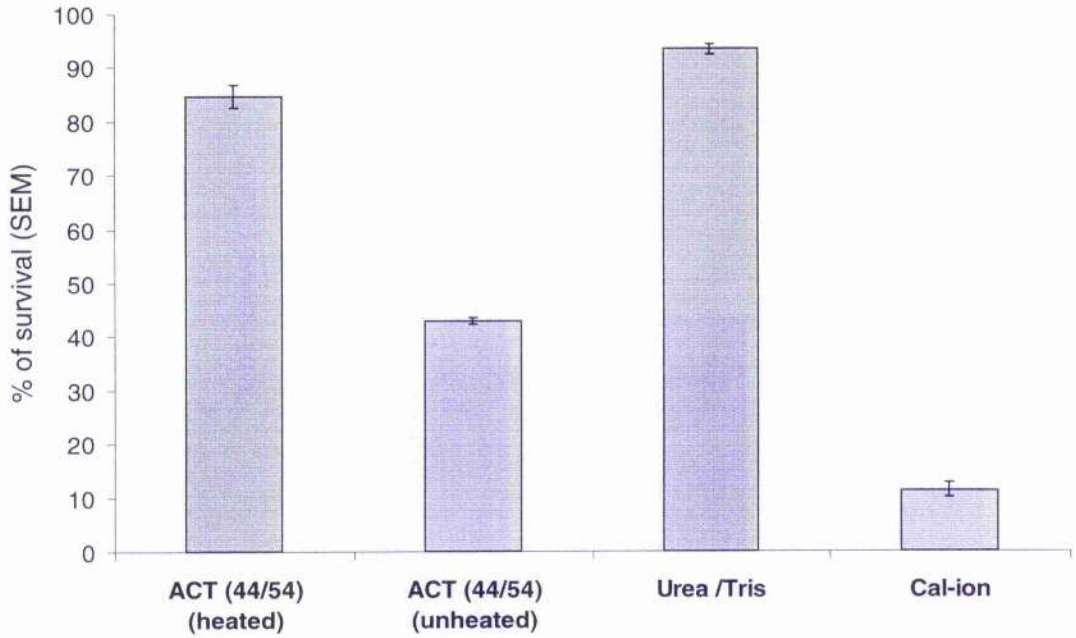
Recombinant ACT protein from *E. coli* is often contaminated with lipopolysaccharide (LPS) due to the high quantities of endotoxin in the cell envelope of this Gram-negative bacterium. This work aimed to investigate whether LPS which is heat-stable, might contribute to the cytotoxicity of rACT by examining the effect of heating on the cytotoxic activity of rACT. The probability of a cytotoxic effect due to LPS on sheep BMMC was examined by using rACT (44/54) which is enzymatically active and invasive at a final concentration of 0.156 µg.ml. One sample of rACT (44/54) suspension was kept at low temperature (icebox) and the another sample of rACT (44/54) at same concentration was heated to 100°C for 15 min. $1x10^6$ cells/ml of sheep BMMC suspension in IMDM medium containing 10 % FCS were treated with these two different rACT preparations. A total amount of 100 µl of mixture was added in triplicate to 96-well plates and incubated for 1 h at 37°C in 5% CO_2. The ATP content of sheep BMMC was estimated by the ATP-bioluminescent assay and the percentage survival was estimated by comparsion with sheep BMMC suspension in IMDM medium containing 10% FCS only. 10^{-6}M of calcium ionophore was used as a control.

0.156 µg/ml of rACT sample treated at 100°C for 15 min gave around 85% survival of sheep BMMC, whereas same concentration of rACT sample kept at low temperature gave around 40% survival of sheep BMMC. 0.03M urea in Tris buffer which was equal to the concentration of the urea/Tris buffer in the rACT preparation gave around 93% survival of sheep BMMC (Fig. 3.4.1b). 10^{-6}M of calcium ionophore was used as a control.

The above results suggested that the reduction in the percentage survival of sheep BMMC treated with unheated rACT (44/54) preparation temperature was significant compared with the reduction in survival of sheep BMMC treated with the heated rACT (44/54) sample. Therefore, these observations suggested either that there is no significant amount of LPS in the rACT sample preparation or there is no significant contribution of LPS to the cytotoxicity of rACT preparation.

Figure 3.4.1b Effect of heating on cytotoxicity of rACT (44/54)

$1x10^6$ cells/ml sheep BMMC were maintained in IMDM medium and treated with either heated rACT (to 100°C) for 15 min, unheated rACT or with the urea diluent for 1 h. At the end of the treatment, survival of sheep BMMC was estimated by the ATP-bioluminescent assay. 10^{-6}M calcium ionophore was used as control (mean of 9 observations ± SEM).


100
90
80
70
60
50
40
30
20
10
0
% of survival (SEM)
ACT (44/54) (heated)
ACT (44/54) (unheated)
Urea /Tris
Cal-ion

3.4.1.2 Effect of rACT on viability of different mammalian cells

The aim of this study was to compare cytotoxic effects of two different forms of rACT on viability and degranulation of sheep BMMC, RBL-2H3 cells and J774A.1 cells. These two forms of rACT:

1- rACT (44/54) which is **enzymatically-active** and **invasive** (AC $^{+}$/hly $^{+}$ or wild type). Both plasmid GW44 (pro-*cyaA*) and GW54 (*cyaC*) are inserted in *E. coli* to produce enzymatically active and invasive ACT.
2- rACT (44-188/54) which is **enzymatically-inactive** and **invasive** (AC $^{-}$/hly $^{+}$ or mutant type). Both plasmid GW44-188 (non-active pro-*cyaA**) and GW54 (*cyaC*) are inserted in *E. coli* to produce enzymatically inactive and invasive ACT.

Any difference in the effects of rACT (44/54) and (44-188/54) could presumably be attributed to the AC enzymic activity of the toxin.

$5x10^5$ cells/ml of sheep BMMC or other cell types (J774A.1 or RBL-2H3) were maintained in different IMDM media: IMDM containing 10% FCS only (as a control), IMDM containing 10% FCS and different concentrations of rACT (44/54) and finally IMDM containing 10% FCS and different concentrations of rACT (44-188/54). In addition, sheep BMMC (or other cell types) maintained in IMDM media containing 10 % FCS and Urea/Tris buffer at a concentration equivalent to that in highest concentration of rACT used in this experiment. Each cell mixture was incubated for 6 h at 37°C in 5 % CO_2. Viability of different mammalian cells was estimated by the ATP-bioluminescence assay. Adenylate cyclase is well known as a toxin which depends for its activity on the conversion of intracellular ATP to cAMP and causes disruption to the cell functions. Therefore, interference of AC enzymic activity with the measurement of ATP levels of mammalian cells in the ATP-bioluminescent assay is possible. Thus, the MTT assay was also used to confirm the effect of ACT on viability of different mammalian cells.

3.4.1.2.1 Sheep BMMC

By the ATP-bioluminescent method, there appeard to be 100% survival of sheep BMMC treated with 0.02 µg/ml of rACT (44/54) which was similar to the effect of the urea buffer diluent used for rACT. A similar effect was obtained with

rACT (44-188/54) (Fig. 3.4.2a). However, by this method of assessing cytotoxicity, the cytotoxic effect of rACT (44/54) toward sheep BMMC appeared to increase with increasing concentration of rACT (44/54). There appeared to be around 50% survival of sheep BMMC at 1.25µg/ml of rACT (44/54). In contrast, there appeared to be around 85% survival of sheep BMMC at same concentration of rACT (44-188/54).

The above result of a cytotoxic effect of rACT (44/54) toward sheep BMMC by the ATP assay was estimated by MTT assay. It observed that sheep BMMC were not killed by different concentrations of rACT as determined by the ATP assay. There was around 99 % survival of sheep BMMC with rACT (44/54) at 1.25 µg/ml as estimated by the MTT assay compared with the apparent 50% survival of sheep BMMC by the ATP assay (Fig. 3.4.2b). This result suggested that rACT depleted the ATP levels of sheep BMMC without affecting the dehydrogenase enzyme activity which is measured by the MTT assay.

Figure 3.4.2a Effect of rACT (44/54) or rACT (44-188/54) on survival of sheep BMMC (ATP assay)

The graph shows a comparison of the effect of different concentrations (μg/ml) of rACT (44/54) and rACT (44-188/54) on survival of sheep BMMC after 6 h incubation. Percentage of survival was estimated by the ATP-bioluminescent assay (means of 6 observations ± SD).

Figure 3.4.2b Effect of rACT (44/54) on survival of sheep BMMC (ATP assay and MTT assay)

The graph shows a comparison of the effect of different concentrations (μg/ml) of rACT (44/54) on survival of sheep BMMC after 6 h incubation. Percentage of survival was estimated by the ATP-bioluminescent assay and the MTT assay (means of 6 observations ± SD).

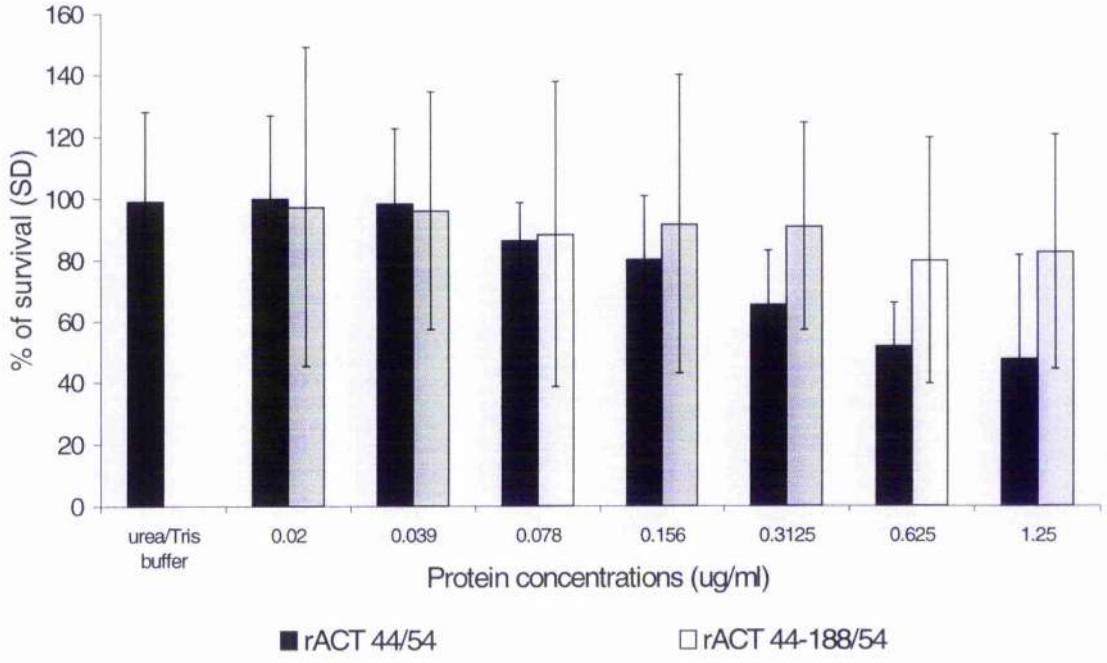


Figure 3.4.2a

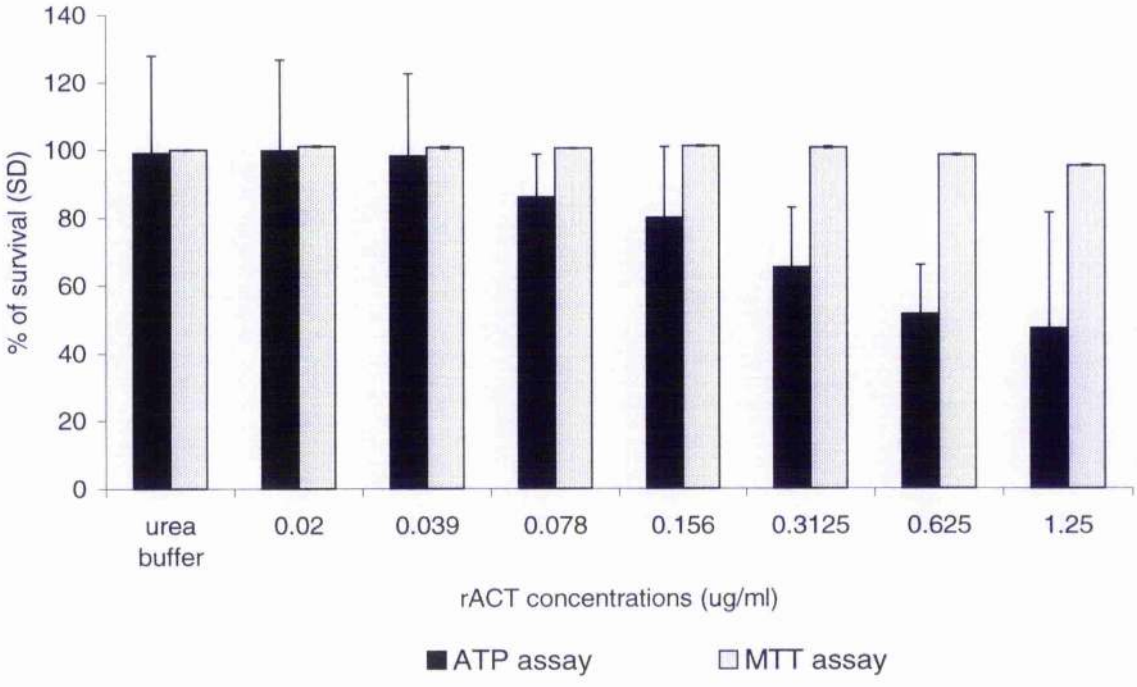


Figure 3.4.2b

3.4.1.2.2 RBL-2H3 cells

Survival of RBL-2H3 cells after incubation for 6 h with different forms of rACT was estimated by the ATP assay (Fig. 3.4.3a). There appeared to be 100% survival of RBL-2H3 cells treated in IMDM medium containing the urea buffer diluent for the rACT preparations. There was 90% survival of RBL-2H3 cells treated with 0.02 μg/ml of rACT (44/54) and there was an apparent cytotoxic effect with increasing concentrations of rACT (44/54). There was apparently 45% survival of RBL-2H3 cells at 1.25 μg/ml of rACT (44/54). In contrast, there appeared 85% survival of sheep BMMC at the same concentration of rACT (44-188/54).

The above results result of an apparent cytotoxic effect of rACT (44/54) towards RBL-2H3 cells by the ATP assay was again not confirmed by the MTT assay. These results by MTT assay showed that survival of RBL-2H3 cells gradually declined with increasing concentrations of rACT (44/54) (Fig. 3.4.3b). There was appeard to be 80% survival of RBL-2H3 cells as estimated by the MTT assay at 1.25μg/ml of rACT (44/54). In contrast, there was apparently around 45% survival of RBL-2H3 cells as estimated by the ATP assay. As in the previous section (4.1.2.1), the result suggests that rACT depleted the ATP levels of RBL-2H3 cells without affecting the dehydrogenase enzyme activity which is measured by the MTT assay.

Figure 3.4.3a Effect of rACT (44/54) or rACT (44-188/54) on survival of RBL-2H3 cells (ATP assay)

The graph shows a comparison of the effect of different concentrations (μg/ml) of rACT (44/54) and rACT (44-188/54) on survival of RBL-2H3 cells after 6 h incubation. Percentage of survival was estimated by the ATP-bioluminescent assay (means of 6 observations ± SD).

Figure 3.4.3b Effect of rACT (44/54) on survival of RBL-2H3 cells (ATP assay and MTT assay)

The graph shows a comparison of the effect of different concentrations (μg/ml) of rACT (44/54) on survival of RBL-2H3 cells after 6 h incubation. Percentage of survival was estimated by the ATP-bioluminescent assay and the MTT assay (means of 6 observations ± SD).

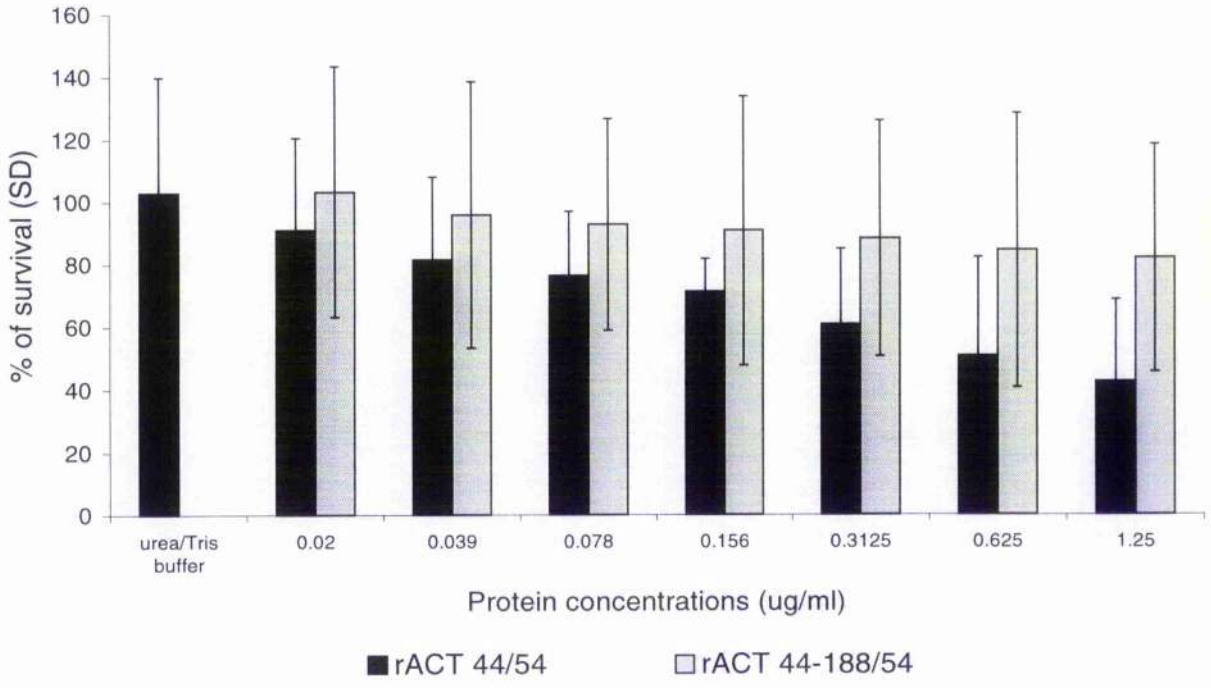


Figure 3.4.3a

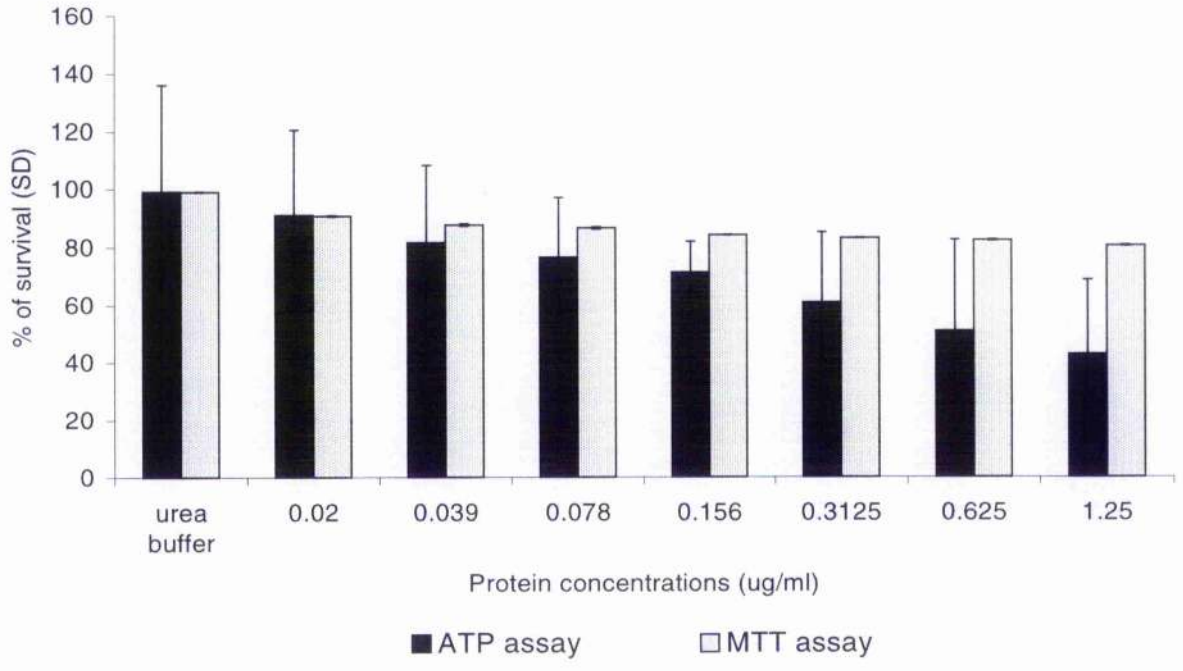


Figure 3.4.3b

3.4.1.2.3 J774A.1 cells

The percentage of survival of J774A.1 cells was apparently dramatically reduced at lower concentrations of rACT (44/54) compared with the percentage of survival of both sheep BMMC and RBL-2H3 cells at the same concentrations of rACT (44/54) when estimated by the ATP assay. There was apparently around 15% survival of J774A.1 cells at 0.02 µg/ml of rACT (44/54) compared with more than 95 % survival of sheep BMMC (Fig. 3.4.2a) and RBL-2H3 cells (Fig. 3.4.3a) at the same concentrations of rACT (44/54). The percentage of survival of J744A.1 cells declined with increased in concentrations of rACT (44/54). On the other hand, there was around 75% survival of J774A.1 cells due to the effect of 0.02 µg/ml of rACT (44-188/54). The effect of rACT (44-188/54) on depleting ATP levels of J774A.1 cells was increased with increasing of concentrations of rACT (44-188/54) (Fig. 3.4.4a).

The above effect of both rACT (44/54) and rACT (44-188/54) on viability of J774A.1 cells was also determined by the MTT assay (Fig. 3.4.4b). There was around 95 % survival of J774A.1 cells at 0.02-0.078µg/ml of rACT (44/54) estimated by MTT assay. Then, there was a dramatic decline in survival of J774A.1 cells at 0.156 µg/ml of rACT (44/54) and there was around 10% survival of J774A.1 cells at 1.25 µg/ml of rACT (44/54). In contrast, there was around 85 % survival of J774A.1 cells at 1.25 µg/ml of rACT (44-188/54).

The above observations suggest that rACT (44/54) had a significant effect on depletion of ATP levels of J774A.1 cells at low concentrations, as estimated by ATP assay but, with the MTT assay, there was only a significant cytotoxic effect of rACT (44/54) at concentrations of rACT at 0.156 µg/ml and above.

In all previous work (4.1.2.1, 4.1.2.2 and 4.1.2.3) the possibility that this killing effect may have been due to an effect of the Urea/Tris buffer diluent in the rACT preparations was excluded by using a concentration of Urea/Tris buffer equal to that in the highest concentration of rACT suspension. No significant reduction in viability of the different mammalian cell types was seen with this concentration.

These observations show that rACT (44/54) which is enzymatically-active and invasive had a much greater cytotoxic effect than rACT (44-188/54) and these effects were dose dependent. Thus, the enzymic activity of rACT is mainly responsible for its cytotoxicity, rather than its invasive (pore-forming) activity.

Figure 3.4.4a Effect of rACT (44/54) or rACT (44-188/54) on survival of J774A.1 cells (ATP assay)

The graph shows a comparison of the effect of different concentrations (μg/ml) of rACT (44/54) and rACT (44-188/54) on the apparent survival of J774A.1 cells after 6 h incubation. Percentage of survival was estimated by the ATP-bioluminescent assay (means of 6 observations ± SD).

Figure 3.4.4b Effect of different concentrations of rACT (44/54) or rACT (44-188/54) on survival of J774 cells estimated by the MTT assay

The graph shows a comparison of different concentrations of rACT (44/54) and rACT (44-188/54) on survival of J774A.1 cells at end of incubation for 6 h. Percentage of survival was estimated by the MTT assay (mean of 9 observations ± SD).

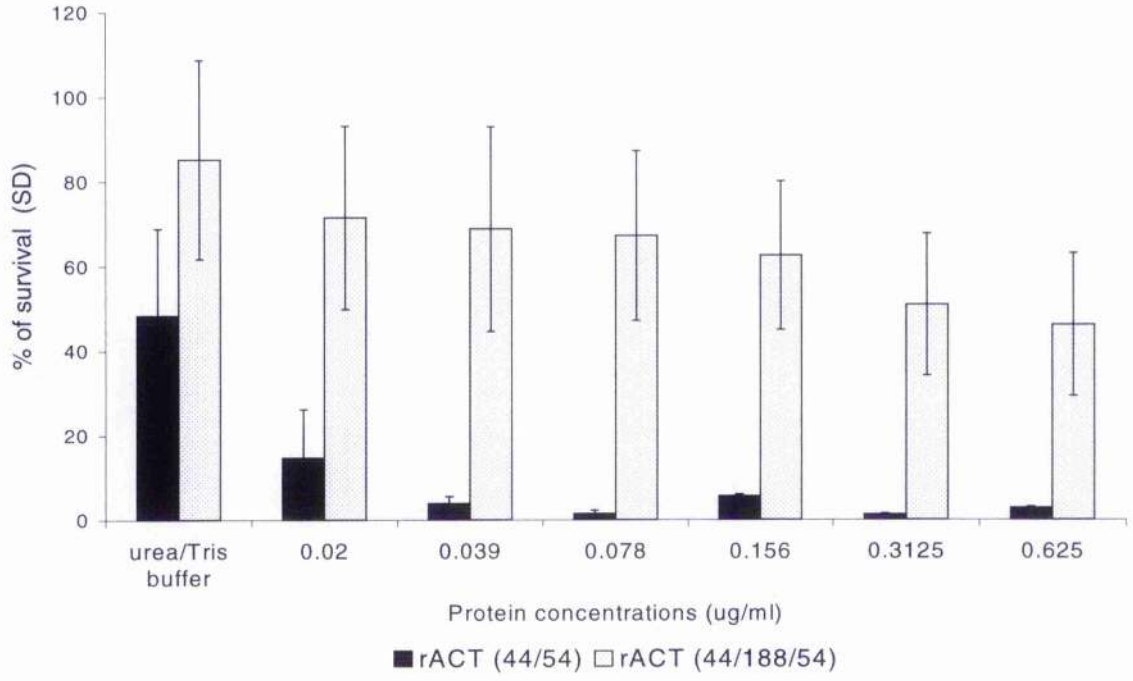


Figure 3.4.4a

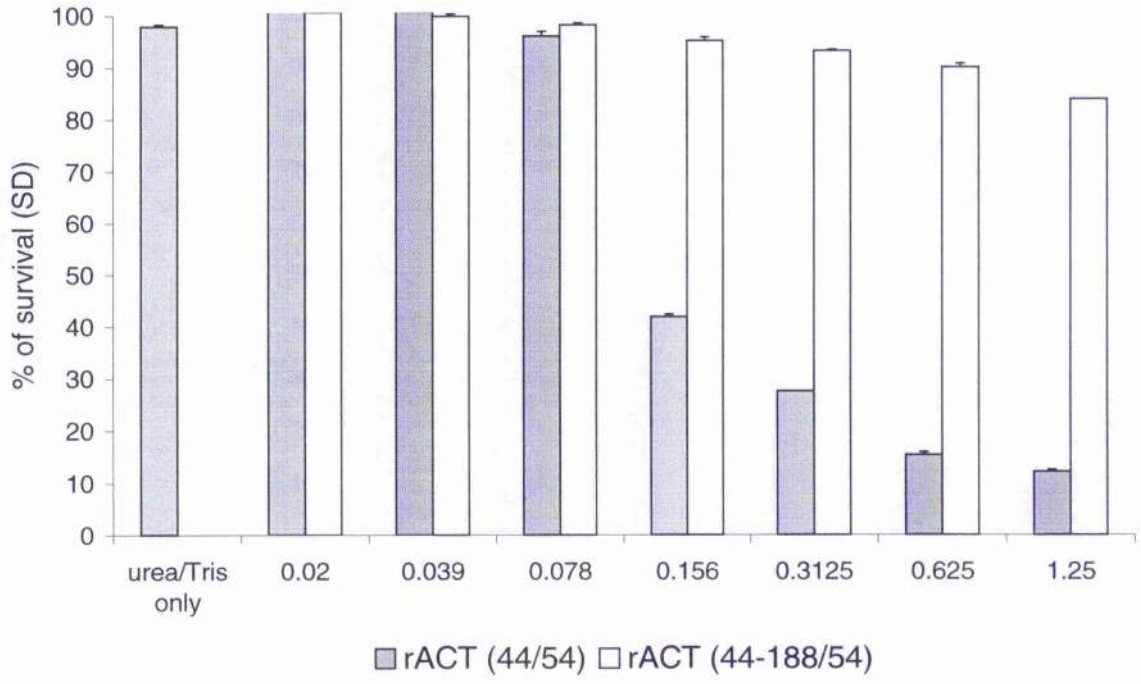


Figure 3.4.4b

3.4.1.3 Effect of rACT on caspase 3/7 activity

The ability of rACT (44/54) to activate caspase 3/7 in sheep BMMC was compared with other mammalian cells. Apoptosis (or cell-programmed death) was assessed by measuring the activity of caspase 3/7 (cysteinyl aspartate-specific proteinases) which have key effector roles in apoptosis. Briefly, caspase 3/7 present in apoptotic cells cleave a pro-fluorescent substrate to produce a fluorescent signal. The amount of fluorescent product generated is proportional to the amount of caspase 3/7 cleavage activity present in the sample and therefore reflects apoptotic activity. $5x10^5$ cells/ml of sheep BMMC or other cell types (J774A.1 or RBL-2H3) were maintained in IMDM medium containing 10% FCS with different concentrations of either rACT (44/54) or rACT (44-188/54) and incubated for 6 h at 37°C in 5 % CO_2. Also, sheep BMMC and other cell types were maintained in IMDM medium containing 10 % FCS as a control. At the end of the incubation time, the amount of fluorescent product generated, which is proportional to the amount of caspase 3/7 substrate cleavage was estimated as relative fluorescent light units (RFLU). Figures 3.4.5a (sheep BMMC), 3.4.5b (RBL-2H3) and 3.4.5c (J774A.1) show caspase 3/7 activity due to the effect of different forms of rACT activity.

Figure 3.4.5a shows little if any activity of caspase 3/7 in sheep BMMC after incubation with two different forms of rACT and no dose response effect with either form of rACT. Thus, this assay provided no evidence that rACT induces apoptosis in sheep BMMC (Fig. 3.4.5a). In contrast, rACT (44/54) showed a significant effect in activating caspase 3/7 activity cleavage in RBL-2H3 cells (Fig. 3.4.5b). It can be seen from this figure that there was very high induction of caspase 3/7 activity due to treatment with different concentrations of rACT (44/54) whereas, with rACT (44-188/54) there was a lower but significant effect. These observations suggested that the enzymatic activity of rACT is the most important factor to promote caspase 3/7 activity in RBL-2H3 cells. The most important finding is that there is very high caspase activity with rACT (44/54) and it increased with increasing concentrations of rACT (44/54).

In contrast, the effect of different forms of rACT on caspase activity was very different on J774A.1 cells (Fig. 3.4.5c). The rACT (44/54) showed a peak effect at

0.078 μg/ml, but this effect decreased with increasing concentrations of rACT (44/54). Similar to sheep BMMC and RBL-2H3 cells, rACT (44-188/54) showed a very low effect on caspase 3/7 activity of J774A.1 cells and no dose response effect. These observations suggested that, at low concentration of rACT (44/54), there was an apoptotic effect in J774A.1 macrophage cells but, with increasing in concentrations of rACT (44/54), there was a possibility of necrotic effect.

These observations showed, three different effects on three cell types. The effect of rACT (44/54) in promoting caspase 3/7 activity (and possibility a poptosis) depends on cell type, and this activity was dose-dependent. Activation of caspase 3/7 in RBL-2H3 cells and J774A.1 cells depended primarily on the enzymatic activity of rACT (possessed by 44/54), rather than on its pore-forming activity (a property of both forms). The possibility that this caspase 3/7 activity may have been due to an effect of the urea in the preparation was excluded by the results obtained by using the two forms of rACT which contained the same concentrations of Urea/Tris buffer.

Figure 3.4.5a Effect of rACT on caspase 3/7 activity of sheep BMMC

The graph shows the effect of different concentrations of rACT (44/54) or rACT (44-188/54) on $5x10^5$ cells/ml of sheep BMMC after incubation for 6 h. Caspase 3/7 activity was estimated as relative fluorescent light units (RFLU) (mean for 6 observations ± SEM).

Figure 3.4.5b Effect of rACT on caspase 3/7 activity of RBL-2H3 cells

The graph shows the effect of different concentrations of rACT (44/54) or rACT (44-188/54) on $5x10^5$ cells/ml of RBL-2H3 cells after incubation for 6 h. Caspase 3/7 was estimated as relative fluorescent light units (RFLU) (mean for 6 observations ± SEM).

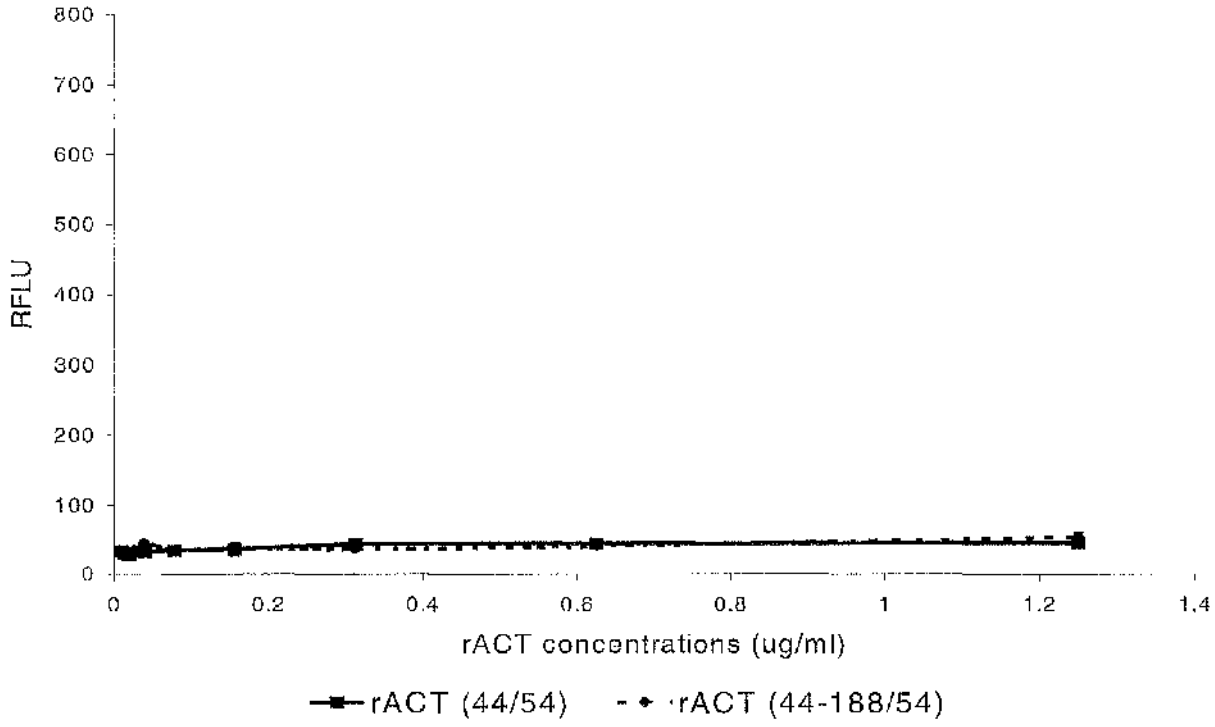


Figure 3.4.5a

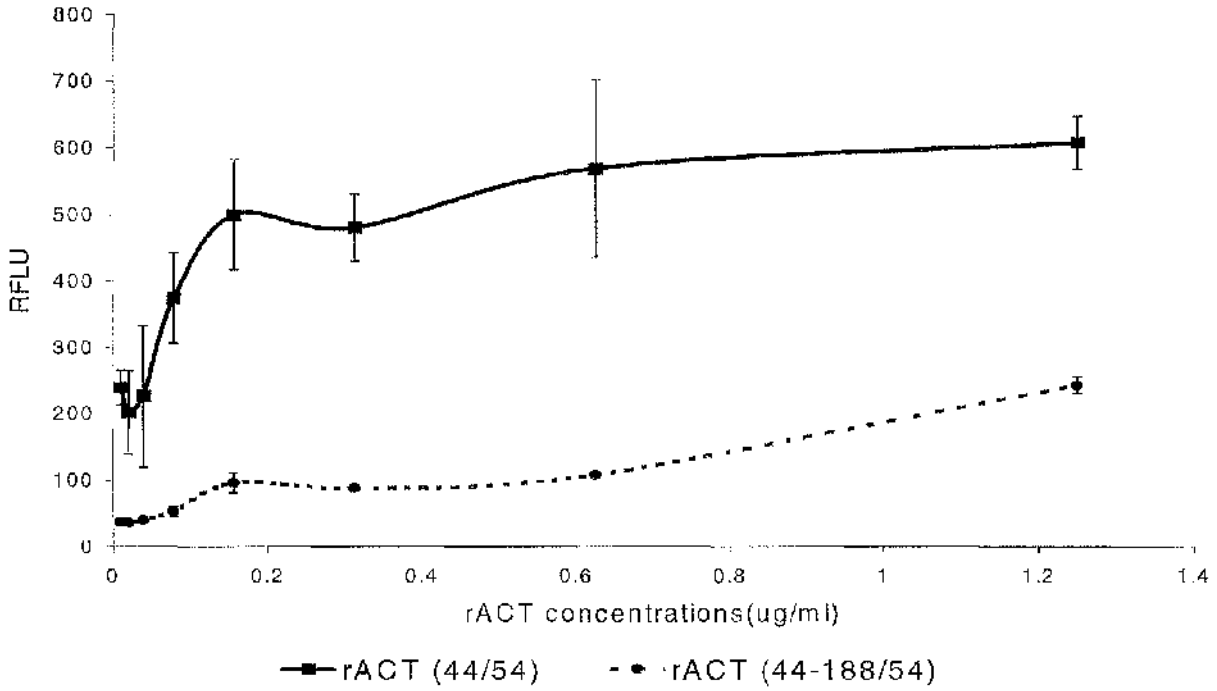
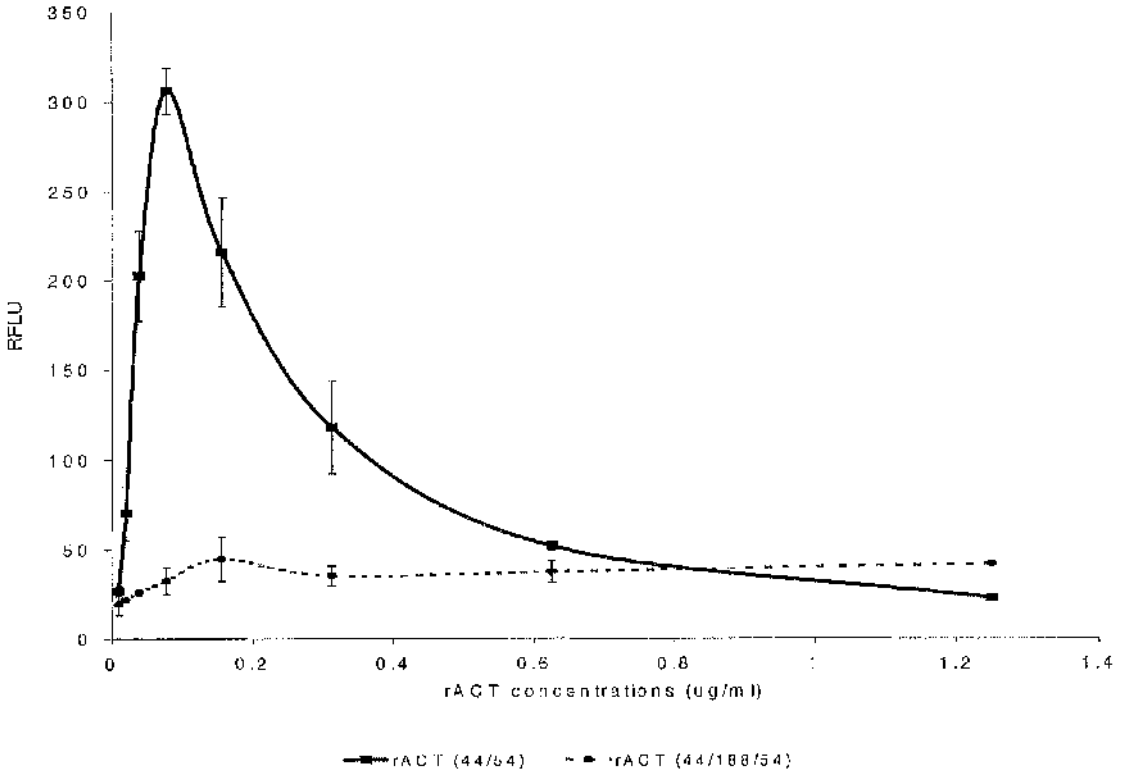


Figure 3.4.5b

Figure 3.4.5c Effect of rACT on caspase 3/7 activity of J774A.1 cells

The graph shows the effect of different concentrations of rACT (44/54) or rACT (44-188/54) on $5x10^5$ cells/ml of J774A.1 cells after incubation for 6 h. Caspase 3/7 was estimated as relative fluorescent light units (RFLU) (mean for 6 observations ± SEM).


350
300
250
200
150
100
50
0
RFLU
0
0.2
0.4
0.6
0.8
1
1.2
1.4
rACT concentrations (ug/ml)
rACT (44/54)
rACT (44/188/54)

3.4.1.4 Effect of rACT on DNA fragmentation

Apoptosis was further assessed by studying the effect of the two different forms of rACT on genomic DNA and its fragmentation, which is another hallmark of apoptosis. $1x10^6$ cells/ml of sheep BMMC (or RBL-2H3 cells) were maintained in IMDM medium containing 10 % FCS and either 0.078 µg/ml or 0.39 µg/ml (as final concentrations) of either rACT (44/54) or rACT (44-188/54). Cell mixtures were incubated for 6 h at 37°C in 5% CO_2. At the end of the incubation, DNA was extracted and separated by agarose gel electrophoresis. DNA fragmentation was visualized by staining with ethidium bromide (Fig. 3.4.6a).

3.4.1.4.1 Sheep BMMC

There was evidence of DNA fragmentation with sheep BMMC after incubation with 0.078 µg/ml of rACT (44/54) (Lane 8), but not with the higher concentration of rACT (44/54) (Lane 7). In contrast, there were similar, but faint bands with sheep BMMC after incubation with different concentrations of rACT (44-188/54) (Lanes 9 and 10). Little if any fragmentation effect due to the Urea/Tris buffer was observed (Lane 6) (Fig. 3.4.6a).

3.4.1.4.2 RBL-2H3 cells

0.39 µg/ml (lane 2) and 0.078 µg/ml (lane 3) of rACT (44/54) showed marked DNA fragmentations with RBL-2H3 cells. In contrast, similar concentrations of rACT (44-188/54) did not show any DNA fragmentation with RBL-2H3 cells (Lanes 4 and 5). Also, the concentration of urea/Tis buffer which was equal to the Urea/Tris buffer present in the highest concentration of rACT (44/54) as used in this experiment did not show any DNA fragmentation with RBL-2H3 cells (Lane 1) (Fig. 3.4.6a).

3.4.1.4.3 J774A.1 cells

Furthermore, the effect of 0.078 µg/ml of rACT (44/54) on genomic DNA of J774A.1 macrophage cells was investigated and compared with that on genomic DNA of sheep BMMC. Figure 3.4.6b shows no evidence for genomic DNA fragmentation from J774A.1 cells after incubation with 0.078 µg/ml of rACT (44/54) for 6 h (Lane

4) and no evidence for DNA fragmentation in J774A.1 cells due to the Urea/Tris buffer was observed (Lane 5). In contrast, there were faint bands (lane 2) in sheep BMMC after incubation with 0.078 µg/ml of rACT (44/54) and no bands of DNA fragmentation in sheep BMMC due to the Urea/Tris buffer (Lane 3).

The above observations suggested that the enzymatic activity of rACT (44/54) is important factor to promoting caspase activity in RBL-2H3 cells and also genomic DNA fragmentation in RBL-2H3 cells compared with the effect of non-enzymatically active rACT (44-188/54). In contrast, with the low concentration of rACT (44/54), there was evidence for genomic DNA fragmentation in sheep BMMC, even though these cells gave little caspase activity. J774A.1 cells showed high caspase activity with low concentrations of rACT (44/54) but this was not reflected in strong DNA fragmentation activity after gel electrophoresis of genomic DNA.

Figure 3.4.6a The effect of rACT (44/54) or (44-188/54) on genomic DNA of sheep BMMC or RBL-2H3 cells

The following figures shows the effect of 0.078 µg/ml or 0.39 µg/ml of rACT (44/54) or (44-54/188) for 6 h on the DNA structure of sheep BMMC or RBL-2H3 cells.

1: urea/Tris buffer on RBL-2H3

2: 0.39 µg/ml of rACT (44/54) on RBL-2H3

3: 0.078 µg/ml of rACT (44/54) on RBL-2H3

4: 0.39 µg/ml of rACT (44-188/54) on RBL-2H3

5: 0.078 µg/ml of rACT (44-188/54) on RBL-2H3

6: urea/Tris buffer on Sheep BMMC

7: 0.39 µg/ml of rACT (44/54) on Sheep BMMC

8: 0.078 µg/ml of rACT (44/54) on Sheep BMMC

9: 0.39 µg/ml of rACT (44-188/54) on Sheep BMMC

10: 0.078 µg/ml of rACT (44-188/54) on Sheep BMMC

11: DNA marker (control).

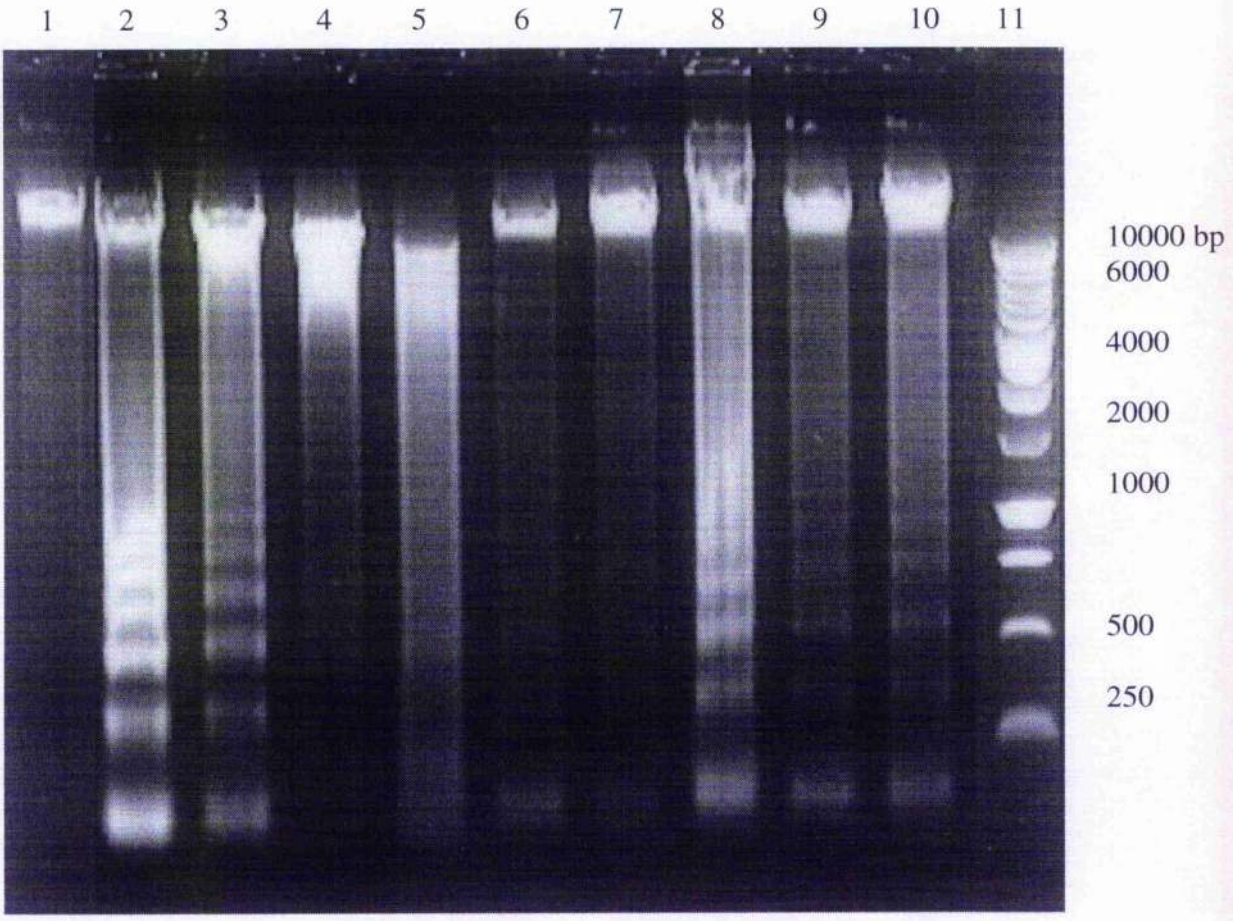

1
2
3
4
5
6
7
8
9
10
11
10000 bp
6000
4000
2000
1000
500
250

Figure 3.4.6b Effect of rACT on genomic DNA of sheep BMMC and J774A.1 cells

0.078 µg/ml of rACT (44/54) was incubated for 6h with 10^6 cells/ml of sheep BMMC and J774 cells. DNA was extracted and separated by agarose gel electrophoresis and DNA fragmentation was visualized by staining with ethidium bromide.

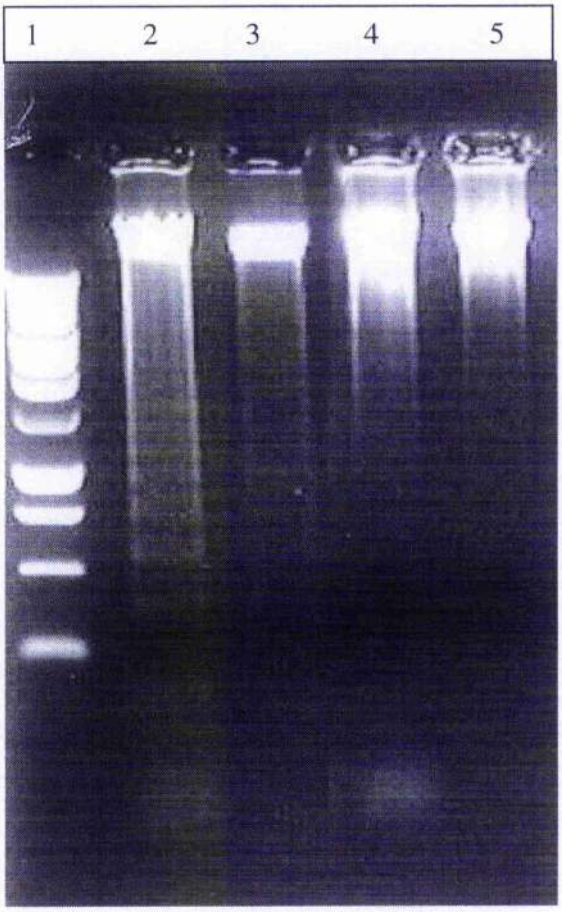
1) DNA marker

2) 0.078 µg/ml of rACT (44/54) with sheep BMMC

3) urea buffer with sheep BMMC

4) 0.078 µg/ml of rACT (44/54) with J774A.1 cell line

5) urea buffer with J774 cell line


1
2
3
4
5

3.4.1.5 Effect of rACT on β-hexosaminidase release

Unfortunately, at this stage in the project, the supply of sheep BMMC was stopped and further observations had to be done with either RBL-2H3 or J774A.1 cells. The effect of rACT on degranulation of RBL-2H3 cells was investigated. Three different forms of rACT were available:

44/54: **enzymatically-active** and **invasive**.

44-188/54: **enzymatically-inactive** and **invasive**.

44-188: **enzymatically-inactive** (AC^-).

$1x10^6$ cells/ml of RBL-2H3 were maintained in IMDM medium containing 10 % FCS (as control) or were treated with different forms of rACT at 0.1μg/ml. In addition, RBL-2H3 cells were treated with the Urea buffer diluent equivalent to that in the rACT (44/54) preparation. The RBL-2H3 cell suspensions were incubated for 3 h at 37°C in 5% CO_2. At the end of the incubation time, β-hexosaminidase release was estimated.

Figure 3.4.7 shows the effect of different forms of rACT. The spontaneous release of β-hexosaminidase was apparently inhibited from RBL-2H3 as a result of incubation with 0.1μg/ml rACT 44/54 indicated by the negative value for this sample but there was spontaneous release from RBL-2H3 when incubated with other forms of rACT (44/188) or (44-188/54) or buffer containing the 0.04M Urea/Tris concentration equivalent to that in the rACT preparation. There was a statistically-significant difference between the inhibitory effect of rACT (44/54) and the effect of rACT (44/188) (student *t*-test, p-value ≤ 0.05).

These observations again show the importance of enzymic activity of rACT (44/54) in inhibition of degranulation of the RBL-2H3 cells.

Figure 3.4.7 Effect of different forms of rACT on release of β-hexosaminidase from RBL-2H3 cells

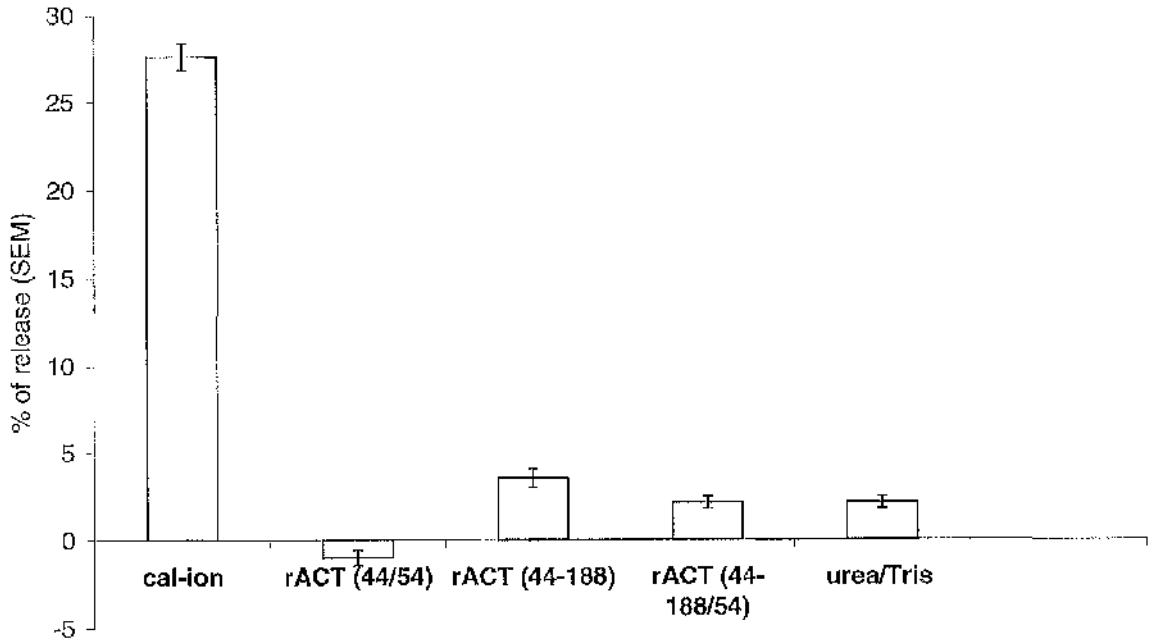
This graph shows the percentage release of β-hexosaminidase from RBL-2H3 cells ($1x10^6$ cells/ml) in IMDM medium due to incubation with 0.1 μg/ml of different forms of rACT for 3 h (mean of 9 observations ± SEM). These forms were

rACT (44/54): enzymatically-active and invasive

rACT (44-188): enzymatically-inactive and non-invasive

rACT (44-188/54): enzymatically-inactive and invasive

cal-ion : 10^{-6}M of Calcium-ionophore


30
25
20
15
10
5
0
-5
% of release (SEM)
cal-ion
rACT (44/54)
rACT (44-188)
rACT (44-188/54)
urea/Tris

3.4.2 Cytotoxic effect of different purified virulence factors of *Bordetella* toward RBL-2H3 cells and J774A.1 cells

In these exeriments, RBL-2H3 cells were used as a model for mast cells and J774A.1 macrophage cells were used as controls to study whether different purified *Bordetella* virulence factors have any cytotoxic effects on viability (by ATP bioluminescence assay and MTT assay), on apoptosis (caspase 3/7 activity and DNA fragmentation) or on degranulation (β-hexosaminidase release). RBL-2H3 cells (and J774A.1 cells) maintained in IMDM medium containing 10 % FCS were treated with 0.1 μg/ml or 0.01 μg/ml (as a final concentrations) of PT or FHA or PRN and the mixtures incubated for different times (3 h and 6 h) at 37°C in 5% CO_2. In some experiments, the effect of these different *Bordetella* factors was compared with the effect of rACT (44/54) and 10^{-6}M of calcium ionophore as controls.

3.4.2.1 RBL-2H3 cells

Figure 3.4.8a shows the effect of 0.01 μg/ml and 0.1 μg/ml of PT, FHA or PRN on the ATP content (by ATP-bioluminescent assay) of RBL-2H3 cells at end of incubation for 3 h and 6 h and compared with the effect of 10^{-6}M of calcium ionophore. Neither concentrations of PT had any major influence on the ATP content of RBL-2H3 cells after incubation for 3 h and 6 h. With 0.1μg/ml FHA, there appeared to be 15 % reduction in the survival of RBL-2H3 cells at end of incubation for 3 h and appeared to be 13 % reduction in survival at end of incubation for 6 h. At 0.1μg/ml PRN, there appeared to be 10 % reduction survival of RBL-2H3 cells at end incubation for 3 h but, no major influence was appeared at end of incubation for 6 h. These observations with both FHA and PRN on survival of RBL-2H3 cells could suggest that the initial apparent reduction was more an effect on metabolic activity of RBL-2H3 cells than killing.

The above results on RBL-2H3 cells were further investigated by MTT assay (Fig. 3.4.8b). As in the ATP assay, there was no major influence of 0.01 μg/ml of PT on the percentage survival of RBL-2H3 cells at 3 h and 6 h. Both FHA and PRN showed similar effect (i.e. there was around 5-10 % reduction in the percentage

survival of RBL-2H3 cells at 3 h with 0.01 μg/ml of both FHA and PRN, but these reductions were not observed at 6 h). With 0.1 μg/ml of PT there was less than 10 % reduction in survival of RBL-2H3 cells at end of incubation for 3 h and 6 h. These results of PT suggested that a possible small killing effect of PT is dose and time dependent. There was no significant killing effect of either concentration of FHAor PRN (0.1 μg/ml and 0.01μg/ml) at 3 h and 6 h.

Figure 3.4.8a Effect of different purified virulence *Bordetella* factors on ATP content of RBL-2H3 cells (ATP-assay)

RBL-2H3 cells were maintained in IMDM and treated with 0.01 µg/ml and 0.1 µg/ml of different purified *Bordetella* factors (PT, FHA and PRN) and incubated for different times (3 h and 6 h). ATP level of RBL-2H3 cells was estimated with ATP-bioluminescent assay and the percentage survival was calculated by comparison with RBL-2H3 cells in IMDM medium. 10^{-6}M of calcium ionophore was used as a control. (mean of 9 observations ± SEM)

Figure 3.4.8b Effect of different purified virulence *Bordetella* factors on viability of RBL-2H3 cells (MTT-assay)

RBL-2H3 cells were maintained in IMDM and treated with 0.01 µg/ml and 0.1 µg/ml of different purified *Bordetella* factors (PT, FHA and PRN) and incubated for different times (3 h and 6 h). The percentage of survival of RBL-2H3 cells was calculated by comparison with RBL-2H3 cells in IMDM medium (MTT assay). 10^{-6}M of calcium ionophore was used as a control. (mean of 9 observations ± SEM)

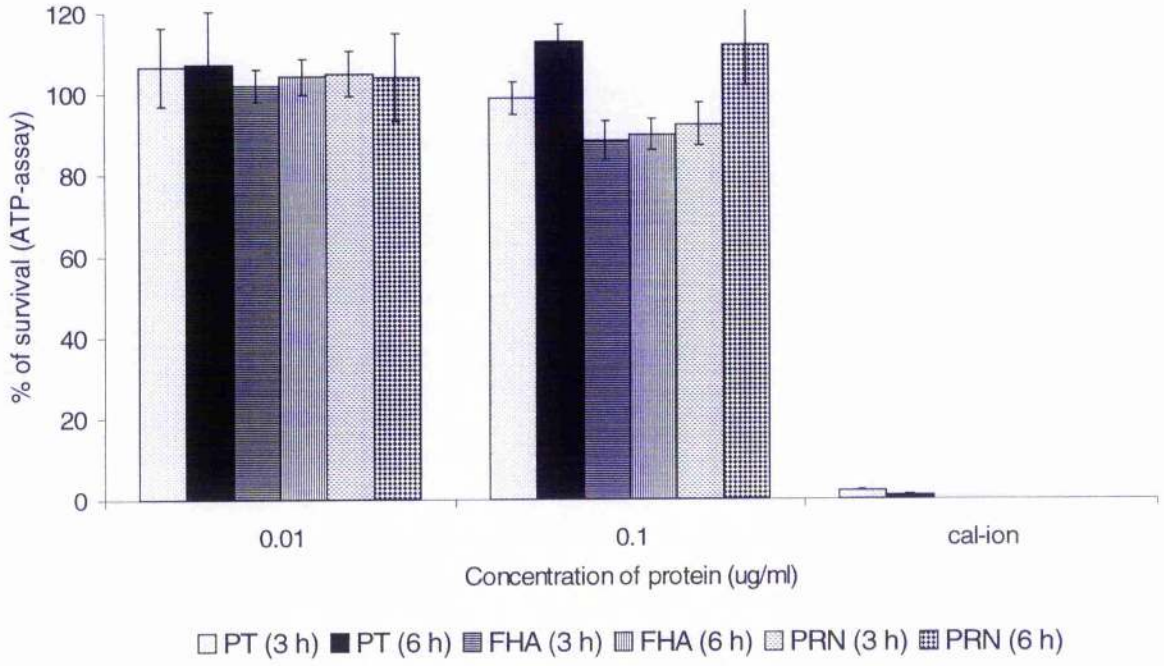


Figure 3.4.8a

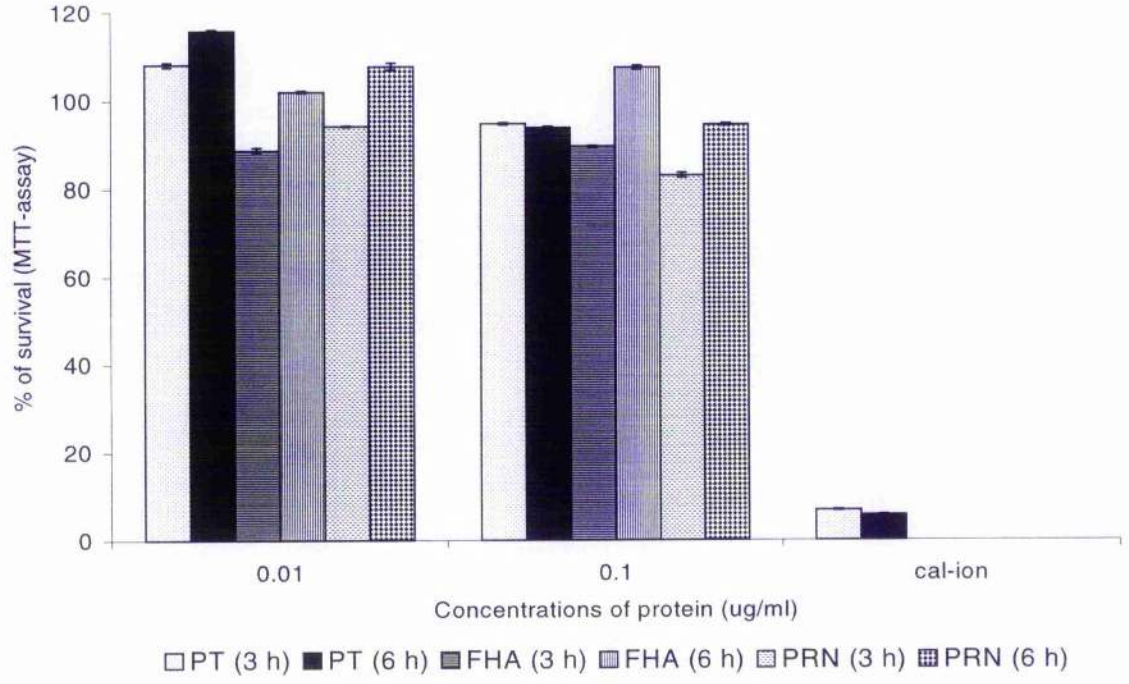


Figure 3.4.8b

3.4.2.2 J774A.1 cells

Figure 3.4.8c shows that 0.01 µg/ml of PT caused around 15 % reduction in the ATP level at both 3 h and 6 h. There was no effect observed at 0.01µg/ml of FHA at 3 h incubation, but there was around 10 % reduction at 6 h. With PRN, there was around 20 % reduction in ATP contents 3 h and this reduction was increased to 30 % in 6 h. In contrast, 0.1µg/ml of PT and PRN significantly reduced the ATP level compared with the effect of 0.1µg/ml of FHA. These observations suggested that the effect of both PT and PRN is dose and time depndent. In contrast, 0.1µg/ml of FHA showed similar observation to 0.01µg/ml after 3 h and 6 h.

The above observations on J774A.1 cells were further investigated by the MTT assay (Fig. 3.4.8d). There was around 10 % reduction in the percentage survival at 3 h and 6 h due to 0.01µg/ml of PT. 0.01µg/ml and 0.1µg/ml of FHA did not show any influence in survival of J774A.1 cells as determined by MTT assay. There appeared to be 20 % reduction in survival due to 0.01µg/ml of PRN, but no effect was observed after 6 h. But, with 0.1µg/ml PRN, there was 20 % reduction in survival after 3 h and 30 % reduction after 6 h. 10^{-6} M of calcium ionophore showed time dependent killing effect in the percentage survival of J774A.1 cells by both assays.

The above observations suggested that the effect of different purified *Bordetella* virulence factors (PT, FHA and PRN) in the survival of RBL-2H3 cells and J774A.1 cells were different. These differences could be due to the availability of receptors on the cell surface of the two cell types and further work should be done to investigate such differences.

Figure 3.4.8c Effect of different purified virulence *Bordetella* factors on ATP content of J774A.1 cells (ATP-assay)

J774A.1 cells were maintained in IMDM and treated with 0.01 µg/ml and 0.1 µg/ml of different purified *Bordetella* factors (PT, FHA and PRN) and incubated for different times (3 h and 6 h). The ATP content of J774A.1 cells was estimated with the ATP-bioluminescent assay and the percentage survival of J774A.1 cells was calculated by comparison with J774A.1 cells in IMDM medium. 10^{-6}M of calcium ionophore was used as a control. (mean of 9 observations ± SEM)

Figure 3.4.8d Effect of different purified virulence *Bordetella* factors on viability of J774A.1 cells (MTT-assay)

J774A.1 cells were maintained in IMDM and treated with 0.01 µg/ml and 0.1 µg/ml of different purified *Bordetella* factors (PT, FHA and PRN) and incubated for different times (3 h and 6 h). The percentage of survival of J774A.1 cells was calculated by comparison with J774A.1 cells in IMDM medium estimated (MTT assay). 10^{-6}M of calcium ionophore was used as a control. (mean of 9 observations ± SEM)

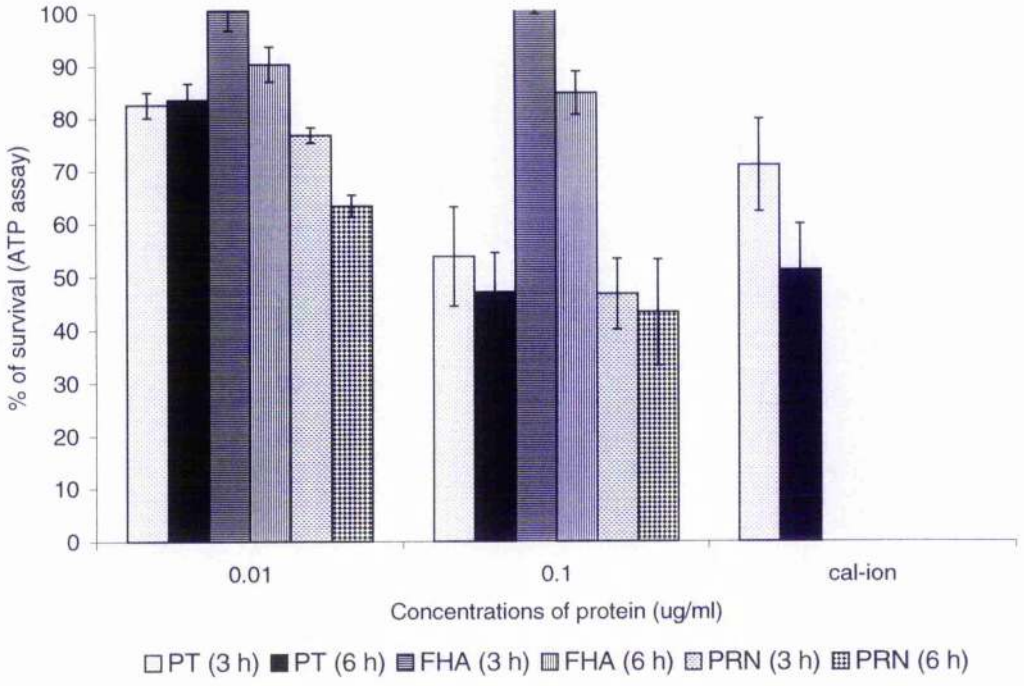


Figure 3.4.8c

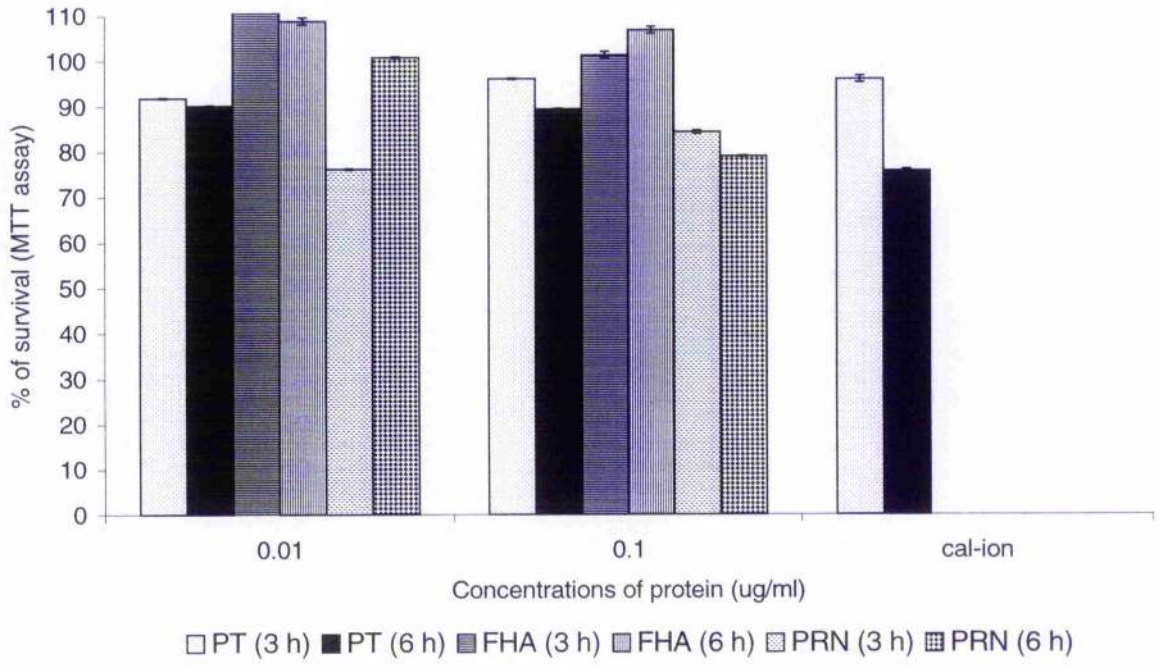


Figure 3.4.8d

3.4.2.3 Effects of different purified factors on caspase 3/7 activity

The effect of different concentrations 0.001 µg/ml, 0.01 µg/ml and 0.1 µg/ml of different purified virulence of *Bordetella* factors (PT, FHA and PRN) on activation of caspases 3/7, which drives the effector phase of apoptosis in different mammalian cells was studied. $5x10^5$ cells/ml of sheep BMMC, RBL-2H3 and J774A.1 were maintained in IMDM medium containing 0.001 µg/ml or 0.01 µg/ml or 0.1 µg/ml (as final concentrations) and incubated for 6 h at 37°C in 5% CO_2. At the end of incubation, caspase 3/7 activity was estimated as RLFU. Figure 3.4.9 shows PT increased caspase 3/7 activity only in the RBL-2H3 cells compared with sheep BMMC and J774A.1 cells. Sheep BMMC or J774A.1 cells showed only basal caspase 3/7 activity with different concentrations of PT. The three cell types showed basal caspase 3/7 activity with different concentrations of FHA and PRN (data not shown).

3.4.2.4 Effect of PT on DNA structure of RBL-2H3 cells

In order to confirm the above findings, DNA fragmentation in RBL-2H3 cells due to incubation with PT and rACT (44/54) was evaluated. 10^6 cells/ml of RBL-2H3 cells were maintained in IMDM medium containing 10% FCS, mixed with 0.1 µg/ml of PT or 0.39 µg/ml of rACT (44/54) and incubated for 6 h. In addition, RBL-2H3 cells mixed with 0.1 µg/ml of PT were incubated for 1 h, then 0.39 µg/ml of rACT (44/54) was added to the mixture and incubated for another 5 h (total incubation time was 6 h). At the end of incubation, DNA was extracted and separated by agarose gel electrophoresis and DNA fragmentation was visualized by staining with ethidium bromide. Figure 3.4.10, shows distinct nuclear DNA fragmentation in RBL-2H3 cells (Lane 4 and Lane 5) after incubation for 6 h with either concentration of PT or rACT (44/54) or both together. These observations suggest that PT activates caspase 3/7, which drives the effector phase of apoptosis of RBL-2H3 cells.

Figure 3.4.9 Effect of PT on caspase 3/7 activity of different mammalian cells (apoptosis assay)

The graph shows the effect of different concentrations, 0.001, 0.01 and 0.1 μg/ml of PT on caspase 3/7 activity of sheep BMMC, RBL-2H3 and J774A.1 cells at $5x10^5$ cells/ml and incuabted for 6 h. (Mean of 9 observations ± SEM).

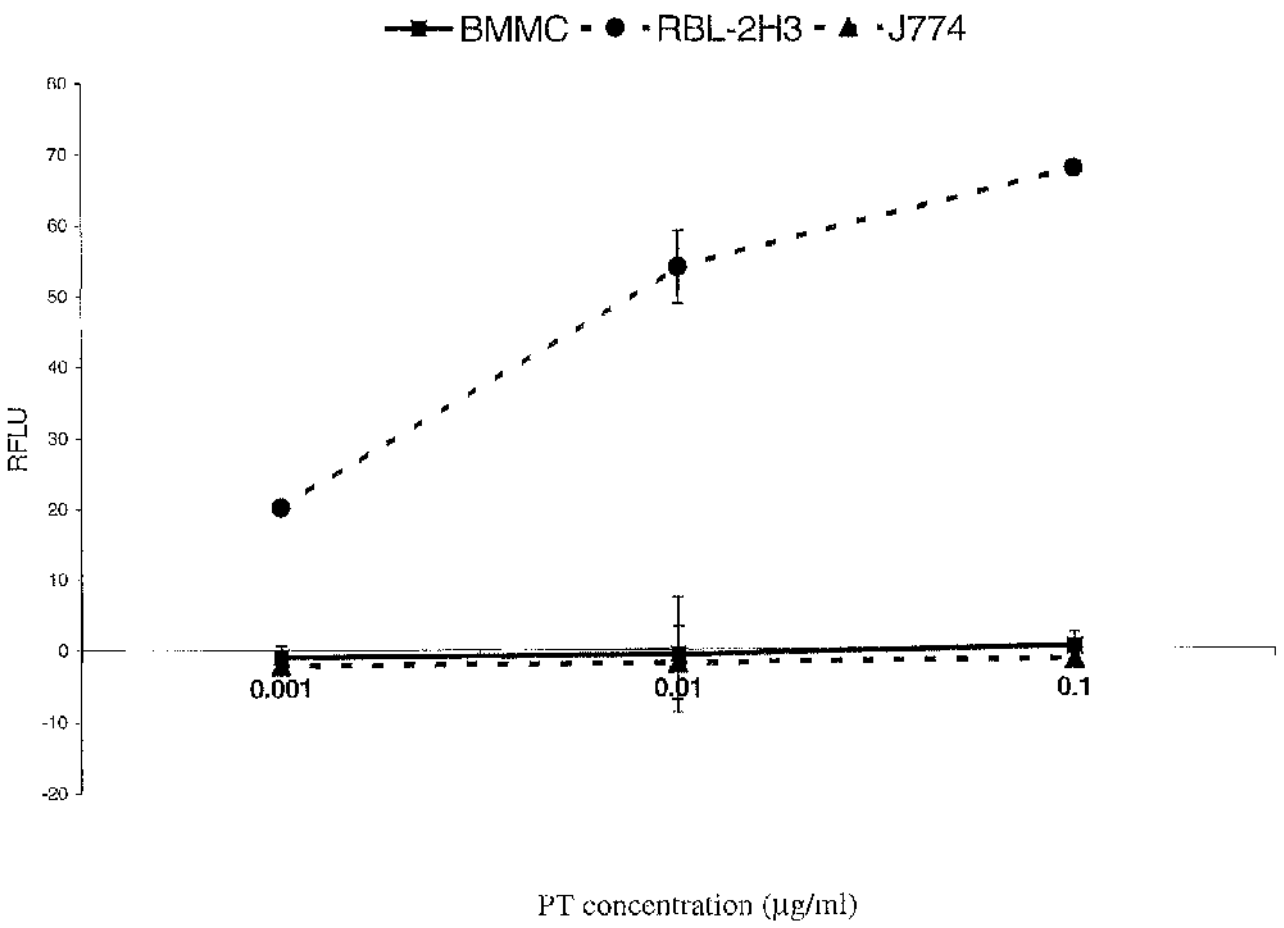
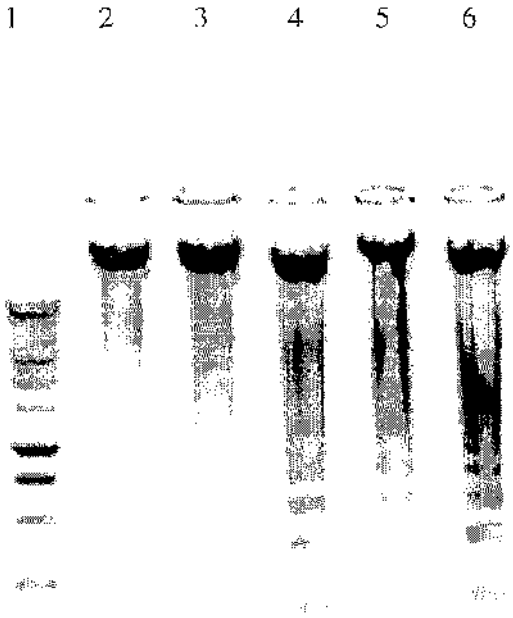

BMMC
RBL-2H3
J774
RFLU
80
70
60
50
40
30
20
10
0
-10
-20
0.001
0.01
0.1
PT concentration (μg/ml)

Figure 3.4.10 Effect of PT and rACT on genomic DNA of RBL-2H3

10^6 cells/ml of RBL-2H3 cells were mixed with 0.1 µg/ml of PT or 0.39 µg/ml of rACT (44/54) and incubated for 6h. In addition, RBL-2H3 cells were mixed with 0.1 µg/ml of PT and incubated for 1h, then 0.39 µg/ml of rACT (44/54) was added to the mixture and incubated for another 5h (total incubation time was 6h).

1- DNA marker
2- RBL-2H3 with IMDM medium
3- RBL-2H3 with Urea/Tris buffer
4- RBL-2H3 with 0.39 µg/ml of rACT (44/54)
5- RBL-2H3 with 0.1 µg/ml of PT
6- RBL-2H3 with 0.1 µg/ml of PT then 0.39 µg/ml of rACT


1
2
3
4
5
6

3.4.2.5 Effects on degranulation (β-hexosaminidase assay)

In the following experiments and for the first time, β-hexosaminidase release was investigated in J774A.1 cells due to the effect of different purified virulence factors of *Bordetella*. $5x10^5$ cell/ml of RBL-2H3 cells and J774A.1 cells were maintained in IMDM medium containing 1% of FCS and either 0.01 μg/ml or 0.1 μg/ml of different purified factors as a final concentrations. 200 μl volume of total mixtures was added in triplicate to 96-well plates. After incubation for 3 h at 37°C in 5% CO_2, β-hexosaminidase release was estimated. 10^{-6}M of calcium ionophore was used as a control (Fig. 3.4.11).

3.4.2.5.1 RBL-2H3 cells

None of the purified factors (PT, FHA and PRN) induced β-hexosaminidase releasefrom RBL-2H3 cells, unlike the effect of 10^{-6}M of calcium ionophore seen here or with *B. bronchiseptica* (Bvg^+ phase) in previous experiments. In fact, it appeared that all three factors (PT, FHA and PRN) inhibited spontaneous release from RBL-2H3 cells, as indicated by the negative values for percentage release in the figure.

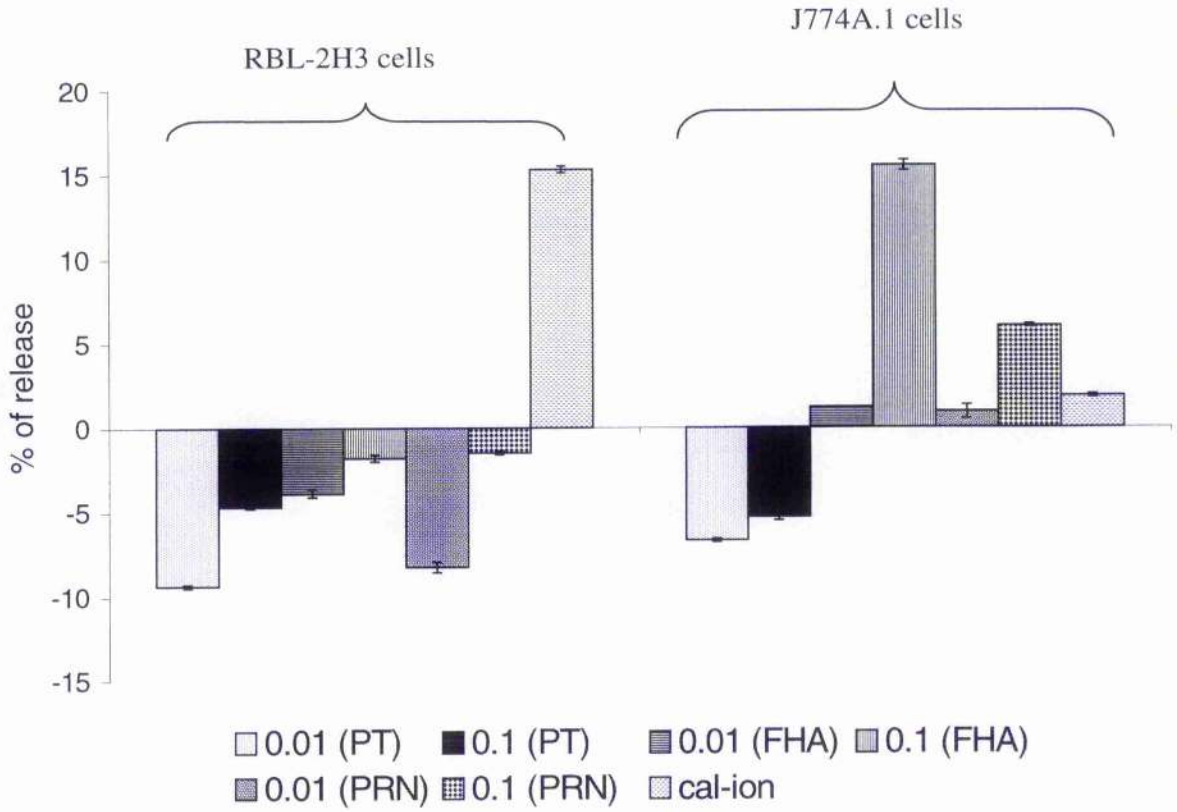
3.4.2.5.2 J774A.1 cells

Surprisingly, both FHA and PRN induced a significant ampunt of β-hexosaminidase release from J774A.1 cells in a dose-dependent manner. In contrast, 0.01 μg/ml and 0.1 μg/ml of PT also inhibited β-hexosaminidase from J774A.1 cells. As with previous observation of 10^{-6}M of calcium ionophore, there was little effect on J774A.1 degranulation.

The above observations suggested that PT inhibited β-hexosaminidase release from both cell types. FHA and PRN do not stimulate β-hexosaminidase release and possibly may inhibit spontaneous release from RBL-2H3 cells. But, with J774A.1 cells, both factors stimulate β-hexosaminidase release.

Figure 3.4.11 Effect of different purified virulence factors of *Bordetella* on β-hexosaminidase release

RBL-2H3 cells and J774A.1 cells were maintained in IMDM medium containing 1% of FCS and either 0.01 μg/ml or 0.1 μg/ml of different purified factors and incubated for 3 h. β-hexosaminidase release was estimated at end of incubation time (mean of 9 observations ± SEM)


RBL-2H3 cells
J774A.1 cells
20
15
10
5
0
-5
-10
-15
% of release
0.01 (PT)
0.1 (PT)
0.01 (FHA)
0.1 (FHA)
0.01 (PRN)
0.1 (PRN)
cal-ion

3.4.3 Effect of pre-treatment RBL-2H3 cells with rACT and PT on β-hexosaminidase release due to calcium ionophore or *B. bronchiseptica*

The previous observations (Fig. 3.4.7b and Fig. 3.4.11) suggested that both PT and rACT were able to inhibit spontaneous β-hexosaminidase release from RBL-2H3 cells. Therefore, it was of interest to investigate the influence of both factors in β-hexosaminidase release from RBL-2H3 cells pre-treated with either PT or rACT and then treated with either calcium ionophore or *B. bronchiseptica* (Bvg^+ phase), both of which are potent release agents.

3.4.3.1 Calcium ionophore

The effect of two different forms of rACT on the degranulation effect of calcium ionophore was studied. RBL-2H3 cells ($1x10^6$ cells/ml) maintained in IMDM containing 10% FCS and 0.1 μg/ml of rACT (44/54) or rACT (44-188/45) and incubated for 3 h at 35°C in 5% CO_2. 10^{-6} M of calcium ionophore was added to the pre-treated RBL-2H3 cell suspensions and incubated for 1 h. Then, β-hexosaminidase release was estimated. 10^{-6}M of calcium ionophore caused around 27% β-hexosaminidase release from untreated RBL-2H3 cells (Fig. 3.4.12a). 0.1 μg/ml of rACT (44/54) showed inhibition of spontaneous β-hexosaminidase release from RBL-2H3 as in previous observations (Fig. 3.4.7b). But, there was a significant β-hexosaminidase release from RBL-2H3 pre-treated with 0.1 μg/ml of rACT (44/54) for 3 h and challenged with 10^{-6}M of calcium ionophore for 1 h compared with the effect of 10^{-6} M of calcium ionophore alone. In contrast, 0.1 μg/ml of rACT (44-188/54) showed very little effect in spontaneous β-hexosaminidase release from RBL-2H3 cells after treatment for 3 h. But, there was a significant β-hexosaminidase release from pre-treated RBL-2H3 cells with 0.1 μg/ml of rACT (44-188/54) for 3 h and challenged with 10^{-6} M of calcium ionophore for 1 h compared with the effect of 10^{-6} M calcium ionophore alone (student *t*-test, *p*-value ≤ 0.05).

Similar experiments were done with using PT. $1x10^6$ cells/ml RBL-2H3 cells maintained in IMDM containing 1% FCS and 0.1 µg/ml of PT was incubated for 3 h at 35°C in 5% CO_2. 10^{-6} M of calcium ionophore was added to the pre-treated RBL-2H3 cell suspensions and incubated for 1 h then β-hexosaminidase release was estimated. 0.1 µg/ml of PT showed inhibition of spontaneous β-hexosaminidase release from RBL-2H3 as in previous observations (Fig. 3.4.11). But, there was a significant β-hexosaminidase release from RBL-2H3 cells pretreated with 0.1µg/ml of PT and then treated with 10^{-6}M calcium ionophore, similar to that from RBL-2H3 cells treated with 10^{-6} M calcium ionophore only (student *t*-test, *p*-value > 0.05) (Fig. 3.4.12b).

The above observations suggested that pretreatment with PT and rACT (44/54) failed to inhibit β-hexosaminidase release from RBL-2H3 cells treated with 10^{-6}M calcium ionophore.

Figure 3.4.12a Effect of calcium ionophore on degranulation of RBL-2H3 pre-treated with rACT

$1x10^6$ cells/ml RBL-2H3 cells were incubated in IMDM containing 10% FCS and 0.1 µg/ml of rACT (44/54) or rACT (44-188/45) for 3 h at 35°C in 5% CO_2. 10^{-6}M of calcium ionophore was added to the pre-treated RBL-2H3 cell suspensions and incubated for 1 h then β-hexosaminidase release was estimated (mean of 9 observations ± SEM)

Figure 3.4.12b Effect of calcium ionophore on degranulation of RBL-2H3 pre-treated with PT

$1x10^6$ cells/ml RBL-2H3 cells were incubated in IMDM containing 10% FCS and 0.1 µg/ml of PT for 3 h at 35°C in 5% CO_2. 10^{-6}M of calcium ionophore was added to the pre-treated RBL-2H3 cell suspensions and incubated for 1 h then β-hexosaminidase release was estimated (mean of 9 observations ± SEM)

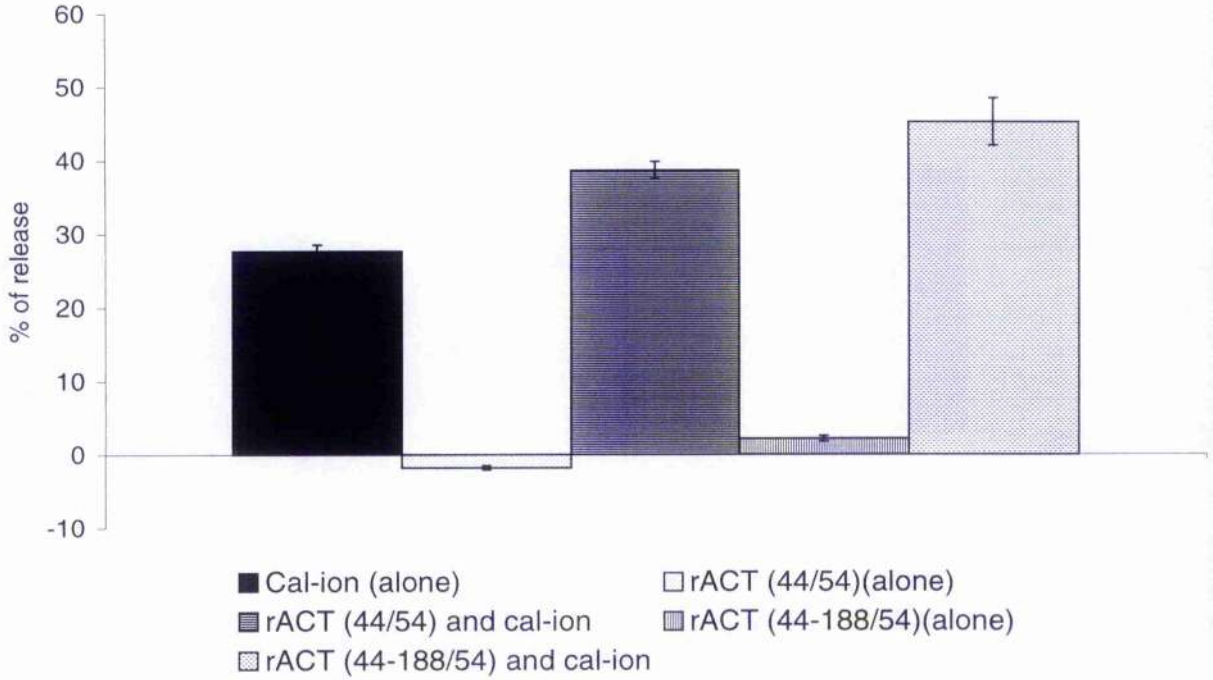


Figure 3.4.12a

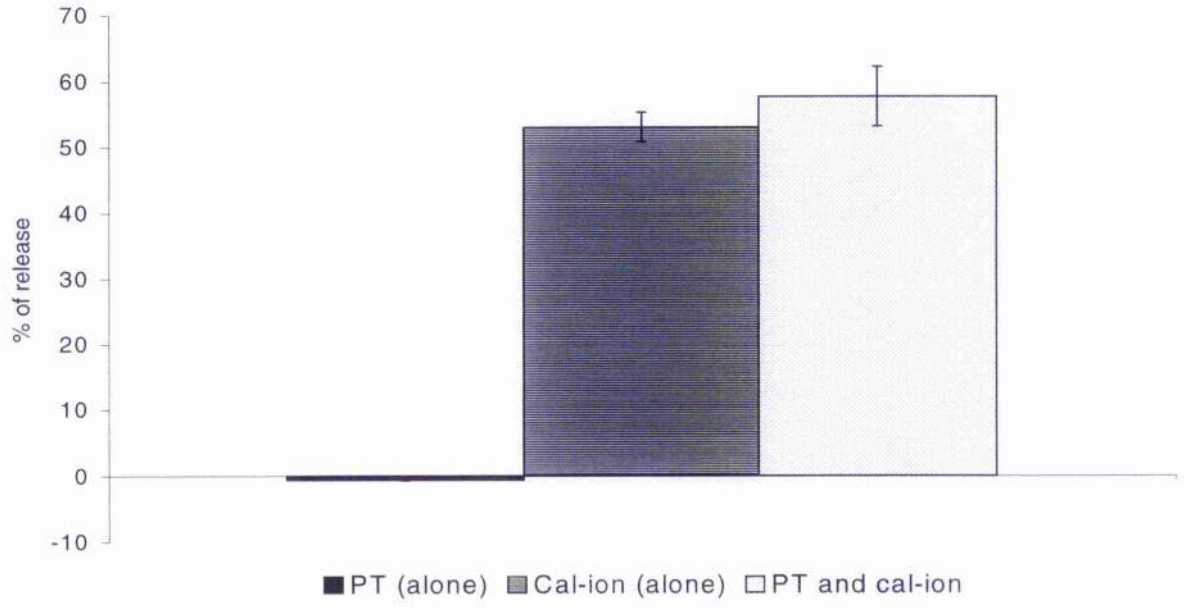


Figure 3.4.12b

3.4.3.2 *B. bronchiseptica* (Bvg^+ phase)

RBL-2H3 cells at 10^6 cells/ml were maintained in IMDM medium containing either 0.1 µg/ml of rACT (44/54) or 0.1 µg/ml PT and incubated for 1 h, 3 h and 6 h at 35°C in 5% CO_2. *B. bronchiseptica* (Bvg^+) grown on BG agar was then added at an infection ratio of 1:500 and reincubated for another 1 h under the previous conditions. At the end of incubation, β-hexosaminidase was estimated in the supernates of the different mixtures.

B. bronchiseptica (Bvg^+ phase) gave around 8% of total β-hexosaminidase release from RBL-2H3 cells after incubation for 1 h (Fig. 3.4.13a). Urea/Tris buffer at concentration equal to that in 0.1 µg/ml of rACT did not show any effect on β-hexosaminidase release. In contrast, 0.1 µg/ml of rACT (44/54) showed considerable inhibition of spontaneous β-hexosaminidase release from RBL-2H3 cells, which agrees with previous observations (Fig 3.4.12a). However, such an inhibitory effect on spontaneous β-hexosaminidase release appears to be temporary in that the effect decreased with increasing time of incubation. It has been reported that once rACT gets inside cells, it casues an increase in cAMP but it is degraded and (Hanski and Farfel, 1985). However, these observations suggested that rACT is able to inhibit β-hexosaminidase release induced by *B. bronchiseptica* from infected RBL-2H3 cells if cells are pre-treated just before (1 h or 3 h) expoure to *B. bronchiseptica*.

In addition, the effects of pretreated with PT on β-hexosaminidase release from RBL-2H3 cells by *B. bronchiseptica* (Bvg^+ phase) was studied (Figure 3.4.13b). As in previous observations, 0.1µg/ml PT showed inhibition of spontaneous β-hexosaminidase release, but this was a slow-developing effect and was more evident with a longer (6 h) incubation period. When, pre-treated RBL-2H3 cells were infected with *B. bronchiseptica* (Bvg^+ phase), there was a statistically significant reduction in β-hexosaminidase release (student *t*-test, p-value ≤ 0.05) compared with non-infected cells at all incubation times, but, this inhibitory effect was greatest after 6 h of pre-treatment. Thus, the inhibitory effect of PT appeared to be time-dependent and, with increased incubation time, a greater inhibitory effect was observed.

Figure 3.4.13a Effect of pre-treatment with rACT on β-hexosaminidase release from RBL-2H3 cells in the presence of *B. bronchiseptica* (Bvg $^+$)

RBL-2H3 cells at $1x10^6$ cells/ml were pre-treated with 0.1 μg/ml of rACT (44/54) for different time (1 h, 3 h and 6 h) and then infected with *B. bronchiseptica* (Bvg $^+$) for 1 h. β-hexosaminidase release was then estimated (mean of 9 observations ± SEM)

A) Effect of *B. bronchiseptica* (Bvg$^+$) on RBL-2H3 cells in IMDM alone.

B) Effect of *B. bronchiseptica* (Bvg $^+$) on RBL-2H3 cells pre-treated with IMDM medium containing 0.04M urea/Tris buffer (urea control).

C) Effect of 0.1 μg/ml rACT (44/54) alone on spontaneous β-hexosaminidase release from RBL-2H3 cells.

D) Effect of *B. bronchiseptica* (Bvg$^+$) on RBL-2H3 cells pre-treated with IMDM medium containing 0.1 μg/ml rACT (44/54).

Figure 3.4.13b Effect of pre-treatment with PT on β-hexosaminidase release from RBL-2H3 cells in the presence of *B. bronchiseptica* (Bvg $^+$)

RBL-2H3 cells at $1x10^6$ cells/ml were pre-treated with 0.1 μg/ml of PT for different times and then infected with *B. bronchiseptica* (Bvg$^+$) for 1 h. β-hexosaminidase release was then estimated (mean of 9 observations ± SEM)

A) Effect of *B. bronchiseptica* (Bvg $^+$) on RBL-2H3 cells in IMDM alone.

B) Effect of 0.1 μg/ml PT alone on spontaneous β-hexosaminidase release from RBL-2H3 cells.

C) Effect of *B. bronchiseptica* (Bvg $^+$) on RBL-2H3 cells pre-treated with IMDM medium containing 0.1 μg/ml PT.

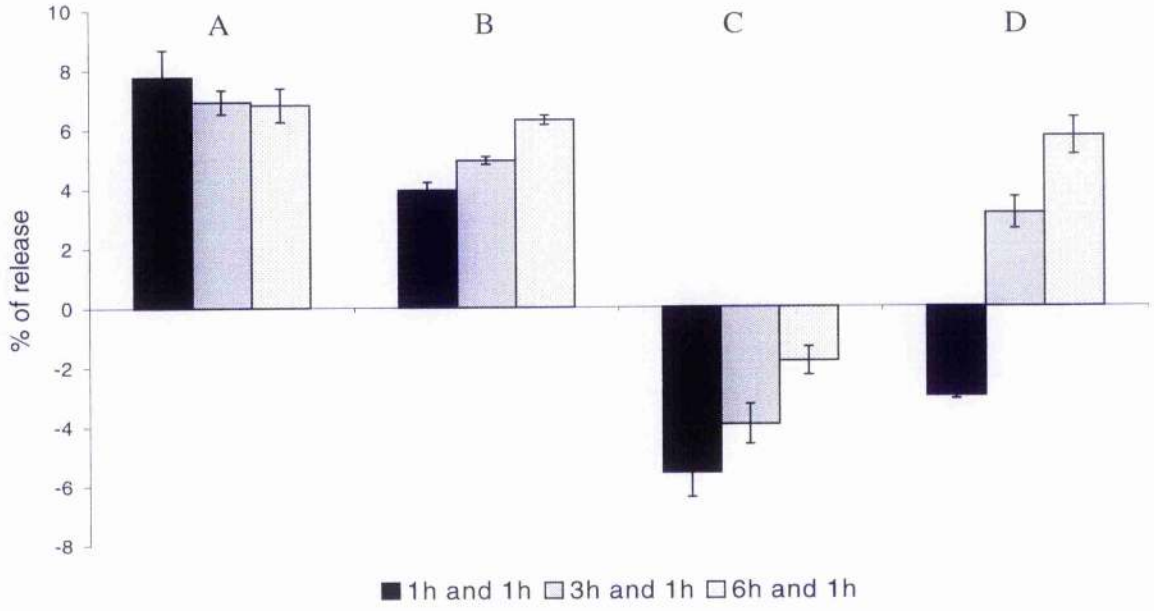


Figure 3.4.13a

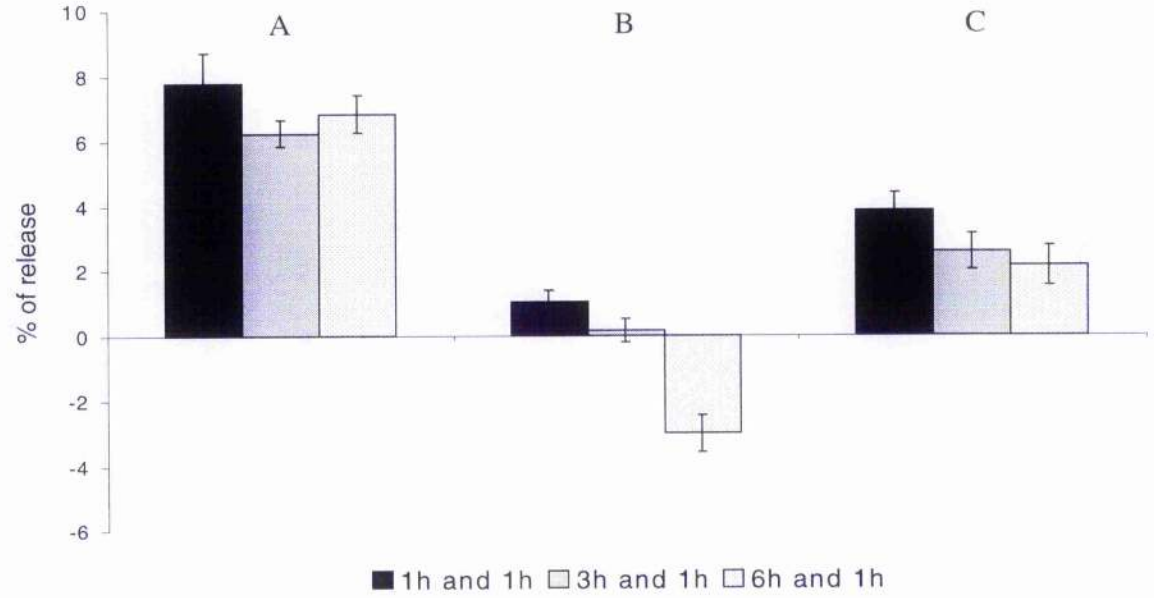


Figure 3.4.13b

3.4.4 Influence of rACT and PT on survival and phagocytic function of J774A.1 cells infected with *B. bronchiseptica*

The previous results determined different effects of ACT (normally present in *B. bronchiseptica* and *B. pertussis*) and PT (present only in *B. pertussis*). For example, a significant reduction in viability of different mammalian cell types have been observed after incubation with ACT but, there was no significant influence of PT on viability of these cell types. In addition, both purified factors have shown inhibitory effect on β-hexosaminidase release from RBL-2H3 but, such inhibitory effect was different when pre-treated RBL-2H3 cells infected with *B. bronchiseptica* (Bvg^- phase) (section 4.3.2). Therfore, it would be more useful to investigate the ability of both factors on phagocytic function of mouse macrophage cell line J774A.1. In this regards, it was reported that PT has been implicated in inhibiting a number of activities of human phagocytic cells including phagocytosis (Hiemstra *et al.*, 1992). Additionally, it was found that the *B. pertussis* ACT was important in crippling the phagocytic functions of neutrophils (Weingart and Weiss, 2000). Therefore, it was interesting to study the influence of both purified factors PT and rACT on survival of J774A.1 cells and numbers of intracellular *B. bronchiseptica* due to interaction with *B. bronchiseptica* (Bvg^+ phase). J774A.1 macrophage cells were used in the following study. Previous observation had shown that RBL-2H3 cells did not take-up *B. bronchiseptica* (Lux 95) (Fig. 3.2.15a and Fig. 3.2.15b), so, these cells were not used.

3.4.4.1 Comparison of the effect of PT and rACT on survival of J774A.1 cells infected with *B. bronchiseptica* (Bvg^+ phase)

0.1 µg/ml of either PT or rACT (44/54) as final concentrations were added into $1x10^6$ cell/ml J774A.1 cells maintained in an antibiotic-free IMDM medium and incubated for 1 h at 37°C in 5% CO_2. Then, *B. bronchiseptica* (Bvg^+) was added at an infection ratio of 1:500 (one J774A.1 cell to 500 bacterial cells) and incubated for another 3 h under 37°C in 5% CO_2. At the end of the incubation period (total time 4 h), the cell suspension was washed three times (to remove any extracellular bacteria) in an antibiotic-free IMDM medium. The viability of J774A.1 cells was estimated by trypan blue exclusion and viability of bacterial cells was estimated by releasing the intracellular or adherent bacteria by adding 100 µg/ml of digitonin to the washed cell

suspension, and plating out. Statistical analysis of the interaction of *B. bronchiseptica* (Bvg^+ phase) with J774A.1 cells treated in different conditions containing 0.1 μg/ml PT or rACT was done by using Student *t*-test. Statistically, collected data were normally distributed, the variance of each sample was similar, and mean and variance were not correlated. A parametric test was chosen and, due to significant values of interaction observed by Student *t*-test, Tukey's test was used (two way ANOVA) to confirm significance values which were done by using Xlstat (www.Xlstat.com) software.

Table 3.4.1, shows that, in general, the viability of J774A.1 cells (mean $\log_{10}$) was reduced due to the presence of *B. bronchiseptica* (Bvg^+) compared with the viability of J774A.1 cells (mean $\log_{10}$) in the absence of *B. bronchiseptica* (Bvg^-) phase (Tukey's test: p-value < 0.0001) which agreed with previous observations (Fig. 3.2.17b). In the absence of bacteria, there was no statistically significant reduction in the viability of J774A.1 cells due to treatment in 0.1 μg/ml PT compared with J774A.1 cells in IMDM alone (Tukey's test p-value > 0.05). But, there was a significant reduction in viability of J774A.1 cells treated with rACT compared with viability of J774A.1 cells treated in IMDM medium alone or in the urea/Tris buffer diluent (Tukey's test p-value < 0.0001). As expected, there was no effect observed in viability of J774A.1 cells due to treatment in Urea/Tris buffer compared with viability of J774A.1 cells treated in IMDM medium only (Tukey's test p-value > 0.05).

Interestingly, there was statistically significant increase in survival of J774A.1 cells pre-treated with 0.1 μg/ml of PT and then infected with *B. bronchiseptica* (Bvg^+ phase) compared with cells in IMDM only (Tukey's test p-value < 0.0001). In contrast, there was a statistically significant decline in survival of J774A.1 cells pre-treated with 0.1 μg/ml of rACT and then infected with *B. bronchiseptica* (Bvg^+ phase) compared with survival cells in IMDM only (Tukey's test p-value < 0.0001).

The above observations suggested that pre-treatment of J774A.1 macrophage cells with PT protected J774A.1 cells from the killing effect of *B. bronchiseptica* (Bvg^- phase), but rACT enhances the killing effect of *B. bronchiseptica* (Bvg^+ phase).

Table 3.4.1: Survival of J774A.1 cells pretreated with PT and rACT and infected with *B. bronchiseptica* (Bvg^+ phase)

J774A.1 cells treated (for 1 h) in different conditions (containing IMDM alone, Urea/Tris buffer, 0.1μg/ml PT or rACT) and infected with *B. bronchiseptica* (Bvg^+ phase) for 3 h. Viability of infected J774A.1 cells (mean Log_{10}) was estimated by trypan blue exclusion. **IMDM:** medium only, **Urea:** Urea buffer diluent for rACT, **SD**: Standard deviation (mean of 6 observations)

J774A.1 cells treatment in	Bacteria	Mean (Log_{10}) number of viable cells/ml	SD
IMDM	Absent	5.854	0.043
Urea		5.831	0.047
0.1 μg/ml (PT)		5.758	0.041
0.1 μg/ml (rACT)		5.091	0.175
IMDM	Present	5.076	0.169
Urea		5.237	0.095
0.1 μg/ml (PT)		5.458	0.060
0.1 μg/ml (rACT)		4.659	0.169

3.4.4.2 Effect of PT and rACT on uptake of *B. bronchiseptica* (Bvg $^+$ phase)

Previous observation suggested that 0.1 µg/ml PT prevents killing of J774A.1 cells by *B. bronchiseptica* (Bvg $^+$ phase). Therefore, it was interesting to investigate the ability of J774A.1 cells for uptake of or adherence to the surrounding *B. bronchiseptica* (Bvg$^+$ phase).

Figure 3.4.14 shows the total number of viable intracellular/adherent *B. bronchiseptica*. As can be seen, there was no significant differences between viability of *B. bronchiseptica* in the case of J774A.1 cells maintained in IMDM alone or Urea/Tris buffer (*t*-test: p-value > 0.05). Numbers were lower in J774A.1 cells treated with 0.1 µg/ml PT compared with J774A.1 cells in IMDM alone (*t*-test: p-value ≤ 0.05). In addition, numbers were much lower in J774A.1 cells treated with 0.1 µg/ml rACT compared with numbers in the Urea/Tris buffer diluent for the rACT preparation (*t*-test: p-value ≤ 0.05).

Figure 3.4.15 shows the numbers of viable intracellular/adherent *B. bronchisptica* per viable J774A.1 cell (i.e. Log $_{10}$ number of intracellular *B. bronchiseptica* – Log $_{10}$ number of viable J774A.1 cells). As can be seen, there was a decrease in the number of viable intracellular/adherent *B. bronchiseptica* per viable J774A.1 cell pre-treated with 0.1 µg/ml of PT compared with numbers in control cells or in cells pre-treated with 0.1µg/ml rACT (*t*-test: p-value ≥ 0.05).

This observation could suggest that pre-treatment of J774A.1 cells with 0.1µg/ml PT prevent killing of J774A.1 cells and could prevent uptake of *B. bronchiseptica* by J774A.1 cells. ACT, however, was cytotoxic for the J774A.1 cells, hence there were fewer bacteria recovered. However, those J774A.1 cells that had taken up bacteria contained similar numbers to those in the control, untreated cells.

Figure 3.4.14 Intracellular survival of *B. bronchiseptica* in J774A.1 cells pretreated with PT or rACT

J774A.1 cells at $1x10^6$ cells/ml were treated in different conditions (IMDM alone, urea/Tris buffer and 0.1 μg/ml PT or rACT) for 1 h and then infected with *B. bronchiseptica* (Bvg $^+$ phase) at MOI of 1:500 for 3 h. At the end of incubation time (total 4 h), extracellular *B. bronchiseptica* were removed by washing and survival of intracellular/adherent *B. bronchiseptica* in J774A.1 was determined by plating on BG agar (mean of 6 observations ± SEM)

Figure 3.4.15 Numbers of *B. bronchiseptica* per J774A.1 cell pretreated in different conditions

Survival of intracellular *B. bronchiseptica* per J774A.3 cell have been calculated by subtracting Log_{10} number of intracellular/adherent *B. bronchiseptica* from viable of Log_{10} number of viable J774A.1 cell for each conditions.

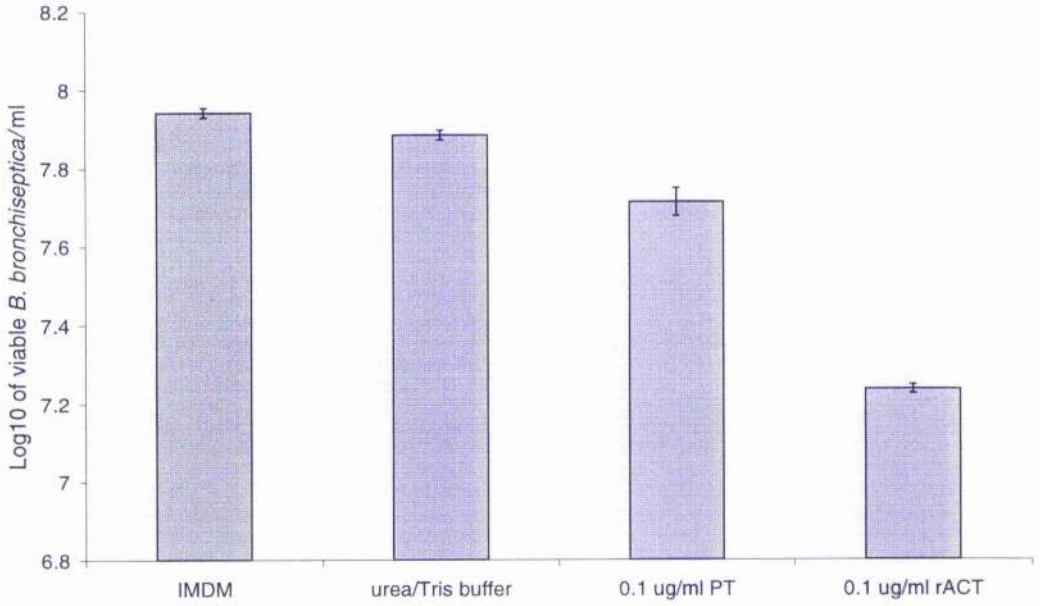


Figure 3.4.14

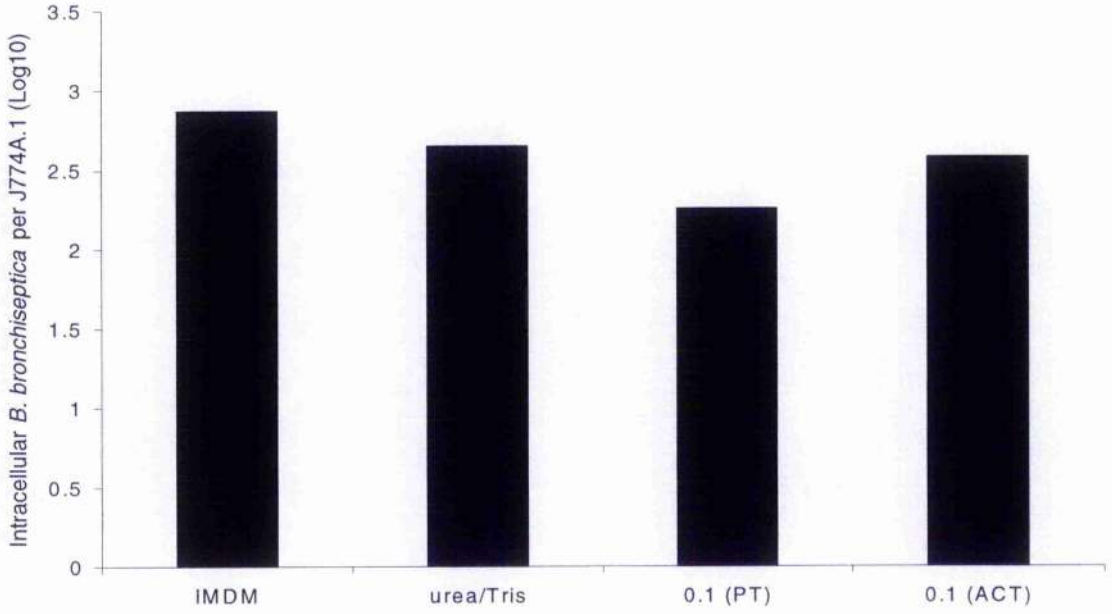


Figure 3.4.15

3.4.4.3 Effect of *B. bronchiseptica* on viability of J774A.1 cells pre-treated with rACT

B. bronchiseptica (Bvg^+ phase) produces ACT, which is well known that causes an increase in intracellular cAMP in target cells. This increase in cAMP is thought to modulate cell functions, for example to inhibit the respiratory burst in neutrophils (Wang *et al.*, 1998). Therefore, it was of interest to study the effect of low doses of rACT (44/54) either to prevent the killing effect of *B. bronchiseptica* (Bvg^+ phase) toward J774A.1 cells or to modulate the uptake of surrounding *B. bronchiseptica*.

J774A.1 cells at $1x10^6$ cells/ml were maintained in antibiotic-free IMDM medium and treated with either; IMDM alone, urea buffer diluent, 0.025 µg/ml, 0.05 µg/ml or 0.1 µg/ml of rACT and incubated for 1 h at 37°C in 5% CO_2. Then each group of pre-treated J774A.1 cells was infected with *B. bronchiseptica* (Bvg^+ phase) at an infection ratio of 1:500 (one J774A.1 cell to 500 bacterial cells) and incubated for another 3 h under the previous conditions. At the end of incubation (total time 4 h), bacteria-cell suspension was washed three times (to remove any extracellular bacteria) in an antibiotic-free IMDM medium. The viability of J774A.1 cells was estimated by trypan blue exclusion and the viability of bacterial cells was estimated by releasing the intracellular or adherent bacteria by adding 100 µg/ml of digitonin into the washed cell suspension, and plating out. Statistical analysis of the interaction of *B. bronchiseptica* (Bvg^+ phase) with J774A.1 cells treated in different conditions was done by using Student *t*-test. Statistically, collected data were normally distributed, the variance of each sample was similar, and mean and variance were not correlated. A parametric test was chosen and, due to significant values of interaction observed by Student *t*-test, Tukey's test was used (two way ANOVA) to confirm significance values which were done by using Xlstat (www.Xlstat.com) software.

Table 3.4.2, shows that, in general, viability of J774A.1 cells (mean $\log_{10}$) was reduced due to the presence of *B. bronchisptica* (Bvg^+) compared with the viability of J774A.1 cells (mean $\log_{10}$) in the absence of *B. bronchiseptica* (Bvg^-) phase (Tukey's test: p-value < 0.0001) which agreed with previous observations (Fig. 2.17b).

There was a significant reduction in viability of J774A.1 cells at all three concentrations of rACT (44/54) compared to the viability of J774A.1 cells in urea buffer or IMDM medium only (Tukey's test: p-value < 0.0001). These observations confirm the highly cytotoxic effect of the high doses of rACT (0.1 µg/ml) which confirmed our previous observation (Fig. 3.4.14).

As can be seen in Table 3.4.2, the viability of J774A.1 cells pre-treated with 0.025 µg/ml of rACT and infected with *B. bronchiseptica* (Bvg^+ phase) was high compared with the viability of J774A.1 cells treated in urea buffer diluent in presence of *B. bronchiseptica* (Bvg^+ phase). This observation was significant (Student t-test p-value ≤ 0.05), but, when analysed with Tukey's test, it was not significant (p-value > 0.05). The last observations suggested that pre-treatment of J774A.1 cells with the lower concentrations of rACT (0.025 µg/ml) may have prevented the killing effect of *B. bronchiseptica* (Bvg^+ phase) toward J774A.1 cells compared with higher doses.

Table 3.4.2: Survival of pretreated J774A.1 cells with rACT and infected with *B. bronchiseptica* (Bvg^+ phase)

J774A.1 cells treated (for 1 h) in different conditions (containing different concentrations of rACT) and infected with *B. bronchiseptica* (Bvg^+ phase) for 3 h. Viability of infected J774A.1 cells (mean Log_{10}) was estimated by trypan blue exclusion. **IMDM:** medium only, **u:** urea buffer diluent, **0.025, 0.05 and 0.1:** different concentrations of rACT (μg/ml) (mean of 6 observations).

Cells treatment in	Bacteria	Mean (Log_{10}) no. of viable cells/ml	SD
IMDM	**Absent**	5.722	0.043
u/T		5.683	0.046
0.025		5.426	0.103
0.05		5.284	0.052
0.1		5.084	0.126
IMDM	**Present**	4.476	0.105
u/T		4.449	0.094
0.025		4.591	0.064
0.05		4.370	0.106
0.1		4.128	0.139

3.4.4.4 Effect of pre-treatment of J774A.1 cells with rACT (44/54) on survival of intracellular/adherent of *B. bronchiseptica*

The previous observation suggested that 0.025 μg/ml rACT may have reduced the killing effect of *B. bronchiseptica* (Bvg^+ phase) towards J774A.1 cells. Therefore, intracellular/adherent *B. bronchiseptica* (Bvg^- phase) was investigated in each condition of J774A.1 cells pretreated with different concentrations of ACT.

Figure 3.4.16 shows the total number of viable intracellular/adherent *B. bronchiseptica*. As can be seen, there was no significant difference between viability of *B. bronchiseptica* in the case of J774A.1 cells maintained in IMDM alone or Urea/Tris buffer (*t*-test: *p*-value > 0.05). Numbers were lower in J774A.1 cells treated with 0.025 μg/ml rACT compared with J774A.1 cells in IMDM alone (*t*-test: *p*-value > 0.05). In addition, numbers were much lower in J774A.1 cells treated with higher doses of rACT compared with numbers in the Urea/Tris buffer diluent for the rACT preparation (*t*-test: *p*-value ≤ 0.05).

Figure 3.4.17 shows the numbers of viable intracellular/adherent *B. bronchisptica* per viable J774A.1 cell (by substracting Log_{10} number of intracellular/adherent *B. bronchiseptica* from the Log_{10} number of viable J774A.1 cell). As can be seen, there was a decrease in the number of viable intracellular/adherent *B. bronchiseptica* per viable J774A.1 cell pre-treated with 0.025 μg/ml of rACT compared with numbers in control cells or in cells pre-treated with other higher doses of rACT (*t*-test: *p*-value ≥ 0.05).

This observation could suggest that pre-treatment of J774A.1 cells with low concentrations of rACT (0.025 μg/ml) reduced the cytotoxic effect of *B. bronchiseptica* (Bvg^+ phase) towards J774A.1 cells by reducing the ability of J774A.1 cells for uptake of surrounding *B. bronchiseptica*.

Figure 3.4.16 Intracellular survival of *B. bronchiseptica* in J774A.1 cells pretreated with different concentrations of rACT

J774A.1 cells at $1x10^6$ cells/ml were treated in different conditions (IMDM alone, urea/Tris buffer and different concentrations of rACT) for 1 h and infected with *B. bronchiseptica* (Bvg $^+$ phase) at MOI of 1:500 for 3 h. At the end of incubation time (total 4 h) extracellular *B. bronchiseptica* were removed by washing and survival of intracellular/adherent *B. bronchiseptica* in J774A.1 was determined by plating on BG agar (mean of 6 observations ± SEM).

Figure 3.4.17 Numbers of *B. bronchiseptica* per J774A.1 cell pretreated in different conditions

Survival of intracellular *B. bronchiseptica* per J774A.3 cell were calculated by subtracting the Log_{10} number of intracellular/adherent *B. bronchiseptica* from viable of Log_{10} number of viable J774A.1 cell for each conditions.

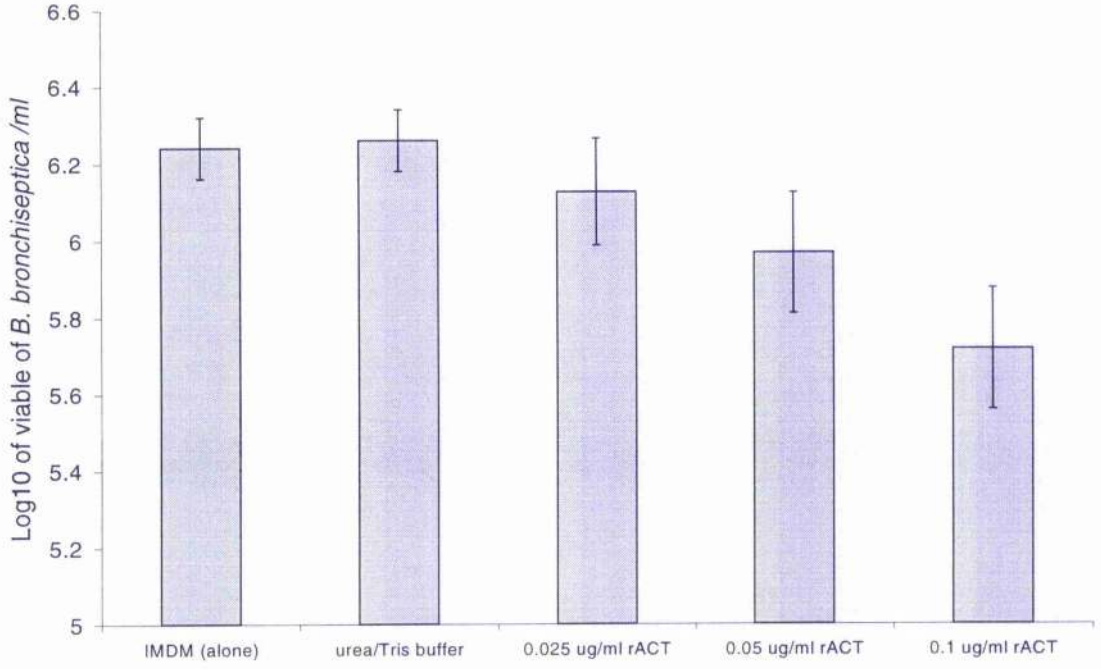


Figure 3.4.16

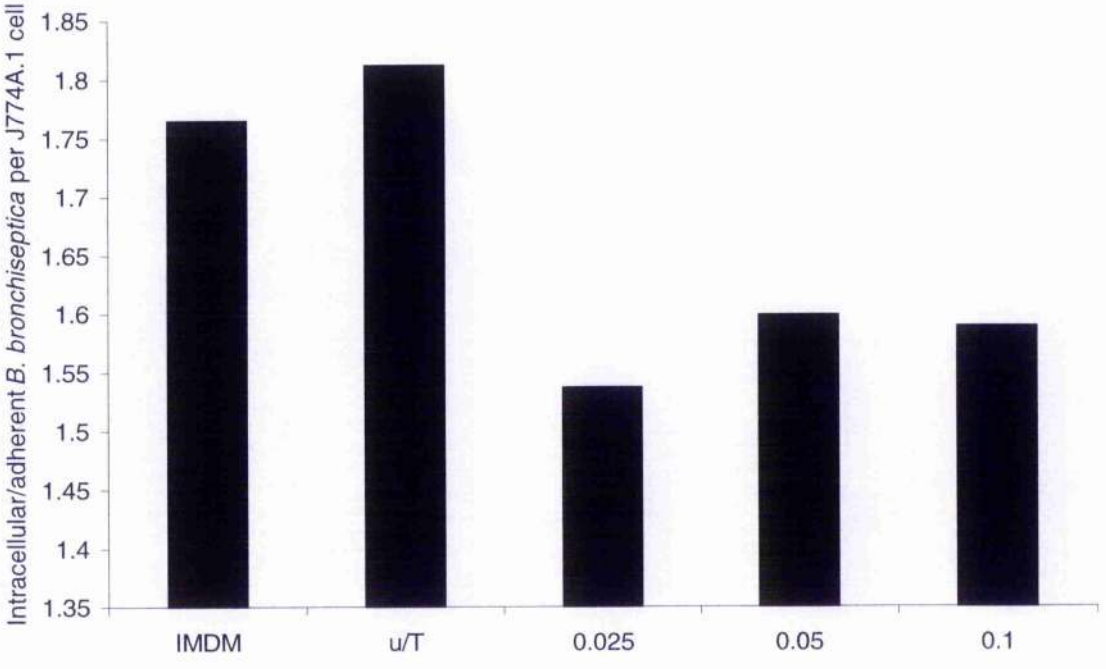


Figure 3.4.17

CHAPTER FOUR
GENERAL DISCUSSION AND CONCLUSIONS

4.1 General Discussion

4.1.1 Advantages and limitation of methods used in this study

Different methods were applied to study the interaction of sheep BMMC and other mammalian cell types (RBL-2H3 and J774A.1) with *B. bronchiseptica* and different *Bordetella* purified factors. Uptake of bacteria by sheep BMMC was studied by using a bioluminescent construct of *B. bronchiseptica* (Lux 95) and a bioluminescent *E. coli*. The bioluminescent method was shown to offer an easy and simple method of measuring the viability of the bacteria in real-time. Based on *in vitro* invasion assays, the kinetics of interaction between sheep BMMC and *B. bronchiseptica* (Lux 95) was followed. The data indicated the presence of viable bioluminescent *B. bronchiseptica* within sheep BMMC, and this observation was confirmed by both viable count and TEM.

It is important to realise that intracellular bacteria within sheep BMMC might include both viable *B. bronchiseptica* surviving the invasion/phagocytic process and bacteria killed by the intracellular bactericidal mechanisms. Therefore, in future studies, it would be useful to apply a method which could help to detect and differentiate between viable and dead bacteria. For example, flow cytometry (FC) or fluorescence microscopy methods have the potential to measure the morphological and physiological characteristics of individual cells through labelling of cellular targets with organism-specific fluorescent dyes (Jepras *et al.*, 1995). Several commercial DNA-intercalating fluorescent labels with various excitation and emission spectra have been developed to distinguish viable and non-viable bacteria on the basis of intact versus damaged cellular membranes.

These methods can measure the relative size, relative granularity, internal complexity and relative fluorescent intensity of individual cells. With its great speed, the FC method can individually evaluate several thousand cells per second. This method has been shown to be both sensitive and accurate in its rapid assessment of the viability of Gram-positive and Gram-negative bacteria (Wattanaphansak *et al.*, 2005).

Bordetella species interact with their mammalian hosts primarily at respiratory surfaces where they bind specifically to the cilia of the respiratory epithelium. In this study, interaction of *B. bronchiseptica* with sheep BMMC was studied *in vitro* and their cytotoxicity was compared with the effect of different bacterial species. Comparison of *B. bronchiseptica* (Bvg^+) and (Bvg^-) strains indicated that only the

Bvg^{I} phase *B. bronchiseptica* significantly reduced the viability of sheep BMMC and of other cell types (RBL-2H3 and J774A.1 cells). Viability of mammalian cells was estimated by a bioluminescent assay for ATP which reflects the metabolic activity of the cells. Furthermore, degranulation of mammalian cells after incubation with *B. bronchiseptica* and other bacterial species was estimated. A significant β-hexosaminidase release was estimated after incubation with *B. bronchiseptica* (Bvg^{I}) compared with the effect of the (Bvg^{-}) strain and certain other *Bordetella* species.

In their virulent phase, *B. pertussis* and *B. bronchiseptica* express a common set of surface-associated and secreted molecules involved in colonisation and virulence which include adhesins such as filamentous haemagglutinin (FHA) and pertactin (PRN), as well as the bifunctional adenylate cyclase toxin /haemolysin (ACT). *B. pertussis* also specifically expresses pertussis toxin (PT). Cytotoxicity of these different purified factors towards sheep BMMC were investigated by estimating their viability by MTT and ATP-bioluminescent assays, apoptosis (caspase-3 and -7 and DNA fragmentation assays and by degranulation (β-hexosaminidase release assay).

The methods used in this study depend on studying the effect of a limited number of proteins produced by a limited number of genes in one experiment, for example, when assessing the viability or degranulation of sheep BMMC or other cell types. Thus, the throughput is very limited and the whole picture of alteration of protein production or gene function in sheep BMMC and other cell types is hard to obtain. The effect of *B. bronchiseptica* on sheep BMMC will alter the expression of a large number of proteins. Likewise, the effects of mammlian cells on *B. bronchiseptica* will be complex. For example, the production of most *B. bronchiseptica* virulence factors is under the control of BvgAS system, which is co-regulated with other gene sets (i.e. *vag* genes and *vrg* genes) (Coote, 2001). Thus, monitoring the cascade of reactions and co-regulations that occur between different genes of *B. bronchiseptica* under different environmental conditions (or when interacting with different cell types) could be difficult by traditional methods. Thus, it would be useful to use a method which could monitor different gene sets either in the mammalian cells or in *B. bronchiseptica* at the same time under different conditions, either *in vitro* or *in vivo*. In this regards, the DNA microarray technique would be

useful because it could help to follow many different gene functions in one experiment (Schoolnik, 2002; Call, 2005) (see Conclusions and future work).

4.1.2 Differences in cell types studied

In the present study, the focus of interest was the interaction between different bacterial species (mainly *B. bronchiseptica*) and sheep BMMC and other cell types (RBL-2H3 and J774A.1) *in vitro*, with view to some insight into how this interaction might influence the outcome of infection. Sheep BMMC were of prime interest used in this study (see 4.1.3). It is well known that they are multifunctional and they have been traditionally associated with the allergic response. They are found throughout the body, but are particularly prevalent at sites which interface with the environment such as the skin, airways and gastrointestinal tract and have been viewed as sentinel cells in host defence (Abraham and Malaviya, 2000). However, recent studies have suggested that these cells may be capable of regulating inflammation and innate immunity (Malaviya *et al.*, 2001). Furthermore, a number of studies have reported that MCs play a critical role in host immune defence against bacterial infections. These studies have shown that mast cells have the ability to recognize and engulf bacteria and release a number of inflammatory mediators (Marshall and Jawdat, 2004).

In contrast, the RBL-2H3 cell line is a tumour analogue of mucosal mast cells and widely used as a model for mucosal mast cells work. These cells have high affinity IgE receptors (FcεRI). They can be activated to secrete histamine and other mediators by aggregation of these receptors or with calcium ionophore. This cell line wasgrown and readily available and easy to maintain, and could be grown in the same IMDM medium as sheep BMMC. It was observed that RBL-2H3 cells were metabolically very active and their multiplication rate was particularly high. When cell proliferation started, IMDM medium was renewed each 48h and replaced by fresh IMDM. For cell cultures which would be used for experiments, there was a limit of about 10 passages after which an increase of cell death was observed by trypan blue exclusion. This meant that a new stock culture should be preparedvial from the frozen RBL-2H3 stock.

In contrast, J744A.1 cells are well known as a professional macrophage cell line. J774A.1 is less metabolically active with a slow growth rate, compared with RBL-2H3 cells but higher than that of sheep BMMC. Therefore, when J774a.1 cell

proliferation started, IMDM medium was renewed twice per week and replaced with fresh IMDM. In contrast, the IMDM medium in the sheep BMMC culture was renewed once per week.

Despite these differences between RBL-2H3 and J774A.1 cells, they were considered to be suitable for comparison with sheep BMMC in their interactions with bacteria. In the following sections (4.1.3 - 4.1.5) are described further properties of these cells and their advantages and limitations of their use are described.

4.1.3 Differentiation and characterisation of mast cells obtained from sheep bone marrow cells

The results presented in this study have shown that mast cells with the characteristics of mucosal mast cells proliferate to form the predominant cell type in sheep bone marrow cultures fed with the growth factors recombinant ovine SCF and IL-3. In earlier studies, the cell-free supernatant of conditioned medium has been used to stimulate mast cell growth in cultures of sheep bone marrow cells (Haig *et al.*, 1988). The conditioned medium was prepared from lymphocytes recovered from sheep infected with the nematode *Ostertagia circumcincta*. Washed lymphocytes were maintained in IMDM containing $5x10^{-5}$ M 2-mercaptoethanol, 500 µg/ml bovine serum albumin, 25 µg/ml human transferrin, 1 µg/ml palmitic, oleic and linoleic acids, 100 U/ml penicillin, 50 µg/ml streptomycin and 7.5 µg/ml concanavalin A. Cultures were incubated for 1-5 days at 37°C. Then, lymphocytes were harvested by centrifugation and the cell-free supernatant was used. It was observed that mast cells developed rapidly after 2 weeks of culture and could persist for 2-3 months (Haig *et al.*, 1988).

Haig and his group later reported the effects of rat stem-cell factor (SCF) and interleukin-3 (IL-3), alone or in combination, on the *in vitro* growth of bone marrow-derived mast cells (BMMC). Rat SCF stimulated the growth of both rat connective tissue mast cells (CTMC) and rat bone marrow mast cells (BMMC). IL-3 stimulated BMMC growth to a lesser extent than did SCF, whereas CTMC numbers did not increase in IL-3 alone. These observations suggested that SCF and IL-3 can regulate the growth of rat BMMC and CTMC, and they had a synergistic effect on the growth of both BMMC and CTMC (Haig *et al.*, 1994).

The growth of ovine and caprine mast cells in bone marrow cultures has been achieved using recombinant ovine interleukin-3 (rOvIL-3) and recombinant ovine stem cell factor (rOvSCF) (Macaldowie *et al.*, 1997). After growth for approximately 2-3 weeks in optimal concentrations of either rOvIL-3 alone or a combination of rOvIL-3 and rOvSCF, the majority of the cells produced in bone marrow culture from both species were mast cells. The significant increase in the total numbers of cells and survival times of the cultures when both cytokines were present compared to either alone, indicated synergy between rOvIL-3 and rOvSCF on mast cell growth. Ovine cells grown in rOvIL-3 alone or rOvIL-3 and rOvSCF contained significantly more aryl-sulfatase and serine protease but similar amounts of beta-hexosaminidase compared with caprine cells during the second week of culture. There were no significant differences in the granule-associated mediator content of cells from either species grown in rOvIL-3 alone compared with those grown in rOvIL-3 and rOvSCF during the first 21 days of culture (Macaldowie *et al.*, 1997).

Investigations in the present study showed that the presence of both growth factors in sheep bone marrow cultures induced mast cell differentiation, but mast cell development was slow in cultures over the first 12-14 days. However, over the next 2 weeks, the proportion of mast cells in the cultures increased. In addition, batch-to-batch variation was observed in the quantity of bone marrow mast cells produced. Bone marrow cultures were obtained on different occasions from different lambs and then stimulated by adding rOvIL-3 and rOvSCF. Their differentiation was characterized by increased cytoplasmic granulation, which was observed by both Leishman's stain and electron microscopic study. After 3 weeks, the percentage of mast cells varied from 50% for some cultures to 90% for other cultures of sheep bone marrow cells.

This variation could have been a reflection of the initial bone marrow cells themselves i.e. the differences were due to the animal from which the bone marrow cells were derived rather than the culture conditions. Unfortunately, the reason for this variation is not known, nor can it be predicted (e.g. it is not due to factors like age, breed, sex, or obvious past exposure to infection). So, perhaps the variation of mast cell numbers was just an extension or reflection of the variability of the cells in their mediator content or functional activity.

Isolated sheep mucosal mast cells (MMC) have been used to examine the ability of four neuropeptides, substance P, vasoactive intestinal peptide, beta-

endorphin and somatostatin, to release mediators in the presence or absence of parasite antigen (Stewart *et al.*, 1996). None of the neuropeptides induced the release of sheep mast cell protease (SMCP) or histamine from MMC. The response of mast cells (MC) to non-IgE-mediated stimulation is critically dependent on the population of MC examined. The neuropeptide substance-P (SP) has been reported to activate connective tissue-type MC (CTMC), while MMC are not activated by SP (Karimi *et al.*, 2000). In comparison, the functional and biochemical characterization of rat bone marrow-derived mast cells was confirmed either by staining characteristics or release of the performed granule mediator β-hexosaminidase in response to calcium ionophore A23187 and other secretagogues. It was reported that rat BMMC have the staining characteristics of mucosal mast cells and contain the mucosal mast cell protease. Rat BMMC released the preformed granule mediator beta-hexosaminidase in response to 1 μM calcium ionophore A23187 (net release 21.8 +/- 6.8%), and neither compound 48/80 nor substance-P induced mast cell degranulation (Broide *et al.*, 1988).

The present observations showed that mast cells differentiated from sheep bone marrow in a culture containing both SCF and IL-3 did not react with substance-P whereas they did respond to calcium ionophore, which suggests that these mast cells were likely to be mucosal mast cell-like. For this reasons, these cells would seem to be highly appropriate as a model system to study the interaction of (mucosal) mast cells with the respiratory mucosal pathogen, *B. bronchiseptica* and related organisms.

Regarding culture medium, IMDM medium (Gibco BRL) which has Glutamax–I, has an advantage in that the glutamine is in a form that is not broken down immediately and used up by the cells. Also, IMDM medium contains several non-essential amino acids, thought to be necessary to support mast cell growth in bone marrow cultures. In the first 2 to 3 weeks, IMDM medium was changed every week because colour changes in the medium indicated an increase in acidity (red to yellow), although such changes in culture acidity was slower after 3 weeks.

Purification of sheep mast cells from other cells in bone marrow preparation was done by various procedures. First, centrifugation of the cell suspension at 250g for 5 min helped to remove RBC. Also, RBCs died within the first 10 days of cell culture. Fibroblast cells and macrophages became attached to the plastic surface of the tissue culture flask. Purification by using percoll density gradient (Amersham

Biosciences) was done with some cultures. However, it was observed that during the purification step many mast cells were lost and contamination of the cell culture with percoll was observed. Thus, in the experiments reported here, the cultures were not purified.

Working with sheep BMMC produced many problems. It was difficult to identify sheep BMMC during the first two weeks of growth by using either Leishman's stain or electron microscopic study. But, with increase in culture age, the number of dense intracytoplasmic granules increased, which could be an indication of sheep BMMC maturation. It has been suggested that immature mast cells can be differentiated from other cells by staining with rabbit polyclonal antibodies raised against sheep mast cells protease (Macaldowie *et al.*, 1997).

In the present study, the development of mast cells was studied. It had been shown that IL-3 could give rise to mast cells. But, it was observed that other cell types such as monocytes, eosinophils and macrophages were present at low levels at different ages of the culture (< 5-20% of total population). The presence of these phagocytic cells could have had some influence on the results of the bacterial uptake experiments, for example by luminometery and, for that reason the use of more purified mast cell preparations could be explored further in future work. However, this possible influence was judged to be slight since, by electron microscopy (EM) intracellular bacteria were clearly evident within cells with typical mast cell morphology.

Use of the mast cell cultures in the present work was limited by availability because mast cell numbers in the BMMC cultures decreased after 35 days. It was advised that use be limited to mast cells within 35-40 days because it appeared that, as the older cells are fragile (i.e. centrifugation at low speed may lyse them), such cells may not be suitable for invasion and cytotoxicity studies (personal communication with Dr. John Huntley).

At times, there was a shortage of sheep bone marrow due to several problems. The outbreak of foot and mouth disease (2001) which cut the supply of sheep bone marrow for a few months was one, and another was that the project on sheep bone marrow cells at the Moredun Research Institute was stopped (2004).

In view of the above problems it might have been better to have used a different source of bone marrow such as rats or mice because these are small animals

and genetically homogenous strains are available, perhaps without such a great variation in granule contents as the sheep cells. *B. bronchiseptica* is a respiratory pathogen of many animals including rodents, therefore these types of mast cells would have been as appropriate as sheep cells.

4.1.4 RBL-2H3 cells as a mast cell model

In the present study, the RBL-2H3 cell line used. Rat basophilic leukemia cells (RBL-2H3), a tumour analogue of mucosal mast cells (i.e. homologous to mucosal mast cells) is a well known cell line (Seldin *et al.*, 1985). RBL-2H3 cells can be activated to secrete histamine and other mediators, and have been used for the study of hematopoietic signalling and secretion (Oliver *et al.*, 1988). In the present study, RBL-2H3 cells had a similar pattern of interaction as sheep BMMC with different secretagogues (i.e. calcium ionophore and substance P). For example, there was around 30 % β-hexosaminidase release due to interaction with 10^{-6}M calcium ionophore, but little β-hexosaminidase release (< 5%) was detected due to the effect of 10^{-6}M substance-P (Fig. 3.1.5). Interaction of substance-P with RBL-2H3 cells however, has been reported. RBL-2H3 cells responded to substance-P when they had been primed with substance P (0.5 μM) for one week (Furukawa *et al.*, 2001). In the present work, however interaction with 10^{-6}M calcium ionophore was detected within 5 min by estimating β-hexosaminidase release and maximum release occurred after incubation for 3 h but then there was decline in release. In contrast, sheep BMMC showed a decline in β-hexosaminidase release after incubation with calcium ionophore for more than 1 h. Therefore, RBL-2H3 cells may be used to study the effect of different bacterial and other stimuli on mast cell degranulation. The results of this study demonstrated the ability of *B. bronchiseptica* (Bvg^+) to directly activate RBL-2H3 cells (a mucosal mast cell model) *in vitro*. The ability of *B. bronchiseptica* (Bvg^+) to induce β-hexosaminidase release from mast cells as a result of degranulation effect may offer a potential link between infection by the organism and pathogenesis of bacterial respiratory infectious diseases (see Section 4.1.4).

However, the use of the RBL-2H3 cells in the present work was limited and their ability to take up bacteria may need further investigation. In the present study, RBL-2H3 cells appeared to be unable to phagocytose *B. bronchiseptica* (Result,

section 3.2.8.2). Therefore, it was not possible to use RBL-2H3 cells with bioluminescent *B. bronchiseptica* (Lux 95).

4.1.5 Advantages and limitation to the use of mast cells and other cell types in this study

Despite the above problems (see section 4.1.1), mast cells were the focus of interest in this study for a variety of reasons. In the last few years, there has been a growing number of reports concerning the role of mast cells in host defence against bacterial infections (Malaviya and Abraham, 1995; Marshall and Jawdat, 2004). These studies have shown that mast cells have the ability to recognize and engulf bacteria and release a number of inflammatory mediators (Marshall *et al.*, 2003). In addition, studies have shown that the mast cell membrane is replete with many receptors/molecules, including those that promote the recognition and binding of bacteria. For example, mast cells exhibit two basic mechanisms of microbial recognition: opsonin-dependent (via Fc and C3 receptors) and opsonin-independent (via integrins, CD48 molecule and Toll-like receptors). Moreover, mast cells may phagocytose and kill adherent bacteria. Phagocytosis of bacteria results in the presentation of bacterial antigens via MHC class I to T cells (Malaviya *et al.*, 1996b).

B. bronchiseptica is well known as a respiratory tract pathogen of a wide range of animal species(see Introduction 1.1.7.5). Therefore, sheep BMMC were thought to be appropriate for this study. Also, sheep BMMC were conveniently available and kindly supplied by Dr. J. Huntley (M.R.I.).

In the present study, bacterial internalisation (e.g. Lux 95 of *B. bronchiseptica*) by sheep BMMC was compared with that by RBL-2H3 cells and macrophage cell lines (P388D1 and J774A.1) (see 4.1.5.3). It was shown that sheep BMMC were indeed able to internalise the bacteria but uptake of Lux 95 was less than uptake by the macrophage cells, which are considered as professional phagocytic cells (Further discussion, see section 4.1.5). On the other hand, there was no evidence for bacterial internalisation by RBL-2H3 cells either by the bioluminescence technique or by TEM (see 4.1.2). Therefore, RBL-2H3 cells were not useful cell in the study of bacterial internalisation. But, the effect of different strains of *B. bronchiseptica* and of other related *Bordetella* species showed that RBL-2H3 cells could be a useful model for studying the degranulation effect of different bacterial cells and other stimuli.

Previous studies have suggested that *M. pneumoniae* is capable of producing activation of the rodent mast cell line RBL-2H3. After 4 hours of coculture, morphologic changes indicative of activation were observed by means of electron microscopy, and *M. pneumoniae* was observed. Coculture of RBL-2H3 cells with *M. pneumoniae* for 4 hours resulted in release of β-hexosaminidase and serotonin into the supernatant. Live, but not heat-killed, organisms induced the release of IL-4 into the culture supernatant, with a peak at 4 hours. During coculture with *M pneumoniae*, production of mRNA for IL-4, IL-6, and TNF-alpha was upregulated after 2 hours and had returned to near baseline by 24 hours after infection (Hoek *et al.*, 2002).

β-hexosaminidase release was used as a marker for degranulation of sheep BMMC and RBL-2H3 cells due to interaction with *B. bronchiseptica* and other *Bordetella* species. β-hexosaminidase is well known as a granule-associated factor in mast cells and has been widely used as a marker for degranulation of mast cells by different chemicals (Stewart *et al.*, 1996; Mizota *et al.*, 2005) or bacteria (Munoz *et al.*, 2003). In the present study, there was a significant β-hexosaminidase release from sheep BMMC after incubation with wild type *B. bronchiseptica* (Bvg^+) compared with the avirulent mutant *B. bronchiseptica* (Bvg^-). Furthermore, the effect of PT and rACT on RBL-2H3 cells was observed by estimating β-hexosaminidase release. In this study, it was shown that the inhibitory effect of PT was time-dependent and, with increased incubation time, a greater inhibitory effect was observed (see section 3.4.3.2)

4.1.6 Relevance of mast cells to bacterial respiratory diseases

The firmly established role of mast cells and their products in allergic (type I) hypersensitivity contrasts with the comparatively sparse knowledge, until recently, of their physiological functions and their role in non-allergic diseases. The prominence of mast cells in lesions in multiple sclerosis (Ibrahim *et al.*, 1996) cardiomyopathy (Arbustini *et al.*, 1997) rheumatoid arthritis (Gotis-Graham and McNeil, 1997) and a number of other chronic inflammatory and fibrotic diseases in human subjects (Algermissen *et al.*, 1999) suggested mast cell involvement. The critical importance of mast cells in many of these diseases has recently been validated by animal models (Brightling *et al.*, 2002). It is now clear that mast cells receive and integrate a wide array of non–IgE-driven chemical signals from their environment, permitting

mobilization of their effector properties. It has been suggested that the extent of mast cell involvement in both allergic and non-allergic disease pathophysiology, as well as host defence, is likely to be far greater than would be predicted strictly on the basis of studies of classical IgE-dependent immune responses (Galli, 1993; Boyce, 2003).

In this regard, mast cells are found particularly prevalent at sites which interface with the environment, such as airways and a number of studies have reported that mast cells play a critical role in host immune defence against bacterial infections. These studies have shown that mast cells have the ability to recognize and engulf bacteria and release a number of inflammatory mediators and there is an expanding list of microbes that can cuase their activity (Abraham *et al.*, 1997).

The function of mast cells as sensors of infection and injury through innate recognition receptors and their response to these signals both limits the potential spread of infection (by recruiting neutrophils) and facilitates wound healing, through actions on fibroblasts and other connective tissue elements (Malaviya *et al.*, 1996a).

In the context of TH2-polarized mucosal inflammation, cytokines transduce growth and survival signals to mast cells and their progenitors and alter their profile of gene expression. These alterations both expand the repertoire of mast cell effector mediators (cytokines) and lower their threshold for activation by both IgE-dependent and non–IgE-dependent mechanisms (Galli, 1993; Boyce, 2003). These mechanisms for phenotypic modification of mast cells might have evolved as a result of pressures exerted by helminth infection. They very likely contribute to functional alterations in mast cells in the tissues of allergic individuals. It is possible that these alterations render mast cells capable of triggering sustained or deleterious inflammatory responses to otherwise innocuous stimuli.

The potential for microbes to elicit or exacerbate allergic mucosal inflammation directly through activation of reactive phenotypically altered mast cells requires further investigation. Under such circumstances, the mast cell effector systems normally specialized to protect against pathogens and heal tissue could lead instead to prolonged inflammation and other damage such as fibrosis, angiogenesis, and tissue remodeling (Boyce, 2003).

There are, however, few reports or the role of mast cells in defence against or damage caused by bacterial respiratory diseases. For example, it was reported that mast cells are critical for the containment of and recovery from airway mycoplasma infection (Xu *et al.*, 2006). Mast cell deficient mice had no apparent defects in

adaptive immune responses but may mast cells contribute to innate defences against this important respiratory pathogen. If this is also the case in humans, than pharmaceutical strategies to eliminate mast cells in severely affected asthmatics may weaken defences against mycoplasma and lead to exaggerated inflammatory responses to infection (Xu *et al.*, 2006).

In term of their potential to cause damages mast cells produce histamine and are associated with the allergic response, and MCs lying below the mucosal surface can also stimulate neurons that innervate the gastrointestinal and respiratory tract. Such stimulation could result in increased peristalsis (gastrointestinal tract) or trigger the cough or sneeze response (respiratory tract), but this would have the benefit of aiding in expulsion of invading microorganisms (Salyers and Whitt, 1994).

In the present study, the Gram-negative bacterium *B. bronchiseptica* (Bvg^+) caused a significant cytotoxicity (ie. reduction in ATP content) in sheep BMMC, and β-hexosaminidase release. Such bacteria-induced cytotoxic effects and mediator release from superficially lying mast cells in the airways epithelium might be of great importance in respiratory tract infections. In fact, the balance between the potential beneficial and harmful effects of bacteria-mast cell interactions might have a pivotal role in determining the outcome of the infection. On the one hand, there is the beneficial phagocyte clearance of bacteria by mast cells and other cell types and, on the other hand, the potentially harmful effect resulting from mast cell destruction and mediator release. In this regard, mast cells have become recognised as essential in immune response as other cell types such as neutrophils and alveolar macrophages which are well-known defence mechanisms against bacterial infection (Malaviya and Abraham, 1995).

In the present study, phagocytic activity of MC was studied by using bioluminescent strains of *B. bronchiseptica* and *E. coli* and degranulation was followed by estimating β-hexosaminidase release which is a granule-associated molecule. For the first time, viable intracellular *B. bronchiseptica* (Lux 95) were shown within sheep BMMC. These results establish baseline data for individual and age-related variation in sheep BMMC phagocytic function, and form a basis for further evaluation of other functions such as chemotaxis, oxidative burst and clearance of bacterial infection in mast cells, either in sheep or other animal models. In the present study, internalisation of *B. bronchiseptica* (Lux 95) by sheep BMMC

was observed and β-hexosaminidase release was also detected due to interaction with *B. bronchiseptica* (Bvg^+). These observations demonstrate the ability of sheep BMMC to recognise and take up surrounding bacteria.

B. bronchiseptica is a respiratory tract pathogen (Mattoo and Cherry, 2005). *B. bronchiseptica* is thought to achieve colonisation of this site by producing a variety of putative adhesins, such as filamentous haemagglutinin (FHA), pertactin (PRN) and adenylate cyclase toxin (CyaA) interfering with the normal phagocyte clearance mechanisms. The cytotoxicity of *B. bronchiseptica* toward sheep BMMC, RBL-2H3 cells and macrophage cell lines was compared with that of different *Bordetella* species (*B. pertussis* and *B. parapertussis*) and unrelated bacteria (*S. pneumoniae* and *P. multocida*).

The effect of *B. bronchiseptica* in respiratory infection in swine has been investigated (Brockmeier and Register, 2000). It was reported that bacteria were observed extracellularly as well as intracellularly within swine alveolar macrophages by using immunofluorescent staining. However, more bacteria were found surviving intracellularly after the macrophages were cultured with polymyxin B to kill extracellular bacteria. Similar results were seen in experiments performed with an isogenic Bvg^- phase-locked mutant of *B. bronchiseptica* cultured at 37° or 23° C, indicating that another temperature dependent mechanism in addition to Bvg may play a role in adhesion and intracellular survival. *B. bronchiseptica* was cytotoxic for swine alveolar macrophages in the Bvg^+ phase only. Furthermore, there was no doubt important to escape from immune clearance mechanisms and establish infection, and could leave the host susceptible to secondary respiratory pathogens (Brockmeier and Register, 2000).

In *B. pertussis* infections, there are no reports that mast cells have a role in clearance or pathology, but preliminary findings from a rat model of pertussis infection showed mastocytosis in the trachea, with evidence of mast cell degranulation (personal communication with J. Wastling and R. Parton). Likewise, there are no reports in the literature that mast cells have a role either in defence against *B. bronchiseptica* infection or in contributing to pathology of disease caused by these bacteria. However, in view of the present finding, where *B. bronchiseptica* (Bvg^+) strain and other *Bordetella* species caused a significant degranulation effect on

mast cells (β-hexosaminidase release) *in vitro*, this could suggest that similar effects could occur *in vivo*.

4.1.7 Use of bioluminescent bacteria as an *in vitro* reporter of the interaction between *B. bronchiseptica* and sheep BMMC (mucosal mast cells) and other cell types

4.1.7.1 Cytotoxic effect of *B. bronchiseptica* (Lux 95)

The bioluminescent mutant strain of *B. bronchiseptica* (Lux 95) used in this study (Forde *et al.*, 1998) had been created from a strain of *B. bronchiseptica* 5376 that had been kept in the laboratory culture collection for many years. Characterisation of strain *B. bronchiseptica* (Lux 95) in the present study showed that it had some properties in common with the *B. bronchiseptica* (Bvg^- phase). These properties included a lack of haemolytic effect, lack of haemagglutination effect and lower enzymatic activity of the adenylate cyclase toxin (ACT) associated with the Bvg^+ phase of growth of another *B. bronchiseptica* strain BBC17 (N.B. Lux strain was derived from a different strain to the Bvg^+ and Bvg^- control strain). On the other hand, *B. bronchiseptica* (Lux 95) showed more of a cytotoxic effect than *B. bronchiseptica* (Bvg^-phase) (Fig. 3.3.12). For example, it was observed that there was around 80% survival of RBL-2H3 cells when incubated with *B. bronchiseptica* (Lux 95), but 95% survival with an equal number of *B. bronchiseptica* (Bvg^- phase) bacteria and 30 % survival with *B. bronchiseptica* (Bvg^+ phase) bacteria. Furthermore, it observed that there was greater β-hexosaminidase release from RBL-2H3 cells due to incubation with *B. bronchiseptica* (Lux 95) compared with *B. bronchiseptica* (Bvg^- phase), but less release compared with (Bvg^+ phase)(Fig. 3.3.13). These observations suggested that *B. bronchiseptica* (Lux 95) used in the present study had some properties in common with both *B. bronchiseptica* (Bvg^+ phase) and (Bvg^- phase) and may have been in an intermediate phase (Bvg^i phase)(see Introduction).

The bacterium *B. bronchiseptica* in the Bvg^- virulent phase or mode is known to be cytotoxic and the phase is characterized by the expression of adhesins such as fimbriae, filamentous haemagglutinin (FHA), and pertactin, and toxins such as adenylate cyclase toxin (ACT). The avirulent (or Bvg^-) phase or mode is poorly cytotoxic and is characterized by motility and none of the known adhesins or toxins

are present. Both phases are under the influence of the BvgAS signal transduction system (Coote, 2001) and modulation between the phases (the process known as antigenic modulation) can be induced by different factors. It has been reported that sulphate anion is a modulating agent for *Bordetella* and therefore addition into the growth media could shut off, very rapidly, the BvgAS signal transduction system and the kinetics of transcriptional pathway for production of different virulent factors. However, modulation is a freely-reversible process and, in the absence of $MgSO_4$ in the medium, the organism should readily revert to its original Bvg^+ mode (Gross and Rappuoli, 1989; Martinez de Tejada *et al.*, 1996; Smith *et al.*, 2001). It was reported that *B. bronchiseptica* (Lux 95), used in the present study, was created in a condition containing 10 mM $MgSO_4$ (at the time of minitransposon mutagenesis, for plasmid transfer) (Forde *et al.*, 1998). In the present study, growth in the absence of the modulating agents ($MgSO_4$) in the culture medium (BG agar) did not help in resumption of transcription of virulence factors.

A more likely explanation for the loss of virulence of *B. bronchiseptica* (Lux 95) is phase variation. Upon growth of *Bordetella* in the laboratory, phase variants arise spontaneously, with a strain-dependent frequency, and these variants are no longer able to express the virulence factors owing to spontaneous mutation in the *bvg* locus (Stibitz *et al.*, 1989). Thus, the laboratory strain used to create the *B. bronchiseptica* (Lux 95) strain could already have entered the Bvg^- or Bvg^i phase, or, for some reason, *B. bronchiseptica* Bvg^- or Bvg^i phase variants were selected during the transformation process. *B. bronchseptica* (Lux 95) used in the present work was grown routinely on BG agar to maximise its virulence factor expression, even though various lines of evidence suggested that it was not completely in the Bvg^+ mode.

The phase variation of *B. bronchiseptica* (Lux 95) may suggest that *B. bronchiseptica* (Lux 95) may not have been ideal for study of interaction with different mammalian cells. In future studies, it would be better to create Lux strains from authentic *B. bronchiseptica* (Bvg^+ phase) and *B. bronchiseptica* (Bvg^- phase), which would help to study their different interactions with different mammalian cells. This could be done by using the method of Forde *et al.* (1998) with the same minitransposon probe used to create strain Lux 95. Uptake of bioluminescent Bvg^+ phase might kill the mammalian cells and then in turn the bacteria would be killed by polymyxin B. Uptake of the bioluminescent Bvg^- phase could lead to possible intracellular survival for some time. By inserting the *lux* genes at different points in *B.*

bronchiseptica chromosomes, under the control of promoters of known Bvg-activated or repressed genes, some insight would be gained into the role of these bvg-regulated factors when *B. bronchiseptica* interacts with, and is internalized by, different mammalian cells. Thus, the use of the bioluminescence technique may helping to understand better the intracellular survival of different phases of *B. bronchiseptica* and other bacteria.

4.1.7.2 Evaluation of using *lux* genes in this study

In the present study, the bioluminescent *B. bronchiseptica* (Lux 95) was one of 2000 mutants of *B. bronchiseptica* constructed by Forde *et al.* (1998) with the aid of the minitransposon promoter probe vector pUTmini-Tn*5km*1*ux*. Forde *et al* (1998) observed that expression of bioluminescence varied considerably between the mutants and when they were grown under different conditions, as viewed by CCD imaging. Expression of bioluminescence in three very bright fusion strains was examined on BG agar and BG agar supplemented with $MgSO_4$ or nicotinic acid, because these stimuli are known to affect Bvg-dependent regulation of gene expression *in vitro* in *Bordetella* species. It was found that the level of light output in these three strains was unaffected by the growth medium. Expression of luminescence was also unaffected at low pH (pH 5.5) and in the presence of the superoxide generator methyl viologen. When levels of bioluminescence were measured by automated luminometry, the brightest strain showed light output greater than that found in *X. luminescens*, the original source of the *lux* genes or in *E. coli* DH5α carrying the multicopy plasmid pT7-3 containing the *lux* genes. Consequently, this *B. bronchiseptica* mutant strain (Lux 95) was chosen as a constitutively bioluminescent strain. However, the location of the *lux* genes in *B. bronchiseptica* (Lux 95) is unknown. With the availability of the *B. bronchiseptica* genome sequence (Parkhill *et al.*, 2003) this could be determined. Knowledge of the *B. bronchiseptica* genome sequence also opens up the possibility of a more rational approach to creating Lux strains in which the *lux* genes are placed under the control of different promoters of genes that are differentially (e.g. Bvg-regulated) or constitutively expressed.

In the present study, the bioluminescence level of *B. bronchiseptica* (Lux 95) maintained in different media (IMDM alone, IMDM containing 10% FCS, PBS alone and PBS containing 10% FCS) was monitored by luminometer. It was observed that the bioluminescence output was dependent on the presence of FCS in the different

media. For example, the results in Figure 3.2.2a suggested that the addition of heat-inactivated fetal calf serum into IMDM growth medium increased light output. It could be that the presence in FCS of extra growth factors as exogenous substrate provides an ideal condition for the growth of *B. bronchiseptica* (Lux 95) in IMDM medium.

The correlation between bioluminescence output (RLU) and viability of *B. bronchiseptica* (Lux 95) was monitored. It was observed that there was a linear correlation between the bioluminescent signal and viable numbers of *B. bronchiseptica* (Lux 95) (Figure 3.2.2b). When both relative light units (RLU) and optical density (OD) were monitored in a long-run experiment (> 20 h)(Figure 3.2.3c), it was observed that, within the first 20h, there was a good correlation between them. But, after that there was a poor correlation between the two factors (Figure 3.2.3d). At the end of a long-run experiment, viability of *B. bronchiseptica* (Lux 95) was determined by viable count on BG agar. The combination of reduction in bioluminescence output, recovery of viable *B. bronchiseptica* (Lux 95) suggests that *B. bronchiseptica* (Lux 95) can remain viable but without producing significant light output. In addition, bioluminescence output of *B. bronchiseptica* (Lux 95) was active up to 20 h, during the log phase of bacterial growth, but thereafter, *B. bronchiseptica* (Lux 95) showed reduced bioluminescence activity during the stationary and decline phases.

A bioluminescent *E. coli* was also used in this study and light output was monitored. When RLU and OD were monitored in a short-run experiment (20 h) (Figure 3.2.6), it was observed that, within the first 10 h, there was a good correlation between both bioluminescence output and optical density. But, after that, there was a poor correlation between the two factors. At the end of a short-run experiment (20 h) viability of *E. coli* was determined by viable count on LB agar and again, large numbers of viable cells were obtained but without significant light output.

Comparison between light output of bioluminescent *E. coli* and *B. bronchiseptica* (Lux 95) in a short-run experiment suggested that the correlation between bioluminescence output and optical density of the bioluminescent *E. coli* was not as good as with *B. bronchiseptica* (Lux 95). The difference is probably due to the fact that *E. coli* is a much more rapidly growing organism than *B. bronchiseptica* and will therefore reach stationary phase in a much shorter time. Even so, the differences

in light output were a rapid decline in *E. coli* and a much slower decline in *B. bronchiseptica*.

Monitoring the light emission in bioluminescent bacteria may give an indication of bacterial growth phase. The latter phenomenon corresponds with the observations of other researchers working with reporter genes. For example, during mid to late logarithmic growth, depending on the species and the nutrient composition of the growth medium, light emission will increase dramatically. The increase in luminescence arises by activation of expression of the genes in the *lux* operon. Furthermore, it has been reported that upon entrance into stationary phase and during starvation, there is a decrease in luciferase activity that corresponded to a decrease in the metabolic activity of the population while the number of culturable cells remained relatively stable (Meighen, 1993; Unge *et al.*, 1999).

In this regard, it was reported that the light emission from a bioluminescent *Yersinia enterocolitica* was partly dependent on the growth phase, and in stationary phase there was a significant reduction of light emission (Maoz *et al.*, 2002). Accordingly, the observations in the present study suggest that, with a longer incubation time of *B. bronchiseptica* (Lux 95) or bioluminescent *E. coli*, presumably when the culture reaches stationary phase, the bacteria gradually crease to emit light even though they remain viable. Such older cultures would therefore not be useful for invasion assays.

In addition, it is known that the *lux* genes can rely on a promoter region of the host organism genome for their expression (Meighen, 1991). *B. bronchiseptica* was successfully mutagenized with a promoter probe suicide vector carrying the *lux* genes from *P. luminescens* on a mini-Tn5 derivative (Forde *et al.*, 1998). The *lux* genes were transferred to the chromosome of *B. bronchiseptica* whereas, in *E. coli*, the *lux* genes were carried on a plasmid. In the *E. coli* strain used in the present study, the *lux* genes were carried on plasmid pT7-3 and were under the control of the phi-10 promoter for T7 RNA polymerase. They were transcribed only at a low level by the *E. coli* RNA polymerase (Szittner and Meighen, 1990). Chromosomal insertions of a transposon carrying the *lux* operon in bacteria would dictate the strength of light emission. As discussed previously the *lux* genes are used as reporters of gene expression and light output will depend on exactly where the *lux* genes have inserted in the chromosome. If the *lux* genes are inserted downstream of the promoter of a gene that is only active in the early stages of growth, light output may decline rapidly

as the culture ages. If downstream of a gene that is constitutively expressed, light output may last longer. These differences may explain the differences in light emission of *E. coli* compared with *B. bronchiseptica*, but may need more investigation.

The use of reporter genes (*lux* genes) to study the interaction of mast cells with *B. bronchiseptica* showed that this technique was very sensitive, and there was a linear relationship between of light intensity and the viable numbers of bacteria, at least in short term experiments with actively growing bacteria. Furthermore, there was no need to add any exogenous substrates to observe the reaction, and, there was no biomedical hazard. Availability of a sensitive detector such as the Lucy luminometer used here enabled the direct measurement *in vitro*. Expression of the *lux* genes in different bacteria (*B. bronchiseptica* and *E. coli*) provided a simple and sensitive system for monitoring the growth and intracellular survival of bacteria, bearing in mind the various limitation of the system that are imposed by the growth phase of the bacterium as discussed above. Furthermore, use of *lux* genes under the control of appropriate promoters could indicate the different environmental conditions that the bacteria may be subjected to when interacting with different mammalian cells, either extracellulary or intracellulary.

The correlation between bioluminescence output and viability of *B. bronchiseptica* (Lux 95) and any new *lux* construct should be investigated thoroughly by viable counting at intervals throughout the growth cycle and in different media, and plotting light output against viable cell numbers. Optical density is a good indicator of viable cell numbers during the early stage of growth but does not distinguish between metabolically-active or inactive bacteria during later stages of growth. In addition, the results suggest that bioluminescence output could be a useful tool for short run experiments of up to 20 h for *B. bronchiseptica* (Lux 95). But, for longer term observations, for example for studying prolonged survival intracellulary a more accurate comparison of RLU and viable cell numbers, by using accurate plate counts would be necessary to confirm the relationship between light output and viability. Alternatively, new constructs might be useful when the *lux* genes might be controlled by promoters that are active in stationary phase.

4.1.7.3 Intracellular *B. bronchiseptica* (Lux 95) within sheep BMMC

These studies with bioluminescent *B. bronchiseptica* (Lux 95) demonstrated that sheep BMMC are able to take-up bacteria. Furthermore, bioluminescence signals are indications of metabolic activity, and these studies demonstrated that both viable sheep BMMC and the contents of lysed sheep BMMC are able to reduce the metabolic activity of *B. bronchiseptica* (Lux 95).

Antimicrobial activity of polymyxin B sulphate was used to kill extracellular *B. bronchiseptica*. Polymyxin B sulphate is a cyclic decapeptide antibiotic with surfactant activity which disrupts the bacterial outer and cytoplasmic membranes by incorporation into the phospholipid bilayer (Ouderkirk *et al.*, 2003). A cytolytic reagent, digitonin ($C_{56}H_{92}O_{29}$), is also applied and, because of its mode of action for permeabilization of certain types of mammalian cells (e.g. blood platelets, hepatocytes, yeast, tumour cells and macrophage cell line), does not effect bacterial integrity and viability (Forde *et al.*, 1998). Both polymyxin B and digitonin were used for detection of intracellular *B. bronchiseptica* within sheep BMMC or other cell types. It was observed that 50 µg/ml of polymyxin B killed more than 99.9% of *B. bronchiseptica* (Lux 95) within 2 h and, due to that, caused a sharp decline in bioluminescence output to basal levels where no mammalian cells were present. The optical density was not significantly altered by such treatment. No effect on light output and viability of bacteria was observed with 50 µg/ml of digitonin (Figure 3.2.5).

Some earlier reports have suggested that *B. bronchiseptica* can be found intracellularly in HeLa cells (Savelkoul *et al.*, 1993) and in murine macrophages (Forde *et al.*, 1998). In the present study, the bioluminescent strain of *B. bronchiseptica* (Lux 95) was used to show that it could be found intracellulary in mast cells as well as in macrophage cell lines (P338D1 and J774A.1). At 4 h postinfection of sheep BMMC with *B. bronchiseptica* (Lux 95), the results clearly showed a low level of light output recorded from infected sheep BMMC after polymyxin B treatment. Lysis of the sheep BMMC with digitonin, which has no effect on the viability of *B. bronchiseptica*, produced a rapid decline in this residual light output to basal levels as the internalized bacteria were exposed to the antibiotic polymyxin B in the surrounding medium and were killed. Furthermore, similar studies were done with bioluminescent *E. coli* (Result, section 2.9) and again showed light output from

infected viable sheep BMMC after polymyxin B treatment. Lysis of the sheep BMMC with digitonin produced a rapid decline in this residual light output to basal levels as the internalized bacteria were exposed to the antibiotic polymyxin B in the surrounding medium and were killed.

In the present study, novel finding have been made by the use, for the first time of bioluminescent bacteria with mast cells. In the present study with sheep BMMC, their ability for uptake of *B. bronchiseptica* (Lux 95) increased with increased maturity of the sheep BMMC. Takeup of *B. bronchiseptica* (Lux 95) was observed with mast cell cultures aged between 14 and 30 days. It was observed that the bioluminescent signals from internalized *B. bronchiseptica* (Lux 95) were higher with 29-day cultures than with 14-day cultures.

In addition, a reduction in the bioluminescent signal from *B. bronchiseptica* (Lux 95) was observed due to co-culture with viable sheep BMMC or by mixing with the intracellular contents of lysed sheep BMMC. The results suggest that metabolic activity of bioluminescent *B. bronchiseptica* was reduced due to either the viable cells themselves or the contents of lysed mast cells. Furthermore, the effect of three day-old culture fluid from sheep BMMC (i.e. sheep BMMC grown in antibiotic-free IMDM medium) on *B. bronchiseptica* (Lux 95) was studied and there was no effect on viability, as judged by any alteration in light output compared with control *B. bronchiseptica* culture. The results suggested that there were no antimicrobial products secreted from sheep BMMC during their differentiation (Result, section 2.6).

An eventual reduction in bioluminescence signal was observed from intracellular *B. bronchiseptica* (Lux 95) within sheep BMMC (Figure 3.2.10b). This could be due to killing of the bacteria in the phagosomal vacuoles by fusion with antimicrobial granules or due to mast cells slowly dying under these assay conditions and therefore exposing *B. bronchiseptica* (Lux 95) to polymyxin B. Alternatively, polymyxin B could have been slowly leaking into the mast cells or being taken up by the mast cells and therefore exposing *B. bronchiseptica* (Lux 95) to polymyxin B. Another possibility is that the bacteria are simply becoming less metabolically-active in the intracellular environment even though they remain viable just as they do in old cultures.

The invasion experiments described here have shown that both phases of *B. bronchiseptica* (Bvg^+ and Bvg^-) as well as the bioluminescent strain (Lux 95) were able to invade or be taken up by sheep BMMC. Similar observations were detected

with HeLa cells (Savelkoul *et al.*, 1993). Savelkoul and his group described that there was no difference in the invasiveness of *B. bronchiseptica* when the bacterium was not able to produce either FimX or Fim2 fimbriae. The reason for this phenomenon was probably that *B. bronchiseptica* produces FHA which is the most important adhesin for *B. pertussis* (Saukkonen *et al.*, 1991). The present results showed that the bioluminescent strain of *B. bronchisptica* (Lux 95) was able to invade or be taken up by mast cells as well as the other two phases. Also, the results of the invasion assay had shown, for the first time, different phases of *B. bronchiseptica* within mast cells.

Pretreatment of sheep BMMC with either cytochalasin D (CD) or monodansylcadaverine (MDC) could help to understand whether the uptake of *B. bronchiseptica* by sheep BMMC occurred by microfilament-dependent phagocytosis rather than by invasion of *B. bronchiseptica* (Forde *et al.*, 1998).

4.1.8 Interaction of *B. bronchiseptica* with different mammalian cells

These results of this study demonstrated that *B. bronchiseptica* is able to interact directly with sheep BMMC and other cell types (RBL-2H3, J774A.1 cell lines) in a Bvg-dependent process.

B. bronchiseptica has been shown to exerted a strong cytotoxic effect on HeLa cells. When sulphate anions were added to the growth medium of *B. bronchiseptica*, this effect was not seen because sulphate anions inhibit the BvgAS response (van den Akker, 1997). In the present work, comparative analysis of the cytotoxicity of *B. bronchiseptica* (Bvg^+ phase) grown on BG agar and other media showed that cytoxticity and degranulation effects of *B. bronchiseptica* grown on BG agar were high compared with other media. In addition, activity of ACT from *B. bronchiseptica* grown on BG agar was high compared with CL broth or LB agar. These observations confirm the suggestion that growth medium is an important environmental factor in virulence modulation of *B. bronchiseptica* (Coote, 2001). In this regard, the BvgAS sensory transduction system activates the transcription of genes encoding all the known protein virulence factors expressed by *Bordetella* subspecies (Deora *et al.*, 2001). Expression of different adhesins and toxins are presumably responsible for the cytotoxic effects of *B. bronchiseptica* (Bvg^+ mode).

Cytotoxic factors of *B. bronchiseptica* were investigated by treating *B. bronchiseptica* (Bvg^+) at different temperatures (Result, section 3.5). Survival of the

different mammalian cell types was unaffected or less affected with *B. bronchiseptica* (Bvg^+) treated to 56°C. These observations demonstrated that the cytotoxic factors of *B. bronchiseptica* (Bvg^+) were heat labile and presumably protein in nature.

It was observed that *B. bronchiseptica* (Bvg^+ phase) has a degranulation effect toward sheep BMMC (and RBL-2H3 cells) causing the release of mast cell contents (e.g. β-hexosaminidase) into the surrounding medium.

4.1.8.1 Comparison of cytotoxicity of *B. bronchiseptica* with that of other *Bordetella* species

Many studies have evaluated virulence factor expression of *B. bronchiseptica*, and it was found to be similar to that of *B. pertussis* except for pertussis toxin (PT) which is specific to *B. pertussis* (Arico *et al.*, 1987; Martinez de Tejada *et al.*, 1996; Parkhill *et al.*, 2003). In the present study, different strains of *B. pertussis* were used; wild-type (*B. pertussis* 338), avirulent strain (Bvg^- phase) (*B. pertussis* 347), PT-deficient strain (*B. pertussis* 357) and ACT-deficient strain (*B. pertussis* 348). These strains were incubated with RBL-2H3 cells for 3 h. It was observed that none of the *B. pertussis* strains showed a reduction in the viability of RBL-2H3 cells or even a release of β-hexosaminidase. It was hoped that the wild type strain (338) would show cytotoxicity toward RBL-2H3 cells like *B. bronchiseptica* (Bvg^+) but, surprisingly, this was not observed. The transposon mutant strains (347, 357 and 348) could then have been used to identify whether certain virulence factors were responsible but again, no cytotoxic or degranulation effects were observed. These observations suggest that *B. pertussis* hss low cytotoxicity compared with that of *B. bronchiseptica* toward RBL-2H3 cells. More cytotoxic effect could perhaps have been observed with a higher infection ratio (more than 1:500) or longer incubation (> 3 h).

Cytotoxicity and the degranulation effects of different *Bordetella* species toward different cell types (RBL-2H3 and J774A.1 cells) were compared with *B. bronchiseptica* (Bvg^+) and its mutants (Bvg^- and Lux 95). As before, *B. bronchiseptica* (Bvg^+) showed a more significant cytotoxic effect toward J774A.1 cells than towards RBL-2H3 cells. Survival of both mammalian cell types was high (≥ 70%) after incubation with different *Bordetella* species compared with *B. bronchiseptica* (Bvg^+) (Fig. 3.3.8). These species of *Bordetella* have not yet been well characterized (except *B. pertussis*), and it is not clear which virulence factors they

possess. A recent paper has shown that *B. hinzii* produces ACT (Donato *et al.*, 2005). In contrast to *B. pertussis* ACT, however, ACT from *B. hinzii* is less extractable from whole bacteria, non-haemolytic, has a 50-fold reduction in adenylate cyclase enzymic activity, and is unable to elevate cyclic AMP levels in host macrophages. Furthermore, in the present work, *B. trematum* and *B. hinzii* showed a significant β-hexosaminidase release from RBL-2H3 cells similar to the effect of *B. bronchiseptica* (Bvg^+) and greater than that of the other *Bordetella* species. Such observations have not been reported previously. These results suggest that it will be important to investigate whether these species produce TTSS and corresponding effector proteins like *B. bronchiseptica* (Bvg^+). Also, it would be useful to further investigate the different factors of *B. trematum and B. hinzii* that initiate β-hexosaminidase release. For this purpose, TTSS mutant strains should be generated, and secretion profiles be compared with that of the wild-type strain. For example, BopN, BopD, Bsp22 and BopB have been identified as type III secreted proteins in *Bordetella*. Severe cytotoxicity such as necrosis was induced in L2 cells by infection with the wild-type *B. bronchiseptica*. In contrast, this effect was not observed by the BopB mutant infection. These results demonstrate that *Bordetella* secretes BopB via a type III secretion system during infection. BopB may play a role in the formation of pores in the host plasma membrane which serve as a conduit for the translocation of effector proteins into host cells (Kuwae *et al.*, 2003).

4.1.8.2 Comparison of the cytotoxicity of *B. bronchiseptica* with that of unrelated bacteria

The cytotoxic and degranulation effects of *B. bronchiseptica* (Bvg^+) for different mammalian cell types were compared with the cytotoxic and degranulation effects of different unrelated bacteria, widely used in our laboratory to determine whether these effects were species specific. These bacteria included a virulent phase *S. pneumoniae* and a pneumolysin-deficient derivative. The virulent phase *S. pneumoniae* is a well-known pathogen for a number of diseases including pneumonia, otitis media, and meningitis in humans of all ages (Andersson *et al.*, 1981). Pneumolysin toxin has a range of biological activities such as lysis of mammalian cells *in vitro* (Nollmann *et al.*, 2004). Other bacteria included *P. multocida* serotype B:2 which causes haemorrhagic septicemia (HS), a commonly fatal systemic disease

of cattle and buffaloes in countries of South and Southeast Asia, and the wild-type strain 85020 compared with a derivative containing a deletion in the aroA gene (strain JRMT12) (Tabatabaei *et al.*, 2002).

It has been reported that significant differences were detected in survival time, core body temperature, lung-associated and systemic bacterial loads, mast cell numbers, magnitude and location of cytokine production, lung disruption, and ability of isolated lung cells to release the cytokine tumor necrosis factor (TNF) alpha in vitro following infection of (CBA/Ca) and resistant (BALB/c) inbred mouse strains with *S. pneumoniae* (Kerr *et al.*, 2002). It was suggested that infection with *S. pneumoniae* may be a factor in their elevated susceptibility to pneumococcal pneumonia which reduced expression of TNF in lung airways during early infection. This leads to reduced neutrophil influx, permitting pneumococcal numbers to increase within the lung tissue, and to the initiation of systemic disease. In the present study, there was a statistically significant differences in survival of sheep BMMC, RBL-2H3 cells and J774A.1 cells after incubation with wild-type and the pneumolysin deficient strain of *S. pneumoniae*. The pneumolysin deficient strain caused little cytotoxicity compared with both virulent strains of *S. pneumoniae* and *B. bronchiseptica* (Bvg^+). β-hexosaminidase release was also measured after exposure to both strains of *S. pneumoniae*. However, the β-hexosaminidase gene has been reported in the genome of *S. pneumoniae* (www.tigr.org) and β-hexosaminidase was detected in the culture supernatants of both strains of *S. pneumoniae* (data not shown). Thus, β-hexosaminidase is not a suitable marker for studying the effect of *S. pneumoniae* on degranulation of mast cells, and other degranulation markers such as histamine or mast cell proteases would be more appropriate.

Both strains of *P. multocida* showed little effect on survival of sheep BMMC, RBL-2H3 cells and J774A.1 cells compared with the cytotoxicity of *B. bronchiseptica* (Bvg^+) (Figure 3.3.11b). In addition, both strains showed little effect on β-hexosaminidase release from sheep BMMC and RBL-2H3 cells compared with the potent degranulation effect of *B. bronchiseptica* (Bvg^+) on these cell types. These results suggest that wild-type of *S. pneumoniae* and *P. multocida* have different virulence factors and showed different cytotoxic effect toward sheep BMMC and other cell types. In these regards, it would be useful to study further the secretion

systems in unrelated bacteria, for example in Gram-positive bacteria, and compare them with the TTSS system in *B. bronchiseptica* (Bvg^+) (Mattoo *et al.*, 2004).

4.1.9 Interaction of different mammalian cells with purified factors of *Bordetella* species

Adenylate cyclase toxin (or CyaA) of *Bordetella* species possesses both enzymatic activity and pore-forming activity. It can invade mammalian cells and catalyzes the unregulated production of cyclic adenosine 3′,5′- monophosphate (cAMP) which disrupts the physiological functions of the cells and may cause cell death. The cytotoxic ability of ACT was investigated by using purified recombinant ACT produced in *E. coli*. This was achieved by comparing a rACT which was enzymatically active and invasive with different mutant forms of ACT.

Two different assays were used to assess cytotoxicity, the ATP bioluminescence assay which uses luciferase to measure ATP presence in cell and the MTT assay which relies on intact mitochondrial function to convert tetrazolium dye. In both the MTT and ATP assay, the wild-type rACT (44/54) was shown to be more cytotoxic than the rACT (44-188/54) which is enzymatically inactive (Table 4.1). This suggests that it is the adenylate cyclase conversion of ATP to cAMP that is responsible for cytotoxicity. However, rACT (44-188/54), it was still able to induce cell death in J774A.1 cells (50% survival by ATP assay and 85% survival by MTT assay), albeit at high concentration (0.625 µg/ml). These results suggest that the invasive, pore-forming activity of rACT (44-188/54) may be responsible for such deplation in the ATP contents of J774A.1 cells. Such effects were not observed with other cell types (sheep BMMC or RBL-2H3 cells). When the results of both assays were compared, it appeard that rACT (44/54) affected the ATP content of the different mammalian cells more than the dehydrogenase enzymes measured in the ATP assay. Recombinant ACT (44/54) showed a high level of cytotoxic effect toward J774A.1 cells compared with sheep BMMC and RBL-2H3 cells. These data suggest that sheep BMMC proliferated *in vitro* could express fewer CD11b/CD18 receptors on their surface compared with J774A.1 cells which showed a high interaction with rACT (44/54).

The primary biological function of ACT is presumably to deliver its enzymatic activity into mammalian cells, ultimately resulting in their intoxication by the

depletion of ATP rather than the formation of membrane channels. In addition, results of these studies suggested that the cytotoxic effect of rACT (44/54) on the viability of sheep BMMC and other cell types may depend either on the number or availability of cell receptors (CD11b). It has been reported that CyaA uses the ß2 integrin Mac-1 (CD11b/CD18, CR3) as a cell receptor in that binding of CyaA to various murine cell lines and human neutrophils was specifically blocked by anti-CD11b monoclonal antibodies. The increase of intracellular cAMP level and cell death triggered by CyaA intoxication was also specifically blocked by anti-CD11b monoclonal antibodies. (Guermonprez *et al.*, 2001). The Mac-1 is present on neutrophils, monocytes, NK cells, eosinophils, and basophils. It has also been shown to be weakly expressed on a human leukemic MC line HMC-1(Weber *et al.*, 1997), and in a small fraction (<5%) of in vitro-derived human MCs (Toru *et al.*, 1997), although Mac-1 has not been detected in mature MCs isolated from various human tissues (Valent, 1994). This could be determined by labelling the different cells with specific fluorescent monoclonal antibodies to the receptor (CD11b/CD18) and using FACS.

It has been shown previously that cell death in macrophage caused by ACT is due to the induction of apoptosis (Khelef *et al.*, 1993). It is possible that the AC activity may be responsible as the generation of high levels of cAMP can lead to alterations in protein phosphorylation and a reduction in ATP contents may deregulate protein kinase cascades and result in apoptosis (Duprez *et al.*, 1993). The results of these studies showed that the haemolytic property of ACT may be not mainly responsible for apoptosis, but it may cause a little apoptosis effect as pore formation results in changes in intracellular calcium level which can activate cell death (McConkey *et al.*, 1993). The results of these studies show that rACT (44/54), which is a cAMP elevating agent, induced a rapid increase in caspase 3/7 activity in RBL-2H3 cells which depends on the enzymic activity. rACT (44/54) showed only a low activity of caspase 3/7 in sheep BMMC similar to the effect of rACT (44-188/54) which suggested that these cells may not produce caspase 3/7 activity and may undergo apoptosis by a different mechanism. Further investigation of these mechanisms would be of interest. Also, this result suggests that RBL-2H3 cells appear useful for future studies of cAMP-dependent protein kinase involvement in biological processes. The results of these studies have shown three different types of activation of caspase 3/7 (apoptosis) on three cell types. The extent of DNA degradation in nuclei was investigated in three different cell types. Using the DNA fragmentation

assay, rACT (44/54) was shown to induce cell death in RBL-2H3 cells via apoptosis and the enzymatic activity of ACT was important to induce such effects. In contrast, the rACT (44-188/54) with only pore forming activity did not show either caspase 3/7 activity or DNA fragmentation in RBL-2H3 cells. There was a small effect on DNA fragmentation of sheep BMMC only at low doe of rACT (44/54) as determined after incubation for 6 h. In contrast, J774A.1 cells cultured in IMDM medium containing both low (0.07 μg/ml) and high (0.3 μg/ml) concentrations of rACT failed to show DNA fragmentation. This is probably because all J774A.1 cells die quickly within 6 h of incubation with low and high concentrations of rACT by necrosis rather apoptosis.

The result of these studies showed for the first time that the spontaneous release of β-hexosaminidase was inhibited due to rACT (44/54). RBL-2H3 cells are well known as a tumour analogues of mucosal mast cells, therefore, the inhibitory mechanisms of ACT in RBL-2H3 cells may be similar to those mast cells because it had been reported that the increase in cAMP levels in mast cells lead to inhibition of release (see introduction) and such an effect is different from that in other mammalian cells. The effects of enzymatically-active and invasive rACT (44/54) on sheep BMMC and other cell types are summarized in Table 4.1. Some discrepancies were noted between the different effects. For example, there was evidence of DNA fragmentation at low concentration of rACT (44/54) in sheep BMMC but no caspase 3/7 activity at either concentration. Also, with J774A.1 cells, there was high induction of caspase 3/7 activity with the low concentration of rACT but no DNA fragmentation was seen at this dose.

Table 4.1: Summary of effects of enzymatically-active and invasive rACT (44/54) on different mammalian cells

Mammalian cell	ATP-depletion (ATP bioluminescence assay)	Cytotoxicity (MTT assay)	Apoptosis (Caspase 3/7)	Apoptosis (DNA fragmentation)	β-hexosaminidase release
Sheep BMMC	- (at 0.039 µg/ml) + (at 1.25 µg/ml)	- (at 1.25 µg/ml)	Little caspase 3/7 activity at low and high doses	Evidence for DNA fragmentation at low dose only	ND
RBL-2H3 cell line	- (at 0.039 µg/ml) + (at 1.25 µg/ml)	- (at 1.25 µg/ml)	Good evidence of apoptosis without necrosis, even at high dose	Evidence for DNA fragmentation at low and high doses	Inhibition of spontaneous release
J774A.1 cell line	+++ (at 0.02 µg/ml)	+++ (at 0.02 µg/ml)	Good evidence for apoptosis (at low dose) and necrosis (at high dose)	No evidence for DNA fragmentation at either doses	ND

- (< 10 % of total survival)
+ (≥ 10 % and < 50 % of total survival)
++ (≥ 50 % and < 70 % of total survival)
+++ (> 70 % of total survival)
ND not determined

4.1.9.1 Interaction of PT, FHA or PRN with RBL-2H3 cells and J774A.1 cells

PT is an ADP-ribosylating toxin synthesized and secreted exclusively by *B. pertussis* and extensively studied for its effect on signalling mechanisms on mammalian cells *in vitro* (Smith *et al.*, 2001). PT binds to cell surface receptors and undergoes endocytosis via a cytochalasin D-independent pathway. The S1 subunit (see introduction) in its reduced form has been shown to catalyze the transfer of ADP-ribose from NAD to the subunit of guanine nucleotide-binding proteins (G proteins) in eukaryotic cells (Bokoch and Gilman, 1984). The results of the present studies showed that with J774A.1 cells, a significant reduction in ATP content was caused by incubation with 0.1 μg/ml compared with 0.01 μg/ml of PT. In addition, no significant cytotoxic effect was determined by the MTT assay. In contrast, no significant cytotoxic effect on RBL-2H3 cells, either by ATP or MTT assays, was determined at the end of incubation for 3 h and 6 h. But, there was an increase in caspase 3/7 activity in RBL-2H3 cells and DNA fragmentation was determined at the end of incubation for 6 h. These results suggest that PT caused a depletion in ATP content of J774A.1 cells which may have a cytotoxic effect. In contrast, PT activates programmed cell death in RBL-2H3 cells. Also, PT caused an inhibitory effect on spontaneous β-hexosaminidase release from RBL-2H3 cells. The last results suggest that further work should be done to determine the inhibitory mechanisms of PT in RBL-2H3 cells and whether an increase in cAMP content or to other factors.

The cytotoxic effect of PRN towards macrophage cells has been reported. A PRN mutant strain of *B. bronchiseptica* was less cytotoxic towards murine macrophage-like cells and on primary porcine alveolar macrophages compared with a significant cytotoxic effect of the wild type (Fordc *et al.*, 1999) particularly where tight cell-to-cell contacts were established. The mechanism of eukaryotic cell death was examined, and cell death was found to occur primarily through a necrotic pathway, although a small proportion of the population underwent apoptosis. In another study, infection of monocyte-derived macrophages (MDM) with wild-type *B. pertussis* resulted in apoptosis within 6 h, while infection with an FHA-deficient derivative strain was only 50% as effective (Abramson *et al.*, 2001). The results of the present studies showed that 0.1 μg/ml PRN had an effect on survival of J774A.1 cells as determined by the ATP assay after incubation for 3 h and 6 h. Such effects of PRN have not been reported previously and deserve further investigation particularly as

pertactin is a major component of some acellular pertussis vaccines. On the other hand, there was no significant cytotoxic effect on RBL-2H3 cells due to incubation with PRN. But, and for the first time, β-hexosminidase release was determined in J774A.1 cells due to treatment with PRN. Again, this effect deserve further study.

No significant cytotoxic effect of FHA toward RBL-2H3 and J774A.1 cells was determined either by ATP or MTT assay. The effect of different concentrations of FHA on spontaneous β-hexosaminidase release from RBL-2H3 cells and J774A.1 cells was compared with the effect of PT and calcium ionophore.

4.1.9.2 Modulation of phagocytic function of J774A.1 cells treated with PT or ACT

It has been reported that *B. bronchiseptica* and certain other *Bordetella* species produce ACT. Also, it well known that ACT enters target cells to catalyze the production of supraphysiological levels of intracellular cAMP. The increase in intracellular cAMP levels in mammalian cells lead to disturbance of many biological functions. For example, early studies showed that partially purified ACT inhibited phagocytic functions by impairing chemotaxis, oxidative response, phagocytosis and superoxide generation (Confer and Eaton, 1982). In addition, it is known that *B. pertussis* mutants deficient in the expression of CyaA are impaired in their ability to cause a lethal infection in mice (Weiss and Goodwin, 1989).

To confirm the role of ACT in blocking phagocytosis, a GFP-labelled ACT mutant *B. pertussis* was added directly to neutrophils or mixed with an equal number of unlabeled wild-type bacteria. In the absence of opsonization, neither the GFP-labelled wild-type strain, the GFP-labelled ACT mutant, nor the GFP-labelled ACT mutant mixed with the unlabeled wild-type strain was efficiently phagocytosed. Opsonization significantly increased the phagocytosis of the labelled ACT mutant but reduced phagocytosis of the wild-type strain as well as of the labelled ACT mutant mixed with the unlabeled wild-type strain. These results suggested that the presence of ACT blocks phagocytosis, even when bacteria are not expressing the toxin (Weingart *et al.*, 2000).

It also been reported that *B. pertussis* adenylate cyclase toxin can increase cAMP levels in monocytes without compromising target cell viability or impairing ingestion of particles and that the resultant accumulated cAMP is responsible for the

inhibition of oxidative responses to a variety of stimuli (Pearson *et al.*, 1987). Other evidence for blocking of phagocytosis due to increase in cAMP has been obtained by using alveolar macrophages (AMs), which are critical participants in innate lung immunity, and their uptake of *Klebsiella pneumoniae* and *E. coli*. Prostaglandin E2 (PGE2) suppression of phagocytosis was associated with enhanced intracellular cAMP production (Aronoff *et al.*, 2004).

On the other hand, PT pretreatment had no effect on the adherence of wild-type *B. pertussis*, BP338, to human monocytes, but incubation with PT at 1.0 ng/ml or greater caused a decrease in the phagocytic abilities. In these studies, *B. pertussis* were added at an MOI of 20. No differences in adherence or uptake following pretreatment of monocytes with PT occurred when experiments were conducted at an MOI of 5 (Schaeffer and Weiss, 2001).

In the present study, ACT was purified from a recombinant *E. coli* strain and not from *B. bronchiseptica*, although it is considered unlikely that this would affect its mode of action. Native PT at a low dose was used in this *in vitro* study to compare with rACT at low dose. It was observed that a significant reduction in viability of J774A.1 cells after incubation with *B. bronchiseptica* alone but, at cause of pre-treatment of J774A.1 cells with 0.1 μg/ml PT and mixed with *B. bronchiseptica*, there was a significant increase in survival of pretreated J774A.1 cells and infected with *B. bronchiseptica* (Bvg^+) compared with non-pretreated J774A.1 cells (p-value < 0.05). In addition, there was a significant reduction in the number of intracellular *B. bronchiseptica* per viable J774A.1 cells when pre-treated with 0.1 μg/ml PT compared with the number in non-pretreated J774A.1 cells. Viability of pre-treated J774A.1 with 0.1 μg/ml rACT for 1 h and infected with *B. bronchiseptica* (Bvg^+) was significantly lower than viability of non-pretreated J774A.1 cells. In addition, there was no significant reduction in the number of intracellular *B. bronchiseptica* per viable J774A.1 cells when pre-treated with 0.1 μg/ml rACT compared with the number in pre-treated J774A.1 cells with urea buffer diluent.

These observations suggest that rACT and PT work in different ways. PT appeared to protect J774A.1 cells from the killing effect of *B. bronchiseptica* (Bvg^+) whereas rACT enhanced the killing effect of *B. bronchiseptica*. Also, 0.1 μg/ml PT appeared to modulate phagocytic function of J774A.1 toward *B. bronchiseptica*,

whereas rACT had no significant effect. Therefore, it was important to investigate the effect of lower doses of ACT in modulation of phagocytic function of J774A.1 cells.

A significant reduction in viability of J774A.1 cells was observed after incubation with *B. bronchiseptica* but, in the case of pre-treatment with 0.025 µg/ml rACT, there was a non-significant increase in survival of J774A.1 cells treated with *B. bronchiseptica* (Bvg^+) compared with non-pretreated J774A.1 cells. In addition, there was a little reduction in the number of intracellular *B. bronchiseptica* per viable J774A.1 cells when pre-treated with 0.025 µg/ml rACT compared with the number in non-pretreated J774A.1 cells (Result, section 4.4.4).

These observations suggest that low concentrations of rACT (0.025 µg/ml) could prevent cell death of J774A.1 cells from the killing effect of *B. bronchiseptica* (Bvg^+). In that case, ACT is well known in its ability to increase intracellular cAMP levels, and then lead to inhibition of several physiological functions in macrophage cells including superoxide production, chemotaxis and bacterial killing (Confer and Eaton, 1982). Therefore, increase in cAMP may cause impotence of phagocytic function in moue macrophage cell line J774A.1. This may be how *B. bronchiseptica* overcomes natural defences and cause disease (i.e. colonization on surface of cilia of respiratory tract).

4.2 Conclusions and future work

Certain microorganisms have been found to interact either directly or indirectly with mast cells. This interaction results in mast cell activation and mediator release which elicits an inflammatory response or direct killing leading to bacterial clearance. Recent studies, however, have shown that mast cells have the capacity to modulate the host's innate immune response to bacteria by their ability to phagocytose bacteria. These studies with bioluminescent *B. bronchiseptica* (Lux 95) demonstrated that sheep BMMC are able to take-up bacteria. Furthermore, bioluminescence signals are indications of metabolic activity, and these studies demonstrated that both viable sheep BMMC and the contents of lysed sheep BMMC are able to reduce the metabolic activity of *B. bronchiseptica* (Lux 95). However, production of antimicrobial agents and interaction of bone marrow derived mast cell with bacteria needs further investigations.

These results demonstrated that *B. bronchiseptica* is able to interact directly with sheep BMMC and other cell types (RBL-2H3, J774A.1 cell lines) in a Bvg-dependent process. The modulated Bvg^- phenotype of *B. bronchiseptica*, unable to express Bvg-activated gene products such as ACT, FHA and PRN, was clearly impaired in its ability to interact with sheep BMMC and other cell types. Since different virulence factors of *B. bronchiseptica* are controlled by BvgAS system, interactions of different purified factors of *Bordetella* with different cell types were examined. Recent advances have shown that some Gram-negative bacteria depend on type III secretion for delivering toxic proteins into the cytoplasm of host cells. Furthermore, as it has been shown that the Bvg virulence control system regulates type III secretion in *B. bronchiseptica* (Yuk *et al.*, 1998), it would be important to do a further study dealing with the importance of the type III secretion system on the interaction of *B. bronchiseptica* with sheep BMMC.

The cytotoxicity of recombinant ACT was investigated in the present work to show that the enzymic activity of rACT is important for cytotoxicity of rACT towards sheep BMMC, RBL-2H3 cells (mucosal mast model) as well as J774A.1 macrophage-like cells. Evidence for cytotoxicity of rACT was shown due to depletion of the ATP content of different mammalian cells. By this criterion, the killing effect of rACT towards J774A.1 cells was higher than for sheep BMMC or RBL-2H3 cells which could suggest that the availability of cell receptors (CD11b) on J774A.1 cells is

greater than on sheep BMMC or RBL-2H3 cells. In addition, it could be concluded that programmed host cell death (apoptosis of these cells) depends on the enzymatic activity of rACT rather than just the pore-forming activity. But, it could be assumed that ACT needs its pore-forming activity to get the enzymatic moiety into the mammalian cells.

It is well known that increases in intracellular cAMP would cripple many biological functions of mammalian cells. Spontaneous β-hexosaminidase release was inhibited in RBL-2H3 cells after incubation with enzymatically-active and invasive rACT. RBL-2H3 cells are well known as a tumour analogue of mucosal mast cells. The inhibitory mechanisms of rACT in RBL-2H3 cells may be therefore similar to that mast cells because it had been reported that an increase in cAMP levels in mast cells leads to inhibition of degranulation. For the first time, β-hexosaminidase release was determined in J774A.1 macrophage cells after incubation with FHA and PRN. Moreover, PRN caused a depletion in the ATP content of J774A.1 cells, and such observations require further investigations to determine their mechanisms.

The effect of RBL-2H3 cells pre-treated with either rACT or PT on β-hexosaminidase release induced by stimulation with *B. bronchiseptica* (Bvg^+) showed that these factors work in different ways. rACT was able to inhibit β-hexosaminidase release from RBL-2H3 cells exposed to *B. bronchiseptica* (Bvg^+), but this effect was temporary (i.e. it decreased with increasing time of incubation). In contrast, the effect of PT on β-hexosaminidsae release from RBL-2H3 cells exposed to *B. bronchiseptica* (Bvg^+) appeared to be time-dependent. With increased time of pre-treatment, a greater inhibitory effect was observed.

Other evidence also suggested that rACT and PT work in different ways. PT appeared to protect J774A.1 cells from the killing effect of *B. bronchiseptica* (Bvg^-) whereas rACT enhanced the killing effect of *B. bronchiseptica*. An increase in intracellular cAMP due to low doses of ACT may cause impotence of phagocytic function in J774A.1 cells and then their ability to take up *B. bronchiseptica* (Bvg^+) would be crippled, as observed. This phenomenon suggests how *B. bronchiseptica* may overcome natural defences and cause disease or secondary bacterial infections.

These studies have shown the interaction of *B. bronchiseptica* and different factors (adhesins and toxins) with sheep BMMC and other cell types. Traditional methods, as used in this study, depend on studying the effect of one protein produced

by one gene in one experiment, for example, on the viability or degranulation of sheep BMMC or other cell types. Thus, the throughput is very limited and the whole picture of gene function in sheep BMMC and other cell types is hard to obtain. The effect of *B. bronchiseptica* on sheep BMMC depends on the expression and properties of a large number of proteins. Furthermore, the production and expression of most *B. bronchiseptica* virulence factors is under the control of a central two-component regulatory system (BvgAS), which is co-regulated with other gene sets (i.e. *vag* genes and *vrg* genes). The BvgS (sensor) protein and BvgA (activator) protein are able to induce the expression of the virulence genes (*vag* genes) which then lead to the repression of another set of genes, known as *vrg* genes (Coote, 2001). Thus, monitoring the cascade of reactions and co-regulations that occur between different genes of *B. bronchiseptica* under different environmental conditions (or when interacting with different cell types) could be difficult by traditional methods. Thus, it would be useful to use a method which could monitor different gene sets at the same time (i.e. which could provide information about what genes are ON (up-regulated) and OFF (down-regulated) under different environmental conditions) either *in vitro* or *in vivo*. In this regards, the DNA microarray technique would be useful because it could help to follow many different gene functions in one experiment (Schoolnik, 2002; Call, 2005). For example, it could be used to monitor the activation (or inactivation) of *vags* and *vrgs* by BvgAS of *B. bronchiseptica* in the presence or absence of sheep BMMC or mouse macrophage cells (J774A.1).

DNA microarrays exploit primary sequence data to measure transcript levels simultaneously for every gene which may play a role in the host-microbe interaction (Eskra *et al.*, 2003). This technology also offers the opportunity to examine the changes in gene expression in relation to specific stimuli (Boshoff *et al.*, 2004). DNA microarrays are composed of immobilized DNA probes that are complementary to the gene targets of interest, thus is important that gene sequences have been determined (Call, 2005). Now, that the whole genome sequences of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* have been completed (Parkhill *et al.*, 2003), DNA microarrays could be applied to study the pathogenicity of *B. pertussis* and related specie such as *B. bronchiseptica* (which has more than 95% homology to *B. pertussis*) toward mast cells (or any other mammalian cells) and their virulence mechanisms used to establish infection and cause disease.

DNA microarrays have already led to the discovery of several new *vags* and *vrgs* (Hot *et al.*, 2003). Two hundred selected *B. pertussis* genes have been analysed by using DNA microarray. These different genes were chosen for their potential involvement in virulence as possible members of the Bvg regulon. Also, it was found that some Bvg-regulated genes were modulated differentially by $MgSO_4$ and nicotinic acid, suggesting that the signal transduction triggered by these two modulators operates through two different initial molecular steps. In a similar way, DNA microarrays could be used to study the interaction between *B. bronchiseptica* and sheep BMMC or other cell types and precisely monitor the coregulation of different genes under different conditions of infection with these mammalian cells.

Therefore, it could suggest as a future work that DNA microarray could help to qualify bacterial killing as no attempt was made (in this project) to quantify total uptake of bacteria by sheep BMMC or J774A.1 cells.

The use of DNA microarrays has uncovered two strongly BvgA/S-activated gene in *B pertussis*, named hotA and hotB (for 'homolog of toxin'), the products of which showed high sequence similarities to pertussis toxin subunits (Locht *et al.*, 2004). It was suggested that such studies will help to identify the full array of virulence factors, as well as provide an integrated understanding of bacterial physiology, and to allow the design of attenuated *B. pertussis* strains useful for intranasal vaccination.

DNA microarrays might also be used to monitor modulation of expression of host cell genes stimulated by interaction with *Bordetella* (e.g. in human mast cells or mouse mast cells). For example, when human peripheral blood mononuclear cells (PBMCs) were exposed to live and heat-killed *B. pertussis* (Boldrick *et al.*, 2002), microarray analysis showed that there were distinct differences between PBMC responses to live and killed *B. pertussis*. For example, the genes encoding tumor necrosis factor (TNFα), macrophage inflammatory protein (MIP1β), interleukin-1 (IL-1α) and IL-1β were induced after exposure to either live or killed bacteria. However, while the induction of these genes was sustained in PBMCs treated with killed bacteria, their transcripts rapidly diminished in the cells exposed to live *B. pertussis*. The ability of live *B. pertussis* to suppress expression of these important antimicrobial genes suggests active mechanisms used by the bacteria to influence the host response.

Mast cells are responsible for immediate hypersensitivity responses. Their widespread distribution in the skin and respiratory tract suggests a role as for these cells as a first-line defence against invading pathogens. However, unregulated activation can result in deleterious effects. As such, understanding control of mast cell inflammatory activities is an area of increasing importance. Influences of *B. pertussis* or different purified factors (adhesins or toxins) on viability or degranulation of human mast cells could be investigated by using DNA microarrays. For example, the DNA sequences of different cytokines secreted by mast cells and which influence recruitment of other cells of immune system could be investigated when under the influence of *B. pertussis* or other *Bordetella* factors.

References

Medical Research and Council, **(1959).** VACCINATION against whooping-cough; the final report to the Whooping-Cough Immunization Committee of the Medical Research Council and to the medical officers of health for Battersea and Wandsworth, Bradford, Liverpool, and Newcastle. *British medical journal* **46**, 994-1000.

Abe, M., Kurosawa, M., Ishikawa, O. and Miyachi, Y. (2000). Effect of mast cell-derived mediators and mast cell-related neutral proteases on human dermal fibroblast proliferation and type I collagen production. *Journal of Allergy and Clinical Immunology* **106**, S78-84.

Abraham, S. N. and Malaviya, R. (2000). Mast cell modulation of the innate immune response to enterobacterial infection. *Advances in Experimental Medicine and Biology* **479**, 91-105.

Araham, S. N. and Malaviya, R. (1997). Mast cells in infection and immunity. *Infection and Immunity* **65**, 3501-3508.

Abraham, S. N., Thankavel, K. and Malaviya, R. (1997). Mast cells as modulators of host defense in the lung. *Frontiers Bioscience* **2**, d78-87.

Abramson, T., Kedem, H. and Relman, D. A. (2001). Proinflammatory and proapoptotic activities associated with *Bordetella pertussis* filamentous hemagglutinin. *Infection and Immunity* **69**, 2650-2658.

Akerley, B. J., Cotter, P. A. and Miller, J. F. (1995). Ectopic expression of the flagellar regulon alters development of the Bordetella-host interaction. *Cell* **80**, 611-620.

Alam, J. and Cook, J. L. (1990). Reporter genes: application to the study of mammalian gene transcription. *Analytical Biochemistry* **188**, 245-254.

Allen, A. and Maskell, D. (1996). The identification, cloning and mutagenesis of a genetic locus required for lipopolysaccharide biosynthesis in *Bordetella pertussis*. *Molecular Microbiology* **19**, 37-52.

Algermissen, B., Hermes, B., Feldmann-Boeddeker, I., Bauer, F. and Henz, B. M. (1999). Mast cell chymase and tryptase during tissue turnover: analysis on in vitro mitogenesis of fibroblasts and keratinocytes and alterations in cutaneous scars. *Exp Dermatol* **8**, 193-198.

Alm, E. and Bloom, G. D. (1982). Cyclic nucleotide involvement in histamine release from mast cells--a reevaluation. *Life Sciences* **30**, 213-218.

Alm, P. E. and Bloom, G. D. (1979). Effect of norepinephrine on in vitro histamine release from rat mast cells. *International Archives of Allergy & Applied Immunology* **60**, 60-67.

Anderson, D. M. and Schneewind, O. (1999). Type III machines of Gram-negative pathogens: injecting virulence factors into host cells and more. *Current Opinion Microbiology* **2**, 18-24.

Andersson, B., Eriksson, B., Falsen, E., Fogh, A., Hanson, L. A., Nylen, O., Peterson, H. and Svanborg Eden, C. (1981). Adhesion of *Streptococcus pneumoniae* to human pharyngeal epithelial cells in vitro: differences in adhesive capacity among strains isolated from subjects with otitis media, septicemia, or meningitis or from healthy carriers. *Infection and Immunity* **32**, 311-317.

Arbustini, E., Gavazzi, A., Dal Bello, B., Morbini, P., Campana, C., Diegoli, M., Grasso, M., Fasani, R., Banchieri, N., Porcu, E., Pilotto, A., Ponzetta, M., Bellini, O., Lucreziotti, S. and Vigano, M. (1997). Ten-year experience with endomyocardial biopsy in myocarditis presenting with congestive heart failure: frequency, pathologic characteristics, treatment and follow-up. *G Ital Cardiol* **27**, 209-223.

Arico, B. and Rappuoli, R. (1987). *Bordetella parapertussis* and *Bordetella bronchiseptica* contain transcriptionally silent pertussis toxin genes. *Journal of Bacteriology* **169**, 2847-2853.

Arico, B., Gross, R., Smida, J. and Rappuoli, R. (1987). Evolutionary relationships in the genus *Bordetella*. *Molecular Microbiology* **1**, 301-308.

Arico, B., Miller, J. F., Roy, C., Stibitz, S., Monack, D., Falkow, S., Gross, R. and Rappuoli, R. (1989). Sequences required for expression of *Bordetella pertussis* virulence factors share homology with prokaryotic signal transduction proteins. *Proceedings of the National Academy of Sciences, USA* **86**, 6671-6675.

Arock, M., Ross, E., Lai-Kuen, R., Averlant, G., Gao, Z. and Abraham, S. N. (1998). Phagocytic and tumor necrosis factor alpha response of human mast cells following exposure to gram-negative and gram-positive bacteria. *Infection and Immunity* **66**, 6030-6034.

Aronoff, D. M., Canetti, C. and Peters-Golden, M. (2004). Prostaglandin E2 inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP. *Journal of Immunology* **173**, 559-565.

Arvand, M., Feldhues, R., Mieth, M., Kraus, T. and Vandamme, P. (2004). Chronic cholangitis caused by *Bordetella hinzii* in a liver transplant recipient. *Journal of Clinical Microbiology* **42**, 2335-2337.

Babiuk, L. A., Lawman, M. J. and Ohmann, H. B. (1988). Viral-bacterial synergistic interaction in respiratory disease. *Advnces in Virus Research* **35**, 219-249.

Bachelet, M., Richard, M. J., Francois, D. and Polla, B. S. (2002). Mitochondrial alterations precede *Bordetella pertussis*-induced apoptosis. *FEMS Immunology and Medical Microbiology* **32**, 125-131.

Baldwin, T. O., Christopher, J. A., Raushel, F. M., Sinclair, J. F., Ziegler, M. M., Fisher, A. J. and Rayment, I. (1995). Structure of bacterial luciferase. *Curruent Opinins in Structural Biology* **5**, 798-809.

Barak, M., Ulitzur, S. and Merzbach, D. (1983). The use of luminous bacteria for determination of phagocytosis. *Journal of Immunological Methods* **64**, 353-363.

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

Barry, E. M., Weiss, A. A., Ehrmann, I. E., Gray, M. C., Hewlett, E. L. and Goodwin, M. S. (1991). *Bordetella pertussis* adenylate cyclase toxin and hemolytic activities require a second gene, cyaC, for activation. *Journal of Bacteriology* **173**, 720-726.

Bemis, D. A. and Kennedy, J. R. (1981). An improved system for studying the effect of Bordetella bronchiseptica on the ciliary activity of canine tracheal epithelial cells. *Journal of Infectious Diseases* **144**, 349-357.

Bendixsen, T., Emery, D. L. and Jones, W. O. (1995). The sensitization of mucosal mast cells during infections with *Trichostrongylus colubriformis* or *Haemonchus contortus* in sheep. *International Journal of Parasitology* **25**, 741-748.

Berg, D. E., Weiss, A. and Crossland, L. (1980). Polarity of Tn5 insertion mutations in *Escherichia coli*. *Journal of Bacteriology* **142**, 439-446.

Berggard, K., Johnsson, E., Mooi, F. R. and Lindahl, G. (1997). *Bordetella pertussis* binds the human complement regulator C4BP: role of filamentous hemagglutinin. *Infection and Immunity* **65**, 3638-3643.

Billard, P. and DuBow, M. S. (1998). Bioluminescence-based assays for detection and characterization of bacteria and chemicals in clinical laboratories. *Clinical of Biochemistry* **31**, 1-14.

Bischoff, S. C., Sellge, G., Lorentz, A., Sebald, W., Raab, R. and Manns, M. P. (1999). IL-4 enhances proliferation and mediator release in mature human mast cells. *Proceedings of the National Academy of Sciences, USA* **96**, 8080-8085.

Blackburn P. E. (2000). Characterisation of the virulence-related, outer membrane proteins of *B. pertussis*. PhD thesis, University of Glasgow.

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

Bokoch, G. M. and Gilman, A. G. (1984). Inhibition of receptor-mediated release of arachidonic acid by pertussis toxin. *Cell* **39**, 301-308.

Boldrick, J. C., Alizadeh, A. A., Diehn, M., Dudoit, S., Liu, C. L., Belcher, C. E., Botstein, D., Staudt, L. M., Brown, P. O. and Relman, D. A. (2002). Stereotyped and specific gene expression programs in human innate immune responses to bacteria. *Proceedings of the national academy of sciences of USA* **99**, 972-977.

Boshoff, H. I., Myers, T. G., Copp, B. R., McNeil, M. R., Wilson, M. A. and Barry, C. E., 3rd (2004). The transcriptional responses of Mycobacterium tuberculosis to

inhibitors of metabolism: novel insights into drug mechanisms of action. *The Journal of Biological Chemistry* **279**, 40174-40184.

Bourne, H. R., Lichtenstein, L. M., Melmon, K. L., Henney, C. S., Weinstein, Y. and Shearer, G. M. (1974). Modulation of inflammation and immunity by cyclic AMP. *Science* **184**, 19-28.

Boyce, J. A. (2003). Mast cells: beyond IgE. *Journal of Allergy & Clinical Immunology* **111**, 24-32; quiz 33.

Boyd, A. P., Ross, P. J., Conroy, H., Mahon, N., Lavelle, E. C. and Mills, K. H. (2005). *Bordetella pertussis* adenylate cyclase toxin modulates innate and adaptive immune responses: distinct roles for acylation and enzymatic activity in immunomodulation and cell death. *Journal of Immunology* **175**, 730-738.

Bradding, P. (1996). Human mast cell cytokines. *Clinical and Experimental Allergy* **26**, 13-19.

Bradding, P., Okayama, Y., Howarth, P. H., Church, M. K. and Holgate, S. T. (1995). Heterogeneity of human mast cells based on cytokine content. *Journal of Immunology* **155**, 297-307.

Brightling, C. E., Bradding, P., Symon, F. A., Holgate, S. T., Wardlaw, A. J. and Pavord, I. D. (2002). Mast-cell infiltration of airway smooth muscle in asthma. *N Engl J Med* **346**, 1699-1705.

Brockmeier, S. L. and Register, K. B. (2000). Effect of temperature modulation and bvg mutation of Bordetella bronchiseptica on adhesion, intracellular survival and cytotoxicity for swine alveolar macrophages. *Vet Microbiol* **73**, 1-12.

Brockmeier, S. L., Palmer, M. V., Bolin, S. R. and Rimler, R. B. (2001). Effects of intranasal inoculation with *Bordetella bronchiseptica*, porcine reproductive and respiratory syndrome virus, or a combination of both organisms on subsequent infection with *Pasteurella multocida* in pigs. *American Journal Of Veterinary Research* **62**, 521-525.

Brockmeier, S. L., Register, K. B., Magyar, T., Lax, A. J., Pullinger, G. D. and Kunkle, R. A. (2002). Role of the dermonecrotic toxin of *Bordetella bronchiseptica* in the pathogenesis of respiratory disease in swine. *Infection and Immunity* **70**, 481-490.

Broide, D. H., Metcalfe, D. D. and Wasserman, S. I. (1988). Functional and biochemical characterization of rat bone marrow derived mast cells. *Journal of Immunology* **141**, 4298-4305.

Burns, D. L. (1999). Biochemistry of type IV secretion. *Curruent Opinins of Microbiology* **2**, 25-29.

Burns, V. C., Pishko, E. J., Preston, A., Maskell, D. J. and Harvill, E. T. (2003). Role of *Bordetella* O antigen in respiratory tract infection. *Infection and Immunity* **71**, 86-94.

Call, D. R. (2005). Challenges and opportunities for pathogen detection using DNA microarrays. *Critical Reviews in Microbiology* **31**, 91-99.

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

Caroff, M., Deprun, C., Richards, J. C. and Karibian, D. (1994). Structural characterization of the lipid A of *Bordetella pertussis* 1414 endotoxin. *Journal of Bacteriology* **176**, 5156-5159.

Caroff, M., Brisson, J., Martin, A. and Karibian, D. (2000). Structure of the *Bordetella pertussis* 1414 endotoxin. *FEBS Letters* **477**, 8-14.

Chabot, J. A., Riback, P. S. and Cochrane, D. E. (1981). Elevated cyclic AMP levels fail to inhibit extracellular calcium dependent histamine secretion from rat mast cells. *Life Sciences* **28**, 1155-1162.

Charles, I., Fairweather, N., Pickard, D., Beesley, J., Anderson, R., Dougan, G. and Roberts, M. (1994). Expression of the *Bordetella pertussis* P.69 pertactin adhesin in *Escherichia coli*: fate of the carboxy-terminal domain. *Microbiology* **140** (**Pt 12**), 3301-3308.

Charles, I. G., Dougan, G., Pickard, D., Chatfield, S., Smith, M., Novotny, P., Morrissey, P. and Fairweather, N. F. (1989). Molecular cloning and characterization of protective outer membrane protein P.69 from *Bordetella pertussis*. *Proceedings of the National Academy of Sciences, USA* **86**, 3554-3558.

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

Chhabra, S. R., Stead, P., Bainton, N. J., Salmond, G. P., Stewart, G. S., Williams, P. and Bycroft, B. W. (1993). Autoregulation of carbapenem biosynthesis in Erwinia carotovora by analogues of N-(3-oxohexanoyl)-L-homoserine lactone. *The Journal of antibiotics (Tokyo)* **46**, 441-454.

Cockett, M. I., Bebbington, C. R. and Yarranton, G. T. (1990). High level expression of tissue inhibitor of metalloproteinases in Chinese hamster ovary cells using glutamine synthetase gene amplification. *Biotechnology (N Y)* **8**, 662-667.

Cohan, V., McKenzie-White, J., Triggiani, M., Massey, W., Kagey-Sobotka, A. and Lichtenstein, L. M. (1989). Heterogeneity of human mast cells and basophils. Effects of a putative 5-lipoxygenase inhibitor. *Biochemical Pharmacology* **38**, 4455-4459.

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

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

Cookson, B. T., Vandamme, P., Carlson, L. C., Larson, A. M., Sheffield, J. V., Kersters, K. and Spach, D. H. (1994). Bacteremia caused by a novel *Bordetella* species, "*B. hinzii*". *Journal of Clinical Microbiology* **32**, 2569-2571.

Coote, J. G. (1992). Structural and functional relationships among the RTX toxin determinants of gram-negative bacteria. *FEMS Microbiology Reviews* **8**, 137-161.

Coote, J. G. (2001). Environmental sensing mechanisms in *Bordetella*. *Advances in Microbial Physiology* **44**, 141-181.

Cornelis, G. R. and Van Gijsegem, F. (2000). Assembly and function of type III secretory systems. *Annual Review of Microbiology* **54**, 735-774.

Cotter, P. A. and Miller, J. F. (1994). BvgAS-mediated signal transduction: analysis of phase-locked regulatory mutants of *Bordetella bronchiseptica* in a rabbit model. *Infection and Immunity* **62**, 3381-3390.

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

Cotter, P. A. and DiRita, V. J. (2000). Bacterial virulence gene regulation: an evolutionary perspective. *Annual Reviews of Microbiology* **54**, 519-565.

Cotter, P. A., Yuk, M. H., Mattoo, S., Akerley, B. J., Boschwitz, J., Relman, D. A. and Miller, J. F. (1998). Filamentous hemagglutinin of *Bordetella bronchiseptica* is required for efficient establishment of tracheal colonization. *Infection and Immunity* **66**, 5921-5929.

Craig, S. S., Irani, A. M., Metcalfe, D. D. and Schwartz, L. B. (1993). Ultrastructural localization of heparin to human mast cells of the MCTC and MCT types by labeling with antithrombin III-gold. *Laboratory Investigation* **69**, 552-561.

Crouch, S. P., Kozlowski, R., Slater, K. J. and Fletcher, J. (1993). The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *Journal of Immunological Methods* **160**, 81-88.

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

Cummings, C. A., Brinig, M. M., Lepp, P. W., van de Pas, S. and Relman, D. A. (2004). *Bordetella* species are distinguished by patterns of substantial gene loss and host adaptation. *Journal of Bacteriology* **186**, 1484-1492.

Cundell, D. R., Kanthakumar, K., Taylor, G. W., Goldman, W. E., Flak, T., Cole, P. J. and Wilson, R. (1994). Effect of tracheal cytotoxin from *Bordetella pertussis* on human neutrophil function in vitro. *Infection and Immunity* **62**, 639-643.

Daunert, S., Barrett, G., Feliciano, J. S., Shetty, R. S., Shrestha, S. and Smith-Spencer, W. (2000). Genetically engineered whole-cell sensing systems: coupling biological recognition with reporter genes. *Chemical Reviews* **100**, 2705-2738.

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

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

Donato, G. M., Hsia, H. L., Green, C. S. and Hewlett, E. L. (2005). Adenylate Cyclase Toxin (ACT) from *Bordetella hinzii*: Characterization and Differences from ACT of *Bordetella pertussis*. *Journal of Bacteriology* **187**, 7579-7588.

Donnelly, S., Loscher, C. E., Lynch, M. A. and Mills, K. H. (2001). Whole-cell but not acellular pertussis vaccines induce convulsive activity in mice: evidence of a role for toxin-induced interleukin-1beta in a new murine model for analysis of neuronal side effects of vaccination. *Infection and Immunity* **69**, 4217-4223.

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

Dvorak, A. M. (1992). Basophils and mast cells: piecemeal degranulation in situ and ex vivo: a possible mechanism for cytokine-induced function in disease. *Immunology Series* **57**, 169-271.

Dvorak, A. M., Schleimer, R. P., Schulman, E. S. and Lichtenstein, L. M. (1986). Human mast cells use conservation and condensation mechanisms during recovery from degranulation. In vitro studies with mast cells purified from human lungs. *Laboratory Investigation* **54**, 663-678.

Echtenacher, B., Mannel, D. N. and Hultner, L. (1996). Critical protective role of mast cells in a model of acute septic peritonitis. *Nature* **381**, 75-77.

Ellis, J. A., Haines, D. M., West, K. H., Burr, J. H., Dayton, A., Townsend, H. G., Kanara, E. W., Konoby, C., Crichlow, A., Martin, K. and Headrick, G. (2001). Effect of vaccination on experimental infection with *Bordetella bronchiseptica* in dogs. *Journal of the American Veterinary Medical Association* **218**, 367-375.

Emsley, P., Charles, I. G., Fairweather, N. F. and Isaacs, N. W. (1996). Structure of *Bordetella pertussis* virulence factor P.69 pertactin. *Nature* **381**, 90-92.

Emsley, P., McDermott, G., Charles, I. G., Fairweather, N. F. and Isaacs, N. W. (1994). Crystallographic characterization of pertactin, a membrane-associated protein from *Bordetella pertussis*. *Journal of Molecular Biology* **235**, 772-773.

Enerback, L. (1966). Mast cells in rat gastrointestinal mucosa. I. Effects of fixation. *Acta Pathological etT Microbiologica Ssendniavica* **66**, 289-302.

Ennis, D. P., Cassidy, J. P. and Mahon, B. P. (2005). Acellular pertussis vaccine protects against exacerbation of allergic asthma due to *Bordetella pertussis* in a murine model. *Clinical and Diagnostic Laboratory Immunology* **12**, 409-417.

Eskra, L., Mathison, A. and Splitter, G. (2003). Microarray analysis of mRNA levels from RAW264.7 macrophages infected with *Brucella abortus*. *Infection and Immunity* **71**, 1125-1133.

Ezzell, J. W., Dobrogosz, W. J., Kloos, W. E. and Manclark, C. R. (1981). Phase-shift markers in *Bordetella*: alterations in envelope proteins. *Journal of Infectious Diseases* **143**, 562-569.

Farizo, K. M., Cafarella, T. G. and Burns, D. L. (1996). Evidence for a ninth gene, ptlI, in the locus encoding the pertussis toxin secretion system of *Bordetella pertussis* and formation of a PtlI-PtlF complex. *Journal of Biological Chemistry* **271**, 31643-31649.

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

Fernandez, R. C. and Weiss, A. A. (1998). Serum resistance in bvg-regulated mutants of *Bordetella pertussis*. *FEMS Microbiology Letters* **163**, 57-63.

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

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

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

Fleckenstein, J. M., Kopecko, D. J., Warren, R. L. and Elsinghorst, E. A. (1996). Molecular characterization of the tia invasion locus from enterotoxigenic *Escherichia coli*. *Infection and Immunity* **64**, 2256-2265.

Fodinger, M., Fritsch, G., Winkler, K., Emminger, W., Mitterbauer, G., Gadner, H., Valent, P. and Mannhalter, C. (1994). Origin of human mast cells: development from transplanted hematopoietic stem cells after allogeneic bone marrow transplantation. *Blood* **84**, 2954-2959.

Forde, C. B., Parton, R. and Coote, J. G. (1998). Bioluminescence as a reporter of intracellular survival of *Bordetella bronchiseptica* in murine phagocytes. *Infection and Immunity* **66**, 3198-3207.

Forde, C. B., Shi, X., Li, J. and Roberts, M. (1999). *Bordetella bronchiseptica*-mediated cytotoxicity to macrophages is dependent on bvg-regulated factors, including pertactin. *Infection and Immunity* **67**, 5972-5978.

Foreman, J. C. and Gomperts, B. D. (1975). The relationship between anaphylactic histamine secretion and the permability of the mast cell membrane to calcium. *International Archives of Allergy & Applied Immunology* **49**, 179-182.

Foreman, J. C., Mongar, J. L., Gomperts, B. D. and Garland, L. G. (1975). A possible role for cyclic AMP in the regulation of histamine secretion and the action of cromoglycate. *Biochemical Pharmacology* **24**, 538-540.

Foreman, J. (1980). Receptor-secretion coupling in mast cells. *Trends in Pharmacological Sciences* **1**, 219-474

Francisco, W. A., Abu-Soud, H. M., Baldwin, T. O. and Raushel, F. M. (1993). Interaction of bacterial luciferase with aldehyde substrates and inhibitors. *Journal of Biological Chemistry* **268**, 24734-24741.

Frossi, B., De Carli, M. and Pucillo, C. (2004). The mast cell: an antenna of the microenvironment that directs the immune response. *Journal of Leukocyte Biology* **75**, 579-585.

Fry, N. K., Duncan, J., Malnick, H., Warner, M., Smith, A. J., Jackson, M. S. and Ayoub, A. (2005). *Bordetella petrii* clinical isolate. *Emerging Infectious Diseases* **11**, 1131-1133.

Furukawa, Y., Furuno, T., Teshima, R. and Nakanishi, M. (2001). Calcium signals in rat basophilic leukemia (RBL-2H3) cells primed with the neuropeptide substance P. *Biological & Pharmaceutical Bulletin* **24**, 1060-1063.

Galan, J. E. and Collmer, A. (1999). Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* **284**, 1322-1328.

Galgiani, J. N., Hewlett, E. L. and Friedman, R. L. (1988). Effects of adenylate cyclase toxin from *Bordetella pertussis* on human neutrophil interactions with *Coccidioides immitis* and *Staphylococcus aureus*. *Infection and Immunity* **56**, 751-755.

Galli, S. J. (1993). New concepts about the mast cell. *New England Journal of Medicine* **328**, 257-265.

Gentile, D. A. and Skoner, D. P. (1996). A role for the sodium, potassium adenosine triphosphatase (Na+,K+ ATPase) enzyme in degranulation of rat basophilic leukaemia cells. *Clinical and Experimental Allergy* **26**, 1449-1460.

Gentry-Weeks, C. R., Cookson, B. T., Goldman, W. E., Rimler, R. B., Porter, S. B. and Curtiss, R., 3rd (1988). Dermonecrotic toxin and tracheal cytotoxin, putative virulence factors of *Bordetella avium*. *Infection and Immunity* **56**, 1698-1707.

Gerlach, G., von Wintzingerode, F., Middendorf, B. and Gross, R. (2001). Evolutionary trends in the genus *Bordetella*. *Microbes and Infection* **3**, 61-72.

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

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

Gibson, S., Mackeller, A., Newlands, G. F. and Miller, H. R. (1987). Phenotypic expression of mast cell granule proteinases. Distribution of mast cell proteinases I and II in the rat digestive system. *Immunology* **62**, 621-627.

Glaser, P., Elmaoglou-Lazaridou, A., Krin, E., Ladant, D., Barzu, O. and Danchin, A. (1989). Identification of residues essential for catalysis and binding of calmodulin in Bordetella pertussis adenylate cyclase by site-directed mutagenesis. *The EMBO journal* **8**, 967-972.

Glaser, P., Munier, H., Gilles, A. M., Krin, E., Porumb, T., Barzu, O., Sarfati, R., Pellecuer, C. and Danchin, A. (1991). Functional consequences of single amino acid substitutions in calmodulin-activated adenylate cyclase of *Bordetella pertussis*. *The EMBO journal* **10**, 1683-1688.

Goldman, W. E. and Herwaldt, L. A. (1985). *Bordetella pertussis* tracheal cytotoxin. *Developments in Biological Standardization* **61**, 103-111.

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

Gordon, J. R. and Galli, S. J. (1991). Release of both preformed and newly synthesized tumor necrosis factor alpha (TNF-alpha)/cachectin by mouse mast cells stimulated via the Fc epsilon RI. A mechanism for the sustained action of mast cell-derived TNF-alpha during IgE-dependent biological responses. *Journal of Experimental Medicine* **174**, 103-107.

Gordon, J. R., Burd, P. R. and Galli, S. J. (1990). Mast cells as a source of multifunctional cytokines. *Immunology Today* **11**, 458-464.

Gotis-Graham, I. and McNeil, H. P. (1997). Mast cell responses in rheumatoid synovium. Association of the MCTC subset with matrix turnover and clinical progression. *Arthritis Rheum* **40**, 479-489.

Goyard, S. and Ullmann, A. (1991). Analysis of *Bordetella pertussis* cya operon regulation by use of cya-lac fusions. *FEMS Microbiology Letters* **61**, 251-256.

Gray, M., Szabo, G., Otero, A. S., Gray, L. and Hewlett, E. (1998). Distinct mechanisms for K+ efflux, intoxication, and hemolysis by *Bordetella pertussis* AC toxin. *Journal of Biological Chemistry* **273**, 18260-18267.

Gray, M. C., Ross, W., Kim, K. and Hewlett, E. L. (1999). Characterization of binding of adenylate cyclase toxin to target cells by flow cytometry. *Infection and Immunity* **67**, 4393-4399.

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

Gross, R. and Rappuoli, R. (1989). Pertussis toxin promoter sequences involved in modulation. *Journal of Bacteriology* **171**, 4026-4030.

Guedin, S., Willery, E., Tommassen, J., Fort, E., Drobecq, H., Locht, C. and Jacob-Dubuisson, F. (2000). Novel topological features of FhaC, the outer membrane transporter involved in the secretion of the *Bordetella pertussis* filamentous hemagglutinin. *Journal of Biological Chemistry* **275**, 30202-30210.

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

Gueirard, P., Weber, C., Le Coustumier, A. and Guiso, N. (1995). Human *Bordetella bronchiseptica* infection related to contact with infected animals: persistence of bacteria in host. *Journal of Clinical Microbiology* **33**, 2002-2006.

Gueirard, P., Druilhe, A., Pretolani, M. and Guiso, N. (1998). Role of adenylate cyclase-hemolysin in alveolar macrophage apoptosis during *Bordetella pertussis* infection in vivo. *Infection and Immunity* **66**, 1718-1725.

Guermonprez, P., Khelef, N., Blouin, E., Rieu, P., Ricciardi-Castagnoli, P., Guiso, N., Ladant, D. and Leclerc, C. (2001). The adenylate cyclase toxin of *Bordetella pertussis* binds to target cells via the alpha(M)beta(2) integrin (CD11b/CD18). *Journal of Experimental Medicine* **193**, 1035-1044.

Guiso, N., Rocancourt, M., Szatanik, M. and Alonso, J. M. (1989). *Bordetella* adenylate cyclase is a virulence associated factor and an immunoprotective antigen. *Microbial Pathogenesis* **7**, 373-380.

Gustafsson, L., Hallander, H. O., Olin, P., Reizenstein, E. and Storsaeter, J. (1996). A controlled trial of a two-component acellular, a five-component acellular, and a whole-cell pertussis vaccine. *the New England Journal of Medicine* **334**, 349-355.

Hackett, M., Guo, L., Shabanowitz, J., Hunt, D. F. and Hewlett, E. L. (1994). Internal lysine palmitoylation in adenylate cyclase toxin from *Bordetella pertussis*. *Science* **266**, 433-435.

Haig, D. M., Blackie, W., Huntley, J., Mackellar, A. and Smith, W. D. (1988). The generation of ovine bone marrow-derived mast cells in culture. *Immunology* **65**, 199-203.

Haig, D. M., Stevenson, L. M., Thomson, J., Percival, A. and Smith, W. D. (1995). Haemopoietic cell responses in the blood and bone marrow of sheep infected with the

abomasal nematode *Telodorsagia circumcincta*. *ournal of Comparative Pathology* **112**, 151-164.

Haig, D. M., Huntley, J. F., MacKellar, A., Newlands, G. F., Inglis, L., Sangha, R., Cohen, D., Hapel, A., Galli, S. J. and Miller, H. R. (1994). Effects of stem cell factor (kit-ligand) and interleukin-3 on the growth and serine proteinase expression of rat bone-marrow-derived or serosal mast cells. *Blood* **83**, 72-83.

Hanberger, H., Nilsson, L. E., Kihlstrom, E. and Maller, R. (1990). Postantibiotic effect of beta-lactam antibiotics on *Escherichia coli* evaluated by bioluminescence assay of bacterial ATP. *Antimicrobial Agents and Chemotherapy* **34**, 102-106.

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

Harvill, E. T., Preston, A., Cotter, P. A., Allen, A. G., Maskell, D. J. and Miller, J. F. (2000). Multiple roles for *Bordetella* lipopolysaccharide molecules during respiratory tract infection. *Infection and Immunity* **68**, 6720-6728.

Hastings, J. W. (1996). Chemistries and colors of bioluminescent reactions: a review. *Gene* **173**, 5-11.

Hazenbos, W. L., Geuijen, C. A., van den Berg, B. M., Mooi, F. R. and van Furth, R. (1995). *Bordetella pertussis* fimbriae bind to human monocytes via the minor fimbrial subunit FimD. *Journal of Infecious Diseases* **171**, 924-929.

Hazes, B., Boodhoo, A., Cockle, S. A. and Read, R. J. (1996). Crystal structure of the pertussis toxin-ATP complex: a molecular sensor. *Journal of Molecular Biology* **258**, 661-671.

Heiss, L. N., Lancaster, J. R., Jr., Corbett, J. A. and Goldman, W. E. (1994). Epithelial autotoxicity of nitric oxide: role in the respiratory cytopathology of pertussis. *Proceedings of the National Academy of Sciences, USA* **91**, 267-270.

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

Henderson, J., North, K., Griffiths, M., Harvey, I. and Golding, J. (1999). Pertussis vaccination and wheezing illnesses in young children: prospective cohort study. The Longitudinal Study of Pregnancy and Childhood Team. *British medical journal* **318**, 1173-1176.

Henderson, W. R. and Kaliner, M. (1978). Immunologic and nonimmunologic generation of superoxide from mast cells and basophils. *Journal of Clinical Investigation* **61**, 187-196.

Herrero, M., de Lorenzo, V. and Timmis, K. N. (1990). Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *Journal of Bacteriology* **172**, 6557-6567.

Hewlett, E. L. (1997). Preparation and composition of acellular pertussis vaccines. Consideration of potential effects on vaccine efficacy. *Developments in Biological Standardization* **89**, 143-151.

Hiemstra, P. S., Annema, A., Schippers, E. F. and van Furth, R. (1992). Pertussis toxin partially inhibits phagocytosis of immunoglobulin G-opsonized *Staphylococcus aureus* by human granulocytes but does not affect intracellular killing. *Infection and Immunity* **60**, 202-205.

Hodgson, J. C., Finucane, A., Dagleish, M. P., Ataei, S., Parton, R. and Coote, J. G. (2005). Efficacy of vaccination of calves against hemorrhagic septicemia with a live aroA derivative of *Pasteurella multocida* B:2 by two different routes of administration. *Infection and Immunity* **73**, 1475-1481.

Hoek, K. L., Cassell, G. H., Duffy, L. B. and Atkinson, T. P. (2002). *Mycoplasma pneumoniae*-induced activation and cytokine production in rodent mast cells. *Journal of Allergy and Clinical Immunology* **109**, 470-476.

Hoppe, J. E. (1999). Update on respiratory infection caused by *Bordetella parapertussis*. *Pediatric Infectious Disease Journal* **18**, 375-381.

Hot, D., Antoine, R., Renauld-Mongenie, G., Caro, V., Hennuy, B., Levillain, E., Huot, L., Wittmann, G., Poncet, D., Jacob-Dubuisson, F., Guyard, C., Rimlinger, F., Aujame, L., Godfroid, E., Guiso, N., Quentin-Millet, M. J., Lemoine, Y. and Locht, C. (2003). Differential modulation of *Bordetella pertussis* virulence genes as evidenced by DNA microarray analysis. *Molecular Genetics and Genomics* **269**, 475-486.

Huntley, J. F. (1992). Mast cells and basophils: a review of their heterogeneity and function. *Journal of Comparative Pathology* **107**, 349-372.

Huntley, J. F., Gibson, S., Knox, D. and Miller, H. R. (1986). The isolation and purification of a proteinase with chymotrypsin-like properties from ovine mucosal mast cells. *International Journal of Biochemistry* **18**, 673-682.

Huntley, J. F., Haig, D. M., Irvine, J., Inglis, L., MacDonald, A., Rance, A. and Moqbel, R. (1992). Characterisation of ovine mast cells derived from in vitro culture of haemopoietic tissue. *Veterinary Immunology and Immunopathology* **32**, 47-64.

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

Hussain, R. F., Nouri, A. M. and Oliver, R. T. (1993). A new approach for measurement of cytotoxicity using colorimetric assay. *Journal of Immunological Methods* **160**, 89-96.

Ibrahim, M. Z., Reder, A. T., Lawand, R., Takash, W. and Sallouh-Khatib, S. (1996). The mast cells of the multiple sclerosis brain. *J Neuroimmunol* **70**, 131-138.

Idigbe, E. O., Parton, R. and Wardlaw, A. C. (1981). Rapidity of antigenic modulation of *Bordetella pertussis* in modified Hornibrook medium. *Journal of medical microbiology* **14**, 409-418.

Imaizumi, A., Suzuki, Y., Ono, S., Sato, H. and Sato, Y. (1983). Heptakis(2,6-O-dimethyl)beta-cyclodextrin: a novel growth stimulant for *Bordetella pertussis* phase I. *Journal of Clinical Microbiology* **17**, 781-786.

Irani, A. M. and Schwartz, L. B. (1989). Mast cell heterogeneity. *Clinical of Experimental Allergy* **19**, 143-155.

Irani, A. M., Bradford, T. R., Kepley, C. L., Schechter, N. M. and Schwartz, L. B. (1989). Detection of MCT and MCTC types of human mast cells by immunohistochemistry using new monoclonal anti-tryptase and anti-chymase antibodies. *Journal of Histochemistry & Cytochemistry* **37**, 1509-1515.

Irani, A. M., Craig, S. S., DeBlois, G., Elson, C. O., Schechter, N. M. and Schwartz, L. B. (1987). Deficiency of the tryptase-positive, chymase-negative mast cell type in gastrointestinal mucosa of patients with defective T lymphocyte function. *Journal of Immunology* **138**, 4381-4386.

Irie, Y., Mattoo, S. and Yuk, M. H. (2004). The Bvg virulence control system regulates biofilm formation in *Bordetella bronchiseptica*. *Journal of Bacteriology* **186**, 5692-5698.

Ivanoff, B. and Robertson, S. E. (1997). Pertussis: a worldwide problem. *Developments in Biological Standardization* **89**, 3-13.

Jepras, R. I., Carter, J., Pearson, S. C., Paul, F. E. and Wilkinson, M. J. (1995). Development of a Robust Flow Cytometric Assay for Determining Numbers of Viable Bacteria. *Applied and Environmental Microbiology* **61**, 2696-2701.

Johansen, T. (1980). Histamine release induced from rat mast cells by the ionophore A23187 in the absence of extracellular calcium. *European Journal of Pharmacology* **62**, 329-334.

Jones, W. O., Huntley, J. F. and Emery, D. L. (1992). Isolation and degranulation of mucosal mast cells from the small intestine of parasitized sheep. *International Journal of Parasitology* **22**, 519-521.

Kaliner, M. and Austen, K. F. (1974). Cyclic AMP, ATP, and reversed anaphylactic histamine release from rat mast cells. *Journal of Immunology* **112**, 664-674.

Kamachi, K., Konda, T. and Arakawa, Y. (2003). DNA vaccine encoding pertussis toxin S1 subunit induces protection against *Bordetella pertussis* in mice. *Vaccine* **21**, 4609-4615.

Karimi, K., Redegeld, F. A., Blom, R. and Nijkamp, F. P. (2000). Stem cell factor and interleukin-4 increase responsiveness of mast cells to substance P. *Experimental Hematology* **28**, 626-634.

Karimova, G. and Ullmann, A. (1997). Characterization of DNA binding sites for the BvgA protein of *Bordetella pertussis*. *Journal of Bacteriology* **179**, 3790-3792.

Kashimoto, T., Katahira, J., Cornejo, W. R., Masuda, M., Fukuoh, A., Matsuzawa, T., Ohnishi, T. and Horiguchi, Y. (1999). Identification of functional domains of *Bordetella dermonecrotizing* toxin. *Infection and Immunity* **67**, 3727-3732.

Keil, D. J., Burns, E. H., Kisker, W. R., Bemis, D. and Fenwick, B. (1999). Cloning and immunologic characterization of a truncated *Bordetella bronchiseptica* filamentous hemagglutinin fusion protein. *Vaccine* **18**, 860-867.

Kemp, T., Pearce, N., Fitzharris, P., Crane, J., Fergusson, D., St George, I., Wickens, K. and Beasley, R. (1997). Is infant immunization a risk factor for childhood asthma or allergy? *Epidemiology* **8**, 678-680.

Kennerly, D. A., Sullivan, T. J. and Parker, C. W. (1979a). Activation of phospholipid metabolism during mediator release from stimulated rat mast cells. *Journal of Immunology* **122**, 152-159.

Kennerly, D. A., Secosan, C. J., Parker, C. W. and Sullivan, T. J. (1979b). Modulation of stimulated phospholipid metabolism in mast cells by pharmacologic agents that increase cyclic 3',5' adenosine monophosphate levels. *Journal of Immunology* **123**, 1519-1524.

Kerr, A. R., Irvine, J. J., Search, J. J., Gingles, N. A., Kadioglu, A., Andrew, P. W., McPheat, W. L., Booth, C. G. and Mitchell, T. J. (2002). Role of inflammatory mediators in resistance and susceptibility to pneumococcal infection. *Infection and Immunity* **70**, 1547-1557.

Khelef, N., Zychlinsky, A. and Guiso, N. (1993). *Bordetella pertussis* induces apoptosis in macrophages: role of adenylate cyclase-hemolysin. *Infection and Immunity* **61**, 4064-4071.

Kimbrough, T. G. and Miller, S. I. (2002). Assembly of the type III secretion needle complex of *Salmonella typhimurium*. *Microbes and Infection* **4**, 75-82.

Kimura, A., Mountzouros, K. T., Relman, D. A., Falkow, S. and Cowell, J. L. (1990). *Bordetella pertussis* filamentous hemagglutinin: evaluation as a protective antigen and colonization factor in a mouse respiratory infection model. *Infection and Immunity* **58**, 7-16.

Kirshenbaum, A. S., Goff, J. P., Semere, T., Foster, B., Scott, L. M. and Metcalfe, D. D. (1999). Demonstration that human mast cells arise from a progenitor cell population that is CD34(+), c-kit(+), and expresses aminopeptidase N (CD13). *Blood* **94**, 2333-2342.

Kitamura, Y., Go, S. and Hatanaka, K. (1978). Decrease of mast cells in W/Wv mice and their increase by bone marrow transplantation. *Blood* **52**, 447-452.

Kitamura, Y., Yokoyama, M., Matsuda, H., Ohno, T. and Mori, K. J. (1981). Spleen colony-forming cell as common precursor for tissue mast cells and granulocytes. *Nature* **291**, 159-160.

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

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

Kuwae, A., Ohishi, M., Watanabe, M., Nagai, M. and Abe, A. (2003). BopB is a type III secreted protein in *Bordetella bronchiseptica* and is required for cytotoxicity against cultured mammalian cells. *Cellular Microbiology* **5**, 973-983.

Ladant, D. (1988). Interaction of *Bordetella pertussis* adenylate cyclase with calmodulin. Identification of two separated calmodulin-binding domains. *Journal of Biological Chemistry* **263**, 2612-2618.

Ladant, D., Glaser, P. and Ullmann, A. (1992). Insertional mutagenesis of *Bordetella pertussis* adenylate cyclase. *Journal of Biological Chemistry* **267**, 2244-2250.

Lasfargues, A., Caroff, M. and Chaby, R. (1993). Structural features involved in the mitogenic activity of Bordetella pertussis lipopolysaccharides for spleen cells of C3H/HeJ mice. *FEMS Immunol Med Microbiol* **7**, 119-129.

Lawrence, A. J., Coote, J. G., Kazi, Y. F., Lawrence, P. D., MacDonald-Fyall, J., Orr, B. M., Parton, R., Riehle, M., Sinclair, J., Young, J. and Price, N. C. (2002). A direct pyrophosphatase-coupled assay provides new insights into the activation of the secreted adenylate cyclase from *Bordetella pertussis* by calmodulin. *The Journal of Biological Chemistry* **277**, 22289-22296.

Lax, A. J. (1985). Is phase variation in *Bordetella* caused by mutation and selection? *Journal of General Microbiology* **131**, 913-917.

Le Blay, K., Gueirard, P., Guiso, N. and Chaby, R. (1997). Antigenic polymorphism of the lipopolysaccharides from human and animal isolates of *Bordetella bronchiseptica*. *Microbiology* **143 (Pt 4)**, 1433-1441.

Lee, C. A. (1997). Type III secretion systems: machines to deliver bacterial proteins into eukaryotic cells? *Trends in Microbiology* **5**, 148-156.

Leef, M., Elkins, K. L., Barbic, J. and Shahin, R. D. (2000). Protective immunity to *Bordetella pertussis* requires both B cells and CD4(+) T cells for key functions other than specific antibody production. *Journal of Experimental Medicine* **191**, 1841-1852.

Legarda, D., Klein-Patel, M. E., Yim, S., Yuk, M. H. and Diamond, G. (2005). Suppression of NF-kappaB-mediated beta-defensin gene expression in the mammalian airway by the *Bordetella* type III secretion system. *Cellular Microbiology* **7**, 489-497.

Leininger, E., Roberts, M., Kenimer, J. G., Charles, I. G., Fairweather, N., Novotny, P. and Brennan, M. J. (1991). Pertactin, an Arg-Gly-Asp-containing *Bordetella pertussis* surface protein that promotes adherence of mammalian cells. *Proceedings of the National Academy of Sciences, USA* **88**, 345-349.

Leininger, E., Bowen, S., Renauld-Mongenie, G., Rouse, J. H., Menozzi, F. D., Locht, C., Heron, I. and Brennan, M. J. (1997). Immunodominant domains present on the *Bordetella pertussis* vaccine component filamentous hemagglutinin. *Journal of Infectious Diseases* **175**, 1423-1431.

Lewis, J. C., Feltus, A., Ensor, C. M., Ramanathan, S. and Daunert, S. (1998). Applications of reporter genes. *Analytical Chemistry* **70**, 579A-585A.

Li, J., Fairweather, N. F., Novotny, P., Dougan, G. and Charles, I. G. (1992). Cloning, nucleotide sequence and heterologous expression of the protective outer-membrane protein P.68 pertactin from *Bordetella bronchiseptica*. *Journal of General Microbiology* **138 Pt 8**, 1697-1705.

Li, L. J., Dougan, G., Novotny, P. and Charles, I. G. (1991). P.70 pertactin, an outer-membrane protein from *Bordetella parapertussis*: cloning, nucleotide sequence and surface expression in *Escherichia coli*. *Molecular Microbiology* **5**, 409-417.

Locht, C. and Keith, J. M. (1986). Pertussis toxin gene: nucleotide sequence and genetic organization. *Science* **232**, 1258-1264.

Locht, C., Bertin, P., Menozzi, F. D. and Renauld, G. (1993). The filamentous haemagglutinin, a multifaceted adhesion produced by virulent *Bordetella* spp. *Molecular Microbiology* **9**, 653-660.

Locht, C., Antoine, R., Raze, D., Mielcarek, N., Hot, D., Lemoine, Y. and Mascart, F. (2004). *Bordetella pertussis* from functional genomics to intranasal vaccination. *Internaional Journal of Medical Microbiology* **293**, 583-588.

Luker, K. E., Tyler, A. N., Marshall, G. R. and Goldman, W. E. (1995). Tracheal cytotoxin structural requirements for respiratory epithelial damage in pertussis. *Molecular Microbiology* **16**, 733-743.

Lynch, M. J., Swift, S., Kirke, D. F., Keevil, C. W., Dodd, C. E. and Williams, P. (2002). The regulation of biofilm development by quorum sensing in Aeromonas hydrophila. *Environ Microbiol* **4**, 18-28.

Lynch M. J. (1999), Investigation of quorum sensing in *Aeromonas* biofilms. PhD thesis, University of Nottingham.

Macaldowie, C. N., Huntley, J. F., Mackellar, A., McInnes, C. and Haig, D. M. (1997). The effects of recombinant ovine interleukin-3 and recombinant ovine stem cell factor on the growth and mediator expression of caprine and ovine bone marrow-derived mast cells. *Vet Immunol Immunopathol* **60**, 97-110.

Macdonald-Fyall J. (2002). The protective and immunomodulatory properties of *B. pertussis* adenylate cyclase toxin. PhD thesis, University of Glasgow.

Macdonald-Fyall, J., Xing, D., Corbel, M., Baillie, S., Parton, R. and Coote, J. (2004). Adjuvanticity of native and detoxified adenylate cyclase toxin of *Bordetella pertussis* towards co-administered antigens. *Vaccine* **22**, 4270-4281.

Magyar, T., Chanter, N., Lax, A. J., Rutter, J. M. and Hall, G. A. (1988). The pathogenesis of turbinate atrophy in pigs caused by *Bordetella bronchiseptica*. *Veterinary Microbiology* **18**, 135-146.

Mahon, B. P., Sheahan, B. J., Griffin, F., Murphy, G. and Mills, K. H. (1997). Atypical disease after *Bordetella pertussis* respiratory infection of mice with targeted disruptions of interferon-gamma receptor or immunoglobulin mu chain genes. *Journal of Experimental Medicine* **186**, 1843-1851.

Makhov, A. M., Hannah, J. H., Brennan, M. J., Trus, B. L., Kocsis, E., Conway, J. F., Wingfield, P. T., Simon, M. N. and Steven, A. C. (1994). Filamentous hemagglutinin of *Bordetella pertussis*. A bacterial adhesin formed as a 50-nm monomeric rigid rod based on a 19-residue repeat motif rich in beta strands and turns. *Journal of Molecular Biology* **241**, 110-124.

Malaviya, R. and Abraham, S. N. (1995). Interaction of bacteria with mast cells. *Methods in Enzymology* **253**, 27-43.

Malaviya, R., Ikeda, T., Ross, E. and Abraham, S. N. (1996a). Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha. *Nature* **381**, 77-80.

Malaviya, R., Twesten, N. J., Ross, E. A., Abraham, S. N. and Pfeifer, J. D. (1996b). Mast cells process bacterial Ags through a phagocytic route for class I MHC presentation to T cells. *Journal of Immunology* **156**, 1490-1496.

Malaviya, R., Twesten, N. J., Ross, E. A., Abraham, S. N. and Pfeifer, J. D. (1996c). Mast cells process bacterial Ags through a phagocytic route for class I MHC presentation to T cells. *J Immunol* **156**, 1490-1496.

Malaviya, R., Ross, E. A., MacGregor, J. I., Ikeda, T., Little, J. R., Jakschik, B. A. and Abraham, S. N. (1994). Mast cell phagocytosis of FimH-expressing enterobacteria. *Journal of Immunology* **152**, 1907-1914.

Malbec, O., Malissen, M., Isnardi, I., Lesourne, R., Mura, A. M., Fridman, W. H., Malissen, B. and Daeron, M. (2004). Linker for activation of T cells integrates positive and negative signaling in mast cells. *Journal of Immunology* **173**, 5086-5094.

Maoz, A., Mayr, R., Bresolin, G., Neuhaus, K., Francis, K. P. and Scherer, S. (2002). Sensitive in situ monitoring of a recombinant bioluminescent *Yersinia enterocolitica* reporter mutant in real time on Camembert cheese. *Applied and Environmental Microbiology* **68**, 5737-5740.

Marshall, J. S. and Jawdat, D. M. (2004). Mast cells in innate immunity. *Journal of Allergy and Clinical Immunology* **114**, 21-27.

Marshall, J. S., King, C. A. and McCurdy, J. D. (2003a). Mast cell cytokine and chemokine responses to bacterial and viral infection. *Curr Pharm Des* **9**, 11-24.

Marshall, J. S., King, C. A. and McCurdy, J. D. (2003b). Mast cell cytokine and chemokine responses to bacterial and viral infection. *Current Pharmaceutical Design* **9**, 11-24.

Martin, T. W. and Lagunoff, D. (1979). Inhibition of mast cell histamine secretion by N-substituteed derivatives of phosphatidylserine. *Science* **204**, 631-633.

Martinez de Tejada, G., Miller, J. F. and Cotter, P. A. (1996). Comparative analysis of the virulence control systems of *Bordetella pertussis* and *Bordetella bronchiseptica*. *Molecular Microbiology* **22**, 895-908.

Martinez de Tejada, G., Cotter, P. A., Heininger, U., Camilli, A., Akerley, B. J., Mekalanos, J. J. and Miller, J. F. (1998). Neither the Bvg- phase nor the vrg6 locus of Bordetella pertussis is required for respiratory infection in mice. *Infection and Immunity* **66**, 2762-2768.

Masuda, M., Minami, M., Shime, H., Matsuzawa, T. and Horiguchi, Y. (2002). In vivo modifications of small GTPase Rac and Cdc42 by *Bordetella* dermonecrotic toxin. *Infection and Immunity* **70**, 998-1001.

Matherne, C. M., Steffen, E. K. and Wagner, J. E. (1987). Efficacy of commercial vaccines for protecting guinea pigs against *Bordetella bronchiseptica* pneumonia. *Laboratory animal science* **37**, 191-194.

Mattoo, S. and Cherry, J. D. (2005). Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies. *Clinical Microbiology Reviews* **18**, 326-382.

Mattoo, S., Miller, J. F. and Cotter, P. A. (2000). Role of *Bordetella bronchiseptica* fimbriae in tracheal colonization and development of a humoral immune response. *Infection and Immunity* **68**, 2024-2033.

Mattoo, S., Yuk, M. H., Huang, L. L. and Miller, J. F. (2004). Regulation of type III secretion in *Bordetella*. *Molecular Microbiology* **52**, 1201-1214.

Mazengia, E., Silva, E. A., Peppe, J. A., Timperi, R. and George, H. (2000). Recovery of *Bordetella holmesii* from patients with pertussis-like symptoms: use of pulsed-field gel electrophoresis to characterize circulating strains. *Journal of Clinical Microbiology* **38**, 2330-2333.

McConkey, D. J., Orrenius, S., Okret, S. and Jondal, M. (1993). Cyclic AMP potentiates glucocorticoid-induced endogenous endonuclease activation in thymocytes. *Faseb Journal* **7**, 580-585.

McGuirk, P. and Mills, K. H. (2000). Direct anti-inflammatory effect of a bacterial virulence factor: IL-10-dependent suppression of IL-12 production by filamentous hemagglutinin from *Bordetella pertussis*. *European Journal of Immunology* **30**, 415-422.

McInnes, C., Haig, D. and Logan, M. (1993). The cloning and expression of the gene for ovine interleukin-3 (multi-CSF) and a comparison of the in vitro hematopoietic activity of ovine IL-3 with ovine GM-CSF and human M-CSF. *Experimintal Hematology* **21**, 1528-1534.

McMillan, D. J., Shojaei, M., Chhatwal, G. S., Guzman, C. A. and Walker, M. J. (1996). Molecular analysis of the bvg-repressed urease of *Bordetella bronchiseptica*. *Microbial Pathogenesis* **21**, 379-394.

Mecheri, S. and David, B. (1997). Unravelling the mast cell dilemma: culprit or victim of its generosity? *Immunology Today* **18**, 212-215.

Meighen, E. A. (1991). Molecular biology of bacterial bioluminescence. *Microbiological Reviews* **55**, 123-142.

Meighen, E. A. (1993). Bacterial bioluminescence: organization, regulation, and application of the lux genes. *Faseb J* **7**, 1016-1022.

Mekori, Y. A. and Metcalfe, D. D. (2000). Mast cells in innate immunity. *Immunological Rreviews* **173**, 131-140.

Metcalfe, D. D., Baram, D. and Mekori, Y. A. (1997). Mast cells. *Physiological Reviews* **77**, 1033-1079.

Mielcarek, N., Hornquist, E. H., Johansson, B. R., Locht, C., Abraham, S. N. and Holmgren, J. (2001). Interaction of *Bordetella pertussis* with mast cells, modulation of cytokine secretion by pertussis toxin. *Cellular Microbiology* **3**, 181-188.

Miller, H. R. (1996). Mucosal mast cells and the allergic response against nematode parasites. *Veterinary Immunology and Immunopathology* **54**, 331-336.

Mills, K. H., Barnard, A., Watkins, J. and Redhead, K. (1993). Cell-mediated immunity to *Bordetella pertussis*: role of Th1 cells in bacterial clearance in a murine respiratory infection model. *Infection and Immunity* **61**, 399-410.

Mitchell, T. J. and Andrew, P. W. (1997). Biological properties of pneumolysin. *Microbial drug resistance* **3**, 19-26.

Mitsui, H., Furitsu, T., Dvorak, A. M., Irani, A. M., Schwartz, L. B., Inagaki, N., Takei, M., Ishizaka, K., Zsebo, K. M., Gillis, S. and et al. (1993). Development of human mast cells from umbilical cord blood cells by recombinant human and murine c-kit ligand. *Proceedings of the National Academy of Sciences of the USA* **90**, 735-739.

Mizota, K., Yoshida, A., Uchida, H., Fujita, R. and Ueda, H. (2005). Novel type of Gq/11 protein-coupled neurosteroid receptor sensitive to endocrine disrupting chemicals in mast cell line (RBL-2H3). *British Journal of Pharmacology* **145**, 545-550.

Mock, M. and Ullmann, A. (1993a). Calmodulin-activated bacterial adenylate cyclases as virulence factors. *Trends Microbiol* **1**, 187-192.

Mock, M. and Ullmann, A. (1993b). Calmodulin-activated bacterial adenylate cyclases as virulence factors. *Trends in Microbiology* **1**, 187-192.

Montaraz, J. A., Novotny, P. and Ivanyi, J. (1985). Identification of a 68-kilodalton protective protein antigen from *Bordetella bronchiseptica*. *Infection and Immunity* **47**, 744-751.

Muller, S., Feldman, M. F. and Cornelis, G. R. (2001). The Type III secretion system of Gram-negative bacteria: a potential therapeutic target? *Expert Opinion on Therapeurtic Targets* **5**, 327-339.

Munoz, J. J., Arai, H. and Cole, R. L. (1981). Mouse-protecting and histamine-sensitizing activities of pertussigen and fimbrial hemagglutinin from *Bordetella pertussis*. *Infection and Immunity* **32**, 243-250.

Munoz, S., Hernandez-Pando, R., Abraham, S. N. and Enciso, J. A. (2003). Mast cell activation by *Mycobacterium tuberculosis*: mediator release and role of CD48. *Journal of Immunology* **170**, 5590-5596.

Naylor, L. H. (1999). Reporter gene technology: the future looks bright. *Biochemical Pharmacology* **58**, 749-757.

Nelson, S. M., Cooper, A. A., Taylor, E. L. and Salisbury, V. C. (2003). Use of bioluminescent *Escherichia coli* O157:H7 to study intra-protozoan survival of bacteria within Tetrahymena pyriformis. *FEMS Microbiology Letters* **223**, 95-99.

Newlands, G. F., Knox, D. P., Pirie-Shepherd, S. R. and Miller, H. R. (1993). Biochemical and immunological characterization of multiple glycoforms of mouse mast cell protease 1: comparison with an isolated murine serosal mast cell protease (MMCP-4). *Biochemical Journal* **294 (Pt 1)**, 127-135.

Nilsson, L., Gruber, C., Granstrom, M., Bjorksten, B. and Kjellman, N. I. (1998). Pertussis IgE and atopic disease. *Allergy* **53**, 1195-1201.

Nogawa, H., Kuwae, A., Matsuzawa, T. and Abe, A. (2004). The type III secreted protein BopD in *Bordetella bronchiseptica* is complexed with BopB for pore formation on the host plasma membrane. *Journal of Bacteriology* **186**, 3806-3813.

Nollmann, M., Gilbert, R., Mitchell, T., Sferrazza, M. and Byron, O. (2004). The role of cholesterol in the activity of pneumolysin, a bacterial protein toxin. *Biophysical Journal* **86**, 3141-3151.

Novak, I. and Falus, A. (1997). Molecular biology and role of histamine in physiological and pathological reactions. A review. *Acta biologica Hungarica* **48**, 385-394.

Novotny, P., Chubb, A. P., Cownley, K. and Charles, I. G. (1991). A novel bivalent acellular pertussis vaccine based on the 69 kDa protein and FHA. *Developments in Biological Standardization* **73**, 243-249.

Odent, M. R., Culpin, E. E. and Kimmel, T. (1994). Pertussis vaccination and asthma: is there a link? *The journal of the American Medical Association* **272**, 592-593.

Ohshiro H. Suzuki R. Furuno T. Nakanishi M. (2000). Atomic force microscopy to study direct neurite-mast cell (RBL) communication *in vitro*. *Immunology Letters*. **74**, 211-214.

Oliver, J. M., Seagrave, J., Stump, R. F., Pfeiffer, J. R. and Deanin, G. G. (1988). Signal transduction and cellular response in RBL-2H3 mast cells. *Progress in Allergy* **42**, 185-245.

Orth, K., Palmer, L. E., Bao, Z. Q., Stewart, S., Rudolph, A. E., Bliska, J. B. and Dixon, J. E. (1999). Inhibition of the mitogen-activated protein kinase kinase superfamily by a Yersinia effector. *Science* **285**, 1920-1923.

Ouderkirk, J. P., Nord, J. A., Turett, G. S. and Kislak, J. W. (2003). Polymyxin B nephrotoxicity and efficacy against nosocomial infections caused by multiresistant gram-negative bacteria. *Antimicrob Agents Chemother* **47**, 2659-2662.

Packard E. R. (2004), Sequence variation and conservation in virulence-related genes and expression of adenylate cyclase toxin of *B. pertussis*. PhD thesis, University of Glasgow.

Parkhill, J., Sebaihia, M., Preston, A., Murphy, L. D., Thomson, N., Harris, D. E., Holden, M. T., Churcher, C. M., Bentley, S. D., Mungall, K. L., Cerdeno-Tarraga, A. M., Temple, L., James, K., Harris, B., Quail, M. A., Achtman, M., Atkin, R., Baker, S., Basham, D., Bason, N., Cherevach, I., Chillingworth, T., Collins, M., Cronin, A., Davis, P., Doggett, J., Feltwell, T., Goble, A., Hamlin, N., Hauser, H., Holroyd, S., Jagels, K., Leather, S., Moule, S., Norberczak, H., O'Neil, S., Ormond, D., Price, C., Rabbinowitsch, E., Rutter, S., Sanders, M., Saunders, D., Seeger, K., Sharp, S., Simmonds, M., Skelton, J., Squares, R., Squares, S., Stevens, K., Unwin, L., Whitehead, S., Barrell, B. G. and Maskell, D. J. (2003). Comparative analysis of the genome sequences of *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. *Nature Genetics* **35**, 32-40.

Parton, R. (1985). Effect of prednisolone on the toxicity of *Bordetella pertussis* for mice. *Journal of medical microbiology* **19**, 391-400.

Parton, R. (1988). Differentiation of phase I and variant strains of *Bordetella pertussis* on Congo red media. *Journal of medical microbiology* **26**, 301-306.

Parton, R. (2005) Bordetella. In "Topley and Wilson's Microbiology and Microbial Infections. 10th Edition. Bacteriology Volume, Ch 72. Edward Arnold, London.

Passerini De Rossi, B. N., Friedman, L. E., Darnaud, S., De Torres, R. A. and Franco, M. A. (2001). Characterization of intermediate phenotypes induced by

chemically undefined laboratory media in virulent *Bordetella bronchiseptica* strains. *Journal of General Applied Microbiology* **47**, 39-46.

Pearce, F. L. (1986). On the heterogeneity of mast cells. *Pharmacology* **32**, 61-71.

Pearson, R. D., Symes, P., Conboy, M., Weiss, A. A. and Hewlett, E. L. (1987). Inhibition of monocyte oxidative responses by *Bordetella pertussis* adenylate cyclase toxin. *Journal of Immunology* **139**, 2749-2754.

Pichichero, M. E., Deloria, M. A., Rennels, M. B., Anderson, E. L., Edwards, K. M., Decker, M. D., Englund, J. A., Steinhoff, M. C., Deforest, A. and Meade, B. D. (1997). A safety and immunogenicity comparison of 12 acellular pertussis vaccines and one whole-cell pertussis vaccine given as a fourth dose in 15- to 20-month-old children. *Pediatrics* **100**, 772-788.

Porter, J. F., Parton, R. and Wardlaw, A. C. (1991). Growth and survival of *Bordetella bronchiseptica* in natural waters and in buffered saline without added nutrients. *Applied and Environmental Microbiology* **57**, 1202-1206.

Prasad, S. M., Yin, Y., Rodzinski, E., Tuomanen, E. I. and Masure, H. R. (1993). Identification of a carbohydrate recognition domain in filamentous hemagglutinin from *Bordetella pertussis*. *Infection and Immunity* **61**, 2780-2785.

Preston, A. (2005). *Bordetella pertussis*: the intersection of genomics and pathobiology. *Canadian Medical Association journal* **173**, 55-62.

Preston, A., Parkhill, J. and Maskell, D. J. (2004). The bordetellae: lessons from genomics. *Nature Reviews Microbiology* **2**, 379-390.

Preston, A., Allen, A. G., Cadisch, J., Thomas, R., Stevens, K., Churcher, C. M., Badcock, K. L., Parkhill, J., Barrell, B. and Maskell, D. J. (1999). Genetic basis for lipopolysaccharide O-antigen biosynthesis in *bordetellae*. *Infection and Immunity* **67**, 3763-3767.

Pullinger, G. D., Adams, T. E., Mullan, P. B., Garrod, T. I. and Lax, A. J. (1996). Cloning, expression, and molecular characterization of the dermonecrotic toxin gene of *Bordetella* spp. *Infection and Immunity* **64**, 4163-4171.

Rambow, A. A., Fernandez, R. C. and Weiss, A. A. (1998). Characterization of BrkA expression in *Bordetella bronchiseptica*. *Infection and Immunity* **66**, 3978-3980.

Reed, P. W. and Lardy, H. A. (1972). A23187: a divalent cation ionophore. *The Journal of Biological Chemistry* **247**, 6970-6977.

Regoli, D., Boudon, A. and Fauchere, J. L. (1994). Receptors and antagonists for substance P and related peptides. *Pharmacological reviews* **46**, 551-599.

Relman, D., Tuomanen, E., Falkow, S., Golenbock, D. T., Saukkonen, K. and Wright, S. D. (1990). Recognition of a bacterial adhesion by an integrin: macrophage

CR3 (alpha M beta 2, CD11b/CD18) binds filamentous hemagglutinin of B*ordetella pertussis*. *Cell* **61**, 1375-1382.

Rivera, J., Cordero, J. R., Furumoto, Y., Luciano-Montalvo, C., Gonzalez-Espinosa, C., Kovarova, M., Odom, S. and Parravicini, V. (2002). Macromolecular protein signaling complexes and mast cell responses: a view of the organization of IgE-dependent mast cell signaling. *Molecular Immunology* **38**, 1253-1258.

Roberts, M., Cropley, I., Chatfield, S. and Dougan, G. (1993). Protection of mice against respiratory *Bordetella pertussis* infection by intranasal immunization with P.69 and FHA. *Vaccine* **11**, 866-872.

Rodgers, J. D., McCullagh, J. J., McNamee, P. T., Smyth, J. A. and Ball, H. J. (2001). An investigation into the efficacy of hatchery disinfectants against strains of *Staphylococcus aureus* associated with the poultry industry. *Veterinary Microbiology* **82**, 131-140.

Rogel, A., Schultz, J. E., Brownlie, R. M., Coote, J. G., Parton, R. and Hanski, E. (1989). Bordetella pertussis adenylate cyclase: purification and characterization of the toxic form of the enzyme. *Embo Journal* **8**, 2755-2760.

Roitt I., Brostoff J. and Male D. (1996). Immunology, 4th edition, Chapter two, Mosby, Spain.

Ross, P. J., Lavelle, E. C., Mills, K. H. and Boyd, A. P. (2004). Adenylate cyclase toxin from *Bordetella pertussis* synergizes with lipopolysaccharide to promote innate interleukin-10 production and enhances the induction of Th2 and regulatory T cells. *Infection and Immunity* **72**, 1568-1579.

Roy, C. R., Miller, J. F. and Falkow, S. (1990). Autogenous regulation of the *Bordetella pertussis* bvgABC operon. *Proceedings of the National Academy of Sciences, USA* **87**, 3763-3767.

Saito, H., Ebisawa, M., Tachimoto, H., Shichijo, M., Fukagawa, K., Matsumoto, K., Iikura, Y., Awaji, T., Tsujimoto, G., Yanagida, M., Uzumaki, H., Takahashi, G., Tsuji, K. and Nakahata, T. (1996). Selective growth of human mast cells induced by Steel factor, IL-6, and prostaglandin E2 from cord blood mononuclear cells. *Journal of Immunology* **157**, 343-350.

Salyers, Abigail A. and Whitt, Dixie D. (1994). Bacterial Pathologenesis. A molecular approach. Chapter one. ASM press.

Sato, Y. and Sato, H. (1999). Development of acellular pertussis vaccines. *Biologicals* **27**, 61-69.

Sato, Y., Kimura, M. and Fukumi, H. (1984). Development of a pertussis component vaccine in Japan. *Lancet* **1**, 122-126.

Saukkonen, K., Cabellos, C., Burroughs, M., Prasad, S. and Tuomanen, E. (1991). Integrin-mediated localization of *Bordetella pertussis* within macrophages: role in pulmonary colonization. *Journal of Experimental Medicine* **173**, 1143-1149.

Savelkoul, P. H., Kremer, B., Kusters, J. G., van der Zeijst, B. A. and Gaastra, W. (1993). Invasion of HeLa cells by *Bordetella bronchiseptica*. *Microbial Pathogenesis* **14**, 161-168.

Sawai, N., Koike, K., Mwamtemi, H. H., Kinoshita, T., Kurokawa, Y., Sakashita, K., Higuchi, T., Takeuchi, K., Shiohara, M., Kamijo, T., Ito, S., Kato, T., Miyazaki, H., Yamashita, T. and Komiyama, A. (1999). Thrombopoietin augments stem cell factor-dependent growth of human mast cells from bone marrow multipotential hematopoietic progenitors. *Blood* **93**, 3703-3712.

Schaeffer, L. M. and Weiss, A. A. (2001). Pertussis toxin and lipopolysaccharide influence phagocytosis of *Bordetella pertussis* by human monocytes. *Infection and Immunity* **69**, 7635-7641.

Schaeffer, L. M., McCormack, F. X., Wu, H. and Weiss, A. A. (2004). *Bordetella* pertussis lipopolysaccharide resists the bactericidal effects of pulmonary surfactant protein A. *Journal of Immunology* **173**, 1959-1965.

Schneider, B., Stubs, D. and Gross, R. (2002). Identification and genomic organization of gene loci negatively controlled by the virulence regulatory BvgAS two-component system in *Bordetella bronchiseptica*. *Molecular Genetics & Genomics* **267**, 526-535.

Schoolnik, G. K. (2002). Microarray analysis of bacterial pathogenicity. *Advances in Microbial Physiology* **46**, 1-45.

Schuster, A., Hofmann, A. and Reinhardt, D. (1993). Does pertussis infection induce manifestation of allergy? *Clinical Investigator* **71**, 208-213.

Schwartz, L. B., Austen, K. F. and Wasserman, S. I. (1979). Immunologic release of beta-hexosaminidase and beta-glucuronidase from purified rat serosal mast cells. *Journal of Immunology* **123**, 1445-1450.

Sebo, P., Glaser, P., Sakamoto, H. and Ullmann, A. (1991). High-level synthesis of active adenylate cyclase toxin of *Bordetella pertussis* in a reconstructed *Escherichia coli* system. *Gene* **104**, 19-24.

Seldin, D. C., Adelman, S., Austen, K. F., Stevens, R. L., Hein, A., Caulfield, J. P. and Woodbury, R. G. (1985). Homology of the rat basophilic leukemia cell and the rat mucosal mast cell. *Proceedings of the National Academy of Sciences, USA* **82**, 3871-3875.

Sher, A., Hein, A., Moser, G. and Caulfield, J. P. (1979). Complement receptors promote the phagocytosis of bacteria by rat peritoneal mast cells. *Laboratory Investigation* **41**, 490-499.

Shin, J. S., Gao, Z. and Abraham, S. N. (2000). Involvement of cellular caveolae in bacterial entry into mast cells. *Science* **289**, 785-788.

Small, D., Levenstein, M., Kim, E., Carow, C., Amin, S., Rockwell, P., Witte, L., Burrow, C., Ratajczak, M. Z., Gewirtz, A. M. and et al. (1994). STK-1, the human homolog of Flk-2/Flt-3, is selectively expressed in CD34+ human bone marrow cells and is involved in the proliferation of early progenitor/stem cells. *Proceedings of the National Academy of Sciences of the USA* **91**, 459-463.

Smith, A. M., Guzman, C. A. and Walker, M. J. (2001). The virulence factors of *Bordetella pertussis*: a matter of control. *FEMS Microbiology Reviews* **25**, 309-333.

Spears, P. A., Temple, L. M., Miyamoto, D. M., Maskell, D. J. and Orndorff, P. E. (2003). Unexpected similarities between *Bordetella* avium and other pathogenic *Bordetellae*. *Infection and Immunity* **71**, 2591-2597.

Stainer, D. W. and Scholte, M. J. (1970). A simple chemically defined medium for the production of phase I *Bordetella pertussis*. *Journal of General Microbiology* **63**, 211-220.

Stassen, M., Hultner, L. and Schmitt, E. (2002). Classical and alternative pathways of mast cell activation. *Critical Reviews in Immunology* **22**, 115-140.

Stefanelli, P., Mastrantonio, P., Hausman, S. Z., Giuliano, M. and Burns, D. L. (1997). Molecular characterization of two *Bordetella bronchiseptica* strains isolated from children with coughs. *Journal of Clinical Microbiology* **35**, 1550-1555.

Stevenson, A. and Roberts, M. (2002). Use of a rationally attenuated *Bordetella bronchiseptica* as a live mucosal vaccine and vector for heterologous antigens. *Vaccine* **20**, 2325-2335.

Stewart, M. J., Emery, D. L., McClure, S. J. and Bendixsen, T. (1996). The effects of four neuropeptides on the degranulation of mucosal mast cells from sheep. *Immunology and Cell Biology* **74**, 255-257.

Stibitz, S. and Yang, M. S. (1991). Subcellular localization and immunological detection of proteins encoded by the vir locus of *Bordetella pertussis*. *Journal of Bacteriology* **173**, 4288-4296.

Stibitz, S., Aaronson, W., Monack, D. and Falkow, S. (1989). Phase variation in *Bordetella pertussis* by frameshift mutation in a gene for a novel two-component system. *Nature* **338**, 266-269.

Stockbauer, K. E., Foreman-Wykert, A. K. and Miller, J. F. (2003). Bordetella type III secretion induces caspase 1-independent necrosis. *Cell Microbiology* **5**, 123-132.

Strachan, D. P., Butland, B. K. and Anderson, H. R. (1996). Incidence and prognosis of asthma and wheezing illness from early childhood to age 33 in a national British cohort. *British medical journal* **312**, 1195-1199.

Sture, G. H., Huntley, J. F., MacKellar, A. and Miller, H. R. (1995a). Ovine mast cell heterogeneity is defined by the distribution of sheep mast cell proteinase. *Vet Immunol Immunopathol* **48**, 275-285.

Sture, G. H., Huntley, J. F., MacKellar, A. and Miller, H. R. (1995b). Ovine mast cell heterogeneity is defined by the distribution of sheep mast cell proteinase. *Veterinary Immunology and Immunopathology* **48**, 275-285.

Suda, T., Suda, J. and Ogawa, M. (1983). Single-cell origin of mouse hemopoietic colonies expressing multiple lineages in variable combinations. *Proceedings of the National Academy of Sciences of the USA* **80**, 6689-6693.

Swift, S., Throup, J. P., Williams, P., Salmond, G. P. and Stewart, G. S. (1996). Quorum sensing: a population-density component in the determination of bacterial phenotype. *Trends in biochemical sciences* **21**, 214-219.

Szittner, R. and Meighen, E. (1990). Nucleotide sequence, expression, and properties of luciferase coded by lux genes from a terrestrial bacterium. *The Journal of Biological Chemistry* **265**, 16581-16587.

Tabatabaei M. (2000), Construction and characterisation of attenuated derivatives of *Pasteurella multocida* : serotype B:2 strains. PhD thesis, University of Glasgow.

Tabatabaei, M., Liu, Z., Finucane, A., Parton, R. and Coote, J. (2002). Protective immunity conferred by attenuated aroA derivatives of *Pasteurella multocida* B:2 strains in a mouse model of hemorrhagic septicemia. *Infection and Immunity* **70**, 3355-3362.

Taube, C., Wei, X., Swasey, C. H., Joetham, A., Zarini, S., Lively, T., Takeda, K., Loader, J., Miyahara, N., Kodama, T., Shultz, L. D., Donaldson, D. D., Hamelmann, E. H., Dakhama, A. and Gelfand, E. W. (2004). Mast cells, Fc epsilon RI, and IL-13 are required for development of airway hyperresponsiveness after aerosolized allergen exposure in the absence of adjuvant. *Journal of Immunology* **172**, 6398-6406.

Toru, H., Kinashi, T., Ra, C., Nonoyama, S., Yata, J. and Nakahata, T. (1997). Interleukin-4 induces homotypic aggregation of human mast cells by promoting LFA-1/ICAM-1 adhesion molecules. *Blood* **89**, 3296-3302.

Tsai, M., Takeishi, T., Thompson, H., Langley, K. E., Zsebo, K. M., Metcalfe, D. D., Geissler, E. N. and Galli, S. J. (1991). Induction of mast cell proliferation, maturation, and heparin synthesis by the rat c-kit ligand, stem cell factor. *Proceedings of the National Academy of Sciences of the USA* **88**, 6382-6386.

Uhl, M. A. and Miller, J. F. (1996). Central role of the BvgS receiver as a phosphorylated intermediate in a complex two-component phosphorelay. *The Journal of Biological Chemistry* **271**, 33176-33180.

Umetsu, D. T., McIntire, J. J., Akbari, O., Macaubas, C. and DeKruyff, R. H. (2002). Asthma: an epidemic of dysregulated immunity. *Nature immunology* **3**, 715-720.

Unge, A., Tombolini, R., Molbak, L. and Jansson, J. K. (1999). Simultaneous monitoring of cell number and metabolic activity of specific bacterial populations with a dual gfp-luxAB marker system. *Applied and Environmental Microbiology* **65**, 813-821.

Utsumi, S., Sonoda, S., Imagawa, T. and Kanoh, M. (1978). Polymorphonuclear leukocyte-inhibitory factor of *Bordetella pertussis*. I. Extraction and partial purification of phagocytosis- and chemotaxis-inhibitory activities. *Biken journal* **21**, 121-135.

Valent, P. (1994). The phenotype of human eosinophils, basophils, and mast cells. *Journal of Allergy and Clinical Immunology* **94**, 1177-1183.

van den Akker, W. M. (1997). *Bordetella bronchiseptica* has a BvgAS-controlled cytotoxic effect upon interaction with epithelial cells. *FEMS Microbiology Letters* **156**, 239-244.

van den Akker, W. M. (1998). Lipopolysaccharide expression within the genus Bordetella: influence of temperature and phase variation. *Microbiology* **144 (Pt 6)**, 1527-1535.

van den Berg, B. M., Beekhuizen, H., Willems, R. J., Mooi, F. R. and van Furth, R. (1999). Role of *Bordetella pertussis* virulence factors in adherence to epithelial cell lines derived from the human respiratory tract. *Infection and Immunity* **67**, 1056-1062.

van Loo, I. H., Heuvelman, K. J., King, A. J. and Mooi, F. R. (2002). Multilocus sequence typing of *Bordetella pertussis* based on surface protein genes. *Journal of Clinical Microbiology* **40**, 1994-2001.

Vandamme, P., Heyndrickx, M., Vancanneyt, M., Hoste, B., De Vos, P., Falsen, E., Kersters, K. and Hinz, K. H. (1996). *Bordetella trematum* sp. nov., isolated from wounds and ear infections in humans, and reassessment of Alcaligenes denitrificans Ruger and Tan 1983. *International journal of systematic bacteriology* **46**, 849-858.

von Wintzingerode, F., Schattke, A., Siddiqui, R. A., Rosick, U., Gobel, U. B. and Gross, R. (2001). *Bordetella petrii* sp. nov., isolated from an anaerobic bioreactor, and emended description of the genus *Bordetella*. *International Journal of Systematic & Evolutionary Microbiology* **51**, 1257-1265.

Walker, K. E. and Weiss, A. A. (1994). Characterization of the dermonecrotic toxin in members of the genus *Bordetella*. *Infection and Immunity* **62**, 3817-3828.

Wang, J. P., Raung, S. L., Huang, L. J. and Kuo, S. C. (1998). Involvement of cyclic AMP generation in the inhibition of respiratory burst by 2-phenyl-4-quinolone (YT-1) in rat neutrophils. *Biochemical Pharmacology* **56**, 1505-1514.

Wardlaw, A. C., Parton, R. and Hooker, M. J. (1976). Loss of protective antigen, histamine-sensitising factor and envelope polypeptides in cultural variants of *Bordetella pertussis*. *Journal of medical microbiology* **9**, 89-100.

Wattanaphansak, S., Gebhart, C., Olin, M. and Deen, J. (2005). Measurement of the viability of *Lawsonia intracellularis*. *Canadian Journal of Veterinary Research* **69**, 265-271.

Weber, S., Babina, M., Feller, G. and Henz, B. M. (1997). Human leukaemic (HMC-1) and normal skin mast cells express beta 2-integrins: characterization of beta 2-integrins and ICAM-1 on HMC-1 cells. *Scandinavian journal of immunology* **45**, 471-481.

Weingart, C. L. and Weiss, A. A. (2000). *Bordetella pertussis* virulence factors affect phagocytosis by human neutrophils. *Infection and Immunity* **68**, 1735-1739.

Weingart, C. L., Mobberley-Schuman, P. S., Hewlett, E. L., Gray, M. C. and Weiss, A. A. (2000). Neutralizing antibodies to adenylate cyclase toxin promote phagocytosis of *Bordetella pertussis* by human neutrophils. *Infection and Immunity* **68**, 7152-7155.

Weiss, A. A. and Falkow, S. (1984). Genetic analysis of phase change in *Bordetella pertussis*. *Infection and Immunity* **43**, 263-269.

Weiss, A. A. and Hewlett, E. L. (1986). Virulence factors of *Bordetella pertussis*. *Annual Review of Microbiology* **40**, 661-686.

Weiss, A. A. and Goodwin, M. S. (1989). Lethal infection by *Bordetella pertussis* mutants in the infant mouse model. *Infection and Immunity* **57**, 3757-3764.

Weiss, A. A., Hewlett, E. L., Myers, G. A. and Falkow, S. (1983). Tn5-induced mutations affecting virulence factors of *Bordetella pertussis*. *Infection and Immunity* **42**, 33-41.

Weiss, A. A., Patton, A. K., Millen, S. H., Chang, S. J., Ward, J. I. and Bernstein, D. I. (2004). Acellular pertussis vaccines and complement killing of *Bordetella pertussis*. *Infection and Immunity* **72**, 7346-7351.

Westrop, G. D., Hormozi, E. K., Da Costa, N. A., Parton, R. and Coote, J. G. (1996). *Bordetella pertussis* adenylate cyclase toxin: proCyaA and CyaC proteins synthesised separately in *Escherichia coli* produce active toxin in vitro. *Gene* **180**, 91-99.

Westrop, G. D., Campbell, G., Kazi, Y., Billcliffe, B., Coote, J. G., Parton, R., Freer, J. H. and Edwards, J. G. (1994). A new assay for the invasive adenylate cyclase toxin of *Bordetella pertussis* based on its morphological effects on the fibronectin-stimulated spreading of BHK21 cells. *Microbiology* **140 (Pt 2)**, 245-253.

Weyant, R. S., Hollis, D. G., Weaver, R. E., Amin, M. F., Steigerwalt, A. G., O'Connor, S. P., Whitney, A. M., Daneshvar, M. I., Moss, C. W. and Brenner, D. J. (1995). *Bordetella holmesii* sp. nov., a new gram-negative species associated with septicemia. *Journal of Clinical Microbiology* **33**, 1-7.

White, E. H., Rapaport, E., Hopkins, T. A. and Seliger, H. H. (1969). Chemi- and bioluminescence of firefly luciferin. *Journal of the American Chemical Society* **91**, 2178-2180.

Whitehead, N. A., Barnard, A. M., Slater, H., Simpson, N. J. and Salmond, G. P. (2001). Quorum-sensing in Gram-negative bacteria. *FEMS Microbiology Reviews* **25**, 365-404.

Willems, R., Paul, A., van der Heide, H. G., ter Avest, A. R. and Mooi, F. R. (1990). Fimbrial phase variation in *Bordetella pertussis*: a novel mechanism for transcriptional regulation. *Embo Journal* **9**, 2803-2809.

Willems, R. J., Kamerbeek, J., Geuijen, C. A., Top, J., Gielen, H., Gaastra, W. and Mooi, F. R. (1998). The efficacy of a whole cell pertussis vaccine and fimbriae against *Bordetella pertussis* and *Bordetella parapertussis* infections in a respiratory mouse model. *Vaccine* **16**, 410-416.

Williams, C. M. and Galli, S. J. (2000). The diverse potential effector and immunoregulatory roles of mast cells in allergic disease. *Journal of Allergy and Clinial Immunology* **105**, 847-859.

Wjst, M., Dold, S., Reitmeir, P., Fritzsch, C., von Mutius, E. and Thiemann, H. H. (1994). Pertussis infection and allergic sensitization. *Annals of Allergy* **73**, 450-454.

Woodbury, R. G. and Neurath, H. (1980). Structure, specificity and localization of the serine proteases of connective tissue. *FEBS Letters* **114**, 189-196.

Woolfrey, B. F. and Moody, J. A. (1991). Human infections associated with *Bordetella bronchiseptica*. *Clinical Microbiology Reviews* **4**, 243-255.

Woolley, D. E. and Tetlow, L. C. (2000). Mast cell activation and its relation to proinflammatory cytokine production in the rheumatoid lesion. *Arthritis research* **2**, 65-74.

Xu, X., Zhang, D., Lyubynska, N., Wolters, P. J., Killeen, N. P., Baluk, P., McDonald, D. M., Hawgood, S. and Caughey, G. H. (2006). Mast cells protect mice from *Mycoplasma pneumonia*. *American Journal of Respiratory & Critical Care Medicine* **173**, 219-225.

Yamaguchi, M., Lantz, C. S., Oettgen, H. C., Katona, I. M., Fleming, T., Miyajima, I., Kinet, J. P. and Galli, S. J. (1997). IgE enhances mouse mast cell Fc(epsilon)RI expression in vitro and in vivo: evidence for a novel amplification mechanism in IgE-dependent reactions. *Journal of Experimental Medicine* **185**, 663-672.

Yanagida, M., Fukamachi, H., Ohgami, K., Kuwaki, T., Ishii, H., Uzumaki, H., Amano, K., Tokiwa, T., Mitsui, H., Saito, H., Iikura, Y., Ishizaka, T. and Nakahata, T. (1995). Effects of T-helper 2-type cytokines, interleukin-3 (IL-3), IL-4, IL-5, and IL-6 on the survival of cultured human mast cells. *Blood* **86**, 3705-3714.

Yuk, M. H., Harvill, E. T. and Miller, J. F. (1998). The BvgAS virulence control system regulates type III secretion in *Bordetella bronchiseptica*. *Molecular Microbiology* **28**, 945-959.

Yuk, M. H., Harvill, E. T., Cotter, P. A. and Miller, J. F. (2000). Modulation of host immune responses, induction of apoptosis and inhibition of NF-kappaB activation by the *Bordetella* type III secretion system. *Molecular Microbiology* **35**, 991-1004.

Appendices

A) Media

A-1) Luria-Bertani

A-1-1) LB (broth)

Tryptone	10 g
Yeast extract	5 g
Sodium chloride	5 g

Made up to 1 litre in distilled water

Dissolved and autoclaved at 121°C for 15 min

A-1-2) LB (agar)

For previous, add 1.2% agar

Dissolved and autoclaved at 121°C for 15 min

A-2) Bordet-Gengou Agar (BG) (pH 7.4)

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

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

A-3) Stainer Scholte (SS) liquid medium (pH 7.6)

L-glutamate	10.7 g
L-proline	0.24 g
Sodium chloride	2.5 g
Potassium di-hydrogen orthophosphate	0.5 g
Potassium chloride	0.2 g
Magnesium chloride ($6H_2O$)	0.1 g
Calcium chloride	0.02 g
Tris	6.1 g

Made up to 1 litre in distilled water

A-3-1) Vitamin solution/SS-supplements

L-cysteine	0.04 g
Iron sulphate ($7H_2O$)	0.01 g
Nicotinic acid	0.004 g
Glutathione	0.15 g
Ascorbic acid	0.4 g

Dissolve in 10ml distilled water, sterile-filtered then added to 1 l of CL medium.

A-4) Cyclodextrin iquid (CL) medium

For CL medium, prepared solution as described above and supplemented with casamino acids (10 g) (Difco) and methyle-β-cyclodextrin 0.25 g.

Made up to 1 litre in distilled water. Solution is prepared to pH 7.6 then autoclaved.

B) DNA Solution

B-1) Stop solution

Sodium dodecyl sulphate (SDS)	0.5 % (w/v)
Tris	50 mM
EDTA	0.4 M

B-2) Tris-Borate-EDTA (TBE) buffer (5X 1L)

Tris	54 g
Boric acid	27.5 g
EDTA (0.5M)	20 ml

B-3) 6X DNA loading buffer

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

C) ACT enzymic assays

C-1) Bicine buffer (pH to 8.0)

100 mM Bicine (pH 8)	25ml
100 mM Magnesium acetate	3.75 ml
100 mM ATP	(pH 71.25 ml (0.5 mM)/2.5 ml (1mM)

Made to 250 ml in deionised distilled water

D) β-hexosaminidase assay buffers

D-1) Citrate buffer for substrate (pH 4.5)

Solution A	
Citric acid	31.5 g
Sodium hydroxide (300ml)	IM
Distilled water	700ml

Solution B

HCl	0.1M

For use add together 67.8ml Solution A to 32.2ml Solution B.

D-2) 0.2M Glycine NaOH (pH 10.7)

Solution A	
Glycine	7.5 g
Sodium Cholride	5.8 g
Distilled water	1000 ml
Solution B	
Sodium hydroxide	0.2M

For use add together 52.8ml Solution A to 47.2 ml Solution B.

D-3) Substrate buffer

5mM p-nitrophenol-N-acetyl-β-D-glucosamide in citrate buffer (pH 4.5)

Add 1.7mg of p-nitrophenol-N-acetyl-β-D-glucosamide (Sigma) to 1ml of citrate buffer (pH 4.5).