THE ROLE OF HUMORAL AND CELLULAR DEFENCES OF THE PIG AGAINST *ACTINOBACILLUS PLEUROPNEUMONIAE*

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To my parents, Moira and John.

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ABBREVIATIONS

ACP	alternative complement pathway
AHS	absorbed human serum
AP-CH50	50% haemolysis unit (ACP)
Apx I	*Actinobacillus pleuropneumoniae* toxin type I
Apx II	*Actinobacillus pleuropneumoniae* toxin type II
Apx III	*Actinobacillus pleuropneumoniae* toxin type III
ATP	adenosine triphosphate
CCP	classical complement pathway
CFU	colony forming units
CH50	50% haemolysis unit (CCP)
cm	centimetre
D.H_2O	distilled water
DNA	deoxyribonucleic acid
EA	antibody sensitised sheep red blood cells
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethyleneglycol-bis-(beta-aminoethyl ether)
ELISA	enzyme linked immunosorbent assay
FCS	fetal calf serum
FHS	filtered human serum
Fig.	figure
g	grams
H_2O	water
HBSS	Hanks balanced salt solution
HHS	heated human serum
Hly I	haemolysin I
Hly II	haemolysin II
HPS	heated pig serum
HRP	horseradish peroxidase
Ig	immunoglobulin
i.m.	intramuscularly
IPS	immune pig serum
IRP	immune rabbit serum
i.v.	intravenously
kDa	kilodaltons
L	litre
LPS	lipopolysaccharide

mA	milliamps
Mab	monoclonal antibody
MAC	membrane attack complex
MEM	Minimum Essential Medium
μg	micrograms
mg	milligrams
μl	microlitre
ml	millilitres
mm	millimetre
NAD	ß-nicotinamide adenine dinucleotide
NHS	normal human serum
nm	nanometers
NPS	normal pig serum
NRS	normal rabbit serum
^{o}C	degree centigrade
OD	optical density
OMP	outer membrane protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDA	piperazine di-acrylamide
pH	negative logarithmic concentration of hydrogen ions
PMN	polymorphonuclear leukocytes
Ptx	pleurotoxin
RRBC	rabbit red blood cells
roH_2O	reverse osmosis water
rpm	rotations per minute
RTX	repeat in structural toxin
SDS	sodium dodecyl sulphate
SPF	specific pathogen free
spp.	species
SRBC	sheep red blood cells
TEMED	tetramethylethylene-diamine
Tris	tris (hydroxymethyl) amino methane
TSB	tryptone soya broth
V	volts
VBS	veronal buffered saline

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DECLARATION

The studies described in this thesis were carried out in Department of Veterinary Pathology at the University of Glasgow Veterinary School between April 1988 and April 1991. The author was responsible for all results except where it is stated otherwise.

No part of this thesis has been presented to any university but it has been reproduced in parts in the following scientific publications and abstracts:

Rycroft, A.N., & Cullen, J.M. 1990a. Complement resistance in *Actinobacillus (Haemophilus) pleuropneumoniae* infection in swine. Am. J. Vet. Res. 51: 1449-1453.

Cullen, J.M., & Rycroft, A.N. 1990. Evasion of the complement-mediated bactericidal activity of pig serum by *Actinobacillus pleuropneumoniae*. Proc. Int. Pig. Vet. Soc. Congr. 11: 4.

Rycroft, A.N., & Cullen, J.M. 1990b. The secreted toxin is distinct from the haemolysin. Proc. Int. Pig. Vet. Soc. Congr. 11: 22.

Rycroft, A.N., Williams, D., Cullen, J.M., & MacDonald, J. 1991. The cytotoxin of *Actinobacillus pleuropneumoniae* (pleurotoxin) is distinct from the haemolysin and is associated with a 120 kDa polypeptide. J. Gen. Microbiol. 137: 561-568.

Cullen, J.M., & Rycroft, A.N. 1992a. Identification and cross reactivity of the haemolytic and cytotoxic proteins of *Actinobacillus pleuropneumoniae* and related pathogens by the use of monoclonal antibodies. Proc. Int. Pig. Vet. Soc. Congr. 12: 198.

Cullen, J.M., & Rycroft, A.N. 1992b. Phagocytosis of *Actinobacillus pleuropneumoniae* serotype 2 strain HK 361 haemolysin and cytotoxin negative mutants by pig alveolar macrophages. Proc. Int. Pig. Vet. Soc. Congr. 12: 205.

Frey, J., Bosse, J.T., Chang, Y.F., Cullen, J.M., Fenwick, B., Gerlach, G.F., Gygi, D., Haesebrouck, F., Inzana, T.J., Jansen, R., Kamp, E.M., MacDonald, J., MacInnes, J.I., Mittal, K.R., Nicolet, J., Rycroft, A., Segers, R.P.A.M., Smits, M.A., Stenbaek, E., Struck, D.K., van Den Bosch, J.F., Willson, P.J., & Young, R. 1993. *Actinobacillus pleuropneumoniae* RTX-toxins: Uniform designations of haemolysins, cytolysins, pleurotoxin and their genes. J. Gen. Microbiol. 139: 1723-1728.

Cullen, J.M., & Rycroft, A.N. 1993. Phagocytosis by pig alveolar macrophages of *Actinobacillus pleuropneumoniae* serotype 2 mutant strains defective in haemolysin II (Apx II) and pleurotoxin (Apx III). J. Gen. Microbiol. (in press).

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SUMMARY

The main objective of this research was to assess the defensive roles of both the humoral and cellular defences of the pig against *Actinobacillus pleuropneumoniae (A. pleuropneumoniae) in vitro*. The aims were firstly to establish the effects of complement on the survival of *A. pleuropneumoniae* and secondly, to study the ability of alveolar macrophages to phagocytose *A. pleuropneumoniae*.

In Chapter 3 the effect of complement on the survival of *A. pleuropneumoniae* was examined. All strains of *A. pleuropneumoniae* tested were found to be resistant to pig complement. The strains were also found to be resistant to both immune pig serum and hyperimmune rabbit serum containing specific antibodies against *A. pleuropneumoniae*. Three of the four strains were found however to have a delayed sensitivity to normal human serum.

The mode of complement resistance in *A. pleuropneumoniae* was compared to a known serum resistant *Escherichia coli* strain using the outer membrane disorganising chemical polymyxin B. All *A. pleuropneumoniae* strains tested could not be sensitised to complement bactericidal activity by this method. This was in contrast to the serum resistant *E. coli* which was sensitised to the effects of complement following treatment with polymyxin B.

In Chapter 4 the method by which *A. pleuropneumoniae* avoided complement-mediated damage was investigated. *A. pleuropneumoniae* was found to consume large amounts of complement activity via the classical and alternative pathways from both pig and human serum. Activation of the complement component C3 also occurred in both pig and human serum. Activation of factor B, which occurs

via the alternative pathway only, was also demonstrated in human serum during incubation with the bacteria.

In Chapter 5 non-viable *A. pleuropneumoniae* were found not to consume complement activity suggesting the ability of *A. pleuropneumoniae* to consume complement was dependant on bacterial viability. The extracellular secreted toxins of *A. pleuropneumoniae* were found not to affect consumption of complement activity and therefore some other function of viable *A. pleuropneumoniae* appeared to be responsible.

In Chapter 6 the ability of pig alveolar macrophages to phagocytose *A. pleuropneumoniae* was investigated. Both haemolytic and cytotoxic activities are known to be associated with 109 (haemolysin II, Hly II) and 120 (pleurotoxin, Ptx) kDa proteins respectively. Both HK 361 (Hly II^+, Ptx^+) and mutant e (Hly II^-, Ptx^+) were found to rapidly destroy all macrophages when incubated with normal pig serum and any phagocytosis could not be determined. Mutant h (Hly II^-, Ptx^-) was found initially not to cause damage to alveolar macrophages. However, after prolonged incubation, a level of damage was observed, although not to the same extent as seen with HK 361 and mutant e. A few phagocytosed mutant h were observed initially, however the numbers did not increase with time. No reduction of toxicity was seen for macrophages incubated with HK 361 and mutant e in the presence of immune serum. The reduced toxicity of mutant h for alveolar macrophages was diminished following the addition of immune pig serum and the numbers of phagocytosed mutant h greatly increased. The nature of the reduced toxicity of mutant h was compared to killed mutant h. No toxicity of killed mutant h (by heat or formaldehyde) was observed in the presence of normal pig serum, and similar levels of phagocytosed bacteria were seen. This suggested the toxicity associated with mutant

h was both heat labile and neutralised by immune pig serum.

In Chapter 7 the immunological relationships of the haemolytic and cytotoxic proteins (109 and 120 kDa respectively) of *A. pleuropneumoniae* serotype 2 strain HK 361 were investigated. Monoclonal antibodies (Mabs) were raised against both the 109 or 120 kDa protein secreted from HK 361.

Mabs raised against each protein cross-reacted with other proteins on Western blots suggesting they were immunologically related. Cross-reactive proteins were found in all the serotype reference strains of *A. pleuropneumoniae*, including some of the related pathogens. The presence of similar proteins in all serotypes of *A. pleuropneumoniae* suggested a similar virulence factor of *A. pleuropneumoniae*. The cross-reaction seen with related pathogens was in agreement with others who suggested the similarity of the *A. pleuropneumoniae* toxins to the RTX (repeat in structural toxin) toxin-producing family.

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Porcine pleuropneumonia is a disease that has only been acknowledged for the last 30 years and its increase in prevalence appears to be correlated to the increased intensity of swine herds (Sebunya & Saunders, 1983). The disease results in huge economic losses which are caused by deaths due to the peracute disease and the cost of both treatment of individual infected pigs and mass medication of feed and water (Wilson *et al.*, 1986). Porcine pleuropneumonia is considered to be the second most important pig disease in terms of its worldwide location and resultant economic losses.

1.2 History and taxonomy of *A. pleuropneumoniae*

The first detailed description of porcine pleuropneumonia was documented in the early 1960's (Shope, 1964). Shope (1964) was the first to propose the name *Haemophilus pleuropneumoniae* for the causative organism of swine pleuropneumonia isolated from an acute outbreak in Argentina. It had been first thought that the Argentinian outbreak might be swine influenza (Shope, 1964). However, on direct observation the clinical signs and post-mortem appearance, together with failure to detect a virus, suggested it was not swine influenza. The morphological appearances and the characteristics of the bacteria isolated on blood agar did however strongly suggest a close similarity to *Haemophilus influenzae suis*. Further examination of the morphological, cultural and biological characteristics confirmed that the organism belonged to the genus *Haemophilus*, and because of its propensity to cause pleuropneumonia in swine was designated by Shope (1964) *Haemophilus pleuropneumoniae*.

Previous observations had been recorded detailing a *Haemophilus*-like organism isolated from pigs (Pittman,

1953; Pattison *et al.*, 1957; Matthews & Pattison, 1961). Those organisms were grouped together with human isolates of either *Haemophilus parainfluenzae* (Rivers, 1922) or *Haemophilus parahaemolyticus* (Pittman, 1953) depending on haemolytic activity (Olander, 1963). A Swiss outbreak, similar to the one described in Argentina, was documented by Nicolet and Konig (1966) and Nicolet (1968 & 1971). These isolates were found to be biochemically similar to that of Shope (1964) (Nicolet & Konig, 1966; Nicolet, 1968; Nicolet, 1971). However by rule of priority of publication, Nicolet (1968) along with Olander (1963), designated them *H. parahaemolyticus*. The same name was adopted in subsequent outbreaks reported in Denmark (Nielsen, 1970a; 1970b), Great Britain (Little, 1970), Canada (Radostits *et al.*, 1963; Schiefer & Greenfield, 1974; Schiefer *et al.*, 1974), Australia (Mylrea *et al.*, 1974), Sweden (Biberstein *et al.*, 1977; Gunnarsson *et al.*, 1977) and the German Democratic Republic (Kiupel, 1975).

In 1976, Kilian (1976a) revealed a number of cultural and biochemical differences between *H. parahaemolyticus* isolated from pigs (Kilian, 1976a) and the human strains designated *H. parahaemolyticus* by Pittman (1953). These porcine strains could be further divided into two groups, named major and minor, on the basis of biochemical characteristics, haemolytic activity and deoxyribonucleic acid (DNA) base composition. The major group comprised of the original strains of Olander (1963) and Shope (1964), which had the ability to ferment ribose, xylose and mannitol, were CAMP reaction positive and had significantly higher guanine and cytosine content. In 1978 Kilian *et al.* proposed the major group of organisms should be designated *Haemophilus pleuropneumoniae* (Matthews & Pattison, 1961) Shope 1964 and the strain Shope 4074 to be the neotype. Pohl *et al.* (1983) proposed the transfer of *H. pleuropneumoniae* along with a *Pasteurella*-like organism

(Bertschinger & Seifert, 1978), which caused porcine necrotic pleuropneumonia, to the genus *Actinobacillus*. The *Pasteurella*-like organism was shown to be phenotypically similar to the V factor dependent strains of *H. pleuropneumoniae* (Matthews & Pattison, 1961) Shope 1964, with the exception of being V factor independent. DNA-DNA hybridisation experiments suggested that the type and reference strains of these two taxa belonged to the same species and that neither should belong to the genus *Haemophilus* as there was no measurable relatedness with the type strain *H. influenzae*. They were however closely related to *Actinobacillus lignieresii*, both phenotypically and on DNA base sequence. The difference in host ranges suggested a new species be adopted, namely *Actinobacillus pleuropneumoniae*. *A. pleuropneumoniae* was divided into two biotypes, biotype one consisting of the V factor dependent strains (Shope 4074[=CCM 5869 (Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia) = ATCC 27088 (American Type Culture Collection, Rockville, Md)]) and biotype two, V factor independent strains (Bertschinger 2008/76 [= Fredenksen P597 = HIM 677-3/4 (Bacteriological Culture Collection, Marburg, Germany)]) (Pohl *et al.*, 1983).

1.3 Aetiology

The aetiological agent of swine pleuropneumonia is a gram-negative, non-sporeforming pleomorphic rod with predominantly coccobacillary forms. It can occur singly, in pairs or short chains with the filamentous form becoming more prominent with increasing age of culture (Olander, 1963; Shope, 1964). Capsules are commonly present (Sebunya *et al.*, 1982; Jacques *et al.*, 1988a). The organism grows rapidly on enriched media forming mucoid, and sometimes waxy, iridescent colonies reaching a diameter of 3mm (Shope, 1964; Nicolet, 1986; Mylrea *et al.*, 1974; Sebunya *et al.*, 1982). Colonies produce a

zone of ß-haemolysis on calf or sheep blood agar (Kilian, 1976b). The zone of haemolysis is increased when the haemolysin acts synergistically with the *Staphylococcus aureus* ß-toxin giving a positive CAMP reaction (Kilian, 1976b).

The organism is facultatively anaerobic and usually microaerophilic on primary isolation (Biberstein *et al.*, 1977). Subcultures normally do not require additional carbon dioxide (Pohl *et al.*, 1983). Growth factor requirements differ between the two biotypes; biotype 1 being V factor (B-nicotinamide adenine dinucleotide) dependent and biotype two V factor independent (Pohl *et al.*, 1983). To date 12 serotypes of biotype 1 have been recognised based on specific antigenic capsular components (Gunnarson *et al.*, 1977; Nielsen, 1985a; Nielsen, 1985b; Nielsen, 1986a; Nielsen, 1986b; Nielsen & O'Connor, 1984; Rosendal & Boyd, 1982).

1.4 Epidemiology

Pleuropneumonia of the pig has been reported worldwide. Increased occurrence appears to be correlated with industrialisation of pig production (Nicolet, 1986). The potential transmission of *A. pleuropneumoniae* is maximised in well stocked herds (Sanford & Josephson, 1981; Davidson & King, 1980). The transfer of the disease is thought to be via the respiratory route by the inhalation of aerosol droplets containing the bacterium (Sebunya & Saunders, 1983; Nicolet, 1986). The introduction of the disease appears to be associated with the importation of infected animals (Greenway, 1981), either by carriers or chronically infected animals (Nicolet, 1986). Moving, mixing and frequency of animals purchased increases the risk of pleuropneumonia in a herd (Rosendal & Mitchell, 1981). Infected animals do not always demonstrate clinical signs although they may carry the bacterium in the nose, tonsil or

sequestered in the lung within lung lesions (Kume *et al.*, 1986a). In an infected herd certain stress factors can promote an outbreak of the disease (MacInnes & Rosendal, 1988). These can include overcrowding, sudden climatic changes and poor ventilation. Seasonal changes have also been reported to influence intensity of the disease (Sanford & Josephson, 1981; Sebunya *et al.*, 1982). Pleuropneumonia can affect pigs of all ages. Losses occur more frequently in the feeder pigs which is thought to be due to additional stress associated with rapid growth (MacInnes & Rosendal, 1988). The morbidity and mortality depends on the immune status of the herd. Elimination of *A. pleuropneumoniae* should be possible as it does not persist in the environment and pigs appear to be the only animal infected under natural conditions.

1.5 Serological typing

To date 12 serotypes of *A. pleuropneumoniae* have been recognised based on heat stable soluble surface polysaccharide or lipopolysaccharide antigens (Nicolet, 1971; Gunnarson *et al.*, 1977; Rosendal & Boyd, 1982; Nielson & O'Connor, 1984; Nielsen, 1985a; Nielsen, 1985b; Nielsen, 1986a; Nielsen, 1986b; Inzana, 1987). The serotypes can be grouped together according to shared type specific polysaccharide or lipopolysaccharide antigenic determinants resulting in the following groups: the 3, 6, 8 group, the 1, 9, 11 group and the 4, 7 group. Serotypes 2, 5, 10 and 12 are homogeneous serotypes sharing no type specific determinants with other serotypes. Serotype 5 can be divided into two subtypes, a and b, based on polysaccharide capsular determinants specific for each subset (Nielsen, 1986b).

Certain serotypes may occur in only one country while others are present in several different regions. In Britain those most commonly found are serotypes 3, 6 ,8

and some untypeable (Hunter *et al.*, 1983; Taylor, 1989). Serotype 2 is dominant in Denmark (Nielsen, 1988). Elsewhere, in Canada for example serotypes 1, 2, 3 and 5 are present and in America, serotypes 1, 2, 3 and 4 (Sebunya & Saunders, 1983). The serotype pattern of a particular region may however change (Nielsen, 1988). This is thought to be due to importation of animals for cross breeding programs. In Denmark for example between 1970 and 1981, only serotypes 2 and 6 were isolated with serotype 2 being dominant. Between 1982 and 1986 other serotypes emerged, in particular types 1, 5, 7, 8, 10 and 12. Animals from imported stock were found to be seropositive to these serotypes suggesting that these imported animals were responsible for the emergence of the new serotypes.

Methods used for serological typing of *A. pleuropneumoniae* have included slide agglutination (Mittal *et al.*, 1982; Rapp *et al.*, 1985a), tube agglutination (Gunnarsson *et al.*, 1977; Mittal *et al.*, 1982), immunodiffusion (Lombin *et al.*, 1985; Nicolet, 1971; Gunnarsson, 1979a), ring precipitation (Mittal *et al.*, 1982; Hunter *et al.*, 1983), indirect haemagglutination (Nielsen & O'Connor, 1984; Mittal *et al.*, 1983b; Nielsen, 1985a), immunofluorescence (Nicolet, 1971; Rosendal & Boyd, 1982; Rapp *et al.*, 1985a), coagglutination (Mittal *et al.*, 1983a; Hunter & Livingstone, 1986; Mittal *et al.*, 1987) and counterimmunoelectrophoresis (Pieffer *et al.*, 1986). The serotype-specific antigens of *A. pleuropneumoniae* are capsular polysacchaccarides and lipopolysaccharides (LPS) (Gunnarsson, 1979b; Neilsen & O'Connor, 1984; Fenwick *et al.*, 1986c; Perry *et al.*, 1990). The various methods employed different antigen preparations. Whole cells were normally used for coagglutination, slide agglutination, and indirect fluorescent antibody and cell extracts for agar gel precipitation, indirect haemagglutination and counterimmunoelectrophoresis

(Nicolet, 1988). None of the methods were completely specific and therefore a combination of methods was normally used. Conflicting results were sometimes obtained using the different antigen preparations and the different methods (Nicolet, 1988) which resulted in different methods being favoured. Gunnarsson *et al.* (1978) found the best routine method to be tube agglutination. Agglutination however could not be used to serotype strains which polyagglutinate or autoagglutinate. Heat treatment of these strains to unmask the neccessary serotype specific antigens also exposed cross reactive common antigens (Mittal *et al.*, 1987). Cross reactions between serotypes 1 and 9 were shown to be due to common epitopes associated with the cell wall antigens (Mittal, 1990). The coagglutination test was simple to preform, reproducible, rapid, easier to read than the agglutination tests (Hunter *et al.*, 1983; Hunter & Livingstone, 1986; Mittal *et al.*, 1987). To standardise serotyping, a working team within the International Pig Veterinary Society (IPVS) was set up to evaluate the various methods. The preliminary recommendations suggested coagglutination for field testing and either agar gel diffusion or indirect haemagglutination for final identification of the heterogeneous serotypes (Nicolet, 1988).

1.6 Pathogenesis

In order to be a successful pathogen there are certain virulence attributes required by invading bacteria. The pathogenicity requirements of *A. pleuropneumoniae* might be expected to include include colonisation of mucous surfaces, invasion of host tissues, survival and multiplication within the host, interference with the host defences and damage to the host. *A. pleuropneumoniae* possesses a variety of components that are thought to aid invasion. These include capsule, adhesion component structures, surface structures

including outer membrane proteins and LPS and also secreted exotoxins. The involvement of these components in the successful production of swine pleuropneumonia has been documented to varying extents.

1.6.1 Lipopolysaccharide

Hani *et al.* (1973) proposed lipopolysaccharide (LPS) as the mediator of the acute pulmonary lesion in *A. pleuropneumoniae* infection. This arose from the similarity of the lesions to those observed subsequent to endotoxic shock. The observation of bilateral renal necrosis in pigs given intravenous (IV) sterile suspension of infected lung tissue led to the hypothesis of generalised Schwartmann's reaction (Hani *et al.*, 1973; Nordstoga & Fjostad, 1967). This was a characteristic feature of endotoxic shock. Evidence for endotoxin was strengthened by a study by Fenwick *et al.* (1986a) in which pigs vaccinated with an *E. coli* rough mutant bacterin (killed bacterial preparation), strain J5, survived infection with *A. pleuropneumoniae*. In contrast 4\5 controls died. It was thought that antibody to lipid A and possibly LPS core antigens stimulated by *E. coli* J5, may have had a protective effect against septicemia and death in *A. pleuropneumoniae* infections. This provided indirect evidence for the role LPS. The protective efficiency of J5 against death had been previously reported in other Gram-negative bacteria (Ziegler *et al.*, 1973). However, failure to protect the animals from *A. pleuropneumoniae* infection suggested the role of other virulence components.

Fenwick *et al.* (1986b) also reported that experimental infection with *A. pleuropneumoniae* in mice genetically altered to give a low response to endotoxin, resulted in less severe clinical signs than in mice with a normal response to endotoxin. 46% of the normal mice died compared to only 10% of the low endotoxin responder mice

suggesting endotoxin may be involved in acute death in *A. pleuropneumoniae* infections. Parallel to infection with *A. pleuropneumoniae*, mice were infected with *E. coli* strain 0111:B4 and its rough mutant J5. No deaths occurred in the mice inoculated with the *E. coli* strains. The different toxicity levels for *E. coli* and *A. pleuropneumoniae* suggested that the effects demonstrated by *A. pleuropneumoniae* infection were not due to LPS alone. The possibility of varying potency of LPS and the usage of live organisms, which may possess a range of pathogenicity factors, suggest that these experiments could not have satisfactorily assessed the involvement of LPS in *A. pleuropneumoniae* infection.

Intrabronchial administration of purified LPS from *A. pleuropneumoniae* induced lesions typical of acute pleuropneumonia in pigs (Fenwick *et al.*, 1986c). This was in contrast to *E. coli* endotoxin which was not capable of producing lesions typical of acute *A. pleuropneumoniae* infection. These LPS fractions provoked dermal Schwartmann's reaction in rabbits and pigs (Fenwick *et al.*, 1986; Maudsley *et al.*, 1986). Rough and smooth LPS from *A. pleuropneumoniae* serotype 5 were compared for toxicity *in vivo* (Fenwick *et al.*, 1986c). Both rough LPS and smooth LPS induced clinical signs typical of endotoxic shock (Kurtz & Quast, 1982). However, rough LPS induced more severe clinical signs at lower doses than smooth LPS. Similar reports in other Gram-negative bacteria have indicated rough LPS also to be more toxic than smooth LPS (Jones *et al.*, 1976).

Differences in LPS structure were postulated to perhaps partly contribute to virulence (Jensen & Bertram, 1986). Comparison of LPS from a virulent *A. pleuropneumoniae* strain (1200) and an avirulent *A. pleuropneumoniae* strain (B8), both of serotype 5, revealed the virulent strain 1200 contained 1\3 more LPS and 12.8 times more galactose than the avirulent B8 strain. This increase in

galactose may be relevant to virulence. Monosaccharide substitution in repeating units or differing repeating units in the 0 side chains of *Salmonella typhimurium* LPS has been proposed to infuence virulence of this *Salmonella typhimurium* (Makela *et al.*, 1973; Valtonen, 1977). Apart from these two strains being unrelated, different haemolytic activities were also observed suggesting these 2 strains could not be compared for pathogenicity solely on LPS structure. The lack of necrosis and haemorrhage observed in mice and pigs inoculated with *A. pleuropneumoniae* LPS compared to intact *A. pleuropneumoniae*, supported the idea that endotoxin alone did not produce all the lung pathology typically observed in the acute form of naturally occurring or induced pleuropneumonia (Udeze *et al.*, 1987; Bertram, 1988).

While *A. pleuropneumoniae* purified LPS appears not to be wholly responsible for the pathology associated with *A. pleuropneumoniae* infection, it does appear, at least in part, to correlate with adhesion of *A. pleuropneumoniae* to the epithelium in tracheal rings. LPS profiles of *A. pleuropneumoniae* serotypes include smooth, rough and semi-rough LPS profiles. Serotypes 2, 4 and 7 being smooth LPS (Rapp *et al.*, 1986; Byrd & Kadis, 1989; Belanger *et al.*, 1990), serotypes 1 & 5, semi-rough LPS (Byrd & Kadis, 1989; Belanger *et al.* 1990) and serotypes 3,6 & 10, rough LPS (Rapp *et al.*, 1986; Byrd & Kadis, 1989). Belanger *et al.* (1990) found that those organisms possessing smooth LPS adhered in large numbers compared to those with semi-rough LPS which adhered poorly. This adhesion could be blocked by antibody to purified LPS confirming the involvement of LPS in adhesion to porcine tracheal rings. *A. pleuropneumoniae* has been shown to produce outer membrane blebs, along with other bacteria (Holt *et al.*, 1980), apparently crossing capsular material and thus exposing the target cell surface to outer membrane components such as LPS (Jacques *et al.*,

1988a). LPS is known to have the capacity to interact with mammalian membranes, in particular membrane phospholipid (Morrison, 1985). These data together with Belanger's observations suggest that cell surface exposed LPS increases ability to adhere *in vitro* to tracheal rings. The significance of adherence appears not to be of primary importance in relation to the virulence of a particular serotype. Serotypes 1 and 5 which were found in acute infection and considered virulent, adhered poorly to tracheal rings. In contrast serotypes 2 and 7, which were considered less virulent, adhered in large numbers. LPS therefore does appear to play a role in lesion development in mice and pigs, and death in mice. However, the involvement of other pathogenic mechanisms seems likely.

1.6.2 Capsule

Capsules are important to the virulence of pathogenic bacteria by providing the organism with a protective barrier against host defences. In many cases encapsulated bacteria, prior to development of specific antibody within a host, generally inhibit the bactericidal and opsonic activity of normal serum. In contrast non-encapsulated isogenic mutants are generally avirulent and are quickly cleared by the host. Capsules of *A. pleuropneumoniae* have been purified and characterised as being negatively charged, high molecular weight, acidic polysaccharide (Altman *et al.*, 1986; 1987a; 1987b; 1987c; 1988; Inzana, 1987).

A. pleuropneumoniae capsules have been reported to be non-toxic and do not produce any clinical signs of illness or lesion development (Fenwick *et al.*, 1986c). It has been suggested that differences in virulence observed between some serotypes (Rosendal *et al.*, 1985; Rogers *et al.*, 1990) may be related to differences in the composition of the capsule. Jensen and Bertram

(1986) found differences in the composition of the structure of capsular material between two non-isogenic serotype 5 strains. One *A. pleuropneumoniae* strain, 1200, was found to be virulent and had a distinct adherent capsule. The other, strain B8 was avirulent and had a fragile easily removed capsule. They proposed that this difference in virulence was related to the difference in capsule thickness between these two strains. However, in addition to being unrelated strains, these two strains also had different LPS structures and haemolytic activities and therefore the results could not be conclusive.

Differences observed in capsule thickness between serotypes 1-10 were also proposed to account for the different virulence levels between some serotypes (Jacques *et al.*, 1988a). However, studies to detect a correlation between capsule thickness and virulence of a particular serotype was not carried out. More recently Rosendal and MacInnes (1990) presented evidence that supported the relationship between capsule thickness and virulence. An *A. pleuropneumoniae* serotype 1 strain, CM5, isolated from a pig with pleuropneumonia and passaged once in vitro, was found to be virulent for pigs. A substrain of CM5 passaged 70 times in vitro, was found to be avirulent in pigs. No difference in LPS structure, production of LPS blebs, cytotoxic activity or resistance to serum could be detected between the 2 strains. The only detectable difference was the capsule thickness. Presence, absence or amount of capsule did not appear to significantly alter the LD 50 in mice (Inzana *et al.*, 1988). These findings were in contrast to reports of other bacteria, *Haemophilus influenzae* type b (Moxon & Vaughn, 1981; Zwahlen *et al.*, 1983) and *E. coli* (Kim *et al.*, 1986; Allen *et al.*, 1987) that nonencapsulation rendered them less virulent than their parent strains. Both the capsulated and nonencapsulated strains reported by Inzana *et al.* (1988) caused death

within 2 days. Only the encapsulated strain was capable of causing bacteraemia in mice indicating that death was not dependant on bacteraemia in the mouse model. It has been suggested that the capsule enables the bacteria to disseminate while toxins produced by the bacteria are responsible for death. Capsular material appears not to be toxic to pigs or involved in lesion development.

1.6.3 Haemolytic and cytotoxic activities

Over the last decade one of the major virulence factors to emerge has been the haemolytic/cytotoxic activity of viable bacteria and their secreted products. The work of Rosendal *et al.* (1980) which described that sonicated bacteria and bacterial culture supernatant induced lung lesions similar to those caused by natural infection of *A. pleuropneumoniae* prompted further research into the properties of the toxic activity and its role in pathogenicity.

Bendixen *et al.* (1981) demonstrated that both a heat-stable activity associated with culture supernatant and a heat-labile activity of viable bacteria of a serotype 1 strain, CM-5, were toxic for both pulmonary lavage cells and cultured blood monocytes. These toxic activities could be neutralised by convalescent pig serum suggesting their secretion *in vivo*. Haemolytic activity was not determined. Nakai *et al.* (1983) was the first to report a heat-stable haemolysin associated only with bacterial supernatant suggesting an extracellular secreted activity. Toxic activity for horse, rabbit, sheep, guinea pigs and pigs red blood cells was unaffected by heat or proteolytic enzymes suggesting it was not a protein or enzyme. Following partial purification of this serotype 2 haemolysin by sucrose density gradients, it was also demonstrated to be lethal for pigs and for a proportion of guinea-pigs. The lack of endotoxin in the preparation ruled out its possible

role in pathogenesis (Nakai *et al.*, 1984). Further analysis of this serotype 2 haemolysin along with serotypes 1-5 haemolysins showed cytotoxic effects on guinea-pig macrophages. The serotype 2 haemolysin was further assessed and found to reduce the phagocytosing ability of cotton rat and pig macrophages but not mouse macrophages (Kume & Nakai, 1986). These cytocidal and anti-phagocytic effects of the haemolysin were associated with a heat-stable carbohydrate (Kume *et al.*, 1986b).

Heat-labile haemolysins were also recorded that were sensitive to pronase and trypsin (Martin *et al.*, 1985; Maudsley & Kadis, 1986; Rosendal *et al.*, 1988; Frey & Nicolet, 1988a; Lalonde *et al.*, 1989;). The requirement of RNA for some *A. pleuropneumoniae* strains to produce detectable extracellular toxic activity (Martin *et al.*, 1985) suggested similarities with both the group A *Streptococcus* haemolysins, streptolysin O (Jeljaszewicz *et al.*, 1978), and the haemolysin produced by *Treponema hyodysenteriae* (Sahed *et al.*, 1981). Both of these haemolysins also required an RNA carrier molecule for extracellular activity. The heat labile haemolysin was also found to be cytotoxic for porcine macrophages (Pijoan, 1986; Udeze & Kadis, 1988; van Leengoed *et al.*, 1989) and both porcine and bovine neutrophils (Rosendal *et al.*, 1988). Supernatant preparations of different *A. pleuropneumoniae* strains appeared to have different cytotoxicity levels for porcine macrophages (Pijoan, 1986). The difference was proposed to be related to virulence. Studies revealed that two highly virulent strains, frequently isolated from field outbreaks, had strong cytotoxic activities for porcine macrophages. In comparison, a strain seldom associated with outbreaks and an avirulent strain incapable of producing lesions in susceptible animals, had an intermediate or no effect on porcine macrophages respectively. Considerable differences in the level of haemolytic and cytotoxic

activities both between and within serotypes was seen by Rosendal *et al.* (1988). Strains tested were either haemolytic and cytotoxic, cytotoxic only or negative for both haemolysin and cytotoxin. Attempts at purifying the haemolytic/cytotoxic activities resulted in copurification of both the haemolytic and the cytotoxic activity (Kume *et al.*, 1986b; Udeze & Kadis, 1988). Udeze & Kadis (1988) found the haemolytic activity corresponded to the first two protein peaks following anion exchange chromatography and eluted very close to the void volume in the Sephacryl S-300 column suggesting it was in an aggregated form. They appeared to recover most of the haemolytic activity following anion exchange chromatography and detected a major protein band of 130 kDa along with three other proteins of lower molecular weights with a combined molecular weight of 133 kDa. Further characterisation of the haemolytic activity revealed its association with a 104, 105, 107, 109 and a 110 kDa protein band respectively (Devenish & Rosendal, 1989; Frey & Nicolet, 1988a; Frey & Nicolet, 1988b; Frey *et al.*, 1988; Lalonde *et al.*, 1989; Rycroft *et al.*, 1991; Fedorka-Cray *et al.*, 1990). Devenish & Rosendal (1989) also identified the haemolysin with two major protein peaks using a Sephadex G-100 column. However they found half the haemolytic activity was lost following ammonium sulphate precipitation and a further 80-90% was lost following gel chromatography with little or no recovery using anion exchange chromatography.

Different serotypes of *A. pleuropneumoniae* had different requirements of Ca^{++} for expression of haemolytic activity (Frey *et al.*, 1988; Frey & Nicolet, 1988b). The haemolytic activities were put into four distinct groups. Serotype 1 required Ca^{++} for the induction of synthesis of haemolytic activity but not for activity. Serotypes 2, 4, 7 & 8 produced a relatively weak haemolysin that required Ca^{++} for its activity but not for induction. Serotypes 5a, 5b, 9, 10, 11 & 12 required

Ca^{++} for both induction and expression of haemolytic activity. Serotype 3 & 6 produced very weak haemolytic activities which could not be further assessed (Frey *et al.*, 1988b). No other bivalent ions could substitute the function of Ca^{++} in haemolysin expression. The threshold concentration of Ca^{++} for haemolysin expression was around 700mM, which is similar to that found free in blood (Frey & Nicolet, 1988b). The requirement of Ca^{++} for expression of haemolytic activity in the serotypes mentioned by Frey *et al.* (1988) would perhaps explain why Rosendal *et al.* (1988) failed to detect haemolytic activity in serotypes 2, 7, & 8. He did detect haemolytic activity in serotype 1, 3, 5, 9 and 10 in which no additional Ca^{++} was added. However the combined Ca^{++} content of 5% heated calf blood added to the tryptone soya broth growth medium may have been sufficient to allow induction of haemolysin in these serotypes. In accordance with Frey & Nicolet (1988) other researchers noted the requirements of Ca^{++} for haemolytic activity (Lalonde *et al.*, 1989; Kamp & van Leengoed, 1989; Devenish & Rosendal, 1989; Rycroft *et al.*, 1991). These differences in requirement of Ca^{++} for haemolytic activity denoted two distinct haemolysins, haemolysin I (Hly I) which required Ca^{++} for its induction and haemolysin II (Hly II) requiring Ca^{++} for expression (Frey & Nicolet, 1990). A relationship between the type of haemolysin produced and virulence was suggested. Strains producing Hly I belonged to serotypes frequently isolated from severe outbreaks and considered more virulent than strains producing Hly II (Frey & Nicolet, 1990). Kamp & van Leengoed (1989) added Ca^{++} in both the growth medium and the haemolysin assays and found haemolytic activity could only be detected in serotypes 1, 5, 9, 10 & 11 and not in serotypes 2, 3, 4, 6 & 7. The differences observed perhaps was due to the different protocols used in the production of the haemolysin and the time lapsed before haemolytic activity was assessed. Kamp & van Leengoed (1989)

assayed their haemolytic activity within 24 hours. Haemolytic activity has been found not to be very stable and perhaps the discrepancies observed were due to the delay before assessment.

The haemolysin has been shown to produce pores in phospholipid membranes (Lalonde *et al.*, 1989). This activity resembles that of the alpha-haemolysin of *E. coli* which together with other organisms includes a growing number of Gram-negative organisms that produce pore forming toxins (Koronakis *et al.*, 1987). Cloning of a haemolysin gene (Chang *et al.*, 1989) of a serotype 5 strain revealed high homology to the *E. coli* alpha haemolysin gene hly A and to the lkt A haemolysin gene of *Pasteurella haemolytica*. Analysis of the cloned genetic material displayed two open reading frames, designated appC and appA. The high homology of these appC and appA genes to both the *E. coli* and *P. haemolytica* hlyCA and lktCA genes respectively, together with the requirement of both the C and A genes for toxic activity placed *A. pleuropneumoniae* haemolysin in the RTX cytotoxin family (Strathdee & Lo, 1989). Isolation and DNA hybridisation of a serotype 1 haemolysin gene of 107 kDa molecular weight (Gygi *et al.*, 1990) revealed a similar close relationship, although with some sequence divergence, with the Hly determinant of other Gram-negative bacteria. These include *Proteus spp.*, *Morganella morganii* (Koronakis *et al.*, 1987; Koronakis & Hughes, 1988; Welch, 1987) and *Actinobacillus actinomycetemcomitans* (Kolodrubetz *et al.*, 1989; Lally *et al.*, 1989). Activation and export of the prohaemolysin gene Hly A by the *E. coli* Hly C, B and D genes confirmed the sustantial sharing of haemolysin structure and function (Gygi *et al.*, 1990). Characterisation of a serotype 9 haemolysin determinant (Smits *et al.*, 1990) showed it was almost identical to the serotype 5 appCA genes (Chang *et al.*, 1989). The lysates containing protein of these clones containing

the serotype 9 DNA demonstrated both haemolytic and cytotoxic properties suggesting that both activities were present on the molecule (Smits *et al.*, 1990). Lian *et al.* (1989) cloned a 29.5 kDA protein and detected haemolytic activity in the *E. coli* host. Its properties included heat-stability and non-regulation by Ca^{++}. Its haemolytic activity was not neutralised by antiserum against the 105 kDa haemolysin. The activity was neutralised by antiserum against the recombinants that contained the 29.5 kDa proteins and also convalescent pig serum. The haemolytic activity was also not neutralised by antiserum of related organisms that produce haemolysins including *E. coli*, *Streptococcus agalactiae* and also purified streptolysin O. They concluded that although haemolytic activity could be detected in the *E. coli* recombinants they could not rule out the possibility that this 29.5 kDa protein was a regulator of haemolytic activity rather than a true haemolysin. The DNA sequencing showed no homology with any of the known haemolysins or toxins but it did have extensive homology with the *E. coli* fnr regulatory gene suggesting its probable role as a regulator.

Identification of a heat-labile cytotoxin distinct from haemolytic activity was next described (Rycroft & Cullen, 1990b; Rycroft *et al.*, 1991; Kamp *et al.*, 1990). Analysis of the crude concentrated cytotoxin by Kamp *et al.*, (1990) revealed the presence of two major bands of 105 and 120 kDa. Monoclonal antibody to the crude formalised cytotoxin reacted only with the 120 kDa protein. Purification of the cytotoxin by a Superose 12 column resulted in loss of cytotoxic activity. Analysis of the fractions showed presence of the 105 but not the 120 kDa protein. They suggested that the 120 kDa protein was the heat-labile cytotoxin of serotype 2 strain 1536 and the 105 kDa protein the inactive form of the 120 kDa protein. This was in contrast to Frey *et al.* (1990), who, using the same serotype 2 strain 1536, detected

haemolytic activity. They did however agree on the presence of two major proteins following purification. They estimated molecular weights to be 105 and 125 kDa. Following neutralisation of the haemolytic activity with antiserum raised against the 105 but not the 125 kDa protein, the 105 kDa protein was identified as the haemolysin. In this laboratory the cytotoxin was also determined to be the 120 kDa protein and the 105 kDa protein the haemolysin (Rycroft & Cullen, 1990b; Rycroft *et al.*, 1991). Mutants deficient in the type II haemolysin of serotype 2 showed concurrent loss of either the 109 kDa protein or both the 109 and the 120 kDa proteins. Mutants lacking only the 109 kDa protein still retained cytotoxic activity for porcine alveolar macrophages showing the two activities to be separate. The lack of both the 105 and the 120 kDa bands in the other mutants correlated with loss of both the haemolytic and the cytotoxic activities. Antiserum raised against the 120 kDa protein recognised the 120 kDa but not the 105 kDa protein. Similarly this antiserum neutralised the cytotoxic activity but not the haemolytic activity. Following the relationship between the presence of the 120 kDa protein and cytotoxicity it was proposed that the 120 kDa protein represented the cytotoxin of *A. pleuropneumoniae*, it was designated pleurotoxin and was distinct from the haemolysin (Rycroft & Cullen, 1990b; Rycroft *et al.*, 1991).

1.6.4 Camp factor

The CAMP factor is associated with a lytic phenomenon whereby a zone of lysis produced by certain bacterial strains cultured on blood agar is enhanced when the haemolysin acts synergistically with the ß-toxin of *Staphylococcus aureus*. This CAMP factor, which is produced by *A. pleuropneumoniae* (Kilian, 1976b), has been proposed to be a virulence factor in other bacteria. Injection (i.v.) of partially purified CAMP

factor from *Streptococcus agalactiae* was found to be lethal for rabbits (Skalka & Smola, 1981). Vaccination with CAMP factor also reduced the lethal dose of group B streptococci (Fehrenbach *et al.*, 1988). Jurgens *et al.* (1987) found similarities to the way both CAMP factor and protein A of *Staphylococcus aureus* bound to immunoglobulins supporting its possible role in virulence. Cloning of total DNA from *A. pleuropneumoniae* revealed a cohaemolysin of 27 kDa (Frey *et al.*, 1989). This protein was a similar size to the CAMP protein of group B streptococci. Cross reaction of the *S. agalactiae* protein B with antiserum raised against the cloned *A. pleuropneumoniae* CAMP protein showed that they were similar. The presence of the CAMP factor in all the *A. pleuropneumoniae* serotypes suggested it could be a common virulence factor. However, haemolysin II and pleurotoxin negative mutants (109^{-}, 120^{-} kDa) were found to possess the CAMP factor and yet possessed no toxicity in their bacterial culture supernatant and therefore the CAMP factor appears not to be responsible for a toxic effect (Rycroft & Cullen, 1990b; Rycroft *et al.*, 1991).

1.6.5 Outer membrane components

Certain outer membrane proteins (OMPs) are important for the expression of virulence. A continued supply of iron is essential for the maintenance of growth by most microorganisms (Weinberg, 1978). A common host defence mechanism against invading pathogens is to withold essential nutrients, including iron. The iron sequestering effect of transferrin or lactoferrin limit the free iron available to the microorganisms. Many pathogenic bacteria have developed high affinity iron uptake systems to combat this host defence mechanism. The two main components of this system include low molecular weight siderophores responsible for chelating iron and iron repressible OMPs which act as receptors for these siderophores (Braun, 1985; McIntosh *et al.*,

1979; Neilands, 1982). Iron-repressible OMPs in the range of 64-76 kDa and 96-105 kDa have been described for *A. pleuropneumoniae* (Deneer & Potter, 1989a; Niven *et al.*, 1989; Gonzalez *et al.*, 1990; Ricard *et al.*, 1991). These have been suggested to serve as receptors for free haeme compounds and mediate transport into the cell (Ricard *et al.*, 1991). Both OMPs are expressed both *in vivo* and under iron restricted conditions *in vitro*, and therefore may be important immunogens. The function of *A. pleuropneumoniae* haemolysin, which lyses erythrocytes, is perhaps to supple an adequate source of iron for *in vivo* growth. *A. pleuropneumoniae* has been shown to possess receptors specific for porcine transferrin only and can only use this species specific transferrin for growth (Niven *et al.*, 1989; Gonzalez *et al.*, 1990; Schryvers & Gonzalez, 1990; Ricard *et al.*, 1991). This strict species specificity for host transferrin has been seen in other pathogens including *Haemophilus influenzae* (Schryvers, 1988). The inability to utilise iron in transferrins from other species has been suggested to perhaps partly explain the inability of certain pathogens to cause infection in other hosts.

A maltose inducible protein has also been described for some strains of *A. pleuropneumoniae* (Deneer & Potter, 1989b). This 42 kDa protein has been proposed to act as a porin in the maltose transport system. Although all the strains tested did not induce the 42 kDa OMP, all strains utilised the maltose as demonstrated by the increased yields when grown in the presence of maltose. The ability to induce this 42 kDa OMP appears not to be essential for pathogenicity since all strains tested were isolated from pigs.

1.6.6 Permeability factor

A permeability factor produced by *A. pleuropneumoniae* has been described which induces dermal oedema in

rabbits (Lallier *et al.*, 1987). The factor was produced at 2 stages of the growth cycle and determined not to be haemolytic or proteolytic. Cytotoxic activity was not determined and antiserum to formalised cells did not neutralise the activity of the permeability factor. It has been found that although antiserum raised against formalised *A. pleuropneumoniae* possesses reactive antibodies, they are not capable of neutralising toxic activities associated with the secreted haemolytic and cytotoxic factors (this thesis, Chapter 6). This may therefore also be true for the permeability factor discussed here.

1.6.7 Adhesion factors

Colonisation of the pig lung is the initial virulence mechanism required to cause infection. Surface properties of *A. pleuropneumoniae* have been suggested to allow bacteria to attach to the nasopharynx in order to colonise the host. These surface properties have included smooth LPS profile (Belanger *et al.*, 1990), haemaggulutination (HA) properties (Jacques *et al.*, 1988b) and the presence of fimbriae (Utrera & Pijoan, 1990). Preliminary evidence for pilus-like structures was documented by Inzana *et al.* (1988) on *A. pleuropneumoniae* phagocytosed by polymorphonuclear cells. Utrera and Pijoan (1990) demonstrated fimbriae on 78% of isolates recovered from experimentally infected pigs after one passage. No fimbriae could be detected after 2 passages on artificial medium or after 1 passage on enriched media, suggesting production of fimbriae is strictly dependent on the environment.

Differing HA activities have been described for *A. pleuropneumoniae* (Jacques *et al.*, 1988b). The ability of bacteria to attach to and agglutinate erythrocytes has been documented to serve as a useful model for bacterial attachment to epithelial cells (Old, 1985). Both

pilation (Duguid *et al.*, 1979) and hydrophobic interactions (Garber *et al.*, 1985) can be involved in HA reactions. *A. pleuropneumoniae* HA activities did not appear to be due to either fimbriae or to hydrophobic reactions. The differences in HA activities could not be attributed to different serotypes and their relationship to virulence was not examined (Jacques *et al.*, 1988b).

1.6.8 Immunoglobulin A proteases

Invading organisms must withstand the mucosal cleansing mechanisms in order to colonise the host. Secretory IgA plays a major role in the immune system at mucosal surfaces. 80% of the antibody secreted at these sites is IgA. Secretory IgA has antibody activity against bacteria, toxins, viruses and enzymes. Certain bacteria secret an IgA protease which inactivates the antibody by cleaving it into intact Fc alpha and Fab alpha fragments. By this mechanism they can avoid the detrimental effects of antibody binding and recognition by the host immune system. Members of the *Haemophilus* species responsible for respiratory disease have been shown to produce IgA protease (Kilian *et al.*, 1979). Non-pathogenic organisms were found to lack this enzyme. Conflicting reports have been documented on the ability of *A. pleuropneumoniae* strains to cleave pig IgA. Kilian *et al.* (1979) detected IgA protease activity in the 2 *A. pleuropneumoniae* strains tested. A later study by Mulks *et al.* (1984) could not confirm the presence of IgA protease activity in any of the strains tested including one previously tested by Kilian *et al.* (1979). Also no homology was shown between the *A. pleuropneumoniae* strains and both *H. influenzae* chromosomal DNA and the gene which codes for IgA protease activity in this species suggesting *A. pleuropneumoniae* does not contain this enzyme. The lack of recognition with the *H. influenzae* DNA probe has been suggested to be due to the *A. pleuropneumoniae* IgA protease being significantly

unique to prevent hybridisation. This would explain the differences observed between the groups on the presence of an IgA protease in *A. pleuropneumoniae*.

1.6.9 Plasmids

Virulence of a number of bacterial pathogens has been shown to be dependent on genes located on extrachromosomal genomes of plasmids or phage, for example the *E. coli* heat labile exotoxin (Stephen & Pietrowski, 1981). No high molecular weight plasmids have been detected in *A. pleuropneumoniae* (Huether *et al.*, 1987; Hirsh *et al.*, 1982). Low molecular weight plasmids conferring resistance to streptomycin and sulfonamide (Willson *et al.*, 1989) and ampicillin, streptomycin and sulfadiazene (Hirsh *et al.*, 1982) have been reported. The lack of genetic material to promote transmission has suggested the appearance of these plasmids was due to enviromental selective pressure (Bertram, 1990; Vaillancourt *et al.*, 1988; Willson *et al.*, 1989). The recent development of a shuttle vector and a conjugative transfer system for *A. pleuropneumoniae* (Lalonde & O'Hanley, 1989) may allow the study of *A. pleuropneumoniae* genetics to develop.

1.7 Pathological description of lung lesions

Lesions induced by *A. pleuropneumoniae* infection are confined to the lung region. In peracute cases the lungs are swollen, firm, dark red in colour (Fig. 1.1b) in contrast to the usual pink colour (Fig. 1.1a). Fluid and blood may ooze from the cut surface. Blood stained froth may be present in the trachea and also the nasal and pleural cavities. Thickening of the interlobular septae and presence of oedema due to fibrinous exudate are common. Pericarditis may also occur. Necrosis and fibrinous pleural adhesions are frequently present. In chronic cases the lesions become encapsulated and can be

Fig. 1.1 Comparison of lungs from pleuropneumonia infected and pleuropneumonia-free pigs.

a) Lungs from a pleuropneumonia-free pig
b) Typical lung lesion from a pig with pleuropneumonia

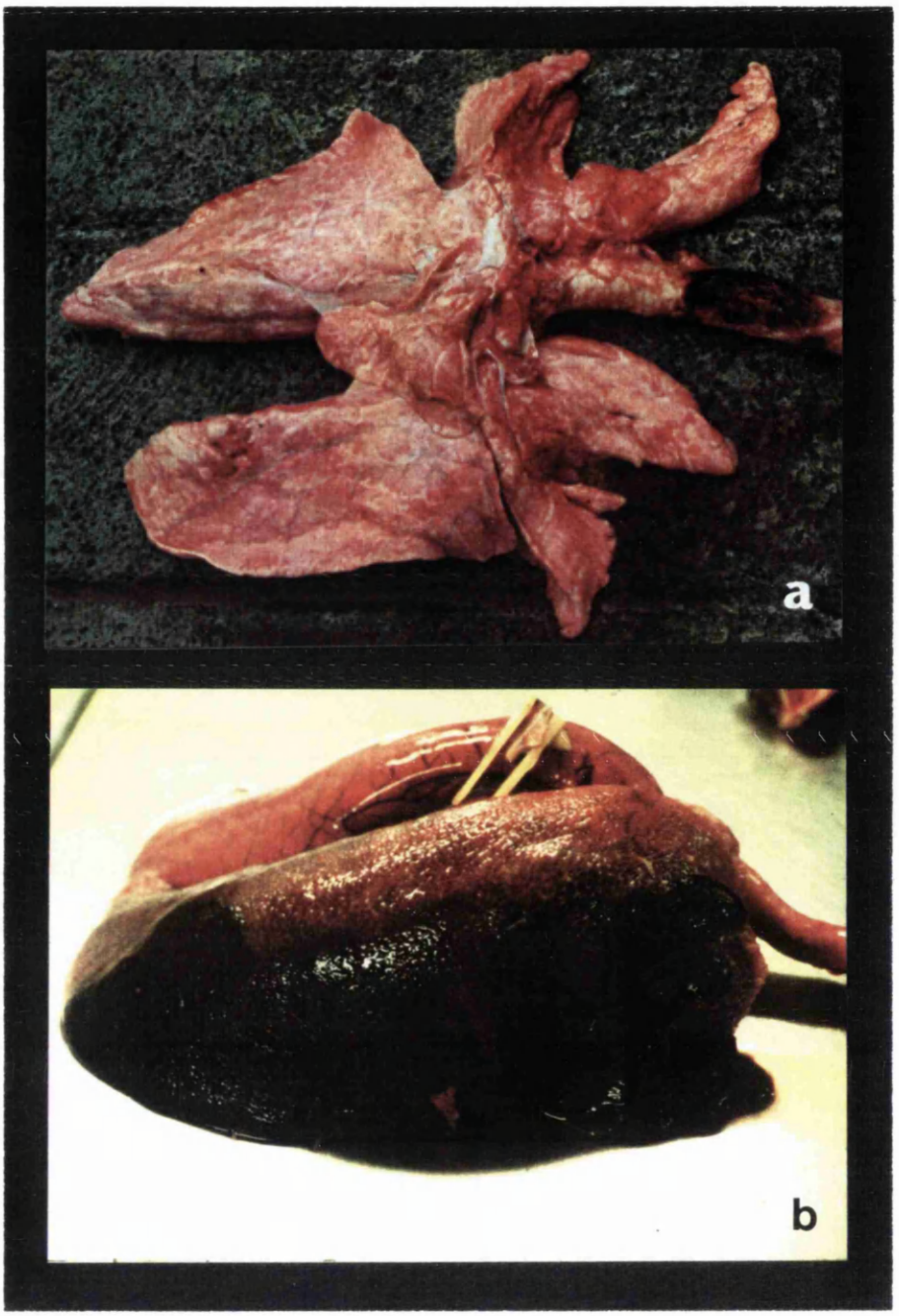


Fig. 1.1

seen as firm greyish nodules (Shope, 1964; Sanford & Josephson, 1981; Bertram, 1988; Blood & Radostits, 1989; Taylor, 1989).

1.8 Lesion development

Liggett and Harrison (1987) followed the development of lung lesions from 3 hours post infection to 7 days. Within 3 hours oedema with multifocal petechial haemorrhages could be seen. Microscopic examination revealed neutrophilic bronchiolitis and alveolitis. There was a predominance of neutrophils in the exudate indicating a major involvement of these cells in early infection. This corresponded with the development of neutropaenia and leucopaenia in the pigs. Lobular haemorrhages and necrotising pneumonia with pleurisy could be seen at 12 to 18 hours. 1 to 2 days post infection there was fibrinous pleurisy and lobar pneumonia together with acute necrotising pneumonia. Lung abscesses and fibrous pleurisy with pleural adhesions were predominant at 4 to 7 days.

In previous studies neutrophils appeared not to be present in the exudate (Shope, 1964; Schiefer *et al.*, 1974; Sanford & Josephson, 1981; Sebunya *et al.*, 1982). Large numbers of degenerate elongated cells in swirling patterns were seen, however their cell type could not be determined. It was suggested that mononuclear cells were primarily involved in clearance of *A. pleuropneumoniae*. However, *A. pleuropneumoniae* produces a potent toxin for alveolar macrophages along with monocytes and neutrophils (Bendixen *et al.*, 1981; Udeze & Kadis, 1988; Rosendal *et al.*, 1988, Rycroft *et al.*, 1991). The toxin-affected macrophages are therefore thought to be incapable of phagocytosing the large influx of neutrophils which are normally responsible for the clearance of these cells and thereby prevent neutrophil-mediated injury. The release of toxic products from both

the degenerate neutrophils and macrophages may also play a role in inflammation and lesion development.

1.9 Clinical signs

The clinical signs of porcine pleuropneumonia can range from chronic to peracute depending on the immune status of the animal (Sebunya & Saunders, 1983; Nicolet, 1986; Blood & Radostits, 1989; Taylor, 1989). Morbidity and mortality can both reach 100%, again depending on immune status of the herd. High mortalities and morbidities are usually seen in herds not previously infected with *A. pleuropneumoniae* (Nicolet, 1971; Nielsen, 1974). The peracute signs include one or more pigs becoming very ill with high fever (41.5^{o}C) being anorexic and apathetic (Fig. 1.2). Some pigs that showed no signs of being ill can be found dead (Shope, 1964). Severe dyspnoea with blood stained froth from the nose and mouth can be seen later. The skin becomes cyanotic initially on the legs, ears and nose and later on the whole body (Nicolet, 1986). Death can occur within 4-36 hours (Shope, 1964; Nicolet, 1968; Nielsen & Mandrup, 1977). Abortions may occur (Sanford & Josephson, 1981; Wilson & Kierstead, 1976) and occasionally septicaemia in young piglets (Thomson & Ruhnke, 1963). The number of pigs noticeably affected with the acute form is greater than the peracute. The raised body temperature, up to 41^{o}C, together with lethargy and inappetance, are characteristic. The animals usually show severe respiratory distress. The acute disease may last for up to five weeks in the herd (Shope, 1964). Following the acute phase animals may die or develop the subacute or chronic forms. Transition from the acute phase to the chronic or subclinical can be both spontaneous (Shope, 1964; Mylrea *et al.*, 1974) and following antibiotic treatment (Schiefer *et al.*, 1974). The course the disease follows will depend on the extent of the lung affected. If sufficient lung tissue is available for

Fig. 1.2 Pig with pleuropneumonia.

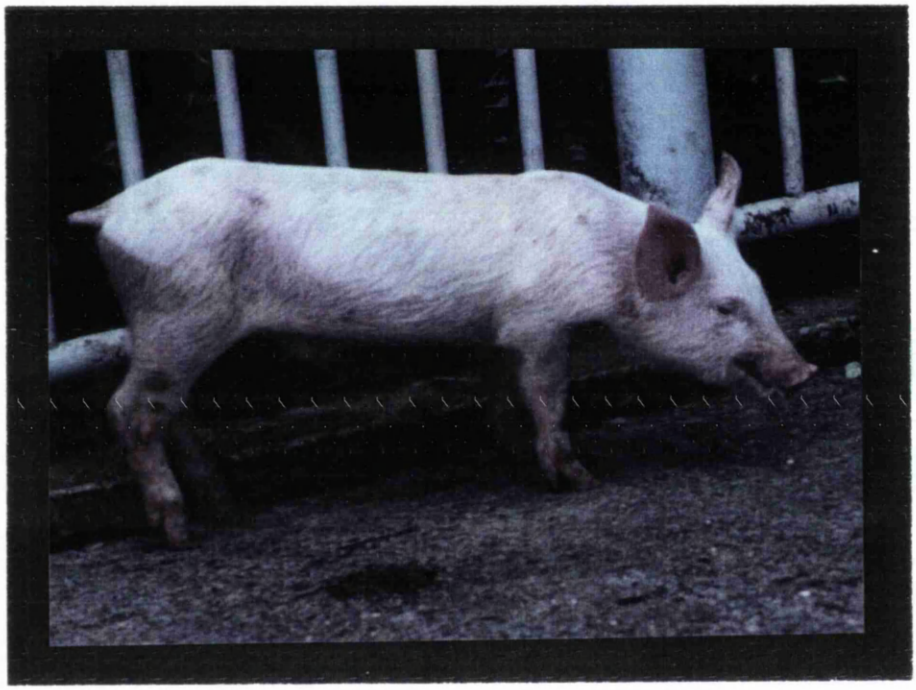


Fig. 1.2

respiration the subacute or chronic forms may follow, if not the animal dies. Chronically infected animals usually show lack of fever, loss of appetite and develop a persistent cough. Some chronically infected animals show very few clinical signs. Subclinically affected animals may show clinical signs following infection with other respiratory pathogens including *Pasteurella multocida* (Nicolet, 1986).

1.10 Diagnosis

1.10.1 Pathological/clinical diagnosis

The clinical signs and pathological findings normally indicate which respiratory disease is present prior to identification of the organism by microbiological culture (Blood & Radostits, 1989; Taylor, 1989). The swiftness of onset together with the clinical symptoms of fever, anorexia, severe dyspnoea and the high mortality differentiates *A. pleuropneumoniae* from most other respiratory diseases of pigs. Swine influenza also has a very rapid onset with 100% of pigs normally being infected, however mortality is much lower, usually less than 1%. This together with lack of response to antimicrobial treatment is indicative of swine influenza. Enzootic pneumonia usually does not produce a fever or respiratory distress. The appearance of the lesions is also distinct. Pasteurellosis is normally associated with a necrotising bronchopneumonia. Mulberry heart disease can display similar clinicial signs however there is no pneumonia. Glasser's disease normally occurs in young pigs and is characterised by serositis, arthritis and meningitis.

1.10.2 Serodiagnostic tests

Serodiagnostic tests are important in establishing the infecting serotype in order that effective prophylaxis

can commence as soon as possible. This is usually in the form of killed bacterial vaccines of the serotypes identified. Although these bacterins do not prevent disease, they do reduce mortality, and because they are serotype-specific, it is important to determine the infecting serotype quickly to prevent further deaths. Serotyping is also important to establish and monitor the epidemiology of the infection.

The complement-fixation test was popular for many years in Denmark, Canada, USA and Switzerland as a means of serotyping *A. pleuropneumoniae* (Nicolet, 1971; Lombin *et al.*, 1982; Gunnarsson, 1979b; Nielsen, 1988). Phenol-water extraction of whole cells gave the most serotype-specific reactions (Gunnarsson, 1979b). Although it was used fairly successfully, cross reactions did occur between some *A. pleuropneumoniae* serotypes and also the related pathogen *Actinobacillus suis* (Rapp *et al.*, 1985b; Rosendal & Mittal, 1985). Cross reactions with *A. suis* were due to shared epitopes on outer membrane proteins (MacInnes & Rosendal, 1987). The 2-mercaptoethanol tube agglutination test was easier to perform and detected active infection in piglets earlier than the complement-fixation test. It also could not detect maternal antibody after 4 weeks and therefore indicated recent infection after this age (Mittal *et al.*, 1984). Enzyme linked immunosorbent assay (ELISA) was found to be as specific and more sensitive than both complement-fixation and 2-mercaptoethanol tube agglutination (Nicolet, 1981; Inzana & Mathison, 1987; Goyette *et al.*, 1986; Willson *et al.*, 1988; Stenbaek, 1990; Nakai *et al.*, 1990). The first ELISA developed employed an EDTA-extracted antigen. Serotypes 1 to 5 were tested and the test was found to be serotype-specific, provided the appropriate antiserum was used (Nicolet, 1981). The later identification of serotypes 6 to 12 have not been tested in this system for cross reactivity. Saline extract at 100^{o}C was found to be as

satisfactory as EDTA-extracted antigen and was more specific due to destruction of heat-labile antigens (Goyette *et al.*, 1986). Cross reactions were still observed between heterogeneous serotypes. This cross reaction was probably due to the fact that the tests employed antibodies to whole cells and crude cellular extracts for detection of antibody. Increased specificity was obtained using purified capsule or monospecific serum to capsule in both ELISA and double-label radio-immunoassay respectively (Inzana & Mathison, 1987; Inzana *et al.*, 1990). Double-label radio-immunoassay could be used to detect both antibody or antigen. This was developed to detect capsular antigens or antibodies to *A. pleuropneumoniae* (Inzana *et al.*, 1990). The test was found to be highly sensitive and serum had to be diluted at least 1:100 to avoid detection of cross reacting antibodies to common antigens. The use of radioisotopes made it unsuitable for field testing and the purification of capsule antigens was not within the capacity of all laboratories.

ELISAs have also been developed using Mabs (Nakai *et al.*, 1990; Stenbaek, 1990). Nakai *et al.* (1990) detected serotype 2 specific antigen using a Mab sandwich ELISA against serotype 2 with no cross reactivity from other serotypes or related gram-negative bacteria. Specific antigen patterns were seen for all 12 serotypes (Stenbaek, 1990). Mab directed against these serotype specific antigens would overcome the cross reactivity seen between the heterogeneous groups of *A. pleuropneumoniae*. Latex agglutination was reported to be as specific and sensitive as the other tests currently used (Mitui *et al.*, 1981; Inzana, 1990), although it was not as sensitive as radioimmunoassay (Inzana *et al.*, 1990). Both cell extract (Mitui *et al.*, 1981) and purified capsule or affinity purified antibody to capsule have been used respectively (Mutui *et al.*, 1981;

Inzana, 1990). The fact that the test can be used for serotyping and identification of infection, together with its rapidity, make it potentially very useful for both field and laboratory testing.

Outer membrane proteins common to all the serotypes of *A. pleuropneumoniae* could also be useful for detection of *A. pleuropneumoniae* infection (Nielsen, 1990). Common proteins in serotypes 1-5 and 7 expressed under iron restricted conditions were demonstrated when probed with convalescent pig serum (Deneer & Potter, 1989a). Ma and Inzana (1990) developed an ELISA system for the detection of the 110 kDa haemolysin protein. This haemolysin protein was common to all 12 serotypes (Frey & Nicolet, 1990; Devenish *et al.*, 1989) and therefore had the potential to screen for *A. pleuropneumoniae* infection. However this haemolysin protein cross reacts with proteins of similar size in other organisms including *A. suis*, *A. pleuropneumoniae* "minor group", the *P. haemolytica* leukotoxin and *E. coli* alpha-haemolysin (Devenish *et al.*, 1989). The use of Mabs in the ELISA system did not increase specificity which is thought to be due to the sequence homology that was demonstrated between these cross reacting organisms (Chang *et al.*, 1989; Gygi *et al.*, 1990).

Both tests incorporating the use of Mabs and latex agglutination have the potential both for increased specificity and speed.

1.10.3 Bacterial isolation

Bacterial culture has also been used to diagnose *A. pleuropneumoniae* infection. This technique is specific but not sensitive. Failure to isolate organisms from infected animals can be due to the presence of small numbers of organisms, for example in recovering animals, or the tissue containing the bacteria is not usually

sampled. Overgrowth by other bacteria and the need sometimes for special culture techniques can result in failure to detect *A. pleuropneumoniae* (Willson *et al.*, 1987). Selective media for isolation of *A. pleuropneumoniae* alone was useful in slaughter pigs where the contamination was higher. There was no advantage when experimentally infected pigs were sampled (Gilbride & Rosendal, 1983).

1.11 Treatment and control

1.11.1 Antibiotic therapy

The value of antibiotic treatment in controlling infection depends largly on administration early on in the clinical disease. Willson and Osborne (1985) studied the effect of different antibiotics in the prevention of *A. pleuropneumoniae* infection and treatment of both acute and chronic disease. Pigs given long-acting oxytetracycline 24 hours prior to challenge showed no mortality or fever. No lung lesions were detected in any of the pigs examined by post-mortem at 2, 6 and 10 weeks post challenge. In contrast treatment with chloramphenicol and/or oxytetracycline only reduced the number of animals that died or developed fever and lung lesions. Antibiotic treatment given following the onset of clinical signs did significantly reduce mortality although lung lesions did occur. Penicillin reduced the number of pigs that became carriers assessed by failure to isolate bacteria as compared to the isolation of bacteria from pigs treated with long-acting oxytetracycline and chloramphenicol. None of the antibiotics tested reduced the numbers of carriers in chronically infected animals or improved the average daily weight gain. Increase in average daily weight gain and reduction of lesions was demonstrated in infected pigs following water mediation using Tiamulin (Schultz *et al.*, 1983). Numerous antibiotics have been used in *A.*

pleuropneumoniae infection. These include ampicillin, amoxycillin, cephalexin, chloramphenicol, enrofloxacin, lincospectin, gentamicin, penicillin, oxytetracycline, trimethoprim sulphonamide, tiamulin and chlortetracycline (Taylor, 1989).

The sensitivities of *A. pleuropneumoniae* to certain antibiotics has been shown to vary based on geographical location (Didier *et al.*, 1984; Gilbride & Rosendal, 1984). Plasmid mediated resistance to antibiotics has been documented (Hirsh *et al.*, 1982; Huether *et al.*, 1987; Willson *et al.*, 1989). Antibiotic sensitivities of the infecting strain of *A. pleuropneumoniae* should be evaluated prior to commencement of treatment. Antibiotic treatment can therefore help reduce mortality and lung lesions in pigs already infected with *A. pleuropneumoniae*. The problem to date has been the inability to eradicate the organism from chronically infected animals which remain a source of continued infection. Recently, antibiotics which appear to eliminate as well as help prevent *A. pleuropneumoniae* infection have been described. Therapeutic effectiveness of cephalosporin (Naxcel^{R}) was shown after three treatments in pigs with pleuropneumonia and it was also very successful in the prevention of infection (Bilic & Sudaric-Grgurec, 1990). Treatment with ceftiofur in experimentally infected animals resulted in failure to isolate the organism (Hus *et al.*, 1990) and it may therefore prove useful in chronically infected animals. Antibiotic treatment alone is not thought to the best long term solution to control pleuropneumonia on the basis of cost, antibiotic resistance and the increased public awareness about the effects of antibiotic residues (MacInnes & Rosendal, 1988).

1.11.2 Vaccines

1.11.2.1 Active immunisation

a) Inactivated whole cell vaccines (bacterins)

Vaccines using killed bacteria as antigen have had various success rates in reducing the mortality and morbidity. Bacterins can prevent mortality although the animals still show severe respiratory disease when challenged with the same serotype (Higgins *et al.*, 1985). Mason *et al.* (1982) showed only a 17% reduction in mortality in piglets vaccinated with a formalin inactivated alum vaccine. Prevention of acute pleuropneumonia was demonstrated in pigs vaccinated with a combination of bacteria and adjuvant (Rosendal *et al.*, 1981). However the adjuvant itself was toxic for some pigs. Mineral oil adjuvants, and to a lesser extent aluminium hydroxide, caused irritation resulting in abscesses and granulomas (Mason *et al.*, 1982; Straw *et al.*, 1985). Bacterins usually do not elicit cross immunity between other infecting serotypes (Nielsen, 1984). However a high degree of cross protection with parenteral killed vaccines was demonstrated between heterologous serotypes 3, 6 and 8 in an experimental study (Nielsen, 1985c). These serotypes are known to possess cross-reacting serotype antigens. This suggests perhaps cross immunity between heterologous serotypes may in fact occur in the field. Their use is limited in the field unless the infecting serotype is known. The rapid onset of clinical symptoms in *A. pleuropneumoniae* infection suggests that efficient preparation and administration is required to have an effect on reducing acute pleuropneumonia.

b) Live vaccines

Pigs intranasally inoculated with live bacteria resisted challenge and showed no clinical signs (Nielsen, 1974). They did however show a range of clinical signs and lung pathology following the initial vaccination. The age of the culture together with the adjuvant used affected the degree of protection obtained. A 6 hour culture in Freunds incomplete adjuvant gave better protection than in an alhydrogel adjuvant or a 18 hour culture in either of the adjuvants (Nielsen, 1976). Intranasal inoculation of live bacteria offered cross protection against clinical symptoms when challenged with other serotypes and reduced the severity of lung lesions (Nielsen, 1979). In contrast, parenteral vaccines with killed bacteria did not elicit cross immunity suggesting a clear difference between immunity to vaccination with killed bacteria and natural infection or experimentally with live organisms (Nielsen, 1984). Vaccination with live *A. pleuropneumoniae* therefore appears to be sufficient to provide protecting against both homologous and heterologous serotypes. The harmful effects of vaccination with live *A. pleuropneumoniae* makes its extensive use in the field unlikely.

1.11.2.2 Passive immunisation

Passive immunisation with hyperimmune rabbit serum to formalised whole cells gave 100% protection in mice against both homologous and heterologous serotypes only when certain serotypes were used (Bhatia *et al.*, 1990). When cross protection studies were carried out with different serotypes, no protection was demonstrated when challenged with heterologous serotypes. Serum raised against formalised cells does not possess neutralising antibody against the secreted toxins of *A. pleuropneumoniae* (Chapter 6) and this may contribute to the lack of protection. The use of the mouse model has

also been shown not to reproduce the disease satisfactorily (Fenwick *et al.*, 1986b). Smith and Lida (1990) found only a reduction in mortality and severity of lesion following challenge in pigs previously having received immunisation with Mab to the challenge strain. No protection was seen following challenge with a heterologous serotype. The amount of antibody given via passive immunisation is perhaps important. Piglets receiving colostrum from chronically infected sows are completely protected against infection by both homologous and heterologous strains until the decline of maternal antibody. Similarly, passive transfer of serum raised against live bacteria results in full protection (Inzana *et al.*, 1988). This suggests that in theory, passive immunisation should be sufficient to provide complete protection against both homologous and heterologous serotypes providing the material possesses all the important immunogens.

1.11.2.3 Active/passive immunisation with cellular components

A cell extract containing protein, carbohydrate and endotoxin that exhibited both haemolytic and cytotoxic activities conferred partial protection against the homologous serotype by decreasing the severity of clinical signs and lung lesions in pigs (Fedorka-Cray *et al.*, 1990). Bhatia *et al.* (1990) also demonstrated partial protection in mice actively immunised with crude capsular material or crude lipopolysaccharide against both homologous and heterologous serotypes 1 and 5. Partial protection was obtained by passive immunisation of hyperimmune serum to capsule, lipopolysaccharide or haemolysin. Heat labile components were also important as demonstrated by the inability of boiled or autoclaved bacteria to protect. Byrd & Kadis (1990) also demonstrated partial protection against mortality and development of lung lesions in pigs immunised with a

combination of LPS, capsular material and haemolysin. Antibodies specific for *A. pleuropneumoniae* LPS were not capable of providing immunity to *A. pleuropneumoniae* infection in mice (Inzana *et al.*, 1988). In contrast partial protection against *A. pleuropneumoniae* infection was demonstrated in pigs immunised with *E. coli* J5 LPS (Fenwick *et al.*, 1986c). These discrepancies were thought to be due to differences in immunising antigens or alternatively that immunisation with *E. coli* J5 may induce a non-specific protective immune factor not induced by *A. pleuropneumoniae* LPS (Warren *et al.*, 1987). Protection against mortality and reduction in lesion size was shown in pigs following 4 parenteral exposures to a liposome-LPS vaccine (Bertram, 1988). This would suggest that LPS does play a part role in *A. pleuropneumoniae* infection in pigs. Again the amount of antibody received by the animal may determine the level of protection obtained. Antibody to capsular polysaccharide is sufficient to protect the host against disease by a variety of bacterial pathogens (Robbins, 1978). Inzana *et al.* (1988) found that mice were not protected against intranasal challenge with homologous or heterologous serotypes after immunisation with a capsular-protein conjugate vaccine although antibody to capsule was detected. Mice were protected from infection with both homologous and heterologous serotypes after immunisation with live encapsulated and nonencapsulated bacteria. This suggests protection of mice against lethal challenges was not dependent on antibody to capsule, it was not serotype-specific and not induced by killed cells. Passive transfer of mouse IgM Mab to capsule protected 4\11 mice against lethal infection and delayed death in the others (Inzana *et al.*, 1988). This partial protection by passive transfer of antibody to capsule has also been documented by Korvuo *et al.* (1988). The explanation given for these differences suggested mice may have received more antibody to capsule by passive transfer than by active immunisation.

Capsular material was found earlier to be a poor immunogen from *A. pleuropneumoniae* (Inzana, 1987). This has also been found to be true for other organisms (Robbins *et al.* 1980) and could also explain the results found in mice. Non-immune pigs, passively immunised with monospecific serum to capsule, were protected from lethal infection but not from development of lung lesions. The pathology was similar to that seen with immunisation with killed bacteria (Nielsen, 1976). In contrast 2/3 pigs that received serum to live *A. pleuropneumoniae* did not develop lesions and bacteria were not recovered. Both Lenser *et al.* (1988) and Rosendal *et al.* (1986) reported protection against homologous strains using a capsular extract vaccine that contained protein together with carbohydrate. Removal of protein from the vaccine rendered it non-protective (Lenser *et al.*, 1988). Protein carriers have been reported to increase immunogenicity (Inzana *et al.*, 1988; Anderson *et al.*, 1985). Therefore it appears that antibody to capsule provides partial protection against mortality but not infection.

Analysis of outer membrane proteins (Rapp *et al.*, 1986; Rycroft & Taylor, 1987; MacInnes & Rosendal, 1987) demonstrated outer membrane proteins common to heterologous serotypes. An antibody response to outer membrane proteins was demonstrated during infection with *A. pleuropneumoniae* (Rapp & Ross, 1986a). Immunisation with an outer membrane enriched vaccine reduced mortality and severity of pneumonia (Rapp & Ross, 1986b). Following the increased interest in the haemolysins of *A. pleuropneumoniae*, their immunogenicity and protective ability was examined. Pigs vaccinated with the 104 kDa haemolysin protein were all protected from death following challenge. 2/5 of the vaccinated pigs that had neutralising antibody titre of over 1:10,000 to haemolysin, also had no lung pathology. The three remaining vaccinated pigs had increasing lung

pathology that corresponded to decreasing neutralising antibody titres to haemolysin (1:4,800, 1:3,900, 1:3,000) (Devenish *et al.*, 1990a). More recently a subunit vaccine containing the haemolysin protein of 105 kDa and a outer membrane protein of 42 kDa, completely protected pigs against both death and lung lesions when challenged with both homologous and heterologous serotype 1, 5a and 5b (van den Bosch *et al.*, 1990). Vaccination with each of the components singly protected against death but not lung pathology.

Subunit vaccines containing important immunogens therefore appear to be the way forward, providing cross immunity is obtained against all the serotypes. A potentially useful vaccine might contain both the haemolytic and cytotoxic activities, an adhesive structure such as pili and the iron repressible proteins. This would in theory protect against firstly, possible adhesion of the organism, secondly, toxic activity by *A. pleuropneumoniae* and thirdly, curtail the growth of the organism by restricting iron uptake.

1.12 Host immune defences

The first major host defence against invading organisms is the skin and exposed epithelial surfaces. Continuous renewal of the outer most layer of skin and mucosal epithelium of the intestinal, respiratory and genital tracts reduces the number of bacteria colonising these surfaces. *A. pleuropneumoniae* is known to be present on the tonsils of healthy carrier pigs (Kume *et al.*, 1986a). This suggests it is able to overcome the continual renewal of epithial surfaces. The possession of pili by *A. pleuropneumoniae* (Utrera & Pijoan, 1991) may aid in the initial colonisation of the upper respiratory mucosal region. The body also has a range of physiological factors that inhibit bacterial growth. Enzymes such as proteases and lysozyme are capable of

lysing bacteria and disrupting the petidoglycan in bacterial cell walls and therefore allowing osmotic lysis. Low pH found on the skin and in certain parts of the body including the stomach prevent multiplication of some organisms. Following the successful bypass of these first line defences, bacteria next face both specific and non-specific defence mechanisms within body tissues. These mechanisms include the action of complement, antibody, phagocytic cells and the B and T lymphocytes.

1.12.1 Complement

The main functions of complement are in mediating inflammatory responses and controlling bacterial infection. The complement system consists of a group of 20 or so proteins found in tissue fluids, plasma and freshly isolated serum which form a triggered enzyme cascade (Fig. 1.3). Once activated, the complement system can result in opsonisation, cellular activation and lysis of bacteria. In general gram-positive bacteria are resistant to the bactericidal effects of complement and gram-negative bacteria are sensitive. However certain pathogenic gram-negative bacteria are in fact resistant to complement-mediated damage (Taylor, 1983).

Gram-negative bacteria can activate both the alternative and classical complement pathways in the absence of specific antibody (Pluschke & Achtman, 1984). However, increased efficiency of classical pathway activation generally occurs in the presence of specific antibody. Certain bacterial cell wall components can non-specifically activate complement (Morrison & Kline, 1977; Vukajlovich *et al.*, 1987). Activation of complement via either pathway results in C3b deposition on the bacterial surface. This acts as an opsonin stimulating bacterial uptake by phagocytes. Release of anaphylatoxins C4a, C3a and C5a following complement activation chemotactically stimulates PMNs, macrophage

Fig. 1.3 Classical, alternative and lytic complement pathways. Adapted from Roitt *et al.* (1987).

COMPLEMENT PATHWAYS

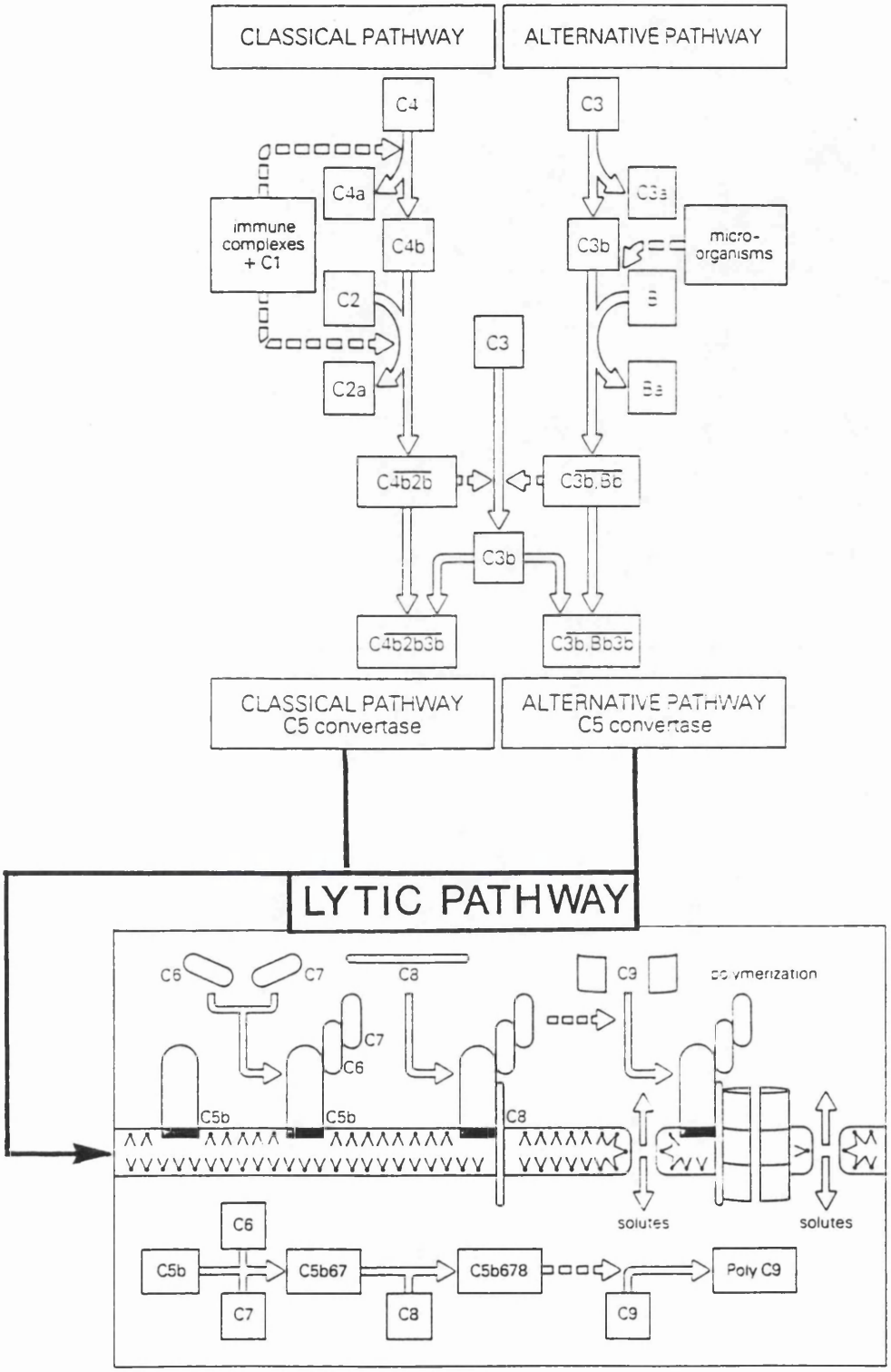


Fig.1.3

and mast cell degranulation together with vasodilation. This results in an influx of these cell types to the site of infection increasing the phagocytic uptake of bacteria by these cells. Bacterial lysis can also occur following complement activation by causing damage to the plasma membrane of the bacteria.

1.12.2 Phagocytes

The second major defence system available in the lung region is the phagocytic system. Following recruitment of phagocytes to the site of inflammation these cells must then be able to recognise the infectious agent in order to eliminate it. These cells have surface receptors capable of recognising various organisms. If these organisms have previously been opsonised their attachment to both PMNs and macrophages which both have receptors for C3b is greatly increased. Activation of the phagocyte membrane following attachment of the organism leads to it being internalised within a phagosome. Fusion with lysosomes to form a phagolysosome follows. The organisms can then be killed by a variety of bactericidal mechanisms.

1.12.3 Antibody

Antibody is involved in numerous mechanisms to neutralise and eliminate both the organism and its products. Antibody binding to the bacteria can disrupt attachment to epithelial surfaces hence preventing colonisation. Antibody efficiently activates the classical complement pathway. Binding of antibody to the bacteria in conjunction with C3b deposition increases the efficiency of bacterial uptake by phagocytes. Antibody can also be involved in neutralisation of toxins or components which interfere with immune function. It can also block the transport of essential

nutrients into the bacterial cell by binding to their receptors.

1.12.4 Cell-mediated immunity

Cell-mediated immunity is usually important in organisms that survive intracellularly where antibody has no effect. Following re-exposure of an antigen, previously sensitised B and T lymphocytes undergo clonal expansion. T lypmhocytes also release lymphokines including macrophage activating factor (MAF) which increases the capacity of macrophages to kill ingested bacteria. Interferons can also be released from the stimulated T cells. This non-specifically enhances killing of infectious agents. B lymphocytes release antibody specific for the stimulating antigen. This increased antibody response enhances opsonisation and neutralisation of the invading bacteria.

Other host defences include the action of fibrin formation and activation of the clotting system which is stimulated following tissue injury by the bacteria. This limits the spread of bacteria to other locations in the body. Stimulated PMNs release lactoferrin which chelates free iron. Iron is essential for microbial growth and hence its proliferation is limited by the action of lactoferrin.

1.13 Bacterial mechanisms of evading the host immune system

1.13.1 Complement

Bacteria employ several different mechanisms to avoid destruction by the complement system and these may be mediated at various stages of the cascade. These mechanisms include failure to activate complement, blockage of activation prior to C5b-9 formation,

formation of a non lytic C5b-9 complex, microbial shedding of molecules that activate or destroy complement, synthesis or acquistion of regulatory molecules, and the microbial use of complement and complement receptors to gain access to obligatory intracellular locations (Joiner, 1988).

1.13.2 Phagocytes

There are several ways that bacteria can avoid being killed by phagocytes (Roitt *et al.*, 1991). Firstly bacteria can prevent phagocytes from arriving on the scene. This can be achieved by releasing molecules which are toxic or can block chemotaxis and inflammatory responses. Other bacteria possess surface coats which can resist phagocyte attachment. If bacteria are phagocytosed, other methods within the phagocyte include prevention of lysosome fusion and phagolysosome formation, and resistance of the cell wall to anti-bacterial components of the phagocytes. Certain bacteria can also escape from the phagolysosome into the cytoplasm where they can no longer be attacked by lysosomes.

1.14 Aims

The overall aim of the work described in this thesis was to study the role of humoral and cellular defences of the pig against *A. pleuropneumoniae*.

The disease caused by *A. pleuropneumoniae* is generally confined to the respiratory tract. Two of the major non-specific host defences available in the lung are the complement system and the phagocytic system. At the onset of the work described here, there had been no information published on the complement system and *A. pleuropneumoniae*. The first aim was therefore to examine the interaction of *A. pleuropneumoniae* and pig

complement. This involved the determination of the resistance or sensitivity of *A. pleuropneumoniae* to complement bactericidal activity. The ability of viable or killed bacteria and secreted bacterial products, to consume or activate complement activity via the classical and alternative pathways was then examined.

The second major host defence system available in the pig lung is the phagocytic system. The role of removing invading organisms from the lung constitutes a major immune defence mechanism of the host. The initial aim was to examine whether *A. pleuropneumoniae* was phagocytosed by porcine alveolar macrophages. There was information available describing the toxicity of *A. pleuropneumoniae* for phagocytic cells (Bendixen *et al.*, 1981; Udeze & Kadis, 1988; Rosendal *et al.*, 1988; van Leengoed *et al.*, 1989). However, there was other information stating that *A. pleuropneumoniae* could be phagocytosed (Inzana *et al.*, 1988; Udeze & Kadis, 1988). It was therefore decided to examine phagocytosis of *A. pleuropneumoniae* both in the presence and absence of these toxic activities using mutants deficient in one or both of the toxic activities.

The final aim was to look at the relationship of the toxic activities associated with *A. pleuropneumoniae* by producing Mabs against them. The relationship between the toxins of *A. pleuropneumoniae* and related pathogens was also examined.

CHAPTER 2
GENERAL MATERIALS AND METHODS

his chapter describes the materials used throughout the tudy. The majority of techniques and materials used ere related to individual studies and are described in he appropriate chapters or appendices.

.1 Bacterial strains

ctinobacillus pleuropneumoniae strain 6664 (serotype 3) as isolated from a typical lesion in a pig belonging to herd chronically infected with pleuropneumonia. *A. leuropneumoniae* strains HK 353, HK 358 (serotype 2) and 66-1920-HAE (serotype 5) were obtained from Professor . Kilian, The Royal Dental Hospital, Denmark (Kilian *et l.*, 1978). *A. pleuropneumoniae* strain HK 361 was btained from the National Collection of Type Cultures Colindale, U.K.). HK 361 mutants, e and h, were roduced within this laboratory (Rycroft *et al.*, 1991). utant e possesses cytotoxin activity only, associated ith a 120 kDa protein (pleurotoxin), and mutant h is eficient in both Hly II (haemolysin type II, 109 kDa rotein) and pleurotoxin. No other phenotypic ifferences were found. Growth rates, colony morphology, uter membrane protein profiles and lipopolysaccharide f both mutants were found to be identical to the parent train. *Escherichia coli* C10 was obtained from the erebrospinal fluid of an infant with meningitis and was vailable within the laboratory (Bjorksten *et al.*, 976). *E. coli* strains RY21, a K1-negative mutant of 10, and RY22, a O7-negative mutant of RY21 were also vailable within the laboratory (Rycroft *et al.*, 1983).

.2 Storage of bacterial strains

. *pleuropneumoniae* strains were stored both lyophilized nd at -70°C in 10% v/v sterile skimmed milk solution. . *coli* strains were maintained on Dorset Egg slopes at oom temperature.

.3 Bacterial culture conditions

outine culture was made on heated blood agar (prepared ith 7%, (w/v), horse blood (Oxoid) and liquid cultures ere grown in Tryptone Soya Broth (TSB; Oxoid) upplemented with 2μg/ml NAD (Sigma Chemical Co Ltd). . *coli* strain RY22 was stored on a Dorset Egg slope. outine culture was made on nutrient agar and liquid ultures were also grown in TSB. *A. pleuropneumoniae* was rown overnight on solid medium followed by overnight ulture with aeration (100 rpm) in TSB containing NAD. ubcultures were then made (1 in 25) into fresh TSB/NAD nd grown for a further 2 hours until logarithmic phase f the growth was reached. Optical densities of acterial cultures were determined by measuring the OD t 650nm using a spectrophotometer (Beckman, DU 64). By revious assessment, an OD_{650} of 0.1 corresponded to 0^8/ml *A. pleuropneumoniae* organisms.

.4 Sera

.4.1 Pig

lood samples were collected from pigs by the aseptic enipuncture of the cranial vena cava. The blood was llowed to clot at 37°C for 30 minutes and then held for 0 minutes on ice. Serum was separated following entrifugation at 2000 x g at 4°C, stored at -70°C and orking volumes were held at 4°C. Pig sera were obtained rom specific-pathogen-free (SPF) (normal pig serum). oth of these sera contained no detectable agglutinating r precipitating antibody against *A. pleuropneumoniae* as etermined by standard tube agglutination of whole acteria (Cruikshank, 1962), and by mmunoelectrophoresis against soluble surface antigens Rycroft *et al.*, 1983). Convalescent pig serum was btained from a pig with a natural chronic infection

aused by serotype 3 strain, 6664 and had an gglutination titre of 1:16 against this strain.

.4.2 Rabbit

yperimmune rabbit sera were raised in New Zealand White abbits by repeated intravenous inoculation using tandard methods (Harlow & Lane, 1988) with formalin-illed cells of either 6664 or HK 361 which had been reated with 0.5% (v/v) formalin overnight, and washed hree times in phosphate-buffered saline. The yperimmune rabbit serum raised against 6664, had a trong precipitation reaction and an agglutination titre f 1:256.

.4.3 Human

ormal human serum was obtained from a pool of healthy olunteers. The sera contained no antibody against *A. leuropneumoniae* as detected by the methods previously sed.

he rabbit and human sera were processed and stored as reviously described for pig serum. Heat-inactivated erum was treated at 56^{o}C for 30 minutes.

CHAPTER 3

IN VITRO SERUM RESISTANCE OF *A. PLEUROPNEUMONIAE*

3.1 INTRODUCTION

Gram-negative bacteria have been reported to be both sensitive and resistant to the bactericidal and bacteriolytic properties of serum. The factor in serum responsible for this is the complement system as discussed in Chapter 1. Serum resistant strains are often associated with their ability to cause infection, and insensitivity to serum has been suggested to be an important virulence mechanism of resistant bacteria (Taylor, 1983). *A. pleuropneumoniae* has the ability to cause disease rapidly (Nicolet, 1986). At the beginning of these experiments there was no published information available regarding the status of *A. pleuropneumoniae* in the complement-mediated bactericidal system. The aim of the work described in this chapter was to assess the sensitivity or resistance of 4 *A. pleuropneumoniae* strains to a variety of sera. Bactericidal activity can occur with and without the presence of antibody. The sera used ranged from that of the host animal, the pig, to those from other mammals including man. The effect of specific antibody against *A. pleuropneumoniae* was also investigated for its ability to induce or enhance bactericidal activity.

The common method used to determine serum bactericidal activity is to expose the organism to serum that contains a suitable concentration of complement with or without antibody. The theoretical basis of this type of assay is straightforward, however there are a number of important parameters that must be maintained to ensure the correct evaluation of true serum sensitivity or resistance. The bacterial inoculum should be in early exponential growth phase. There have been reports of serum susceptible organisms being resistant or having reduced susceptibility to the bactericidal activity of complement if harvested before logarithmic growth phase (Davis & Wedgwood, 1965; DeMatteo *et al.*, 1981). The

method used to wash the bacterial cells may have the opposite effect of making the cells more sensitive to the bactericidal effects of serum. Cells should not be cooled to 0°C to avoid possible temperature shock, and should not be washed excessively (Wright & Levine, 1981; Fierer *et al.*, 1974). A high percentage of serum is advisable to ensure availability of complement components to kill the bacteria. High serum concentrations are also helpful to limit the high pH value of certain serum-buffer mixtures which may be disadvantageous to complement killing (Taylor *et al.*, 1972). The buffer used has also been found to be important and should contain calcium and magnesium ions at concentrations optimum for complement activity. Another important point is the sampling time at which serum sensitivity or resistance is assessed. Certain *E. coli* strains show little difference after 1 hours' incubation, however after 3 hours, less than 1% of the inoculum remains (Taylor, 1974). It is therefore important to study serum resistance over a 3 hour period.

With these points to consider, initial serum resistance studies were carried out using 3 well-defined *E. coli* strains, which represented serum resistance, intermediate sensitivity to serum and full sensitivity to serum in order to optimise the assay.

The second section of the work described in this chapter involves attempts to sensitise those strains of *A. pleuropneumoniae*, found to be serum resistant, to the bactericidal effects of serum. Certain Gram-negative bacteria have been sensitised to the bactericidal effects of serum under environmental conditions which have a disruptive effect on their lipopolysaccharide layer (Reynolds & Pruul, 1971a; Reynolds & Pruul, 1971b; Fierer & Finley, 1979; Pruul & Reynolds, 1972). Polymyxin B, an antibiotic principally active against

Gram-negative bacteria, potentiates sensitivity to serum by disrupting both the outer (Warren *et al.*, 1957) and inner (Teuber, 1969) membranes of Gram-negative bacterial cell envelopes.

Synergy between complement and polymyxin B has been documented in killing bacteria (Davis *et al.*, 1971; Traub & Sherris, 1970; Pruul & Reynolds, 1972; Fierer & Finley, 1979). The conformational changes induced by polymyxin B are thought to make the LPS more susceptible to attack perhaps by exposing the core phospholipid material which may be a target for complement action (Wilson & Spitznagel, 1971).

The mode of serum resistance of *A. pleuropneumoniae* is unknown. The serum resistance of *A. pleuropneumoniae* was compared therefore, with that of other gram-negative bacteria. Various serum resistant strains of *E. coli* have been sensitised to complement following treatment with polymyxin B (Dixon & Chopra, 1986; Fierer & Finley, 1979). This chapter describes the attempts to sensitise *A. pleuropneumoniae* to pig complement via polymyxin B treatment, and to compare it to the sensitisation of a serum resistant *E. coli* strain by the same methods.

3.2 MATERIALS AND METHODS

Details of buffers and solutions can be found in Appendix 1.

3.2.1 Serum bactericidal assay

The ability of bacteria to survive the bactericidal effects of serum was determined using a modification of the method of Taylor *et al.* (1972). Bacterial strains were grown overnight in TSB/NAD at 37^{o}C with aeration (100 rpm) in an orbital incubator. Samples of overnight bacterial cultures were diluted 1:25 in fresh prewarmed

TSB/NAD. The cultures were incubated in static culture at 37°C for 15 minutes, followed by a further 90 minutes with aeration (100 rpm), or until logarithmic growth phase had been reached. 1ml of bacterial culture was sedimented in a sterile Eppendorf tube at 11,600 x g for 1 minute. Following centrifugation the supernatant was discarded and the bacterial pellet resuspended to 10ml in a sterile phosphate, gelatin buffer (buffer M, appendix 1). 0.5ml of this suspension was mixed with 1.5ml of freshly thawed serum which had been warmed to 37°C, and contained 2μg NAD/ml. The suspension was mixed by vortexing and a 50μl sample immediately taken. The sample was diluted 10^{-2} and 10^{-4} in buffer M. 100μl aliquots of these dilutions were mixed with Isosensitest agar (Oxoid), which had been held at 46°C and supplemented with 1μg of NAD/ml, using the pour-plate method. The agar was allowed to set before incubation at 37°C for 24-48 hours. Further samples were taken after 1, 2, 3 and sometimes 4 hours' incubation. Bacterial viability was determined by counting the number of colony forming units following incubation. The log percent viability was determined for each strain over the entire incubation period and compared to any controls that had been included in the experiment.

Samples were taken in duplicate and all *A. pleuropneumoniae* and *E. coli* strains were tested a minimum of 3 times in each of the experiments 1-6 (sections 3.2.4-3.2.9)

3.2.2 Complement inactivation

Serum was held at 56°C for 30 minutes in order to inactivate complement activity.

3.2.3 Serum absorption

Human serum was absorbed by mixing with approximately 2 x 10^{10} fresh, live bacteria/ml (grown in TSB/NAD as previously described for serum bactericidal assays) for 60 minutes at 0°C. Sera were centrifuged at 2,000 x g at 4°C to pellet the bacteria and the supernatant filtered through a filter of 0.22μm pore size (Millipore). Sera were used immediately or stored frozen at -20°C. Control sera were similarly treated but not mixed with bacteria.

3.2.4 Experiment 1 - bacterial viability in normal pig serum

A. pleuropneumoniae strains HK 353, HK 358, 6664 and 266-1920-HAE were assessed for resistance or sensitivity to the bactericidal activity of normal pig serum by the serum bactericidal method described in section 3.2.1. Strains were simultaneously incubated in heat-inactivated serum possessing no complement activity.

In order to confirm the presence of normal bactericidal activity in the pig serum, the serum was tested for bactericidal activity using a known serum sensitive *E. coli* strain, RY22 (Rycroft *et al.*, 1983). This strain has been characterised as being sensitive to human serum. To ensure that the strain would behave similarly in pig serum, it was assessed along with parent strains, *E. coli* RY21 and C10, which are partly serum sensitive and serum resistant respectively.

3.2.5 Experiment 2 - bacterial viability in immune pig serum

The 4 *A. pleuropneumoniae* strains HK 353, HK 358, 6664 and 266-1920-HAE were assessed for viability in serum which was obtained from a pig with natural chronic pleuropneumonia attributable to serotype 3 strain, 6664,

using serum bactericidal assays. This serum contained specific antibodies to that strain and the methods used to determine this are discussed in the general materials and methods in Chapter 2. *E. coli* strain RY22 was also assessed for viability in this serum.

3.2.6 Experiment 3 - bacterial viability in rabbit and human serum

All *A. pleuropneumoniae* strains were tested for survival in normal rabbit serum, hyperimmune rabbit serum raised against serotype 3 strain, 6664, and normal human serum using the serum bactericidal assay. Both normal rabbit serum and normal human serum contained no detectable antibodies against *A. pleuropneumoniae* as described in Chapter 2. Hyperimmune rabbit serum contained antibodies against *A. pleuropneumoniae*.

3.2.7 Experiment 4 - bacterial viability in normal human serum absorbed with *A. pleuropneumoniae*

The 3 strains of *A. pleuropneumoniae* HK 353, HK 358 and 6664 were incubated in 75% of human serum that had previously been absorbed with the corresponding strain using the method described in section 3.2.3, and assessed by the serum bactericidal assay as described previously in 3.2.1. Controls included incubation of *A. pleuropneumoniae* strains with filtered human serum to ensure that any loss of bactericidal activity seen in the absorbed serum was not due to the process of filtration.

Serum sensitive *E. coli* RY22 was also incubated in absorbed human serum to assess for loss of bactericidal activity by the absorption method.

3.2.8 Experiment 5 - bacterial viability in normal pig serum containing additional heated human serum as a source of antibody

To assess whether the absorbable component in human serum was antibody, it was proposed to add a volume of heated human serum, which would contain any antibody present, to normal pig serum. This was done in an attempt to both identify the component in human serum responsible for *A. pleuropneumoniae* sensitivity to this serum, and to sensitise *A. pleuropneumoniae* to pig serum. 10% of heated human serum was added to 65% normal pig serum and the serum bactericidal assay carried out as described in 3.2.1.

3.2.9 Experiment 6 - bacterial viability in absorbed human serum following preincubation with heat-inactivated human serum

The absorbable component in human serum may not be compatible with the pig complement system. To assess whether the lack of sensitisation of *A. pleuropneumoniae* to pig serum containing heated human serum, as a possible source of antibody, was due to incompatibility, it was tested using human serum. Heated human serum was added to absorbed human serum in an attempt to reintroduce the factor responsible for bactericidal activity back into the absorbed human serum.

A modification of the serum bactericidal method previously described in 3.2.1 was used. Bacteria were grown and treated as described for the serum bactericidal assay (3.2.1). 0.5ml of bacteria in buffer M was incubated with 1.5ml of heat-inactivated human serum and incubated at 37^{o}C for 30 minutes. The bacteria were pelleted by centrifugation, washed once with buffer M, and resuspended in 1.5ml of the absorbed human serum and tested in the serum bactericidal assay (3.2.1).

Controls included strains being incubated with heated human serum prior to incubation with normal pig serum. Any difference between the 2 different conditions above would suggest that human antibody is not compatible with the pig complement system. Other controls included a. treatment with absorbed human serum followed by incubation again with absorbed human serum, and b. incubation with normal pig serum prior to assessment of growth in normal pig serum. These were used to determine any differences which the prior incubation with absorbed human serum might impose on the subsequent incubation in the known systems of absorbed human serum and normal pig serum. A final control of incubation with human serum followed by growth assessment in human serum was used to portray the expected sensitivity of *A. pleuropneumoniae* strains incubated in this serum.

3.2.10 Polymyxin B treatment of bacterial strains

In an attempt to localise the area or components of *A. pleuropneumoniae* that are responsible for the observed resistance to pig serum, *A. pleuropneumoniae* was treated with sub-lethal concentrations of a membrane disorganising component, polymyxin B. Providing this is the site responsible for the observed serum resistance, the membrane will become permeable to complement components allowing the complement cascade to be completed and result in the death of the organism. Bacteria were incubated in both normal and heat-inactivated pig serum following treatment with polymyxin B. A reduction of growth rate in normal pig serum could then be said to be due to the action of polymyxin B on the membrane allowing entry of the complement components and thus identifying the site responsible for serum resistance in *A. pleuropneumoniae*.

Initial experiments were aimed at sensitising the serum resistant organism, *E. coli* C10, to pig complement

following exposure to polymyxin B. Although certain *E. coli* strains have successfully been sensitised to serum by polymyxin B treatment, other strains have not (Vaara *et al.*, 1984). The *E. coli* strain being tested here had not been tested for polymyxin B sensitisation in pig serum and the first aim was therefore to establish a suitable control.

Bacteria were grown as described for serum bactericidal assays (3.2.1). 1.0ml of bacterial samples were centrifuged for 1 minute at 11,600 x g (3 spins of 15 seconds each with rotation through 180^{o} for each spin) and the resultant pellet resuspended in 1ml of buffer M. 0.1ml aliquots were mixed with 0.9ml of polymyxin B (Sigma Ltd.) solution at 0^{o}C to give final concentrations of 1.25, 2.5, 5, 10, 20, 40, 80, 160 and 320μg of polymyxin B/ml. Bacteria were immediately pelleted by centrifugation, the supernatant discarded and the pellets resuspended in 1.0ml of buffer M. 50μl of the suspensions were mixed with 200μl of prewarmed normal or heated pig serum. Bottles containing the serum and bacteria were incubated for 30 minutes at 37^{o}C in a water bath. Samples were taken at time 0 and 30 minutes and viable counts determined as previously described for the bactericidal assays (3.2.1). Bacterial counts obtained from incubation with normal pig serum were compared to those obtained during incubation with heated pig serum and expressed as a percentage of the latter.

Duplicate samples were taken at each concentration and experiments were repeated a minimum of 3 times for each strain.

3.3 RESULTS

3.3.1 Experiment 1 - viability of *A. pleuropneumoniae* isolates in normal pig serum

All 4 *A. pleuropneumoniae* strains appeared to be resistant to the bactericidal effects of normal pig serum. Viable bacterial numbers were shown to increase over time during incubation with normal pig serum (Figs. 3.1-3.4). Consistently higher viability was observed for *A. pleuropneumoniae* strains HK 353, HK 358 and 6664 in normal pig serum compared to the control of heat-inactivated pig serum (Figs. 3.1-3.3). Strain 266-1920-HAE demonstrated greater growth in heat-inactivated pig serum than in the normal pig serum after 2 hours incubation (Fig. 3.4).

The 3 *E. coli* strains incubated in pig serum were found to behave in an identical fashion to their described behaviour when incubated with human serum. Serum sensitive *E. coli* strains, RY21 and RY22, were also found to be sensitive to pig serum. The viable bacterial numbers of RY22 were reduced to less than 0.1% of the original inoculum after the first hours' incubation (Fig. 3.5). Serum resistant strain C10, was found to be fully resistant to normal pig serum. The death of the serum sensitive RY22 strain also confirmed that the normal pig serum possessed adequate bactericidal activity.

3.3.2 Experiment 2 - viability of *A. pleuropneumoniae* isolates in immune specific pig serum

All 4 *A. pleuropneumoniae* strains were found to be resistant to immune pig serum that contained specific antibodies against *A. pleuropneumoniae* strain 6664. The 4 strains all increased in bacterial numbers over the 3 hours incubation to a similar extent, and the results

Fig. 3.1 Viability of *A. pleuropneumoniae* HK 353 in normal pig serum

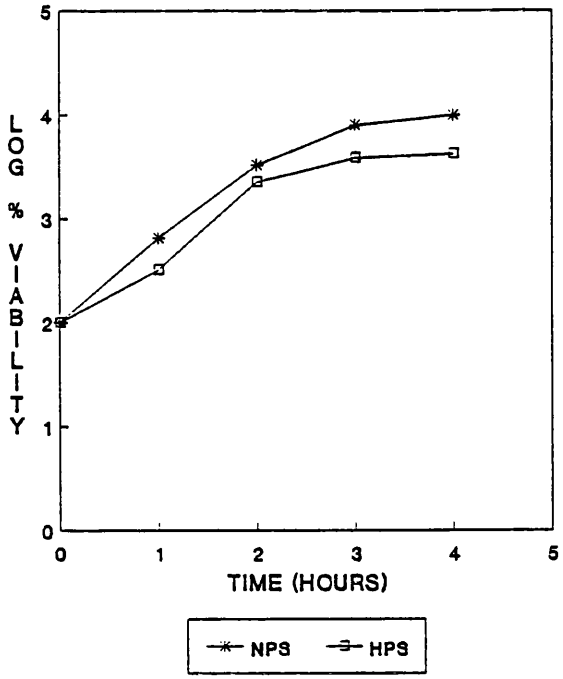
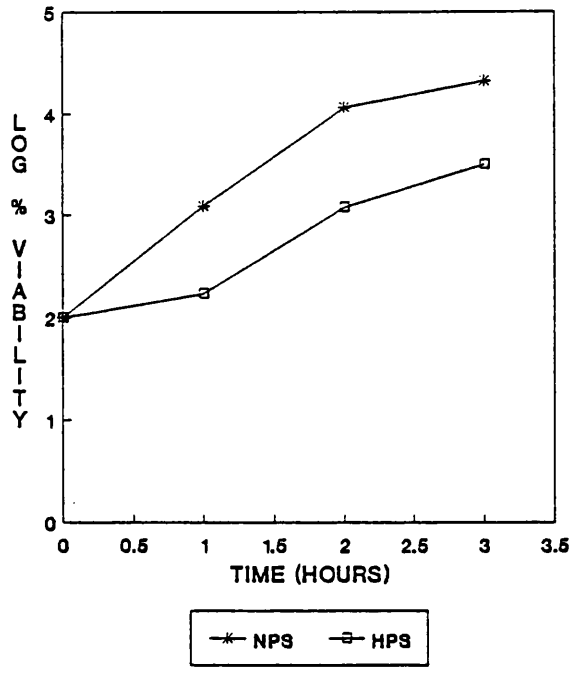


Fig. 3.2 Viability of *A. pleuropneumoniae* HK 358 in normal pig serum



LEGEND

NPS - normal pig serum
HPS - heated pig serum

Fig. 3.3 Viability of *A. pleuropneumoniae* 6664 in normal pig serum

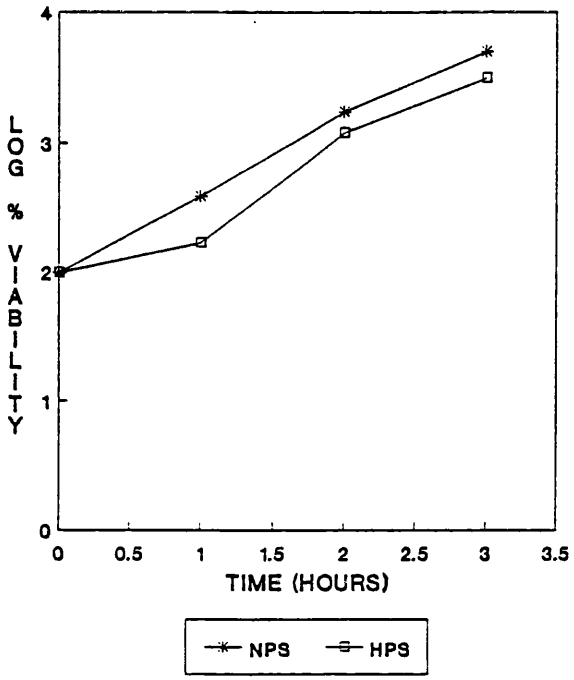
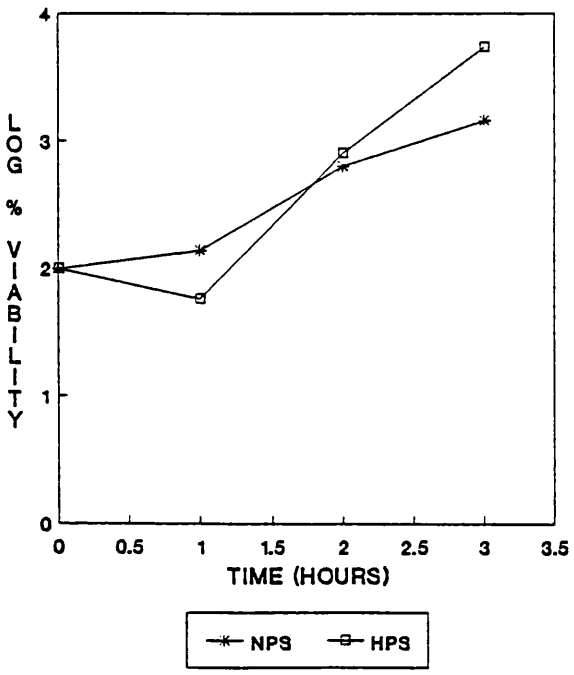


Fig. 3.4 Viability of *A. pleuropneumoniae* 266-HAE-1920 in normal pig serum



LEGEND

NPS - normal pig serum
HPS - heated pig serum

Fig. 3.5 Viability of *E. coli* strains in normal pig serum

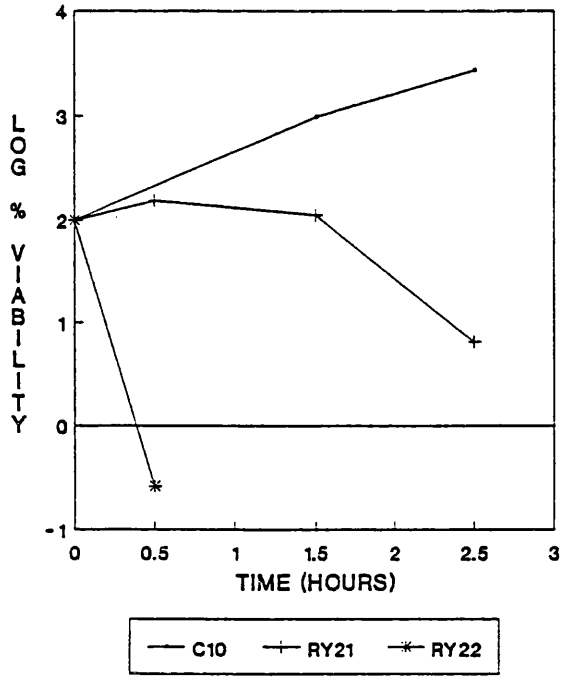
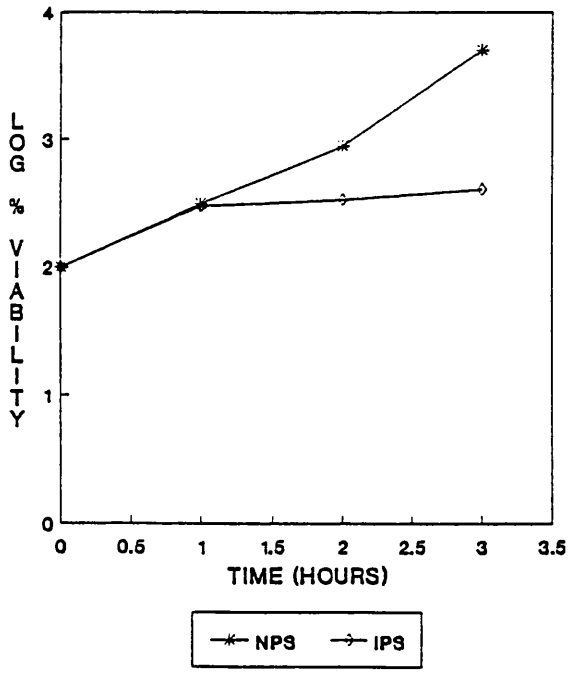


Fig. 3.6 Viability of *A. pleuropneumoniae* strains in immune pig serum



LEGEND

NPS - normal pig serum
IPS - immune pig serum

Fig. 3.7 Viability of *A. pleuropneumoniae* strains in rabbit and human serum

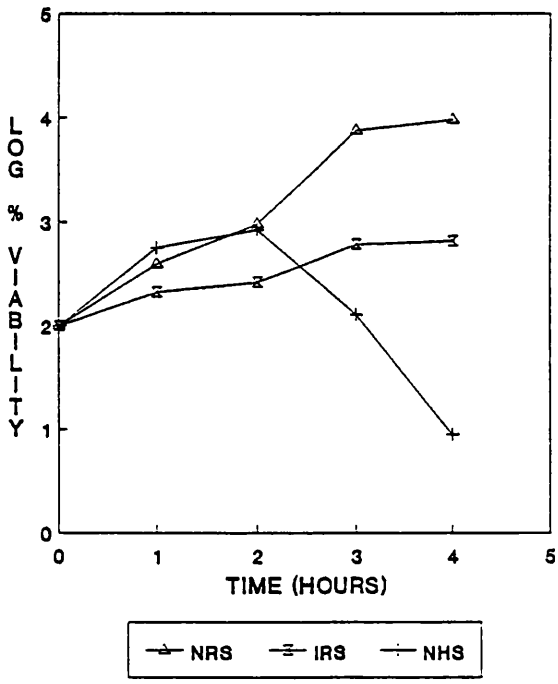
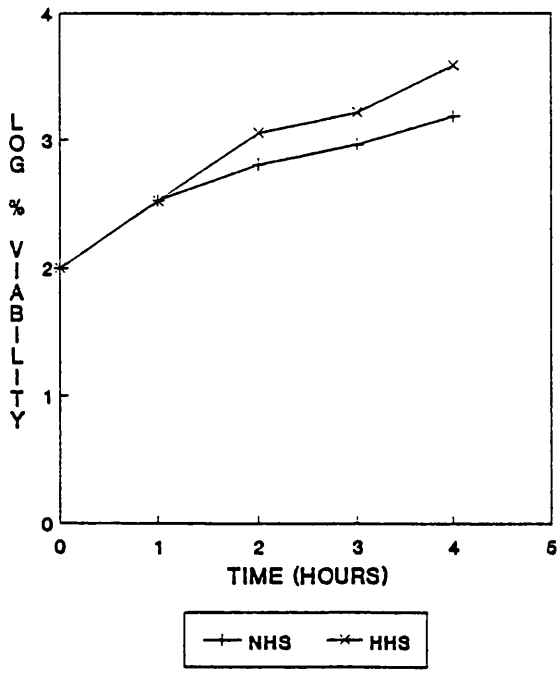


Fig. 3.8 Viability of *A. pleuropneumoniae* 266-HAE-1920 in normal human serum



LEGEND

NRS - normal rabbit serum
IRS - immune rabbit serum
NHS - normal human serum
HHS - heated human serum

fourth strain, 266-1920-HAE, survived in normal human serum and increased in bacterial numbers throughout the 4 hour incubation. This strain also showed greater growth rates in heat-inactivated human serum than in normal human serum (Fig. 3.8). As previously discussed, all sera were tested for bactericidal activity using the serum sensitive *E. coli* strain RY22, and all demonstrated sufficient bactericidal activity against this strain.

3.3.4 Experiment 4 - the role of an absorbable component(s) in the sensitivity of *A. pleuropneumoniae* to human serum

Since 3 of the 4 *A. pleuropneumoniae* strains were found to be sensitive to the bactericidal effects of normal human serum, it was decided to absorb the human serum with each *A. pleuropneumoniae* strain in an attempt to remove any non-specific antibody that may be responsible for this sensitivity seen in human serum.

All 3 strains tested were found to be resistant to the absorbed human serum (Fig. 3.9) compared to normal human serum. This suggested that the absorbed human serum had lost its bactericidal activity. Serum sensitive *E. coli* strain RY22 displayed the same sensitivity to the absorbed human serum as to the normal human serum, indicating complement activity had not been lost during absorption and filtration (Fig. 3.9). To assess whether this loss of bactericidal activity against *A. pleuropneumoniae* was due to absorption alone, or as a result of the filtration process, control serum was similarly held at $0^{o}C$, filtered and tested using the *A. pleuropneumoniae* strains. The strains showed the same sensitivity in filtered human serum as in normal human serum (Fig. 3.9). Fig. 3.9 is representative of strains HK 353, HK 361 and 6664.

Fig. 3.9 Viability of *A. pleuropneumoniae* *E. coli* strains in absorbed human serum

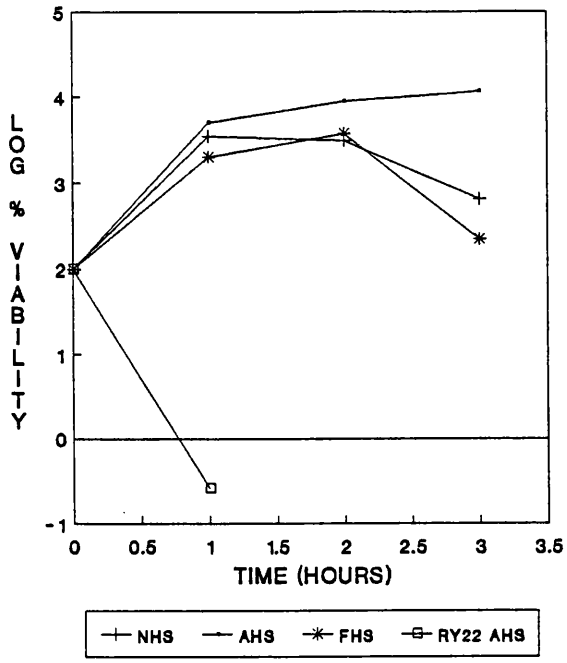
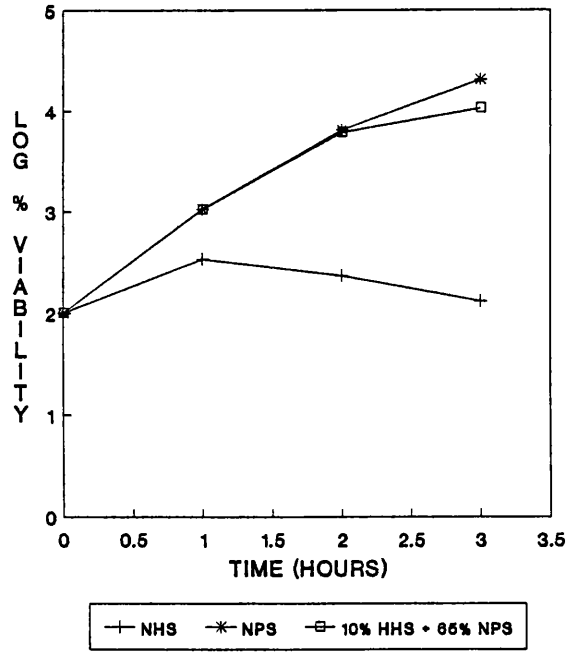


Fig. 3.10 Viability of *A. pleuropneumoniae* strains in pig serum supplemented with absorbed human serum



LEGEND

NHS - normal human serum
AHS - absorbed human serum
FHS - filtered human serum
NPS - normal pig serum
HHS - heated human serum
RY22 AHS - *E. coli* RY22 incubated in absorbed human serum

3.3.5 Experiment 5 - the role of heated human serum added as a source of non-specific antibody to normal pig serum

To assess whether the absorbable component in human serum was antibody, 10% heated human serum was added to 65% normal pig serum prior to the addition of the *A. pleuropneumoniae* strains being tested for viability by the previously described serum bactericidal assays. If the absorbable component in the human serum was antibody, then it should be able to be reintroduced into the serum in the form of heated human serum. The heated serum would still contain any antibody that may be present, however no complement activity would remain. 10% of heated human serum was considered to be an adequate amount, as antibody is only required in extremely small amount to induce antibody-mediated killing by the complement pathways.

All 3 *A. pleuropneumoniae* strains were found to be resistant to pig serum containing 10% heated human serum. There was no difference in the bacterial growth rates seen in pig serum containing heated human serum and normal pig serum alone (Fig. 3.10). The presence of the heat-inactivated human serum also did not retard the growth rates of the *A. pleuropneumoniae* strains, which were equivocal to the pig serum alone (Fig. 3.10). Fig. 3.10 is representative of *A. pleuropneumoniae* strains HK 353, HK 361 and 6664.

3.3.6 Experiment 6 - bacterial viability in absorbed human serum following pretreatment with heat-inactivated human serum

The aim of this experiment was to reintroduce the component depleted in absorbed human serum by preincubation of bacteria with heated human serum prior to incubation in absorbed human serum.

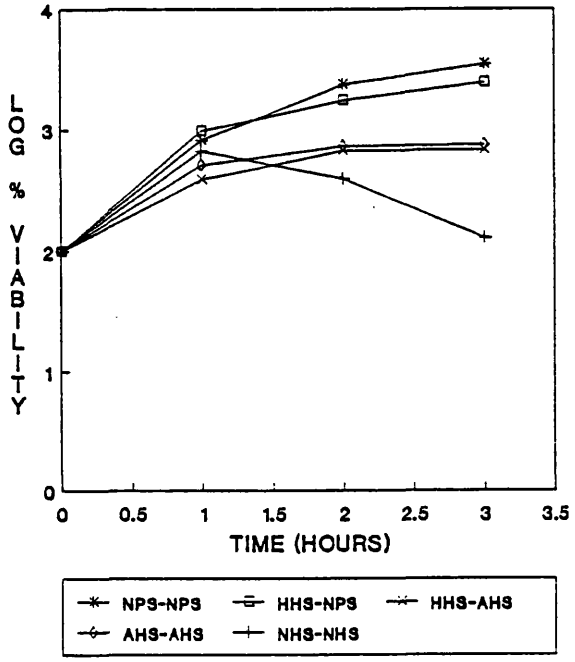
All strains were resistant to all combinations of sera except the pretreatment with normal human serum followed by incubation in normal human serum (Fig. 3.11). Pretreatment with the heated human serum did not sensitise the *A. pleuropneumoniae* strains when incubated with the absorbed human serum, suggesting heat-inactivated human serum did not restore bactericidal activity to the absorbed human serum.

3.3.7 Experiment 7 - bacterial sensitisation to normal pig serum following polymyxin B treatment

Attempts to sensitise *A. pleuropneumoniae* isolates to pig serum following treatment with polymyxin B were not successful. None of the 4 *A. pleuropneumoniae* strains were sensitised to the bactericidal effects of normal pig serum following treatment with sub-lethal concentrations of polymyxin B. *A. pleuropneumoniae* strains HK 353, HK 358 and 6664 were found to have higher viable bacterial counts in normal pig serum than in heat-inactivated pig serum over a range of polymyxin B concentrations (Figs. 3.12-3.14). *A. pleuropneumoniae* strain 266 showed increased viability in both normal pig serum and heat-inactivated pig serum over a range of polymyxin B concentrations (Fig. 3.15).

In contrast serum resistant *E. coli* strain C10 was sensitised to normal pig serum following treatment with a range of polymyxin B concentrations. A reduction in viability in normal pig serum was observed compared to that seen in heat-inactivated pig serum (Fig. 3.16). A summary of the results obtained from *A. pleuropneumoniae* and *E. coli* is shown in Fig. 3.17.

Fig. 3.11 Viability of *A. pleuropneumoniae* in absorbed human serum following pretreatment with heated human serum



LEGEND

NPS-NPS incubation with normal pig serum followed by incubation wih normal pig serum

HHS-NPS incubation with heated human serum followed by incubation with normal pig serum

HHS-AHS incubation with heated human serum followed by incubation with absorbed human serum

AHS-AHS incubation with absorbed human serum followed by incubation with absorbed human serum

NHS-NHS incubation with normal human serum followed by incubation with normal pig serum

Fig. 3.12 Sensitivity of *A. pleuropneumoniae* HK 353 to pig serum following treatment with polymyxin B

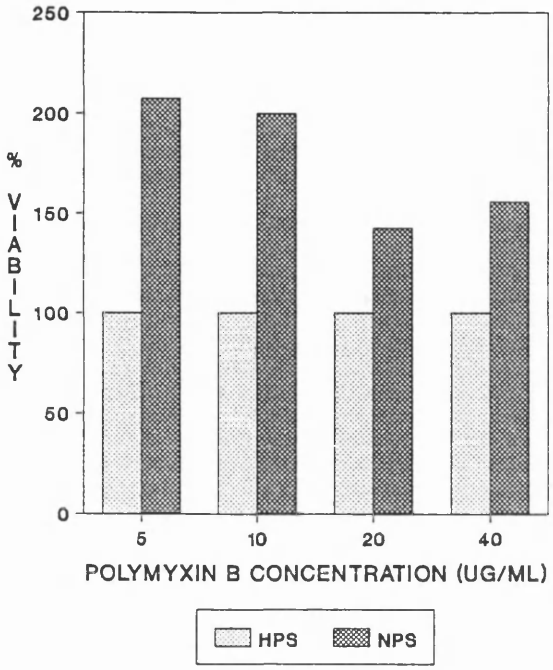
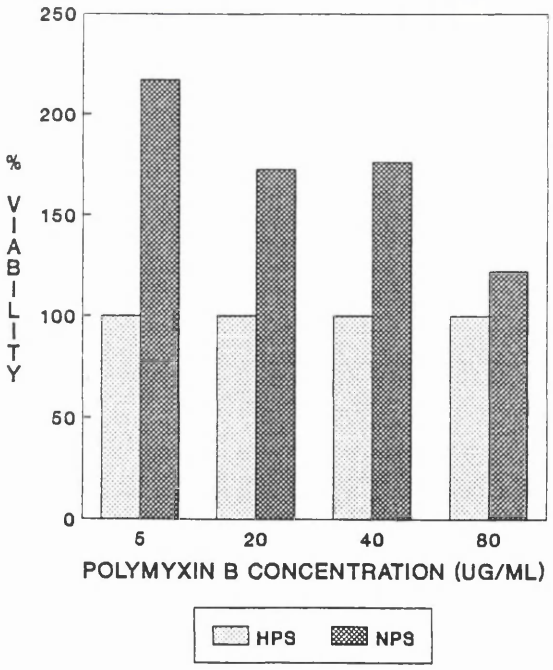


Fig. 3.13 Sensitivity of *A. pleuropneumoniae* HK 358 to pig serum following treatment with polymyxin B



LEGEND

NPS - normal pig serum
HPS - heated pig serum

Fig. 3.14 Sensitivity of *A. pleuropneumoniae* 6664 to pig serum following treatment with polymyxin B

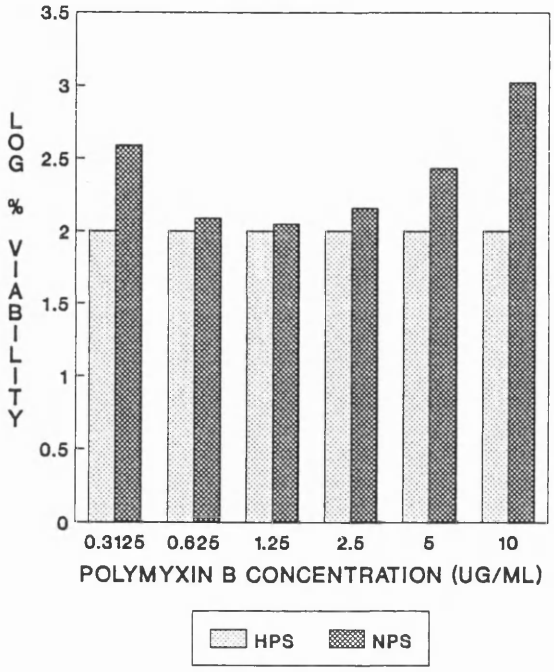
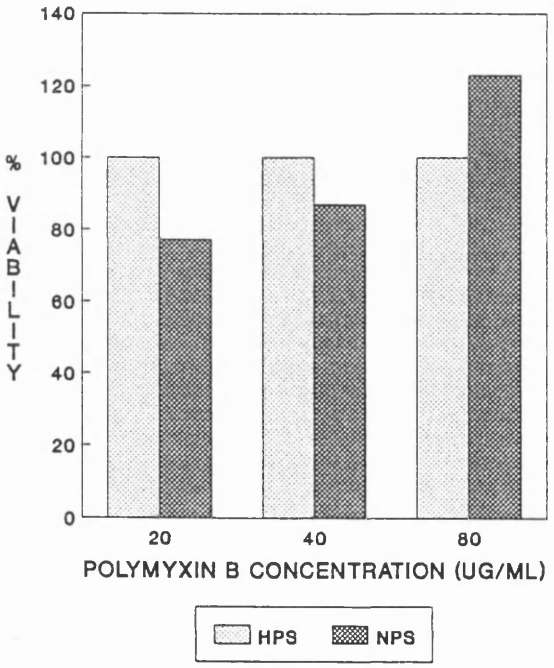


Fig. 3.15 Sensitivity of *A. pleuropneumoniae* 266-HAE-1920 to pig serum following treatment with polymyxin B



LEGEND

NPS - normal pig serum
HPS - heated pig serum

Fig. 3.16 Sensitivity of *E. coli* C10 to pig serum following treatment with polymyxin B

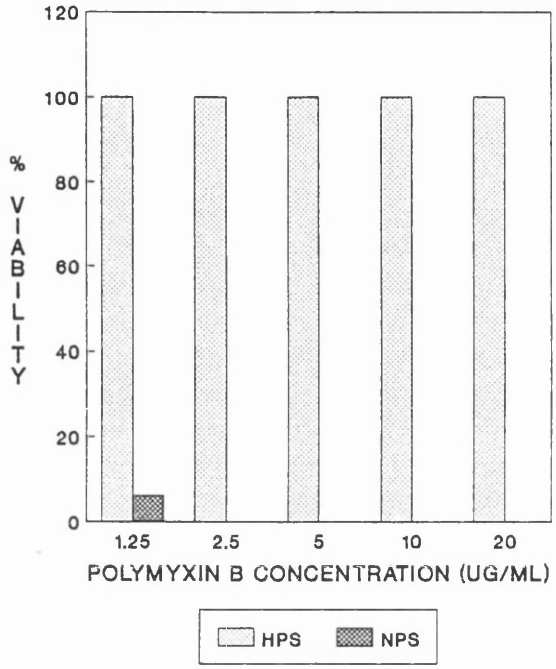
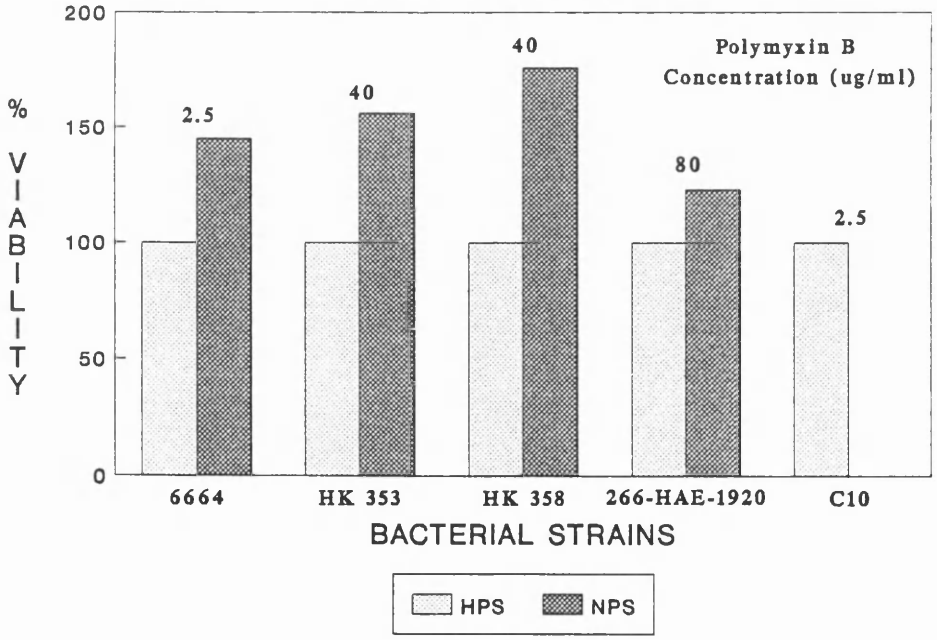


Fig. 3.17 Sensitivity summary of *A. pleuropneumoniae* and *E. coli* strains to pig serum following treatment with polymyxin B



LEGEND

NPS - normal pig serum
HPS - heated pig serum

3.4 DISCUSSION

3.4.1 Serum bactericidal assays

The aim of the work described in this chapter was to determine whether the non-specific humoral defence mechanism, complement, could mediate a bactericidal action against *A. pleuropneumoniae*. All strains of *A. pleuropneumoniae* were found to be resistant to pig serum even in the presence of specific antibody against one of the strains (Figs. 3.1-3.4 & 3.6). This suggests that the pig complement system is not bactericidal against *A. pleuropneumoniae*. Resistance to both complement and specific antibody has been documented in other Gram-negative bacteria including *Salmonella minnesota*. This organism was not killed in any concentration of human serum containing high titres of agglutinating antibody (Joiner *et al.*, 1982a; Joiner *et al.*, 1982b). Since the onset of this work, serum resistance using a serotype 5 strain of *A. pleuropneumoniae* has also been documented (Inzana *at al.*, 1988). Similarly it was also found to be resistant to pig bactericidal activity in the presence of specific antibody against that strain.

The lack of bactericidal activity in the pig serum against *A. pleuropneumoniae* appears not to be due to an inadequate killing system as demonstrated by the rapid death of the *E. coli* sensitive strain in pig sera (Fig. 3.5). The increased growth observed with *A. pleuropneumoniae* strains incubated in normal pig serum compared to the heat-inactivated pig serum, devoid of complement activity, has also been described for other bacteria (Fig. 3.1-3.3). Certain strains of *E. coli* have been reported to increase in numbers in the presence of complement and antibody (Taylor, 1978; Hughes *et al.* 1982) perhaps suggesting the loss of certain heat-labile component(s) which enhance bacterial growth. This trend was observed for *A. pleuropneumoniae* strains HK 353, HK

358 and 6664 throughout the incubation. It was also observed in strain 266-1920-HAE during the first 2 hours incubation after which slightly greater growth was seen in the heated pig serum (Fig. 3.4).

The difference in the degree of resistance to both the immune pig serum and hyperimmune rabbit serum and that of normal pig and rabbit serum, can probably be attributed to the presence of specific antibody against *A. pleuropneumoniae*. Both immune pig serum and the hyperimmune rabbit sera contained detectable antibodies against *A. pleuropneumoniae* and showed similar reduced growth trends compared to the higher growth observed for all the strains in normal pig and rabbit serum, which contained no detectable antibodies against *A. pleuropneumoniae* (Figs. 3.6 & 3.7). The lower increase in growth in the immune pig and rabbit serum may have been due to the effect of agglutinating antibody, which can sometimes lead to the underestimation of survival rates (Melching & Vas, 1970).

3 of the 4 *A. pleuropneumoniae* strains were found to be sensitive to the bactericidal action of human serum, but only after a delay of 2 hours (Fig. 3.7). Delayed killing has also been seen for *Serratia marcescens* which was the result of selective activation of the alternative pathway (Traub & Kleber, 1976). Killing by activation of the alternative pathway has been shown to proceed at a lower rate than that by classical pathway activation (Root *et al.*, 1972). The different results obtained for strain 266-1920-HAE and the other *A. pleuropneumoniae* strains in their sensitivity to human serum was perhaps due to strain 266-1920-HAE having a much slower growth rate than the other strains (personal observations) (Fig. 3.8). Bacterial metabolism has been shown to influence serum sensitivity (Griffiths, 1974; Taylor & Kroll, 1983). These researchers demonstrated that normally serum sensitive bacteria can become serum

resistant when assessed in non-logarithmic growth phase. Taylor and Kroll (1983) found that optimal killing of gram-negative bacteria required bacterial adenosine triphosphate (ATP) and that killing was inhibited by substances such as choramphenicol which inhibit bacterial growth. These results suggested that bacterial growth is required for optimal killing by complement perhaps by exposing sites for the C5b-9 attack complex which may not be present on stationary-phase organisms (Joiner *et al.*, 1984).

The absorption by *A. pleuropneumoniae* of the factor(s) responsible for the delayed sensitivity to human serum suggested that antibody or certain complement components necessary to mediate bactericidal activity against *A. pleuropneumoniae*, were being removed by this process (Fig. 3.9). Serum having undergone the filtration process, without being absorbed with bacteria, retained full bactericidal activity against *A. pleuropneumoniae* strains. This suggested loss of killing activity was attributable to the absorption of component(s) responsible for bactericidal activity rather than by the process of filtration. The absorbed serum was found however to have retained its level of bactericidal activity for the *E. coli* serum sensitive strain (Fig. 3.9), suggesting that the component(s) was not complement. The addition of 10% heat-inactivated human serum, as a source of antibody, to normal pig serum had no effect on the growth rate (Fig. 3.10). This may also suggest that the absorbable component was not antibody, or alternatively that the human component(s) responsible for *A. pleuropneumoniae* serum sensitivity was not compatible with the pig complement system. This phenomenon of incompatibility has been seen previously with serum proteins of one species and antibodies of another (Rowley, 1973).

Bacterial preincubation with heat-inactivated human serum, followed by incubation in absorbed human serum, also did not sensitise *A. pleuropneumoniae* (Fig. 3.11), which suggests that the factor responsible for the killing effect of human serum on *A. pleuropneumoniae* was not present in heat-inactivated human serum. This would also suggest that the component(s) was not antibody as this would still be expected to be present in heated serum. The component(s) responsible for the delayed sensitivity of *A. pleuropneumoniae* therefore appears to be neither antibody nor complement, and is both absorbable and heat-labile. There have been other reports of non-antibody, non-complement factors required for complement killing (Goldman & Austen, 1974; Clas & Loos, 1982). The 21 kDa factor described by Clas & Loos (1982) was also heat-labile and was inactivated by ß-glucosidase, pronase, proteinase K. It could not be replaced by either IgG or IgM directed against the organism, *Salmonella minnesota* Re 595. The extra factor was thought to be required for either C4 or C2 binding. Kawakami *et al.* (1982) also reported a factor that was both heat and 2-mercaptoethanol sensitive, with a molecular weight of 28 kDa that reacted specifically with only some chemotype strains of *Salmonella*. The apparent lack of shared biochemical or functional activity between these factors has been suggested that these non-antibody, non-complement factors that enhance or are required for complement-mediated killing demonstrate organism specificity (Joiner *et al.*, 1984). The different sensitivities observed between both the human and the pig and rabbit serum may suggest that human serum contains an additional factor(s) lacking in both pig and rabbit serum or alternatively that *A. pleuropneumoniae* has adapted to be resistant to the equivalent factor in pig serum.

3.4.2 Polymyxin B sensitisation

Following the observation that the *A. pleuropneumoniae* strains tested were resistant to the bactericidal activity of pig complement, it was decided to compare the mode of serum resistance with other Gram-negative serum resistant bacteria. The organism used for comparison was a serum resistant *E. coli* strain. The mode of serum resistance in certain *E. coli* strains has been attributed to the outer membrane. This has been found following studies using conditions that disrupt the outer membrane, followed by the testing of that strain for sensitivity to complement (Fierer & Finley, 1979; Vaara & Vaara, 1983; Vaara *et al.*, 1984; Vaara & Viljanen, 1985; Viljanen *et al.*, 1986). Methods used to disrupt the outer membrane have involved treatment with polymyxin B.

E. coli strain C10 was found to be a suitable control following its sensitisation to pig serum after treatment with sub-lethal concentrations of polymyxin B (Fig. 3.16). This was in contrast to all 4 *A. pleuropneumoniae* strains which were not sensitised to the bactericidal effects of pig serum following similar exposure to polymyxin B (Figs. 3.12-3.15). *A. pleuropneumoniae* strain 266-1920-HAE was found to be less viable in normal pig serum following exposure to 20 and 40 μg/ml of polymyxin B (Fig. 3.15). However, following exposure to a concentration of 80 μg/ml, higher counts were observed in the normal pig serum suggesting that the bacteria were not sensitised to complement following polymyxin treatment. Also strain 266-1920-HAE has been previously found to have a slower growth rate in normal pig serum compared to heated pig serum (Fig. 3.4), in contrast to the other 3 strains of *A. pleuropneumoniae* (Figs. 3.1-3.3). The lower growth rates seen initially following exposure to 20 and 40 μg/ml to polymyxin B may be due to this effect. The resistance of 266-1920-HAE to

complement activity following treatment with 80 μg/ml of polymyxin B demonstrates that, like the other 3 *A. pleuropneumoniae* strains, it is resistant to sensitisation to pig complement by polymyxin B treatment.

Polymyxin B resistant gram-negative bacterial strains have also been reported including strains belonging to *Proteus*, *Morganella*, *Providencia* and *Neisseria* species (Koike *et al.*, 1969; Teuber, 1969; Atkinson, 1980; Storm *et al.*, 1977) and also many strains of *Serratia marcescens* (Traub, 1982). Resistance to polymyxin B has been shown to be attributed to the outer membrane in the case of *Proteus mirabilis* (Storm *et al.*, 1977). The LPS of *Proteus mirabilis* is completely substituted by 4-aminoarabinose (Sidorczyk *et al.*, 1983) resulting in the LPS being less acidic. This reduced acidity apparently reduces the degree to which polycations are bound by the LPS and this has been suggested to account for the polycation resistance seen with polymyxin B (Vaara & Viljanen, 1985). *Salmonella typhimurium pmrA* mutants were found to contain 4-6 times more 4-aminoarabinose in their lipid A making 60-70% of their LPS in a less acidic form (Vaara *et al.*, 1981). These mutants were found to have a low level of resistance to polymyxin B compared to their sensitive parents and also bound 4 times less polymyxin B (Vaara, 1981; Vaara *et al.*, 1979). The differences in the amount of 4-aminoarabinose in the LPS of *P. mirabilis* and *S. typhimurium* has been suggested to explain the differences in the level of resistance to polymyxin B (Vaara & Viljanen, 1985).

The mechanism of complement resistance following incubation with polymyxin B appears not to be due to resistance to polymyxin B mediated damage as demonstrated by the killing of *A. pleuropneumoniae* at higher concentrations of polymyxin B (personal observations). This is in agreement with Vaara *et al.*

(1984) who reported an *E. coli* strain was resistant to treatment with polymyxin B nonapepetide (PMBN) and serum, but was nevertheless sensitive to the outer membrane permeability increasing action of PMBN. This was demonstrated by showing the strain's sensitivity to fusidic acid following treatment with PMBN.

Disruption of the outer membrane by polymyxin B has been shown not to be lethal in itself. It has been reported that polymyxin B nonapeptide, which lacks the terminal diaminobutyric acid residue and the attached fatty acid of the parent compound, induces similar changes to the outer membrane structure and permeability (Dixon & Chopra, 1986a; Vaara *et al.*, 1984; Viljanen *et al.*, 1986; Vaara & Vaara, 1983). Polymyxin B nonapeptide has drastically reduced or no antibacterial activity (Dixon & Chopra, 1986a; Nikaido & Vaara, 1985; Vaara & Vaara, 1983) compared with its toxic parent, and therefore it has been suggested the outer membrane damage caused by polymyxin B is unlikely to be directly responsible for its bactericidal activity (Dixon & Chopra, 1986b). Since polymyxin B disrupts both the outer and inner membranes, it has been proposed that its lethality arises as a result of cytoplasmic membrane disruption (Dixon & Chopra, 1986b).

Polymyxin B is reported to cause outer membrane damage at similar concentrations to those having a lethal effect on the bacterial cytoplasmic membrane (Viljanen *et al.*, 1986). It is possible that the sub-lethal concentrations used to sensitise *A. pleuropneumoniae* were not sufficient to cause outer membrane damage in this study. This however is doubtful as many serum resistant gram-negative bacteria are sensitised by sub-lethal concentrations of polymyxin B. It is more likely that the mechanism of serum resistance in *A. pleuropneumoniae* is not wholly dependent on the outer membrane acting as a barrier to complement components.

The use of polymyxin B nonapeptide would have overcome the problems of toxicity whilst still retaining the ability to disorganise the outer membrane, however this compound was not commercially available at the time. Also, the results of the experiments indicated that sensitisation of the controls could be achieved using sub-lethal concentrations of polymyxin B and therefore it was found not to be necessary to prepare polymyxin B nonapeptide.

The method of synergy between polymyxin B (& PMBN) and serum has been suggested to be due to its disruptive action on the outer membrane ie. to expose the deep structures of the outer membrane to antibodies, or other components in serum, that lead to the activation of complement, and hence allow the membrane attack complex (MAC) access to the hydrophobic region. It would also allow MAC complexes that had formed at a distance from the hydrophobic region, to have access to it (Vaara *et al.*, 1984).

The resistance of *A. pleuropneumoniae* to the bactericidal activity of normal pig serum, following exposure to sub-lethal concentrations of polymyxin B, suggests that perhaps the outer membrane is not important in the serum resistance of *A. pleuropneumoniae*. This may be due to the lack of complement activation, even in respect of the opening of the outer membrane. Alternatively, complement may be being activated but is blocked at a further stage in the cascade. For example in for some bacteria the MAC forms but incorrectly assembles preventing correct insertion into the membrane to cause sufficient loss of nutrients and eventual death. Lastly, perhaps the nature of the lipopolysaccharide of the strains being studied is such that it is resistant to polymyxin B sensitisation as in the case of *P. mirabilis* (Sidorczyk *et al.*, 1983).

CHAPTER 4

IN VITRO ACTIVATION AND CONSUMPTION OF COMPLEMENT BY *A. PLEUROPNEUMONIAE*

4.1 INTRODUCTION

As discussed in the previous chapter, the *A. pleuropneumoniae* strains tested were resistant to pig complement even in serum that contained antibodies against one of these strains. Resistance to complement-mediated bactericidal activity has been achieved by various bacteria that employ a range of mechanisms enabling them to evade the detrimental actions of complement (Joiner, 1988). One mechanism of evasion used by bacteria is to avoid activating the complement cascade pathways, thereby escaping bactericidal activity.

The aim of this chapter was to determine if the mechanism by which *A. pleuropneumoniae* avoided pig complement damage was by its inability to activate the complement cascade by either or both of the pathways.

Complement-mediated damage of bacteria can proceed via activation of either the classical (CCP) or the alternative (ACP) pathway, resulting in consumption of complement components. Consumption and activation of the complement pathways were assessed in both pig and human serum which had been previously incubated with *A. pleuropneumoniae*. Consumption of complement activity can be assessed using functional haemolytic assays. These functional haemolytic assays (CH50 and AP-CH50 assays) measure the functional activity of both the classical and the alternative pathways respectively including the terminal components (C3-C9). Utilisation of any complement components by activation of either pathway, or by other mechanisms discussed later, may, depending on the degree of usage, deplete that particular component(s). This in turn can result in a gap in the complement pathway, preventing future completion of the cascade. Analysis of activated serum for remaining complement activity by functional haemolytic assays, may

result in an inability or a reduced ability to lyse the blood cells due to a shortage of one or more of the components required for each step in the cascade. This lack of lysis can be expressed as complement consumption of a particular pathway.

The basis of the functional haemolytic complement test is to incubate a fixed quantity of red blood cells with varying amounts of complement and buffer for a fixed period of time. Once the reaction is stopped, the percentage lysis of the red blood cells can be calculated. The percentage lysis and the equivalent volume of serum responsible are plotted to obtain a sigmoid dose-response curve. The central part of the curve, usually between 20 and 80% lysis, is steep and the percentage of cells lysed is sensitive to small changes in the amount of complement in the serum. For this reason the endpoint of the titration is taken as 50% haemolysis = 1 CH50 (AP-CH50) unit. The sigmoid curve can be mathematically described using the van Krogh equation (Whaley, 1985).

$$X = K\ (Y/1-Y)1/n$$

X = amount of serum added to tube (μl)
K = a constant which is the 50% unit of complement

At 50 % haemolysis 1-Y = 1, hence X = K. The value of K can be obtained by plotting log X against log Y/1-Y.

Complement consumption by bacteria in the serum is evaluated by the calculation of the number of CH50 (classical pathway) (AP-CH50, alternative pathway) units and comparing it to the control serum which has not been incubated with bacteria. A reduction in the number of CH50 (AP-CH50) units in the serum previously incubated with bacteria, compared to the bacteria-free control

serum, indicates that consumption of complement has occurred in the bacteria-treated serum.

Immunoelectrophoresis of test serum can be carried out to assess activation of a certain component by the identification of activation cleavage proteins. Immunoelectrophoresis measures the presence of the proteins in question whether they are functionally active or not. Using specific antiserum against these proteins, changes following incubation with bacteria, can be detected either by the presence of additional antigen/antibody precipitation arcs or by the distortion of the original arc. From the results obtained one can assess whether activation of a particular protein has occurred. It can then be concluded that the complement cascade has been activated or partly activated at this particular point.

The human complement system has been well characterised to allow evaluation of the individual components, enabling each to be assessed for reduction and the broken link can therefore be determined. The pig complement system however has not yet been as well characterised, and only one antiserum was commercially available against purified complement component C3. The extent of the information which could be evaluated in the pig serum was firstly, whether complement consumption occurred via either the classical or the alternative pathways using functional haemolytic assays and secondly, whether there was cleavage of C3 which can occur via both pathways and is indicative of C3 activation. As a comparison, *A. pleuropneumoniae* treated human serum was also evaluated for complement consumption and activation of both C3 (via both pathways) and factor B (via ACP only). All assays were also determined using three well characterised *E. coli* strains, C10, RY21 and RY22, which have been described previously in Chapter 2.

4.2 MATERIALS & METHODS

All buffer solutions used in both the functional haemolytic assays and immunoelectrophoresis are described in Appendix 1.

4.2.1 Functional haemolytic complement consumption assays

4.2.1.1 Serum

All serum used in the complement assays was stored at -70°C. Pig and human sera were obtained from healthy pigs and humans, and processed as previously described in Chapter 2. Neither serum was found to possess antibodies against *A. pleuropneumoniae* by the methods described in Chapter 2.

4.2.1.2 Red blood cells

Sheep red blood cells (SRBC) were obtained commercially (Becton Dickinson) and diluted in an equal volume of Alsever's solution prior to storage at 4°C. Rabbit red blood cells (RRBC) were obtained using the method of Whaley (1985). Rabbit blood was collected by bleeding from the marginal ear vein of New Zealand White rabbits. The blood was collected in an equal volume of Alsever's solution, centrifuged at 2,000 x g for 10 minutes at 4°C, and the plasma and buffy coat removed by aspiration. The cells were washed three times in EDTA (10mM) $GVB^{=}$ buffer and resuspended in the same volume. RRBC were then incubated at 37°C for 15 minutes in a water bath, and washed a further three times in Mg-EGTA buffer. The cells were resuspended in the same buffer and stored at 4°C for a maximum of 2 weeks.

4.2.1.3 Serum absorption

Normal pig and human serum was absorbed three times by incubating 1ml of pig or human sera with 0.05ml packed volume of either washed SRBC or RRBC, for 15 minutes at 0°C (Platt-Mills & Ishizaka; 1974). The blood cells were removed by centrifugation (11,600 x g for 5 seconds) and the absorbed sera were either used immediately or stored at -70°C.

4.2.1.4 Antibody sensitisation of SRBC (EA)

Sensitisation of SRBC was performed using a modified method based upon that of Whaley (1985) and Palmer & Whaley (1986). SRBC were removed from the stock suspension in Alsever's solution and centrifuged at 2,000 x g for 10 minutes at 4°C. The supernatant plasma and the buffy coat were removed by aspiration and washed twice in $GVB^{=}$ buffer. The SRBC were adjusted to 1 x 10^9 cells/ml using the following method. 1ml of SRBC suspension was added to 29ml of deionised water, and the optical density of the lysate measured using a spectrophotometer at a wavelength of 541nm. An OD reading of 0.385 corresponds to a concentration of 1 x 10^9 SRBC/ml (Whaley, 1985). The volume of SRBC was adjusted to 1 x 10^9/ml using the equation,

Volume of SRBC required = Initial volume x (OD measured/0.385)

The SRBC were then warmed in a water bath at 37°C. Antibody to SRBC (Gibco) was diluted in an equal volume of $GVB^{=}$ buffer and prewarmed to 37°C. The antiserum was slowly added to the swirling SRBC suspension and the mixture agitated for 20 minutes in a 37°C water bath by hand. The resultant antibody sensitised SRBC (EA) were centrifuged at 2,000 x g for 5 minutes at 4°C. EA were washed twice in $GVB^{=}$ buffer, once in GVB^{2+} buffer and

resuspended in their original volume in GVB^{2+} buffer. EA were stored at 4°C for a maximum of 1 week.

4.2.1.5 SRBC antiserum titration

A modified version of the methods employed by Palmer & Whaley (1986) were used. EA were prepared as previously described using a range of antiserum dilutions prepared in GVB^{2+} buffer in a total volume of 300µl. EA were incubated with 450µl of GVB^{2+} buffer containing a final concentration of 1:20 of either normal pig or human serum. The reaction mixture was incubated for 60 minutes at 37°C in a water bath and shaken once after 30 minutes. 2ml of ice cold saline (0.87% NaCl) was added to all tubes to stop the reaction, followed by centrifugation at 1,000 x g for 5 minutes at 4°C. The optical densities (OD) of the resulting supernatants were read at 541nm, and the percentage lysis of the SRBC calculated. The optimal concentration of antiserum can be calculated by plotting a graph of the concentration of antiserum used against the corresponding percentage lysis. An acceptable curve should produce a plateau when the percentage lysis of the SRBC is not increased with increasing concentrations of antiserum to the SRBC (Palmer & Whaley, 1986). The optimal concentration of antiserum to use corresponds to the second point on the plateau.

4.2.1.6 Bacterial preparation for complement consumption assays

Bacterial strains were grown as previously described for the serum bactericidal assays in Chapter 3. Bacterial numbers were assessed by reading the OD of the bacterial suspension at 650nm. An OD reading of 1.0 corresponds approximately to 1 x 10^9 CFU/ml of *A. pleuropneumoniae* (personal observations). The suspensions were adjusted to give an OD of 0.75 at 541nm which corresponds to 7.5

x 10^8 CFU/ml of bacteria. The bacteria were washed once in either VBS^{2+} buffer for the classical pathway assays or Mg-EGTA buffer for use in the alternative pathways assays, and resuspended in the appropriate buffers.

4.2.1.7 CH50 determination

The generally accepted CH50 unit is the amount of complement that causes 50% lysis of antibody sensitised SRBC via the classical complement pathway. Sera diluted over a range of concentrations with GVB^{2+} buffer in a total volume of 225μl were incubated with 150μl of sensitised SRBC (EA). The mixture was incubated at 37°C in a water bath for 60 minutes and shaken once after 30 minutes. 1ml of ice-cold saline was added to all tubes and the suspensions were centrifuged at 1,000 x g for 5 minutes at 4°C. The OD of the resulting supernatants was assessed at 541nm and the percentage lysis calculated by comparison with the 100% lysis control. The percentage lysis was plotted against the corresponding serum concentration. The amount of serum that caused 50% lysis could be calculated from this graph for both the controls and the test sera. The volume of serum that caused 50% lysis, together with the additional information in Table 4.1, were used to calculate the number of CH50 or AP-CH50 units in the each serum using the formulae described in Table 4.2.

4.2.1.8 Complement consumption by bacteria via the classical pathway

Complement consumption by *A. pleuropneumoniae* isolates HK 361, HK 353 and 6664 of both pig and human serum was assessed using functional haemolytic assays based upon the method of Wilson *et. al.*, (1985). A volume of serum containing 30 CH50 units was mixed with 7.5 x 10^8 bacteria in GVB buffer to a final reaction volume of 225μl, and the mixture held at 37°C for 30 minutes.

	Serum Dilution = 5 CH50(AP-CH50)			
	PIG		HUMAN	
	CCP	ACP	CCP	ACP
A. pleuropneumoniae strains	1/6	1/1.8	1/6	1/5.2
E. coli strains	neat	1/1.8	1/6	1/5.2
Reaction volume(µl)	225	100	225	100

Table. 4.1 - Serum dilutions and reaction volumes used for the classical and alternative pathways titrations.

Table. 4.2 - Formulae to calculate CH50 (AP-CH50) units and percentage complement consumption.

1. Calculation of the number of CH50 (AP-50) units in both control sera and sera incubated with bacteria

a. <u>Calculation of final dilution = 1 CH50 unit.</u>

$$\frac{\text{test volume of serum}}{\text{reaction volume}} \times \text{initial dilution} = \text{final dilution} = \text{1 CH50 unit/ml}$$

b. <u>Calculation of CH50 Units in reaction volume</u>

CH50 units in undiluted serum in reaction volume = dilution factor

c. <u>Calculation of CH50 units in 1ml of undiluted serum.</u>

$$\text{CH50 units/ml in undiluted serum} = \text{Dilution Factor} \times \frac{1000}{\text{reaction volume}}$$

2. Calculation of percentage complement consumption in sera incubated with bacteria compared to a bacteria free control

$$1 - \frac{\text{(Test CH50 units/ml)}}{\text{(Control CH50 units/ml)}} \times 100$$

Following centrifugation at 11, 600 x g for 1 minute to remove the bacteria, the supernatant was diluted 1 in 6 to give a final concentration of 5 CH50 units per original volume (Table 4.1). The residual complement activity of the samples was assessed by the 50% haemolytic titration for complement (CH50) described in section 4.2.1.7. The amount of complement activity remaining in the test sera was expressed as a percentage of the total remaining in the bacteria-free serum control using the following formula, [1-(CH50 test/CH50 control)] x 100. Included in these experiments were 3 *E. coli* control strains, C10, RY21 and RY22, which are described in Chapter 2.

4.2.1.9 AP-CH50 determination

The generally accepted AP-CH50 unit is the amount of complement that causes 50% lysis of RRBC via the alternative complement pathway. This was performed using a modification of the method of Whaley (1985). Serial dilutions of the sera to be tested were prepared in MG-EGTA (10mM) buffer in a final volume of 100μl. 100μl of 1 x 10^8 RRBC/ml were added to each tube and incubated in a 37^{o}C water bath for 90 minutes, and the tubes shaken once after 45 minutes incubation. 1ml of ice cold saline was added to all tubes to terminate the reaction, followed by centrifugation at 1,000 x g for 5 minutes in the cold. The OD of the supernatants was assessed at 414nm. The percentage lysis was calculated, and the number of AP-CH50 units determined as described for the assessment of CH50 units in section 4.2.1.7.

4.2.1.10 Complement consumption by bacteria via the alternative pathway

A modified method based on that of Whaley (1985) was used. Sera containing 5 AP-CH50 units were incubated with 7.5 x 10^8 organisms in Mg-EGTA in a total volume of

350μl. Following incubation at 37°C for 30 minutes, bacteria were removed by centrifugation and samples assessed for residual complement activity by the 50% haemolytic titration for the alternative pathway (AP-CH50) as described in section 4.2.1.9. Consumption of complement via the alternative pathway was assessed in serum preincubated with each of the three *A. pleuropneumoniae* strains, HK 361, HK 353 and 6664 together with *E. coli* control strains, C10, RY21 and RY22.

4.2.1.11 Controls

All individual assays were carried out in duplicate. The results shown are the mean of 2 or more experiments. Positive and negative controls included:

1. 100% lysis - red blood cells in distilled water
2. red blood cells in buffer only
3. buffer and serum only

The positive control, 100% lysis, indicated the maximum OD reading obtained from lysis of all the available red blood cells. The negative control of red blood cells and buffer only, assessed the stability of the red blood cells following all the test procedures. Any lysis above the acceptable level for spontaneous lysis resulted in a fresh batch of red blood cells being tested. Once an acceptable batch of red blood cells was found, the percentage spontaneous lysis was determined and subtracted from both the 100% lysis control and the test samples. The buffer and serum control assessed the total complement activity available following incubation in the absence of bacteria. This was the positve complement control. The buffer and serum control also assessed the OD reading that was attributable to serum colour. Any readings that were high, were again subtracted from the positive control and all the test samples prior to percentage lysis being determined in the test samples.

4.2.2 Immunoelectrophoresis

4.2.2.1 Antiserum

Goat anti-human C3b proactivator (factor B) and goat anti-human C3 (ICN Immunobiologicals) were used at 1:4 and 1:8 dilutions respectively. Rabbit anti-pig C3 (Cappel) was used at 1:25 dilution.

4.2.2.2 Zymosan preparation

This was prepared using the method of Whaley (1985). 100mg zymosan A (Sigma Ltd.) was suspended in 10ml of VBS buffer and boiled for 30 minutes. The suspension was centrifuged at 11,600 x g for 1 minute to pellet the zymosan. The supernatant was discarded and the pellet washed a further twice in VBS buffer. The zymosan was resuspended in 10ml VBS buffer giving a solution of 10mg/ml, and stored at 4^{o}C.

4.2.2.3 Zymosan treated serum

1ml of the zymosan suspension (10mg) was washed twice in VBS buffer and the supernatants discarded. The pellet was resuspended in 10ml of serum and incubated at 37^{o}C for 60 minutes. The mixture was centrifuged at 11,600 x g for 1 minute. The serum was then removed and frozen at -70^{o}C until used.

4.2.2.4 Slide preparation

Standard microscope slides were rinsed in reverse osmosis (ro) water followed by 95% alcohol before use. Slides were precoated with agar solution by adding a few drops of molten agar to the slide, and preparing a smear using a second microscope slide. Precoating prevents the agar strip being detached from the slide during the washing procedure. 2.5ml of molten agar was pipetted

onto the slide and allowed to set at room temperature, followed by refrigeration for 30 minutes. A trough was prepared by removing an agar strip 65mm long by 2mm across. The agar was not removed until after immunoelectrophoresis was performed. 2 wells 4mm in diameter were prepared 4mm from either side of the trough and 25mm from the end of the slide. The agar plugs were removed by aspiration.

4.2.2.5 Determination of C3 activation

Serum preincubated with bacteria was assessed for activation of C3. Bacteria were grown as previously described for the serum bactericidal assays (3.2.1). 5 x 10^8 washed bacteria were suspended in 100μl of VBS buffer and 100μl of either pig or human serum. Following incubation for 30 minutes at 37^{o}C, the bacteria were removed by centrifugation at 11,600 x g for 1 minute and the serum recovered. EDTA was added to the sera to give a final concentration of 10mM (23μl of 86mM EDTA). The sera were either used immediately or stored at -70^{o}C.

Sera, previously incubated with bacteria, were then assessed for the presence of complement activation components by immunoelectrophoresis. The serum was diluted 1 in 4 and 20μl of the diluted sera was then added to one of the two wells on each slide. The other well was filled with control sera that had been incubated for 30 minutes at 37^{o}C without bacteria. The slides were placed in the tank and wet filter paper wicks attached to either end of the slides to make contact with the buffer. Serum samples were electrophoresed for 5 hours at 10V/cm until the free bromophenol blue marker had migrated to within 1cm of the anode wick. The marker consisted of a drop 0.5% bromophenol blue solution which was added to a control well containing serum.

The agar strip was removed from the trough and filled with 150μl of either rabbit anti-pig C3 or goat anti-human C3. The agar slides were placed in a moist enviroment at room temperature for 24-48 hours to allow diffusion to occur. Slides were then washed overnight in saline solution to remove any non-precipitated protein from the gel. This was followed by washing the slides in roH_2O (reverse osmosis water) for 2 hours to remove the salt solution. The slides were covered with 3mm Whatman filter paper and dried under a stream of warm air. The filter paper was removed and the dried slides stained with Coomassie Brilliant Blue R (Sigma) in methanol:H_2O:acetic acid (5:4:1) for 10 minutes and destained in the above solvent solution. The three *E. coli* isolates were also assessed for their ability to activate C3.

4.2.2.6 Determination of factor B activation

Serum preincubated with bacteria was assessed for activation of factor B. Activation of factor B can occur only via the alternative pathway, unlike C3 which can occur via both the classical and the alternative pathways. Factor B activation could only be assessed in human serum because the porcine reagent for the determination of factor B was not available. Activation of factor B in human serum was initially found to proceed in the buffer and serum controls, suggesting that the procedure used was capable of activating factor B. Different incubation times of the serum and buffer controls were assessed for the activation of factor B during the incubation process. Small quantities of factor B were found to be activated during the 20 minute incubation with activation increasing after 30 minutes incubation (Appendix 2, Fig. 4.1a). No activation was seen in the 5 and 10 minute incubation samples. The incubation period for determination of factor B

activation by the bacteria was therefore reduced from 30 to 15 minutes.

Bacteria were grown as described for the serum bactericidal assays (3.2.1). 5 x 10^8 washed bacteria were suspended in 100μl of Mg-EGTA buffer and 100μl of human serum. Following incubation for 15 minutes at 37^{o}C, the bacteria were removed by centrifugation at 11,600 x g for 1 minute and the serum recovered. EDTA was added to the sera to give a final concentration of 10mM (23μl of 86 mM EDTA). The sera were either used immediately or stored at -70^{o}C. Each of the sera incubated with bacteria was then assessed for the presence of factor B activation components by immunoelectrophoresis as described in section 4.2.2.5. Development and staining of the slides was carried out with 150μl of goat anti-human C3b proactivator (factor B) as described above. The three *E. coli* isolates were also assessed for the ability to activate human factor B.

4.2.2.7 Controls

The controls used were serum and buffer alone and this was processed in the same way to those sera incubated with bacteria. To assess whether spontaneous activation of complement occurred in the serum and buffer control, a second control was included in which EDTA was added prior to incubation and immunoelectrophoresis. EDTA chelates Ca^{++} and Mg^{++} ions which are necessary for activation of the classical and alternative pathways. This prevents any activation during the incubation and immunoelectrophoresis procedures. Any differences between these two first controls would suggest activation of complement was occurring in the absence of bacteria invalidating the test results. The third control was zymosan treated serum. Zymosan is an activator of the alternative complement pathway and

therefore provides a reference positive control for complement components C3 and factor B. Controls of serum and buffer alone were added to one of the wells in each slide containing the test serum. Any differences seen between the test and the control could therefore be said not to be due to different electrophoretic conditions. All experiments were carried out a minimum of 3 times and the mean of the results are shown.

4.3 RESULTS

4.3.1 Functional haemolytic assays

4.3.1.1 Consumption of complement via the classical pathway in pig serum incubated with *A. pleuropneumoniae*

All 3 *A. pleuropneumoniae* strains consumed high levels of the classical complement pathway. Figure 4.1 shows the percentage haemolysis of each serum incubated with *A. pleuropneumoniae* treated serum and also of the control serum (incubated without bacteria) over a range of serum volumes. The volume of serum required to produce 50% lysis (1 CH50 unit) of red blood cells, depicted by the arrows, was calculated from the graph and the total number of CH50 units in each sera was assessed as described in Table 4.2. These figures showed a difference in the number of CH50 units present between the controls, which were not incubated with bacteria, and each of the three test sera incubated with *A. pleuropneumoniae* strains (Table 4.3). The percentage consumption of all the sera was calculated as described in Table 4.2. HK 358 test serum consumed 82.2% of the available complement activity. The reduction in complement activity in test serum incubated with both HK 353 and 6664 was such that 50% haemolysis could not be reached even using the maximum reaction volume of serum (Fig. 4.1, denoted # in Tables 4.3, 4.5-4.7). The part of 1 CH50 unit that corresponded to the maximum amount

Fig. 4.1 Titration of classical complement activity remaining in pig serum incubated with *A. pleuropneumoniae*

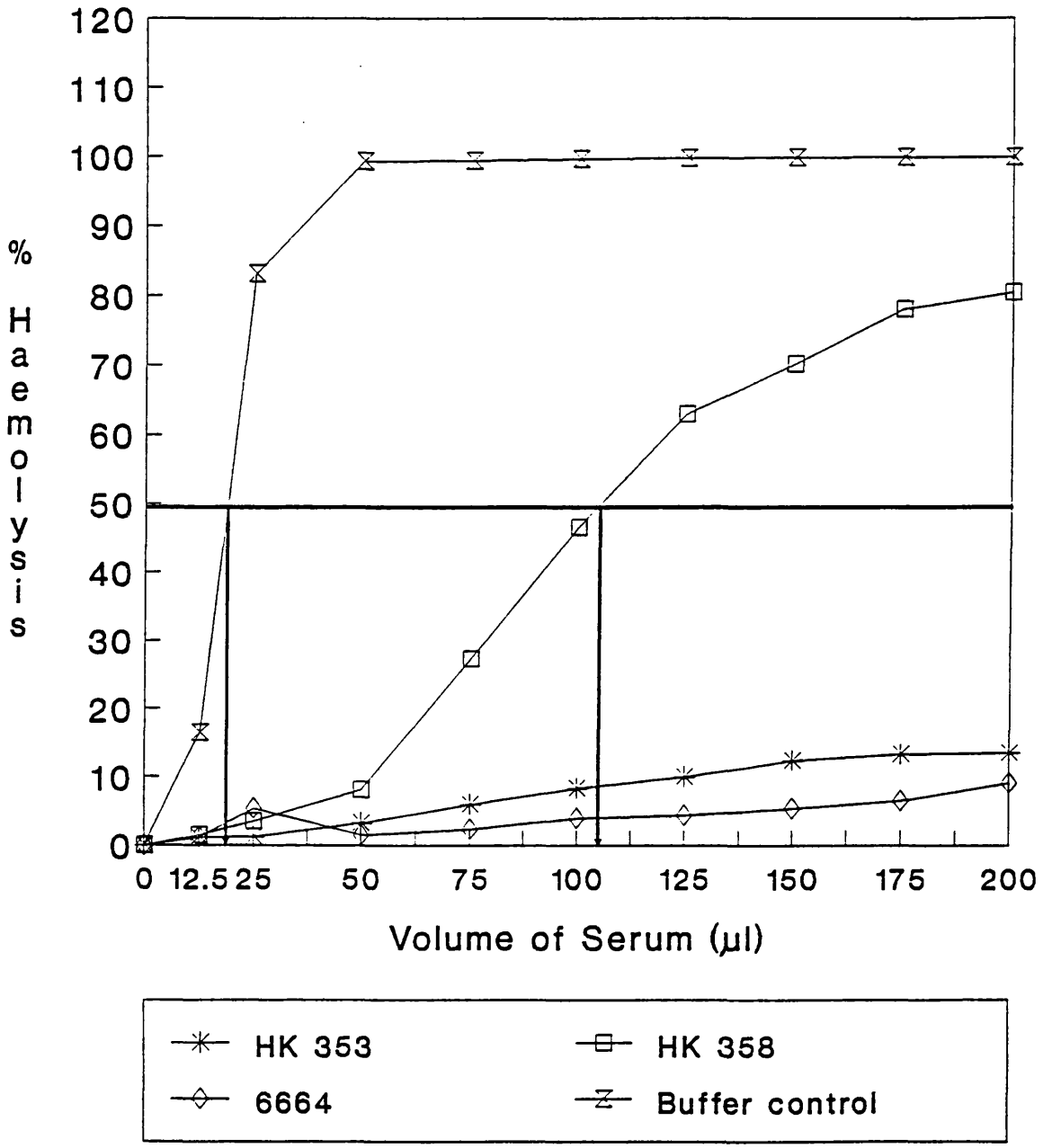


Table. 4.3 Number of CH50 units in pig serum incubated with *A. pleuropneumoniae* and *E. coli*

	Volume of sera (µl) = 1 CH50	Part of 1 CH50 unit=maximum lysis in (#)	No. of CH50 units/ml
Buffer control	18.8		316.0
A. pleuropneumoniae Strains			
HK 353	#	0.268	7.1
HK 358	105.5		56.3
6664	#	0.180	4.8
Buffer control	10.9		91.7
E. coli Strains			
C10	21.9		45.7
RY21	#	0.000	0.0
RY22	#	0.000	0.0

of lysis obtained using the maximum reaction volume of serum for both the HK 353 and the 6664 treated serum was calculated and shown in Table 4.3. From this, the number of CH50 units in 1ml of undiluted serum could be assessed, and the percentage consumption calculated. The percentage consumption of the classical complement pathway for HK 353 and 6664 were found to be 97.8% and 98.5% respectively (Table 4.4).

The 3 *E. coli* control strains consumed complement activity in the pig serum when compared to the control serum incubated without bacteria (Table 4.3). The volume of serum required to produce 50% lysis was assessed (Fig. 4.2) and the corresponding number of CH50 units calculated (Table 4.3). There was however, a difference in the levels of complement consumed between the 3 *E. coli* strains (Table 4.4). The *E. coli* parent strain C10 consumed complement activity but at a reduced rate compared to its two mutant strains, RY21 and RY22. *E. coli* C10 consumed 50.2% of the available complement activity via the classical pathway whilst both C10 mutants, RY21 and RY22, consumed 100% of the complement activity. The consumption of the parent strain *E. coli* C10 test serum was also in sharp contrast to the previously tested *A. pleuropneumoniae* test sera. Consumption for the C10 strain, which was calculated to be 50.2%, is between 30 and 48.3% less than that consumed by the three *A. pleuropneumoniae* strains, HK 361, HK 353 and 6664.

4.3.1.2 Consumption of complement activity via the classical pathway in human serum incubated with *A. pleuropneumoniae*

Again all 3 *A. pleuropneumoniae* strains consumed high levels of available complement activity in the normal human serum, compared to that present in the bacteria-free control serum as depicted by the volumes of serum

Table. 4.4 Percentage of complement activity consumed by bacteria via classical and alternative pathways

A. pleuropneumoniae Strains	COMPLEMENT PATHWAYS			
	Classical		Alternative	
	Pig	Human	Pig	Human
HK 353	97.8	72.7	98.0	96.7
HK 358	82.2	76.4	97.9	96.3
6664	98.5	97.0	100	98.2
E. coli Strains				
C10	50.2	36.3	41.8	97.5
RY21	100	97.4	99.1	97.9
RY22	100	96.5	98.7	97.8

Fig. 4.2 Titration of classical complement activity remaining in pig serum incubated with *E. coli*

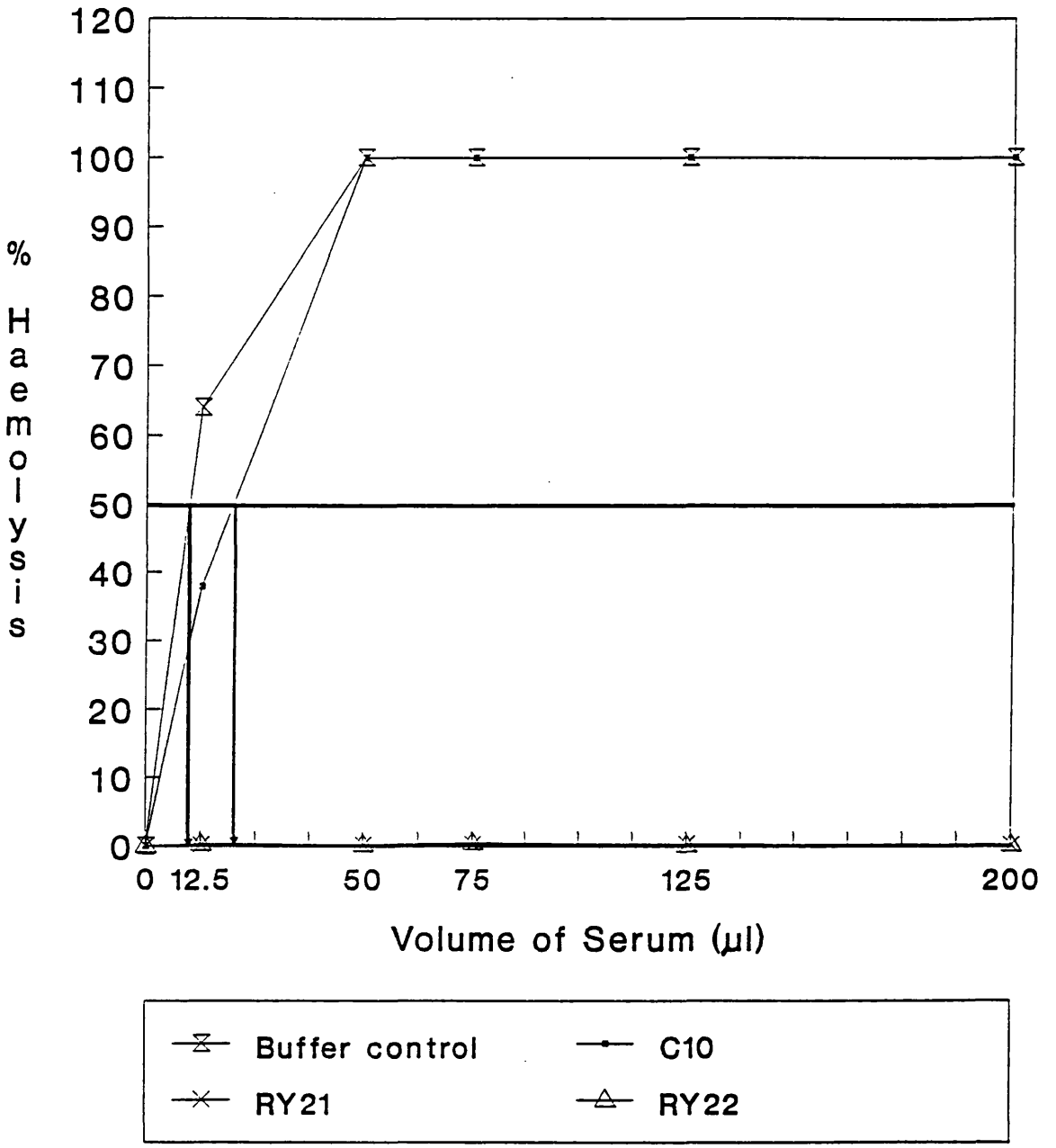
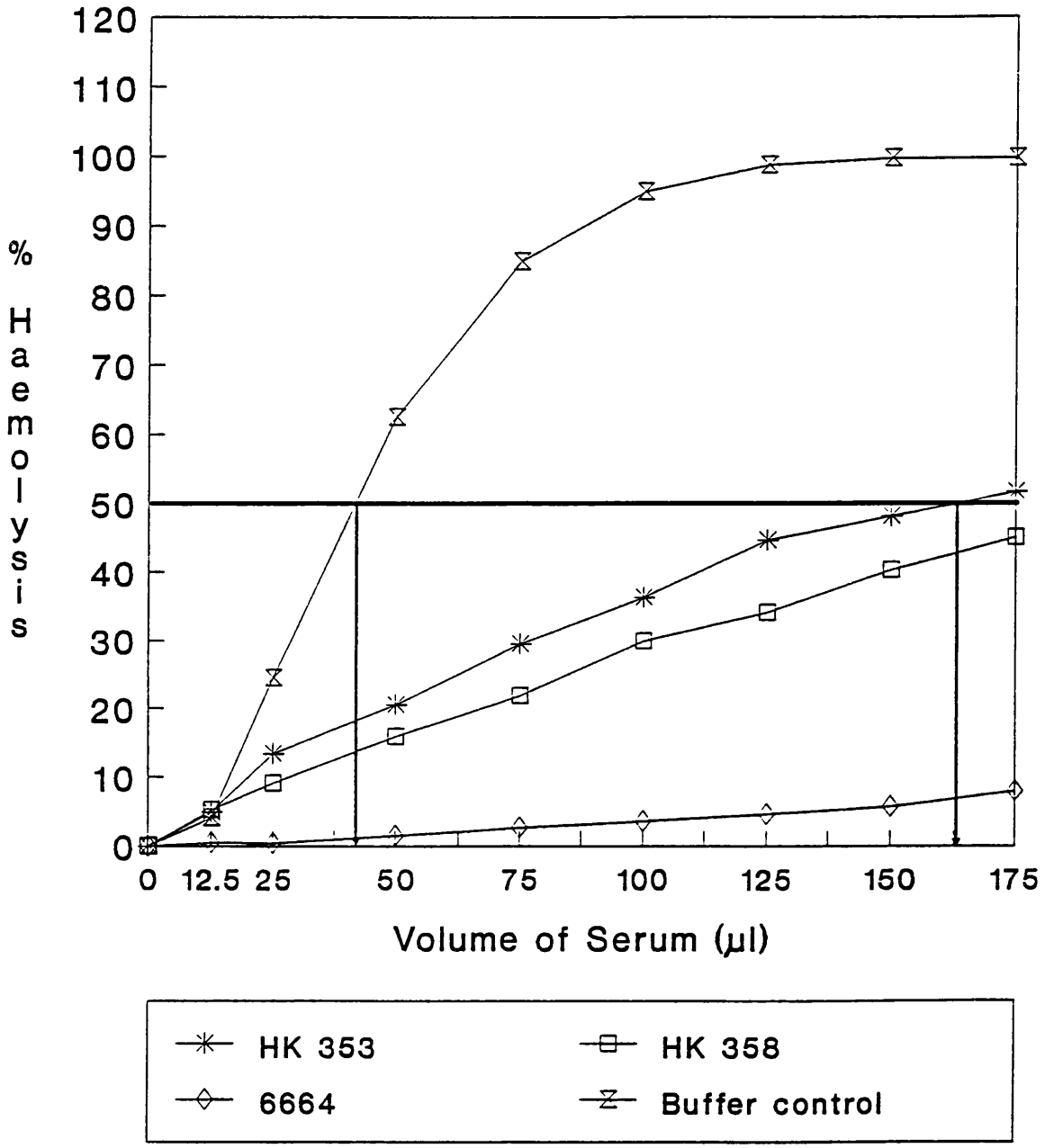


Fig. 4.3 Titration of classical complement activity remaining in human serum incubated with *A. pleuropneumoniae*



required to produce 50% lysis (Fig. 4.3). The number of CH50 units were calculated as described before and are shown in Table 4.5. The percentage consumption was calculated to be 72.7%, 82.3% and 97.0% for HK 353, HK358 and 6664 respectively (Table 4.4). HK 353 had reduced consumption in the human serum, 72.7%, compared to 97.8% in normal pig serum, a reduction of 25.8%. Both the other serotype two strains, HK 358, and 6664 had very similar consumption rates to those in pig serum.

Similarly all 3 *E. coli* control strains consumed a degree of complement activity. The amount of human complement activity consumed by the individual strains was comparable to the amount of pig complement as described in section 4.3.1.1. The volume of serum required to produce 50% lysis was determined (Fig. 4.4) and the corresponding number of CH50 units were calculated and shown in Table 4.5. The corresponding consumption of complement activity (Table 4.4) showed that parent strain C10 did consume complement activity but at a much reduced rate compared to both the *E. coli* mutant strains. RY21 and RY22 had extremely high consumpton rates of 97.4 and 96.5% respectively compared to only 36.3% consumption by C10. The 36.3% consumption rate by *E. coli* C10 was between 36.4 and 60.7% less than the *A. pleuropneumoniae* test human serum. Both the *E. coli* mutant strains showed similar consumption rates to both the *A. pleuropneumoniae* treated pig and human serum.

4.3.1.3 Consumption of complement via the alternative pathway in pig serum incubated with *A. pleuropneumoniae*

All 3 *A. pleuropneumoniae* strains consumed almost all the available alternative complement activity. 50% lysis could not be calculated in any of the *A. pleuropneumoniae* test serum suggesting very little activity was available following incubation with the

Table. 4.5 Number of CH50 units in human serum incubated with *A. pleuropneumoniae* and *E. coli*

	Volume of sera (µl) = 1 CH50	Part of 1 CH50 unit=maximum lysis in (#)	No. of CH50 units/ml
Buffer control	41.7		142.4
A. pleuropneumoniae Strains			
HK 353	163.2		36.4
HK 358	#	0.902	23.8
6664	#	0.158	4.4
Buffer control	61.8		96.1
E. coli Strains			
C10	97.1		61.2
RY21	#	0.092	2.5
RY22	#	0.126	3.4

Fig. 4.4 Titration of classical complement activity remaining in human serum incubated with *E. coli*

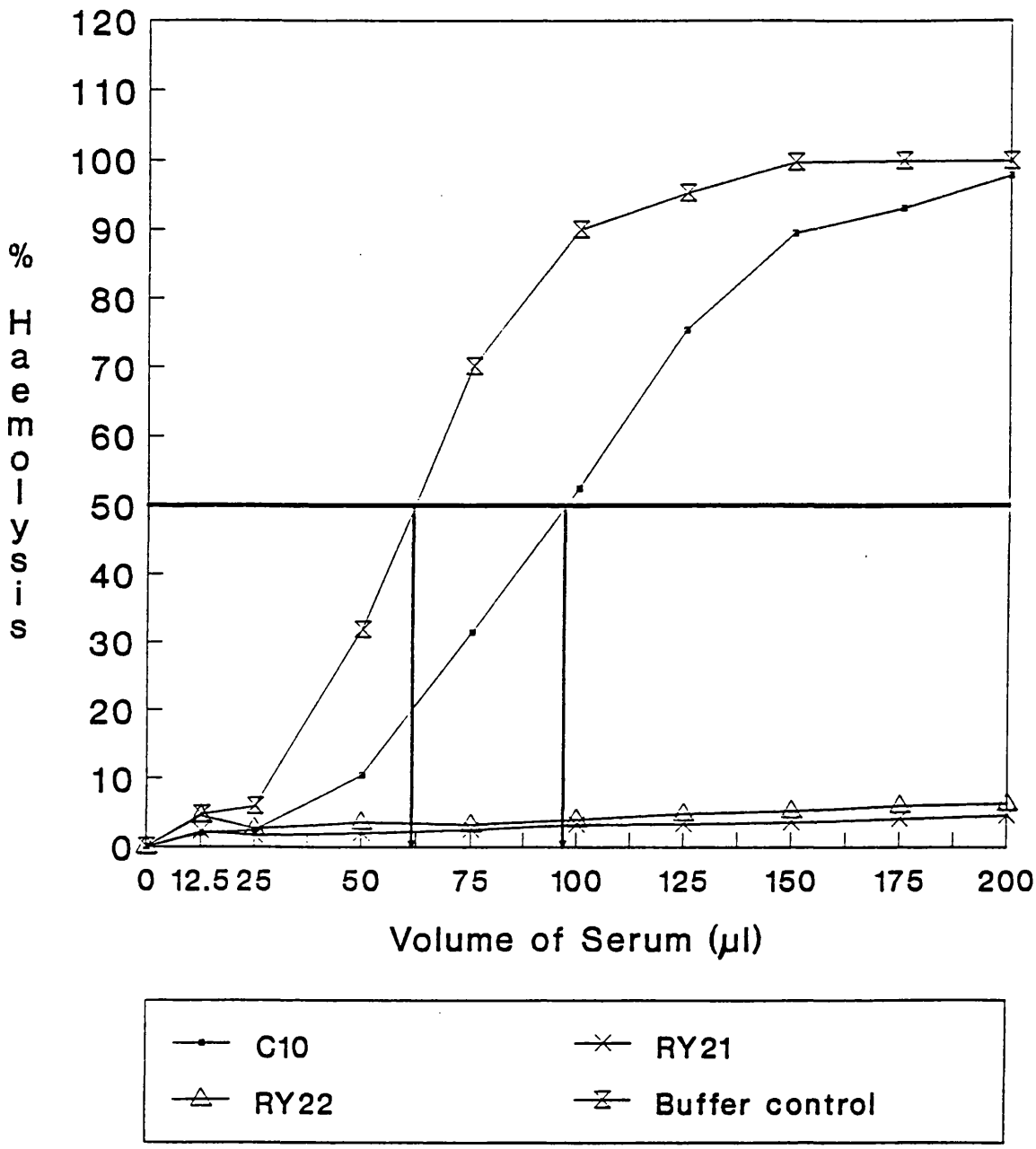
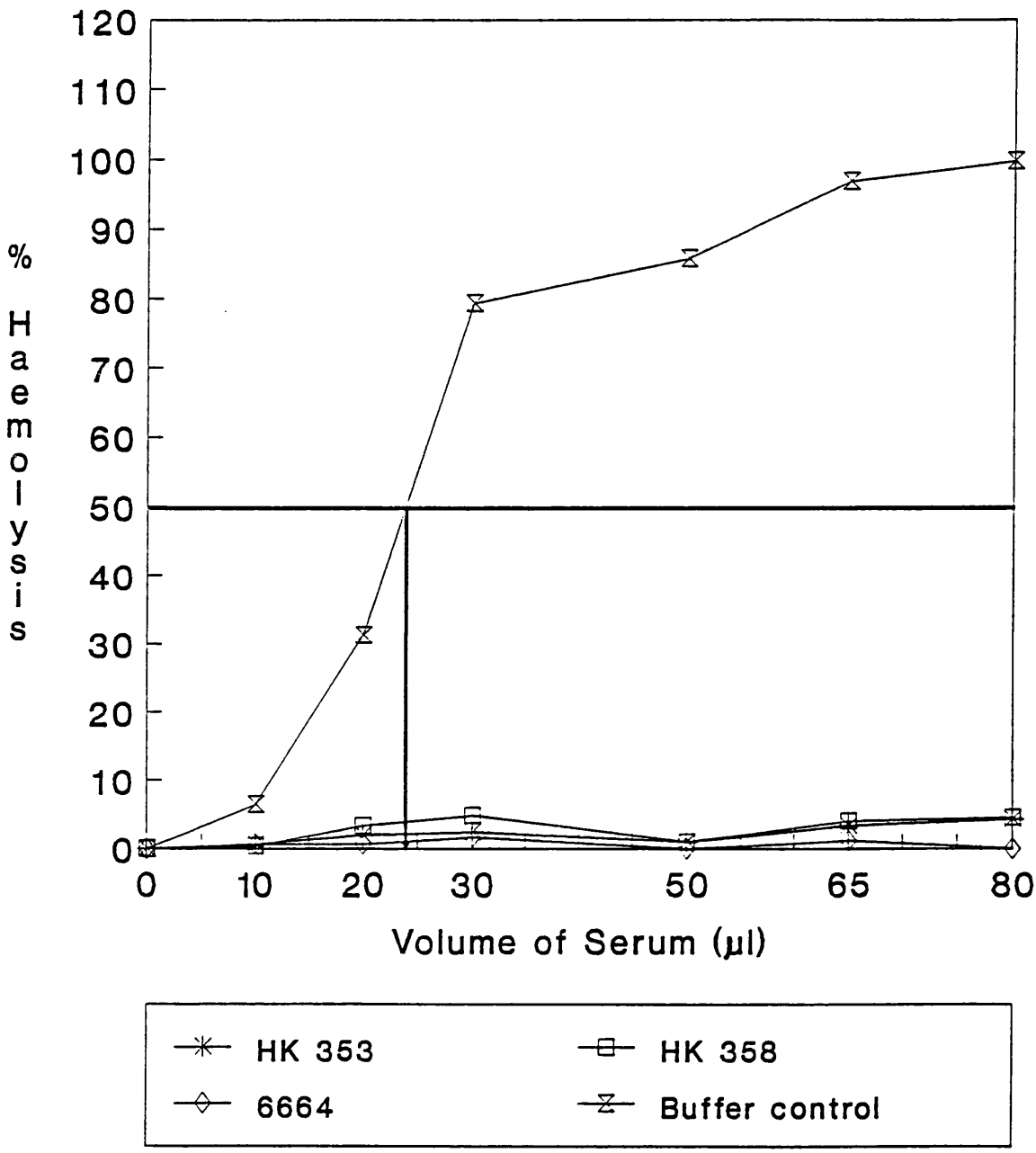


Fig. 4.5 Titration of alternative complement activity remaining in pig serum incubated with *A. pleuropneumoniae*



bacteria (Fig. 4.5). The number of AP-CH50 units was calculated for the control, and the part AP-CH50 unit for the test sera as described previously (Table 4.2) and are shown in Table 4.6. The percentage consumption values demonstrated that all the *A. pleuropneumoniae* strains showed almost complete consumption of the available complement activity in the treated pig serum compared to the control. HK 353, HK 358 and 6664 consumed 98%, 97.9 and 100% respectively (Table 4.4). The consumption rates were slightly higher in all the *A. pleuropneumoniae* strains than those tested in pig serum for classical pathway consumption.

Results similar to those previously demonstrated by the *E. coli* strains in classical complement consumption in both pig and human serum were obtained. All *E. coli* strains consumed complement activity. The volume of serum required by the control to produce 50% lysis was significantly less than all *E. coli* treated serum (Fig. 4.6). This was reflected in the number of CH50 units available in the control serum as compared to the *E. coli* treated serum (Table 4.6). The percentage consumption was 41.8%, 99.1% and 98.7% for C10, RY21 and RY22 treated serum repsectively (Table 4.4). The parent strain C10 again showed the least complement consumption (41.8%), more than 50% less than the mutant strains RY21 and RY22, which displayed almost complete consumption of the available complement activity. The consumption rates of the *E. coli* mutant strains RY21 and RY22 were similar to those observed in *A. pleuropneumoniae* treated pig serum.

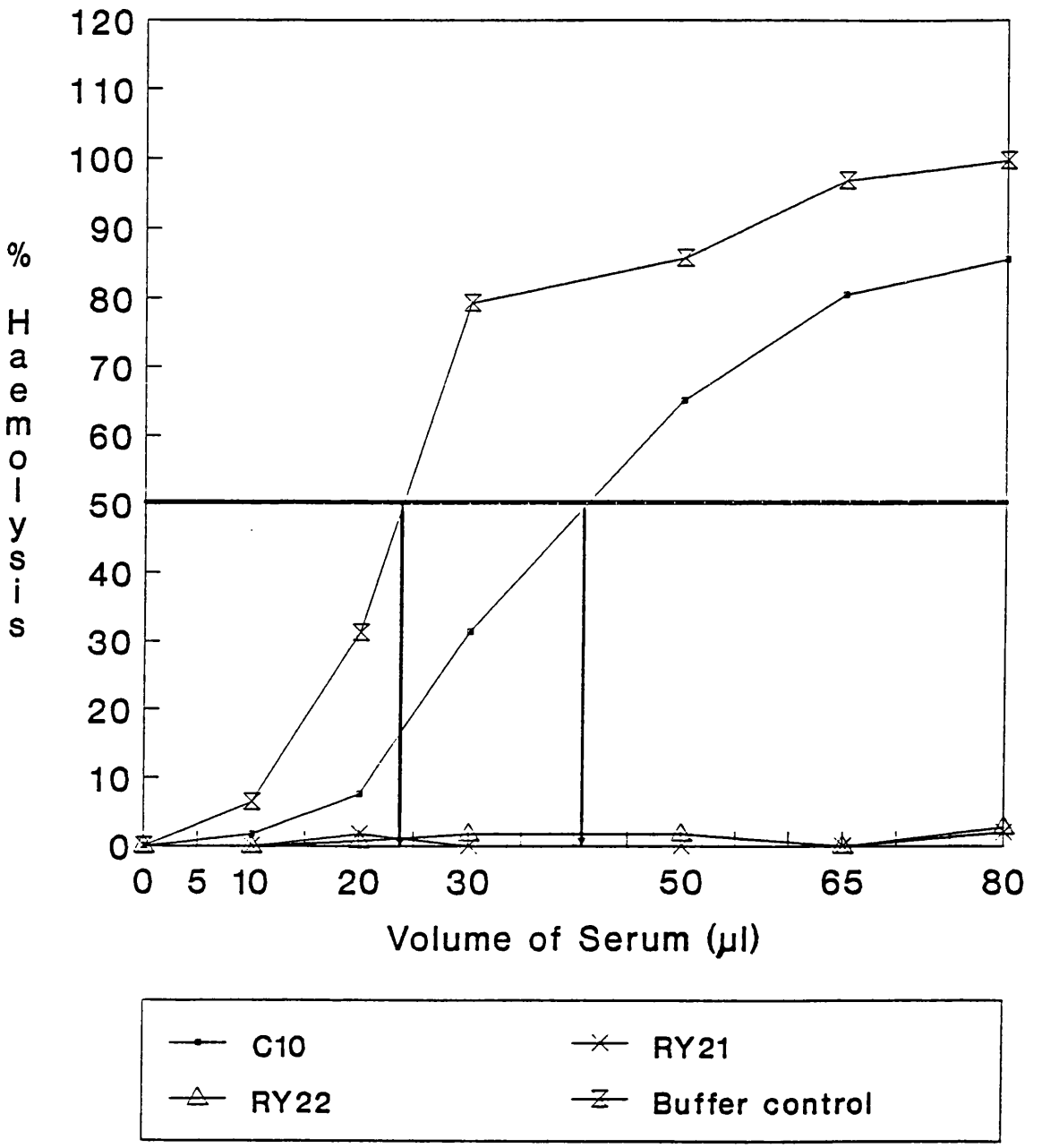
4.3.1.4 Consumption of complement via the alternative pathway in human serum incubated with *A. pleuropneumoniae*

High complement consumption rates where observed in the human serum incubated with the 3 *A. pleuropneumoniae*.

Table. 4.6 Number of AP-CH50 units in pig serum incubated both *A. pleuropneumoniae* and *E. coli*

	Pig serum - Alternative Pathway		
	Volume of sera (µl) = 1 CH50	Part of 1 CH50 unit=maximum lysis in (#)	No. of AP-CH50 units/ml
Buffer control	23.9		75.3
A. pleuropneumoniae Strains			
HK 353	#	0.084	1.5
HK 358	#	0.090	1.6
6664	#	0.000	0.0
E. coli Strains			
C10	41.1		43.8
RY21	#	0.040	0.7
RY22	#	0.054	1.0

Fig. 4.6 Titration of alternative complement activity remaining in pig serum incubated with *E. coli*



50% lysis could only be calculated for the control (Fig. 4.7). The AP-CH50 units were calculated as before (Table 4.2) and are shown in Table 4.7. The consumption rates were 96.7% for HK 353, 96.3% for HK 358 and 98.2% for 6664, demonstrating almost complete consumption of all the complement activity by all three *A. pleuropneumoniae* strains (Table 4.4).

All three *E. coli* strains consumed almost all the activity available. 50% lysis could only be calculated for the control serum and buffer (Fig. 4.8). There was a large difference in the number of available AP-CH50 units between the control and the *E. coli* treated sera (Table 4.7). The *E. coli* parent strain, C10 consumed 97.5% and the mutants, RY21 and RY22 consumed 97.9% and 97.8% of the available activity (Table 4.4). Both the mutant strains RY21 and RY22 showed similar consumption rates in the alternative pathway with those observed in pig serum and also with the *A. pleuropneumoniae* strains in both pig and human serum. Parent strain C10 also displayed similar consumption rates with both the mutant strains in the pig and human serum and with the *A. pleuropueumoniae* strains in both the sera. The high percentage of alternative pathway complement activity consumed by *E. coli* C10 in human serum was different from that previously seen. Both alternative pathway consumption in the pig serum and classical pathway consumption in both pig and human serum showed lower consumption levels of 41.8%, 50.2% and 36.3% respectively.

4.3.2 Immunoelectrophoresis

4.3.2.1 C3 activation in pig serum incubated with *A. pleuropneumoniae*

Activation of pig complement component C3 was demonstrated by the elongation of the original C3 arc.

Fig. 4.7 Titration of alternative complement activity remaining in human serum incubated with *A. pleuropneumoniae*

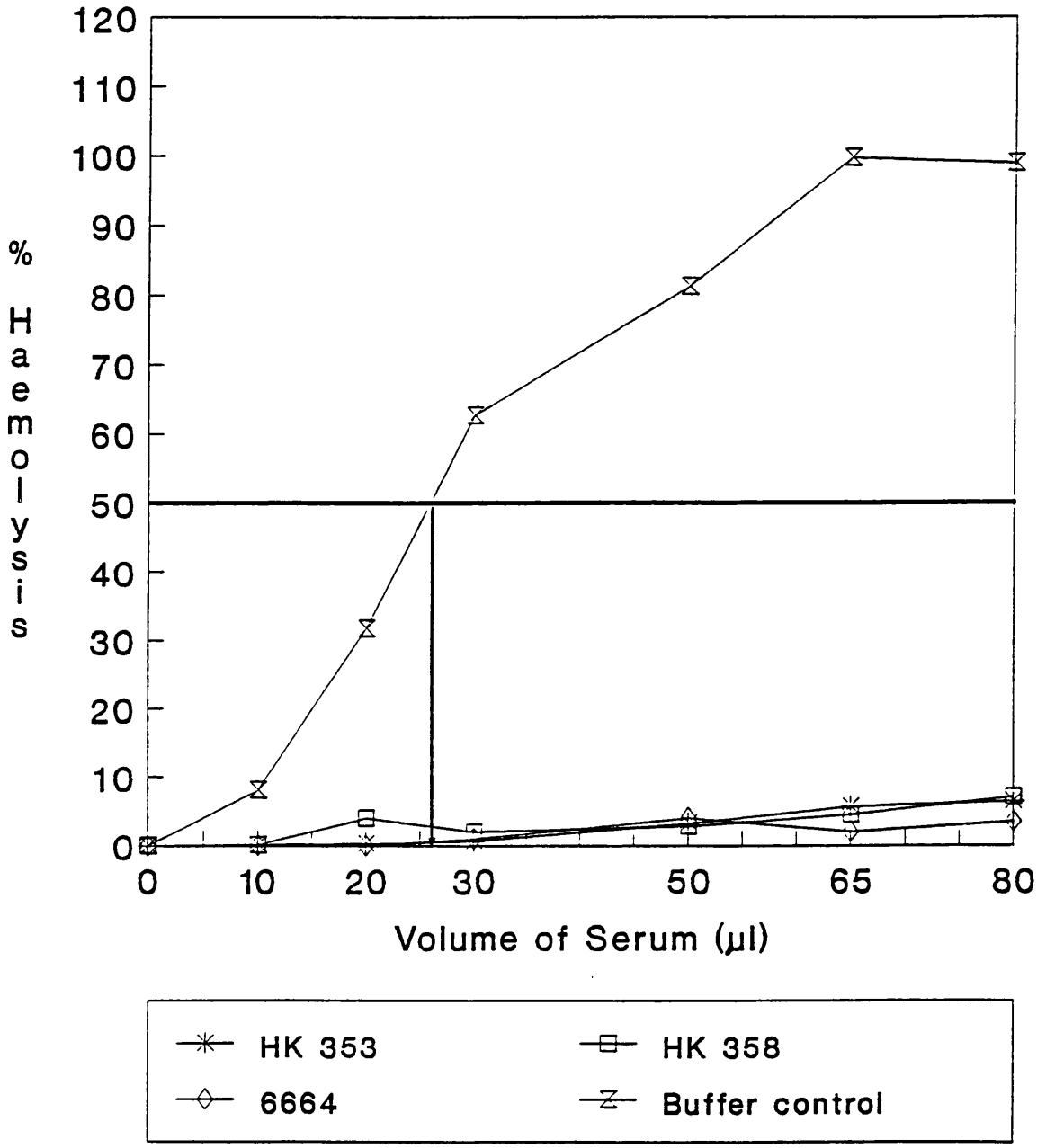
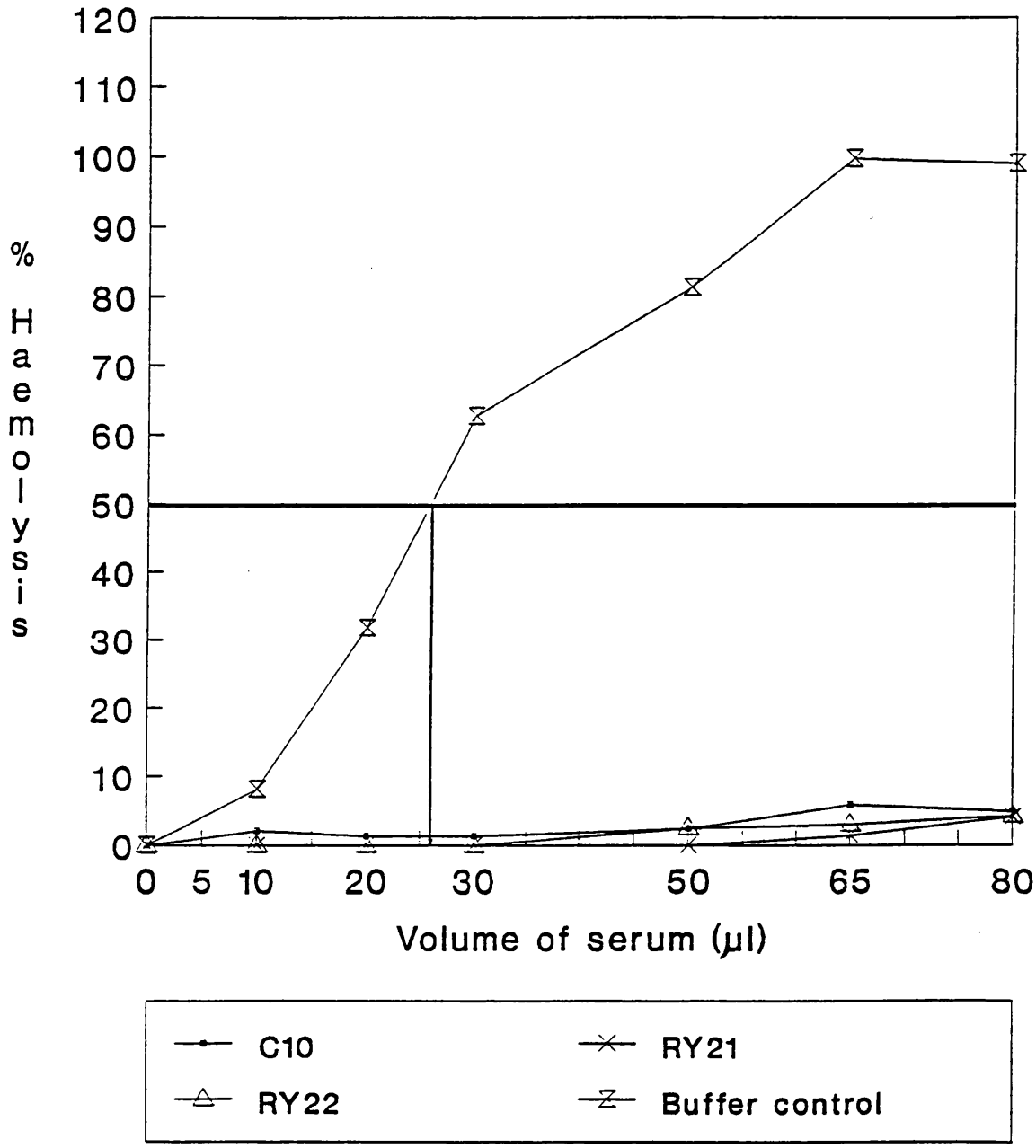


Table. 4.7 Number of AP-CH50 units in human serum incubated with *A. pleuropneumoniae* and *E. coli*

	Human serum - Alternative pathway		
	Volume of sera (ul) = 1CH50	Part of 1CH50 unit=maximum lysis in (#)	No. of AP-CH50 units/ml
Buffer control	25.5		202.0
A. pleuropneumoniae Strains			
HK 353	#	0.128	6.7
HK 358	#	0.142	7.4
6664	#	0.070	3.6
E. coli Strains			
C10	#	0.096	5.0
RY21	#	0.082	4.3
RY22	#	0.084	4.4

Fig. 4.8 Titration of alternative complement activity remaining in pig serum incubated with *E. coli*



This elongation of the arc towards the anode(+) indicated that a part of the C3 molecule had been activated, and hence broken down into smaller proteins, which migrate faster towards the anode. This thereby gives an elongated appearance to the arc. The visualisation of individual arcs produced by the different breakdown components was not possible due to the lack of specificity of the commercial anti-pig C3 serum.

All test sera previously incubated with the three *A. pleuropneumoniae* strains showed an elongation of the C3 arc towards the anode (Fig. 4.9, slides 1-3). This was similar to that observed in the positive control (Fig. 4.9, slide 4). Elongation was not seen in the negative controls of serum and buffer alone incubated in the absence of bacteria shown on the bottom of each slide (slides 1-5). These negative controls of serum and buffer alone also demonstrated that no spontaneous activation of complement C3 had occurred during incubation and immunoelectrophoresis as shown by their similar profiles to the serum and buffer control that contained EDTA prior to electrophoresis (slide 5).

Some degree of elongation was present in the test sera incubated with each of the three *E. coli* strains (Fig. 4.10, slides 1-3) demonstrating at least part of C3 had undergone activation.

4.3.2.2 C3 activation in human serum incubated with *A. pleuropneumoniae*

All test sera incubated with *A. pleuropneumoniae* also demonstrated elongation of the arc suggesting activation of C3 in human serum. Figure 4.11 shows elongation of the upper arc in slides 1-3 for HK 353, HK 358 and 6664 respectively. Controls are as as described above.

Fig. 4.9 Immunoelectrophoretic patterns of pig serum following incubation with *A. pleuropneumoniae* and developed with anti-pig C3.

Slides

1a. Pig serum incubated with *A. pleuropneumoniae* HK 353
2a. Pig serum " " " HK 358
3a. Pig serum " " " 6664
4a. Pig serum incubated with zymosan (positive control)
5a. Addition of EDTA to control pig serum prior to immunoelectrophoresis (negative control)
1-5b. Control pig serum incubated without bacteria (negative control)

Fig. 4.10 Immunoelectrophoretic patterns of pig serum following incubation with *E. coli* and developed with anti-pig C3.

Slides

1a. Pig serum incubated with *E. coli* C10
2a. " " " " " RY21
3a. " " " " " RY22
4a. " " " " zymosan (positive control)
5a. Addition of EDTA to control pig serum prior to immunoelectrophoresis (negative control)
1-5b. Control pig serum incubated without bacteria (negative control)

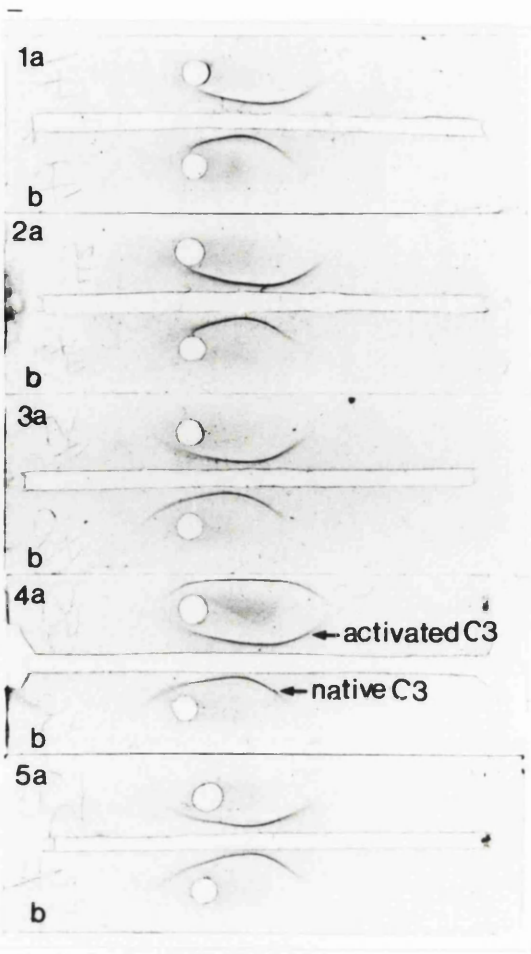


Fig.4.9

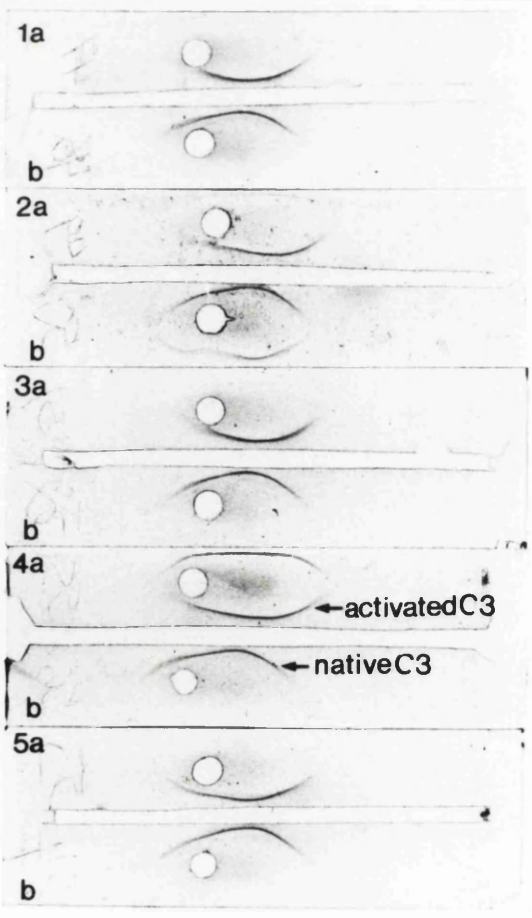


Fig. 4.10

Fig. 4.11 Immunoelectrophoretic patterns of human serum following incubation with *A. pleuropneumoniae* and developed with anti-human C3.

Slides

1a. Human serum incubated with *A. pleuropneumoniae* HK 353
2a. Human serum " " " HK 358
3a. Human serum " " " 6664
4a. Human serum incubated with zymosan (positive control)
5a. Addition of EDTA to control human serum prior to immunoelectrophoresis (negative control)
1-5b. Control human serum incubated without bacteria (negative control)

Fig. 4.12 Immunoelectrophoretic patterns of human serum following incubation with *E. coli* and developed with anti-human C3.

Slides

1a. Human serum incubated with *E. coli* C10
2a. " " " " " RY21
3a. " " " " " RY22
4a. " " " " zymosan (positive control)
5a. Addition of EDTA to control human serum prior to immunoelectrophoresis (negative control)
1-5b. Control human serum incubated without bacteria (negative control)

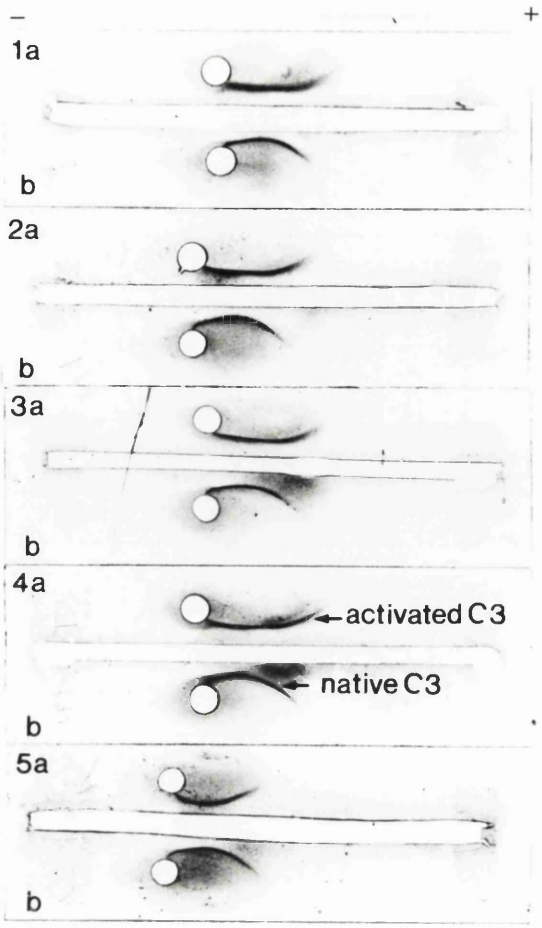


Fig. 4.11

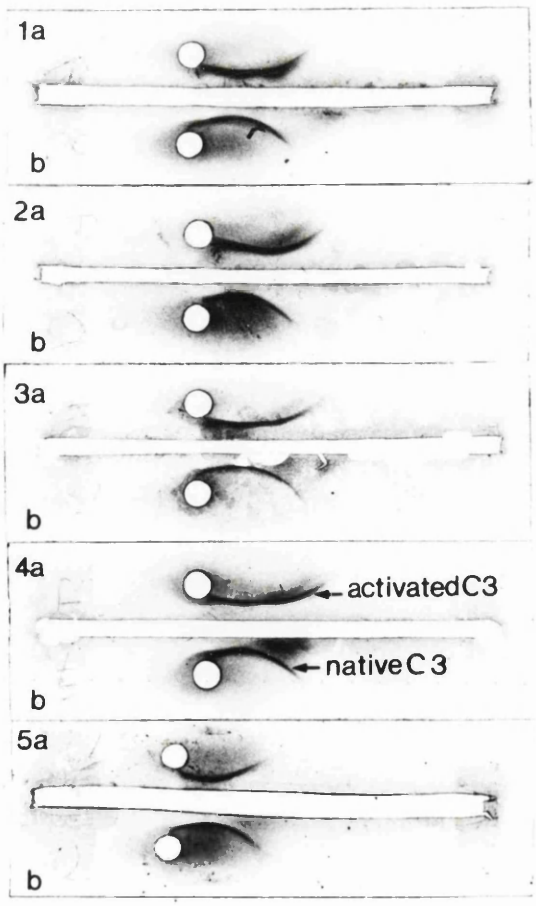


Fig. 4.12

All the test sera incubated with the *E. coli* strains C10, RY21 and RY22 showed partial activation of C3 indicated by the elongation of the arcs (Fig. 4.12).

4.3.2.3 Factor B in human serum incubated with *A. pleuropneumoniae*

Activation of factor B is denoted by the appearance of a second arc (Bb) located at the cathode side of the original nonactivated protein (B), usually together with some of the naive protein remaining. The smaller cleavage protein, Ba, can sometimes be seen overlapping the original factor B protein arc.

The three test sera incubated with each *A. pleuropneumoniae* all demonstrated the presence of additional arcs indicative of activation (Fig. 4.13, slides 1-3) similar to those seen in the zymosan positive control (slide 4). The negative controls of buffer and serum alone, and EDTA, were both negative indicating no spontaneous complement activation (slide 5).

Cleavage proteins arcs developed in slides 1, 2 and 3 (Fig. 4.14) indicated also that factor B activation had resulted during incubation of the human serum with all three *E. coli* isolates. Results for the controls resembled those described above, validating the test results (slides 4 & 5).

4.3.2.4 Determination of the pathway responsible for C3 activation

Attempts to assess by which pathway C3 activation occurred were unsuccessful. The process used to degrade factor B (described in Appendix 2) and thereby eliminating the alternative pathway as a route for C3 activation, also appeared to activate C3. Activation of

Fig. 4.13 Immunoelectrophoretic patterns of human serum following incubation with *A. pleuropneumoniae* and developed with anti-human factor B.

Factor B activation fragments are denoted by Ba and Bb.

Slides

1a. Human serum incubated with *A. pleuropneumoniae* HK 353
2a. Human serum " " " HK 358
3a. Human serum " " " 6664
4a. Human serum incubated with zymosan (positive control)
5a. Addition of EDTA to control human serum prior to immunoelectrophoresis (negative control)
1-5b. Control human serum incubated without bacteria (negative control)

Fig. 4.14 Immunoelectrophoretic patterns of human serum following incubation with *E. coli* and developed with anti-human factor B.

Factor B activation fragments are denoted by Ba and Bb.

Slides

1a. Human serum incubated with *E. coli* C10
2a. " " " " " RY21
3a. " " " " " RY22
4a. " " " " zymosan (positive control)
5a. Addition of EDTA to control human serum prior to immunoelectrophoresis (negative control)
1-5b. Control human serum incubated without bacteria (negative control)

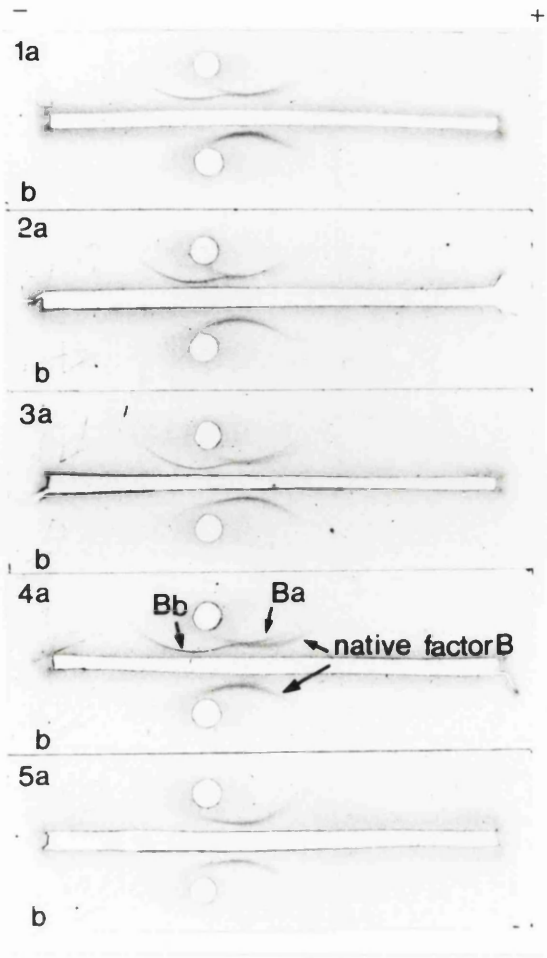


Fig. 4.13

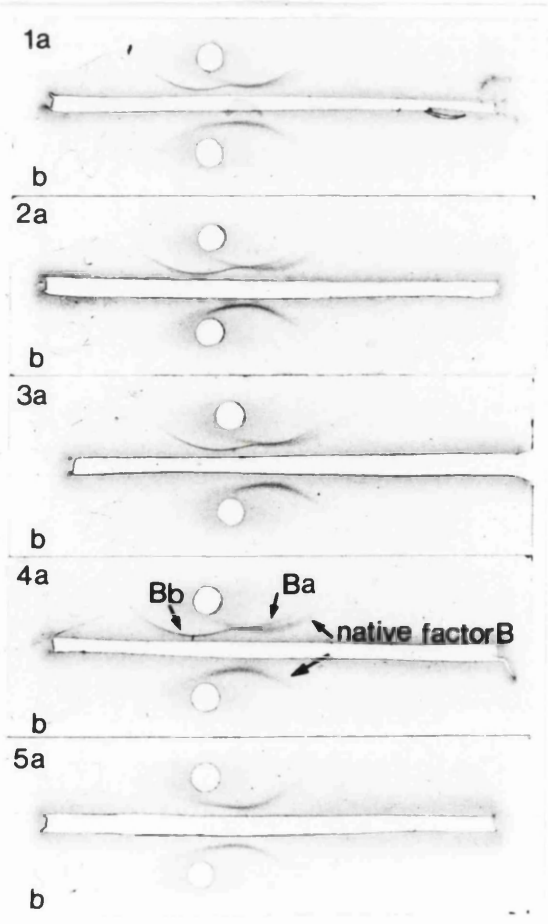


Fig. 4.14

C3 exclusively via the classical pathway could therefore not be determined. The methods used to evaluate C3 activation via alternative pathway activation alone in the pig serum (described in Appendix 2) could not produce reproducible results.

4.3.2.5 Determination of C3 activation exclusively via the classical pathway in human serum

As described for the pig serum, activation of C3 occurred in the factor B depleted serum prior to incubation with bacteria preventing assessment of C3 activation via the classical pathway alone.

4.3.2.6 Analysis of C3 and factor B activation fragments in both human and pig serum by SDS-PAGE and Western blotting

Attempts were made to visualise and quantify the C3 and factor B activation fragments produced by immunoelectrophoresis using SDS-PAGE and Western blotting techniques. These attempts were unsuccessful due to the lack of sensitivity of the detection antisera for use in the above analysis methods.

4.4 DISCUSSION

Many Gram-negative bacteria have been shown to be resistant to the bactericidal effects of the complement system (Taylor, 1983; Joiner *et al.*, 1984; Joiner, 1988). Serum resistance has been related to several factors including the presence of a capsule, composition of lipopolysaccharide, plasmid mediated factors and also certain outer membrane proteins (Taylor, 1983). *A. pleuropneumoniae* has previously found to be resistant to pig complement even in the presence of specific antibody (Chapter 3, Rycroft & Cullen, 1990a; Inzana *et al.*, 1988). The outer membrane appears not to be the site of

the observed resistance (Chapter 3, Rycroft & Cullen, 1990a) however the presence of a capsule is thought to be involved in serum resistance (Inzana *et al.*, 1988). The mode of complement resistance by *A. pleuropneumoniae* is not known.

One of the mechanisms that complement resistant organisms employ to evade the detrimental actions of complement, is to avoid activating the complement system (Joiner, 1988). The first aim of this work was to assess whether this was the mode of complement resistance employed by *A. pleuropneumoniae*.

The loss of activity of the complement pathways, depicted by the non-lysis of red blood cells in the functional haemolytic assays, is usually a result of the absence of one or more component(s) of either the alternative, or the classical pathway, or of the terminal components, which are utilised by both pathways (Whaley, 1985). Classical pathway activation can lead to the consumption of the early components C1, C4 and C2, and alternative pathway activation to B, D and properdin consumption. Activation of either pathway leads to the cleavage of C3, C5 and the incorporation of C6, C7, C8 and C9 into the C5b-C9 membrane attack complex.

The initial aim of the work described in this chapter was to assess whether *A. pleuropneumoniae* strains were capable of activating complement via the classical and the alternative pathways. The very low lysis levels of red blood cells in the functional haemolytic assays, which can be correlated with the amount of complement activity present, found for both the classical and the alternative pathway assays, demonstrated extensive complement consumption in the presence of *A. pleuropneumoniae* (Table 4.4). Similar results in human serum via both the classical and the alternative pathways suggest that *A. pleuropneumoniae* may behave in

a similar fashion in both pig and human serum (Table 4.4).

The *E. coli* K1 positive C10 control behaved as expected, consuming around half of the complement activity via the classical pathway (Table 4.4) (van Dijk *et al.*, 1979). These lower consumption levels in the human (36.3%) serum are in agreement with others who found between 10 and 50% of complement had been consumed via the classical pathway by a K1 positive *E. coli* (van Dijk *et al.*, 1979). This is thought to be due to the presence of a K1 type capsular material which can block the activation of complement by the layer of LPS below the capsule to varying degrees (van Dijk *et al.*, 1979). The 50.2% consumption seen in the pig serum (Table 4.4) is also similar to that previously seen with human serum suggesting that *E. coli* behaves similarly in both sera. The difference in the consumption of alternative pathway by *E. coli* C10 in pig (41.8%) and human (97.5%) serum may represent differences in the alternative pathway between the two sera (Table 4.4). Although the K1 capsule is a homopolymer of sialic acid and thus represents a molecule incapable of activating the alternative pathway, it has been shown that its presence does not completely block subcapsular activation by the LPS. Similar high percentages of alternative pathway consumption by a K1 *E. coli* strain have also been reported (van Dijk, 1979). The *E. coli* RY22 mutant which does not possess capsular material, is a much more efficent activator of complement, consuming most, if not all of the available complement activity, in both the pig and human serum by both pathways and as also seen with RY21 (Table 4.4).

Complement consumption can however occur without activation. One of the important complement components is C3, which can be activated via the classical or the alternative pathways. The activation of this component

would assess whether the complement cascade was functional at least up to the C3 stage. Immunoelectrophoresis confirmed that C3 was activated in both pig and human serum by all the *A. pleuropneumoniae* strains tested (Fig. 4.9 & 4.11). As explained previously, C3 can be activated via the alternative or the classical or both pathways. Factor B activation was assessed only in the human serum due to the availability of reagents. All *A. pleuropneumoniae* strains activated factor B yeilding its two cleavage proteins, Ba and Bb (Fig. 4.13). Treatment to remove the alternative pathway in the human serum to evaluate C3 via the classical, resulted in the non-specific activation of C3. Similar attempts to selectively remove each pathway in the pig serum also non-specifically activated of C3 by the methods used and were therefore abandoned.

In summary each of the three *A. pleuropneumoniae* strains tested consumed the majority of the available complement activity via both the classical and the alternative pathways in both pig and human serum determined using the functional haemolytic assays (Table. 4.4). Activation of the complement component C3, which can be activated via either the classical or the alternative pathway, was demonstrated in both the pig and human serum incubated with each of the *A. pleuropneumoniae* strains (Fig. 4.9 & 4.11). Activation of factor B, indicative of alternative pathway activation, was shown in human serum incubated with all the *A. pleuropneumoniae* strains (Fig. 4.13).

The ability of *A. pleuropneumoniae* to avoid complement mediated bactericidal activity therefore appears not to be a result of non-activation of the complement cascade consumption, at least up to the C3 stage, as shown by the consumption of complement activity and activation of complement up to C3.

CHAPTER 5

THE ROLE OF BACTERIAL VIABILITY AND HAEMOLYTIC ACTIVITY SECRETED BY *A. PLEUROPNEUMONIAE* IN COMPLEMENT CONSUMPTION

5.1 INTRODUCTION

As discussed in the previous chapter, the inability of *A. pleuropneumoniae* to activate complement, at least to the C3 stage, appears not to be the mechanism used to avoid complement bactericidal activity. A second mechanism utilised by certain serum resistant bacteria to avoid complement damage, is to release molecules that can activate, deplete or destroy complement (Joiner, 1988).

Some of the these mechanisms employed by microorganisms to avoid complement mediated damage, may or may not be dependent on cell viability. The first aim was therefore to examine whether viable and non-viable bacteria alike were capable of consuming complement activity. The next question was: if complement consumption was dependent on bacterial viability, were the secreted products of metabolism, which include the haemolytic activity type II (Hly II), involved in complement consumption by *A. pleuropneumoniae*?

5.2 MATERIAL AND METHODS

5.2.1 Total complement-mediated bactericidal capacity

The total complement-mediated bactericidal activity in serum, previously incubated with *A. pleuropneumoniae* (test serum), was assessed by incubating this serum with a serum sensitive *E. coli* strain, RY22. The concentration of serum incubated with *A. pleuropneumoniae*, was the lowest concentration that adequately killed *E. coli* RY22 at a cell concentration of 5 x 10^6 cells in a 1ml reaction volume and after 1 hours' incubation period. Any consumption of complement activity in the test sera following incubation with the *A. pleuropneumoniae* strains, HK 353, HK 361 and 6664, would therefore result in an increase in the number of

CFU of the serum sensitive strain, RY22. Alternatively, if no complement activity had been consumed by preincubation with the *A. pleuropneumoniae* strains, this would result in a decrease or death of the serum sensitive *E. coli* strain, RY22. Heat inactivated serum containing no complement activity, and serum preincubated without *A. pleuropneumoniae*, were used as controls. 100μl of test serum was incubated with 900 μl of buffer M containing 5 x 10^6 *E. coli* RY22. The reaction mixture was incubated at 37^{o}C for 1 hour. Samples were taken at time 0 and 1 hour, and the viability (CFU) of *E. coli* RY22 was assessed by the pour plate technique (3.2.1).

5.2.2 Inactivation of bacteria

Bacteria were grown as previously described in 3.2.1. and inactivated either for 2 minutes at 100^{o}C, or with 0.5% v/v formalin overnight.

5.2.3 Consumption of complement activity by non-viable bacteria

Viable and non viable bacterial cells (grown and processed as described in 3.2.1 and above respectively) were adjusted to 7.5 x 10^8 cells in 100μl of buffer M (3.2.1) and incubated with an equal volume of serum for 30 minutes at 37^{o}C. Following centrifugation to remove the bacteria, 100μl of the test serum was assessed for complement-mediated bactericidal activity against the indicator serum sensitive *E. coli* strain RY22 as described in the assessment of total complement mediated bactericidal activity (5.2.1).

5.2.4 Production of haemolytic activity by *A. pleuropneumoniae* in culture supernatant

A. pleuropneumoniae strains were first grown to logarithmic phase as described in 3.2.1. Bacterial cultures were then subcultured (1/25) into fresh TSB/NAD and grown for a further 2.25 hours in an orbital incubator at 80 rpm. Following centrifugation (10,000 x g for 10 minutes at 4°C) to remove the bacteria, the supernatant was used immediately or stored frozen at -20°C.

5.2.5 Haemolytic activity assessment and quantification

50µl of culture supernatant was diluted and added to 350µl of haemolysin assay buffer in a microcentrifuge. 400µl of 5% (v/v) sheep erythrocytes, washed twice in saline, was added and the mixture incubated at 37°C for 60 minutes. Tubes were then gently mixed, centrifuged for 5 seconds at 11,300 x g, and the absorbance of the resultant supernatants was measured at 541nm.

5.2.6 Complement consumption by the extracellular haemolysin of *A. pleuropneumoniae*

Bacteria-free culture supernatant (100µl) was incubated with an equal volume of serum at 37°C for 30 minutes. Remaining complement bactericidal activity in the test serum was determined as described in the assessment of total complement-mediated bactericidal assay. To ensure that the reduction of *E. coli* RY22 observed in the pig serum treated with haemolytically active supernatant was not due to any detrimental effect of the haemolytic substance, *E. coli* RY22 was incubated in the presence of the haemolytic supernatant only.

5.3 RESULTS

5.3.1 Complement consumption by viable and non-viable bacteria

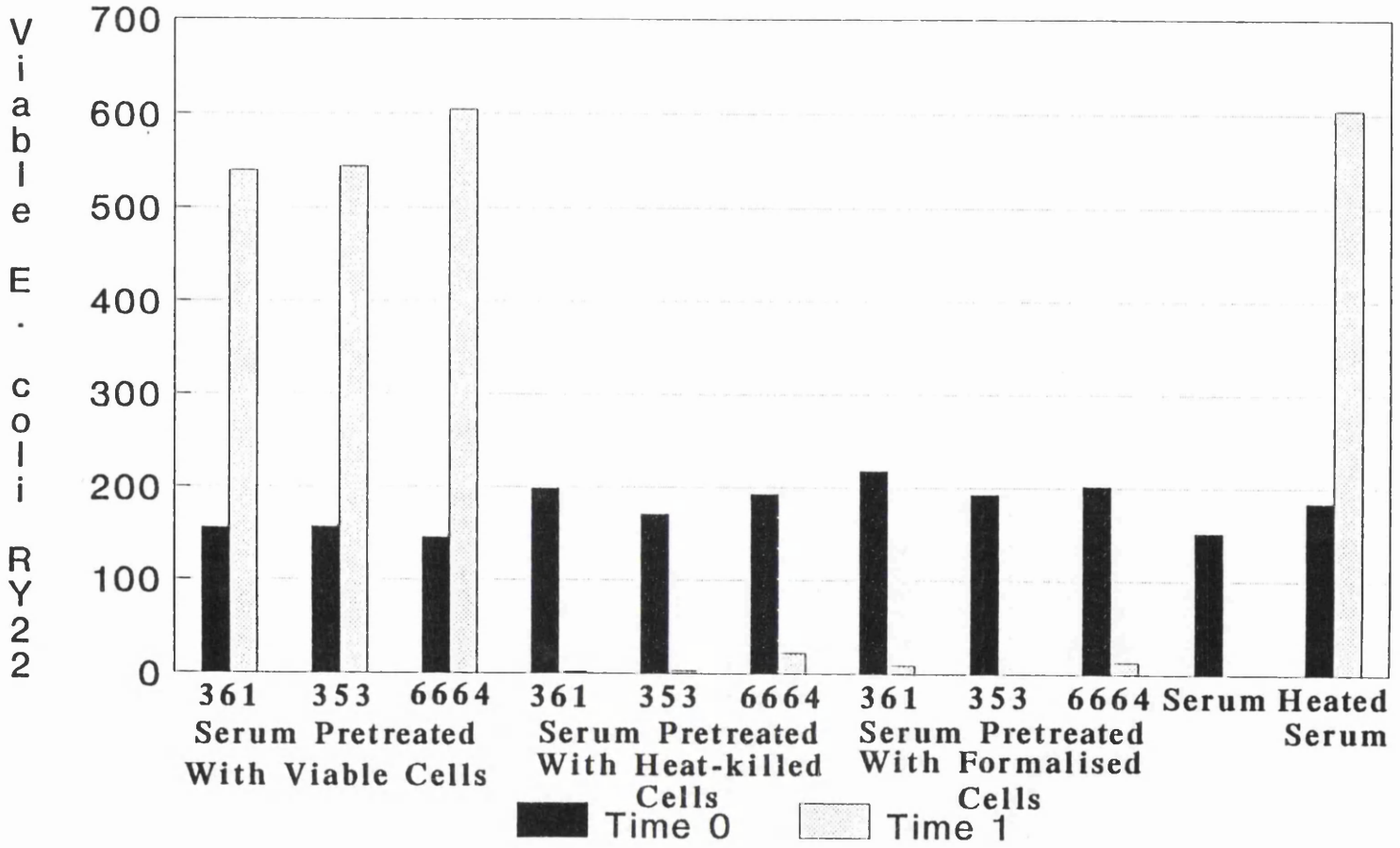
All test sera pretreated with non-viable *A. pleuropneumoniae* bacteria killed the serum sensitive *E. coli* RY22 strain after incubation for 1 hour (Fig. 5.1). The two procedures for rendering cells non-viable gave identical results. Neither resulted in the consumption of complement activity as shown by the death or decline of *E. coli* RY22 (Fig. 5.1). This was in contrast to the test serum preincubated with viable *A. pleuropneumoniae* (as previously demonstrated in the complement consumption assays using viable cells in Chapter 4). The test sera treated with the corresponding viable bacteria resulted in an increase in the survival of serum sensitive *E. coli* RY22. Control serum containing no complement activity, allowed survival of *E. coli* RY22 numbers comparable to those with serum preincubated with viable *A. pleuropneumoniae* strains. Control normal serum preincubated without viable *A. pleuropneumoniae* resulted in the death of *E. coli* RY22 (Fig. 5.1).

5.3.2 Complement consumption by extracellular haemolytic activity

Bacterial supernatants from 3 *A. pleuropneumoniae* strains were assessed for haemolytic activity. *A. pleuropneumoniae* strain, HK 361 produced high levels of haemolytic activity compared to HK 353 and 6664, which both produced very poor detectable levels of haemolytic activity by the same method (Table 5.1). The culture supernatants from all three strains were tested for the ability to consume complement activity.

All sera, pretreated with the *A. pleuropneumoniae* culture supernatants, killed the serum sensitive

Fig. 5.1 Survival of serum sensitive *E. coli* in serum preincubated with non-viable and viable *A. pleuropneumoniae*



A. pleuropneumoniae Strains	OD 541 nm
HK 361	1.388
HK 353	0.091
6664	0.073
Controls	
Saline	0.015
Culture broth	0.023
100% Lysis	1.445

Table. 5.1 Haemolysin levels in *A. pleuropneumoniae* culture supernatants

indicator *E. coli* strain. Both haemolytic and non-haemolytic culture supernatant alike resulted in the complete killing of RY22 after 1 hour (Figs. 5.2, 5.3 & 5.4). This suggested there was no relationship between the amount of complement consumption and the level of haemolytic activity in the culture supernatant. As demonstrated previously in Chapter 4, viable bacteria consumed all the available complement activity resulting in survival of the indicator serum sensitive *E. coli* RY22 (Figs. 5.2, 5.3 & 5.4). The serum control which possessed full complement activity demonstrated the decline of *E. coli* RY22 in this serum (Figs. 5.2, 5.3 & 5.4) and was again in sharp contrast to the large increase seen in the heat inactivated serum which possessed no complement activity (Figs. 5.2, 5.3 & 5.4). No inhibition of *E. coli* RY22 growth was observed when incubated in haemolytically active supernatant for 1 hour (Fig. 5.2). The bacterial growth rate was similar to that in heat-inactivated serum.

The results shown in Figs. 5.1-5.4 and Table 5.1 are representative of 3 separate experiments.

5.4 Discussion

Many pathogenic bacteria are resistant to the bactericidal effects of the complement system. There are a range of mechanisms by which bacteria achieve this resistance to complement activity. Certain bacteria do not activate or consume complement activity. However this was found not to be the mechanism used by *A. pleuropneumoniae* as detailed in the last chapter. A second mechanism used by certain serum resistant bacteria to avoid complement damage is to release molecules that can activate, deplete or destroy complement components (Joiner, 1988).

Fig. 5.2 Survival of serum sensitive *E. coli* in serum preincubated with HK 361 culture supernatant

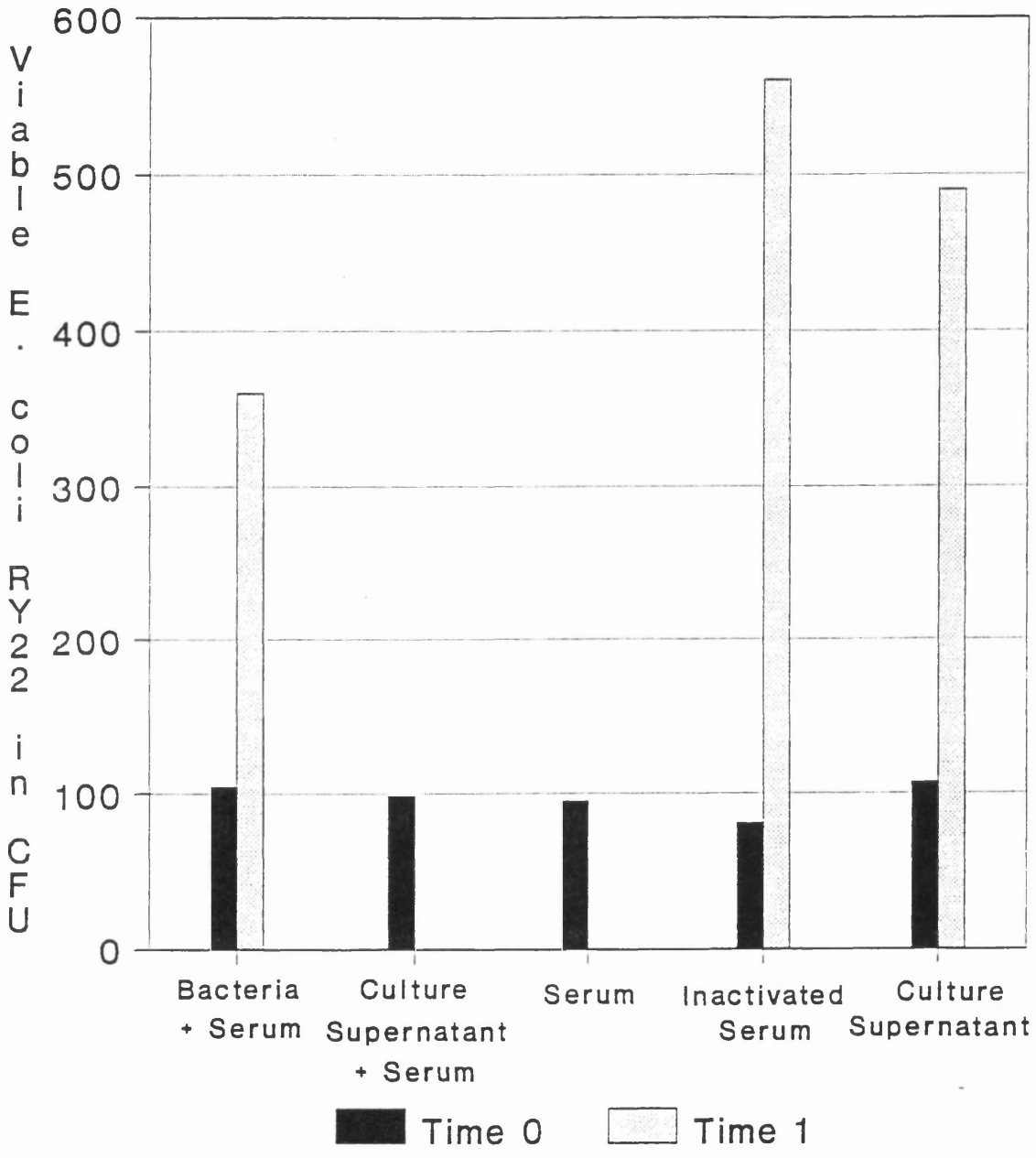


Fig. 5.3 Survival of serum sensitive *E. coli* RY22 in serum preincubated with HK 353 culture supernatant

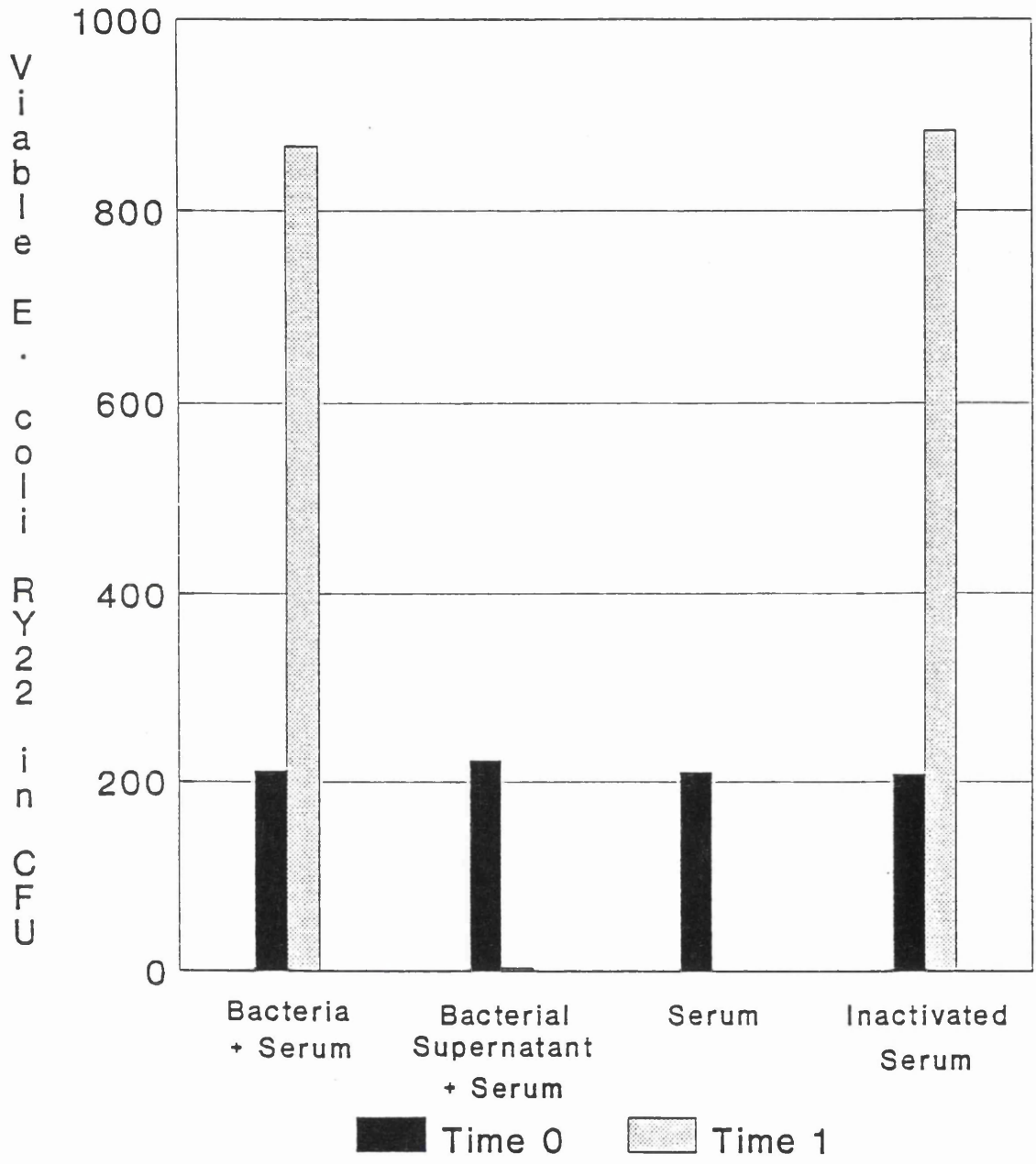
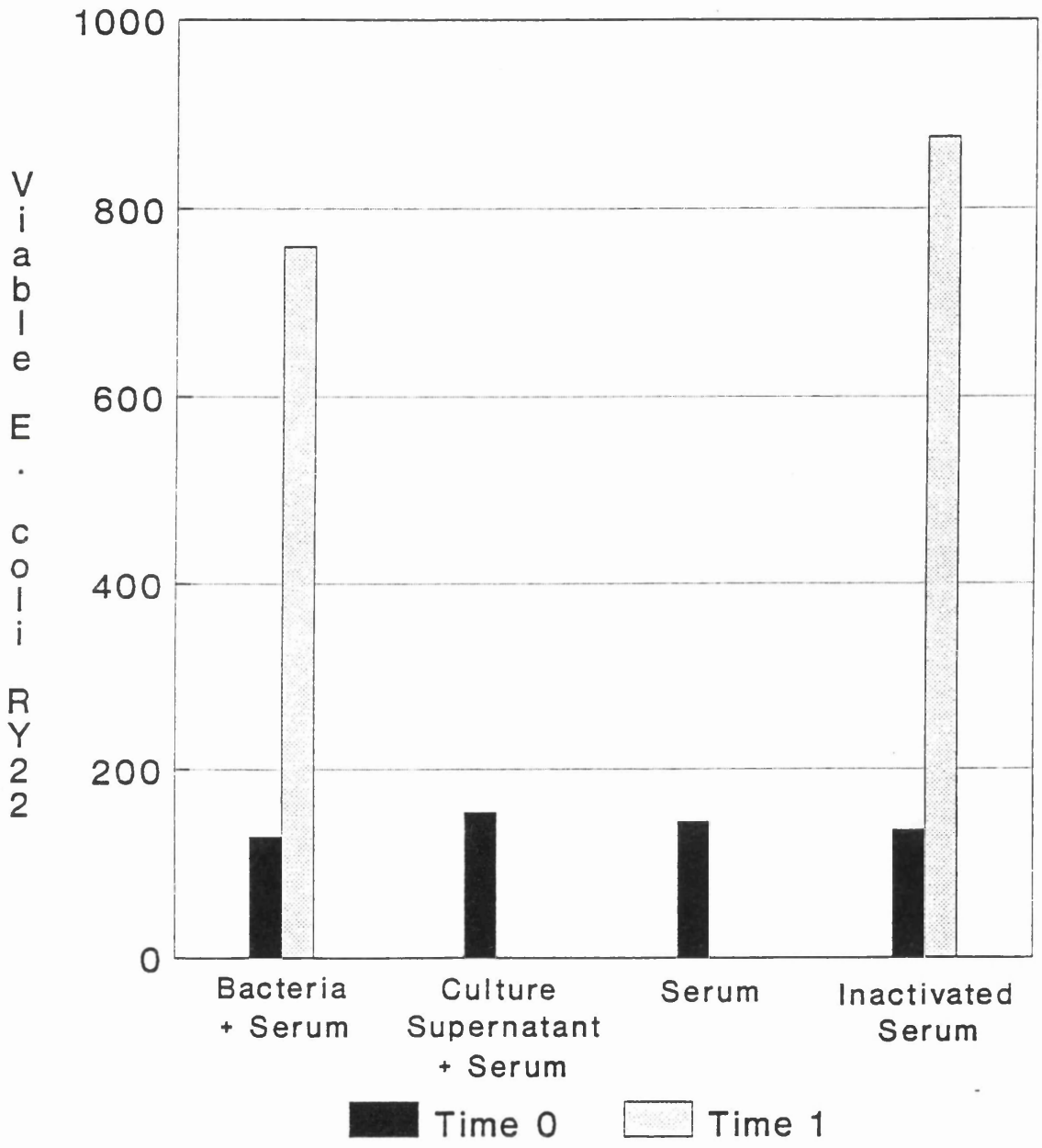


Fig. 5.4 Survival of serum sensitive *E. coli* RY22 in serum preincubated with 6664 culture supernatant



Examples of these 3 different classes of molecules include firstly, components shed by microorganisms that can activate the classical or alternative pathways resulting in the microbial surface becoming inert to the complement cascade. The parasite, *Schistosoma mansoni* has been shown to shed molecules capable of consuming C3 from serum resulting in them being non-activators of the complement cascade (Marikovsky *et al.*, 1986; Rasmussen & Kemp, 1987; Samuelson *et al.*, 1980). The molecules shed from the parasitic surface include residual cercarial glycocalyx, which is a known C3 acceptor (Samuelson & Caulfield, 1986).

Secondly, other microorganisms release substances capable of depleting complement components both close to and at a distance from the cell membrane. Examples of these include the capsule of type Ia group B Streptococci which has been suggested to reduce C1 using this mechanism (Levy *et al.*, 1984) and the mucoexopolysaccharide slime of *Pseudomonas aeruginosa* which activates the alternative pathway cleaving C3 and factor B (Lambris *et al.*, 1982). Soluble techoic acid produced by *Staphylococcus aureus* has also been shown to consume early classical pathway components from immune serum (Joiner, 1988).

Lastly, certain microbial products can also inactivate or destroy complement components other than by conventional complement activation. These include the local high levels of ammonia produced by *P. aeruginosa* (Hostetter *et al.*, 1986). Microbial proteases are also involved in inactivating complement components including elastine from *P. aeruginosa* which cleaves a number of complement molecules (Schultz & Miller, 1974). Other proteases are non-specific, attacking a range of complement and non-complement substrates (Catanese & Kress, 1984; Markham *et al.*, 1979; Nilsson *et al.*, 1985;

Schultz & Miller, 1974; Sundqvist *et al.*, 1985; Ward *et al.*, 1973).

As discussed in the introduction, some of the above mentioned mechanisms employed by the microorganisms may or may not depend on cell viability. It has been previously shown in Chapter 4 that viable *A. pleuropneumoniae* are capable of activating and consuming complement activity. The next aim was therefore to assess whether non-viable *A. pleuropneumoniae* were also able to consume complement activity.

Non-viable *A. pleuropneumoniae*, unlike their viable counterparts, were unable to consume complement activity. This was shown by the decrease in the numbers of CFU of the serum sensitive *E. coli*.

The difference observed between viable and non-viable bacteria in their ability to consume complement activity may suggest that metabolically active bacteria are necessary to consume activity. Alternatively, the component(s) responsible for complement consumption may be located on both viable and non-viable cells alike and treatment with heat or formaldehyde, used to kill the bacteria, may in some way damage or destroy those components rendering them unable to consume complement activity.

Assuming that the difference in the ability of *A. pleuropneumoniae* to consume complement was due to its viability and not to the inactivation process, it was decided to look at the products of metabolism in an attempt to identify the responsible component(s) for complement consumption. Certain *A. pleuropneumoniae* strains have been documented to possess haemolytic activity in their culture supernatant (Nakai *et al.*, 1983; Kume & Nakai, 1986; Rosendal *et al.*, 1988; Frey & Nicolet, 1990). This haemolytic activity has been

associated with toxicity against a range of cell types (Kume & Nakai, 1986; Kume *et al.*, 1986b; Udeze & Kadis, 1988; Rosendal *et al.*, 1988). The culture supernatants of some *A. pleuropneumoniae* strains being studied in this laboratory were also found to possess haemolytic activity (Rycroft *et al.*, 1991). It was therefore decided to assess whether this toxic activity may also be involved in the observed consumption of complement activity by viable *A. pleuropneumoniae*.

It was found however that haemolytically active bacterial supernatant was unable to consume complement activity. This would suggest that the haemolytic activity present in *A. pleuropneumoniae* culture supernatants was not responsible for the observed complement consumption by viable bacterial cells. The two culture supernatants that produced very low, if any haemolytic activity, also did not consume complement activity. The lack of difference in complement consumption observed between haemolytically active and non-active supernatant also confirms the lack of involvement of the haemolytic activity in complement consumption.

These strains also produce a cytotoxin (pleurotoxin) which would also have been present in the supernatants (Rycroft *et al.*, 1991). It can therefore be concluded that neither Hly II nor pleurotoxin is responsible for the apparent consumption of complement. This failure of culture supernatant to consume complement does not necessarily preclude the involvement of other secreted products. Certain secreted components remain intimately associated with the bacterial cell membrane (Joiner, 1988).

The mode of complement evasion of *A. pleuropneumoniae* still remains unknown. Nevertheless, it is clear from the results that both the failure to activate complement

CHAPTER 6

PHAGOCYTOSIS OF *A. PLEUROPNEUMONIAE*

6.1 INTRODUCTION

The process of phagocytosis is a defence mechanism of major importance in removing pathogens from the lung. Since the beginning of the 1980's, *A. pleuropneumoniae* has been reported to have a toxic effect for porcine phagocytic cells. Both viable whole cells and culture supernatants were found to possess toxicity against alveolar and peritoneal macrophages, polymorphonuclear leukocytes (PMN) and peripheral blood monocytes (Bendixen *et al.*, 1981; Kume & Nakai, 1986; Kume *et al.*, 1986b; Pijoan, 1986; Udeze & Kadis, 1988; Rosendal *et al.*, 1988; van Leengoed *et al.*, 1989). Likely candidates as causes of this damage were the secreted extracellular haemolytic and cytotoxic activities of *A. pleuropneumoniae* (Udeze & Kadis, 1988; Rosendal *et al.*, 1988; van Leengoed *et al.*, 1989; Frey & Nicolet, 1990). The mechanism by which these toxins damage phagocytic cells is unknown; however, it has been shown that one haemolysin of serotype 1 strain, 4074, forms pores in phospholipid membranes (Lalonde *et al.*, 1989).

At the start of this work, phagocytosis of *A. pleuropneumoniae* had been documented using porcine PMN and alveolar macrophages (Inzana *et al.*, 1988; Udeze & Kadis, 1988). There had also been reports of damage to alveolar macrophages by culture supernatants of the same *A. pleuropneumoniae* strains (Pijoan, 1986). These contradictory reports of damage to the phagocytic cells, and also to their ability to phagocytose adequately, left the situation unclear as to the actual extent of damage caused by *A. pleuropneumoniae* to the phagocytic cells.

Those phagocytosis studies used strains of *A. pleuropneumoniae* that produced both haemolytic and cytotoxic activity in their culture supernatant (Udeze & Kadis, 1988). These toxic activities have since been

named haemolysin I and II (Hly I and Hly II). Hly I is a 105 kDa protein and has been found to be both strongly haemolytic and cytotoxic and Hly II, a 103 kDa protein, possesses only weakly haemolytic and cytotoxic activity (Kamp *et al.*, 1991). Similar results had also been obtained with certain strains of *A. pleuropneumoniae* in this laboratory. They produced extracellular haemolytic and cytotoxic activities that appeared to be associated with 109 (haemolysin II, Hly II) and 120 kDa (pleurotoxin, PTX) proteins respectively (Fig. 6.1a) (Rycroft *et al.*, 1991).

Phagocytosis of *A. pleuropneumoniae* had therefore been studied only in strains that produce both haemolysins I and II (Inzana *et al.*, 1988; Udeze & Kadis, 1988). We had previously found that a serotype 2 strain, HK 361, produces a potent cytotoxin associated with a 120 kDa protein (Ptx) which was active against alveolar macrophages (Rycroft & Cullen, 1990b; Rycroft *et al.*, 1991). However, it was not known whether macrophages were able to phagocytose prior to being killed. The aim of this study was firstly therefore to evaluate whether phagocytosis by alveolar macrophages occurred in the presence of both Hly II and Ptx, and secondly, whether phagocytosis was occurring in the absence of Hly II and Ptx, using mutants deficient in either Hly II alone or both Hly II and Ptx (Fig. 6.1a).

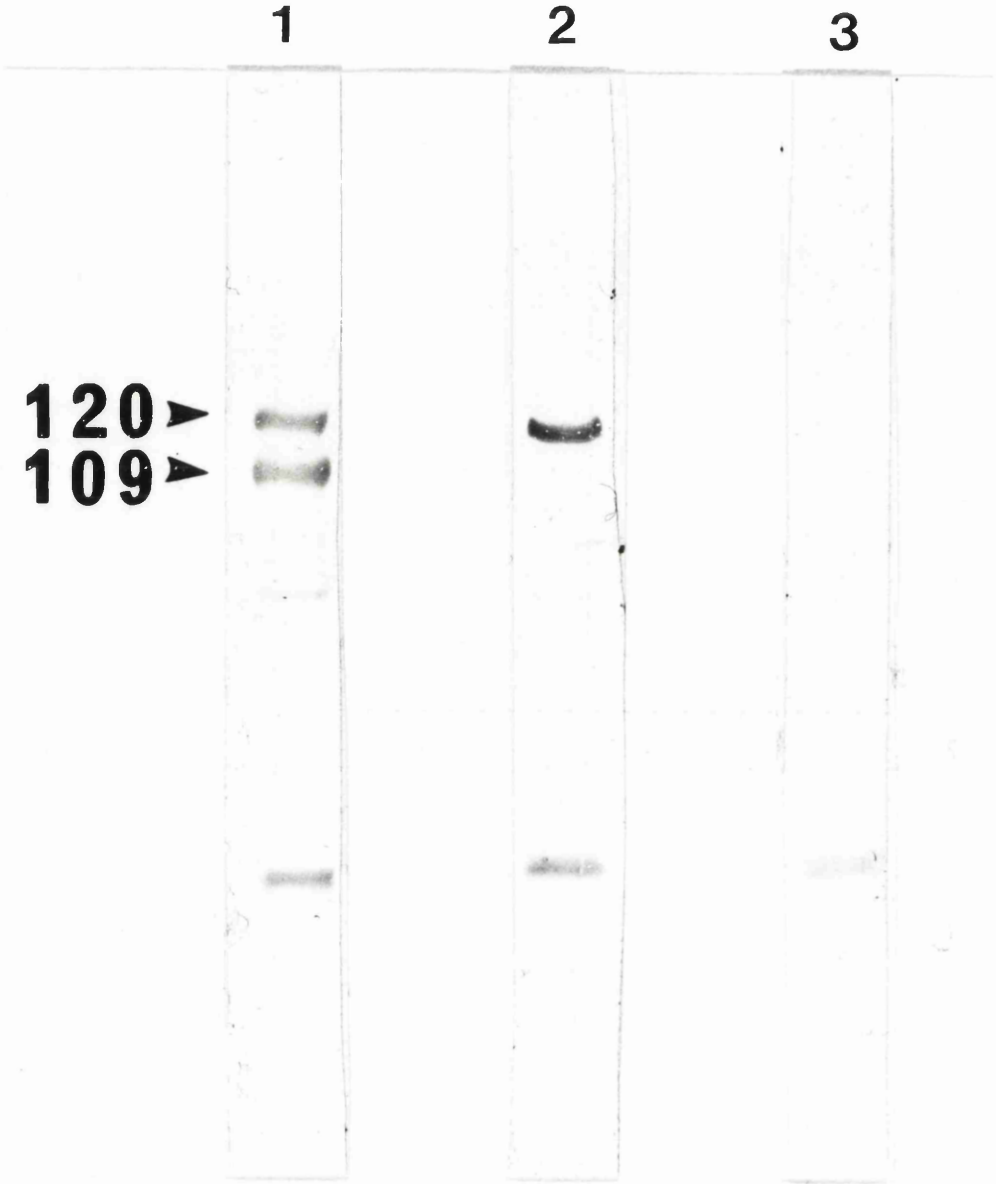
6.2 MATERIALS & METHODS

6.2.1 Bacterial strains

HK 361 serotype 2 mutants were used in the work described in this chapter and have been described in detail in Chapter 2. HK 361 mutant e possesses cytotoxic activity only, associated with a 120 kDa protein (Pleurotoxin), and mutant h is deficient in both Hly II (haemolysin type II, 109 kDa protein) and pleurotoxin.

Fig. 6.1a Comparison of the presence of the toxic 109 and 120 kDa polypeptides in bacteria-free culture supernatant from HK 361 and its mutants, e and h determined by Western blotting and probing with immune pig serum.

1) Parent strain HK 361 produces both 109 and 120 kDa proteins associated with haemolytic and cytotoxic activity respectively
2) Mutant e only produces the 120 kDa cytotoxic protein
3) Mutant h produces neither the 109 or 120 toxic proteins


1
2
3
120
109
HK 361
Mutant e
Mutant h

6.2.2 Bacterial preparation for phagocytosis studies

Bacteria were grown as previously described for serum bactericidal assays (3.2.1). The bacterial cultures were centrifuged at 11,300 x g for 1 minute, washed once with gel-HBSS and resuspended in the same buffer 1 x 10^8 cells/ml (2μg NAD/ml added for *A. pleuropneumoniae* strains).

6.2.3 Sera

The normal pig serum, immune pig serum and hyperimmune rabbit serum have been previously described in Chapter 2.

6.2.4 Trypan blue exclusion test

The vital stain trypan blue is taken up only by dead cells, staining their nuclei blue. Viable macrophage counts were determined by diluting cells in an equal volume of 0.2% (w/v) trypan blue solution and assessing those cells which had not taken up the vital dye using a haemocytometer.

6.2.5 Pig alveolar macrophages

Lungs were obtained from high health status pigs which were killed with barbiturates and used immediately. Lung lavage was performed using Hanks balanced salt solution (HBSS, Gibco, Scotland), which was placed in the trachea by means of a filter funnel (Fig. 6.1). The lungs were inflated to maximum capacity with HBSS and gently massaged. The HBSS was then emptied into a sterile container held on ice and the procedure repeated using fresh HBSS. The suspension collected in this way was then centrifuged at 110 x g for 4 minutes and the pellet washed twice with ice-cold gel-HBSS at 4^{o}C (Leijh *et al.*, 1986, Rycroft *et al.*, 1991). Gelatin was added to

Fig. 6.1 *In vitro* lung lavage of porcine lungs via the trachea.

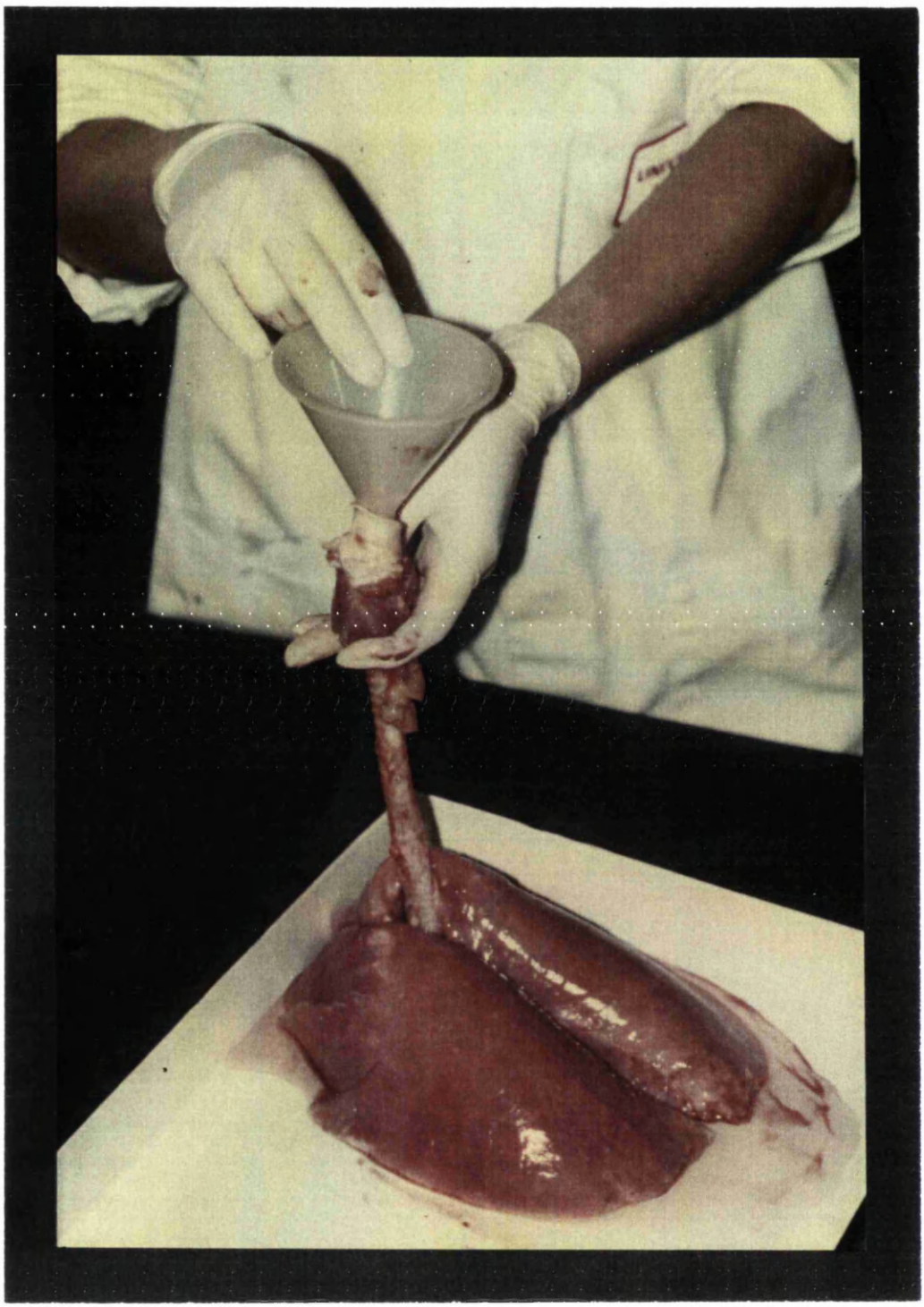


Fig. 6.1

protect the bacteria in the phagocytosis assay, since HBSS alone is bactericidal (Roberts, 1967). Remaining red cells were lysed by the addition of 0.87% (w/v) NH_4Cl for 10 minutes. Following centrifugation, the cell pellet was resuspended in gel-HBSS and the viability of the cell suspension determined by the trypan-blue exclusion test. The cell suspension was adjusted to 1 x 10^7 viable cells/ml and Giemsa-stained cell preparations were examined for cell type by light microscopy.

6.2.6 Cytospin preparation

Cytospins were prepared using ethanol precleaned standard microscope slides and cytospin filter cards (Shandon). 200μl containing 4.5 x 10^5 cells were loaded into the sample chamber and centrifuged for 5 minutes at 55 x g in a cytocentrifuge (Shandon). Slides were allowed to air dry before being fixed with methanol and Giemsa stained.

6.2.7 Preopsonisation of bacteria

Bacteria were grown and washed as previously described in 6.2.1. Bacterial pellets were resuspended in gel-HBSS (5 x 10^7 cells/ml) containing 10% serum and incubated at 37^{o}C under slow rotation (4rpm) for 30 minutes. Preopsonised bacteria were centrifuged at 11,300 x g for 1 minute and washed once in gel-HBSS.

6.2.8 Inactivation of bacteria

Bacteria were grown as previously described and treated either for 2 minutes at 100^{o}C, or with 0.5% v/v formalin overnight to inactivate the bacteria.

6.2.9 Phagocytosis assay

The method used was based upon that of Leijh *et al.*, (1986). Bacteria, grown or treated as previously described, were washed once in gel-HBSS and resupended in the same buffer to 5 x 10^7 cells/ml. A portion (0.5ml) of bacterial suspension was incubated with 0.5ml of macrophages (1 x 10^7 cells/ml) and 0.1ml of serum in non-adherent tubes (Leijh *et al.*, 1986) at 37°C with slow rotation (4 rpm). Samples were taken at 0, 1 and 2 hours. Viable bacterial counts and preparations for cytospins and electron microscopy were carried out by the methods described below.

Bacteriological determination of undigested, unattached viable bacteria was carried out as follows; 100μl samples were transferred to 300μl of ice-cold gel-HBSS to stop phagocytosis and centrifuged at 110 x g for 4 minutes at 4°C. Supernatants were carefully aspirated and stored. Pellets were washed twice more using 300μl of gel-HBSS each time, giving a total of 1ml of washings. A portion (10μl) of this supernatant was diluted 10^{-2} and 10^{-4} in gel-HBSS. Samples (100μl) of each dilution were assessed for bacterial viability by the pour plate method. The pellets were then used to prepare both cytospin smears and sections for examination by electron microscopy. Pellets were resuspended in 400μl of gel-HBSS.

For cytospins, a portion (200μl) (4.5 x 10^5 cells) of each sample was centrifuged for 5 minutes at 55 x g in a cytocentrifuge (Shandon). Slides were air dried, fixed with methanol and stained with Giemsa solution. The remaining 200μl samples were used for electron microscopy. Samples were fixed with 1.3% paraformaldehyde and 1.6% gluteraldehyde in 0.1M sodium cacodylate. The samples were postfixed with osmium tetroxide, sectioned and stained with 20% uranyl acetate

and lead citrate and examined with a transmission electron microscope (Zeiss 109). Electron microscopy sample preparation and photography was performed by the Electron Microscopy Unit in this department.

A. pleuropneumoniae strains HK 361 (Hly II^+, Ptx^+), mutant e (Hly II^-, Ptx^+) and mutant h (Hly II^-, $Ptx,^-$) were assessed for phagocytosis by pig alveolar macrophages along with an *E. coli* control strain, C10.

6.2.10 Different serum conditions

Heat-treated serum was prepared as previously described in Chapter 2. Heated and normal pig serum were used to assess the effects of the absence of complement and specific antibody, and the presence of complement but no specific antibody respectively. Heated and hyperimmune rabbit serum for -complement/+specific antibody and +complement/+specific antibody assessment respectively. The immune pig serum was used as a source of both homologous and heterologous pig antibody. Serum-free controls were included to assess the effects of the absence of both heated or normal serum.

6.2.11 Intracellular survival of *A. pleuropneumoniae*

Bacteria (5 x 10^7 cells/ml) preopsonised with immune pig serum were incubated with 1 x 10^7/ml macrophages for 30 minutes under slow rotation (4rpm). Extracellular bacteria were removed by differential centrifugation (4 minutes at 110 x g) and two washes with gel-HBSS. A portion (0.5ml) of gel-HBSS containing 10μg of gentamicin was added to the macrophages and left at room temperature for 5 minutes. Macrophages were centrifuged (4 minutes at 110 x g) and washed twice with gel-HBSS to remove the gentamicin. The cells were resuspended in 1ml of RPMI medium containing 10% heat-inactivated (56^{o}C/30 minutes) foetal calf serum (FCS) (Gibco). Samples

(100μl) were taken from time 0 to 17 hours (time zero equal to initial preopsonisation stage) and washed as described for the phagocytosis assay. Pellets were resuspended in 400μl of gel-HBSS. Samples (200μl) were lysed either by the detergent Triton X-100 (0.001-1%) or 3 freeze/thaw cycles using liquid nitrogen and a 37°C water bath in the presence of bovine serum albumin (1%). Viability of bacteria released from the macrophages was assessed using the pour plate method with iso-sensitest agar.

Samples for each assay were taken in duplicate or triplicate and each experiment was carried out between 3 and 5 times.

6.3 RESULTS

6.3.1 Phagocytosis of *A. pleuropneumoniae* strain HK 361

A. pleuropneumoniae HK 361, which produces both Hly II and Ptx, was assessed for phagocytosis by alveolar macrophages following incubation with alveolar macrophages in the presence of normal pig serum.

Lung lavage samples were almost entirely composed of alveolar macrophages. Following exposure of the macrophages to *A. pleuropneumoniae* HK 361, in the presence of normal pig serum, the majority of cells observed in the cytospin preparations had already undergone a degree of degeneration compared to the control in the time 0 sample, which was collected immediately after the addition of the bacteria to the macrophage and serum mixture (Fig. 6.2a,b). In the presence or absence of normal pig serum, similar macrophage degeneration occurred. In the presence of serum, bacteria were seen to adhere to the macrophage surface and occasional bacteria were present within phagolysosomes. No bacteria were seen to be either

Fig. 6.2 Giemsa stained cytospin preparations of HK 361 incubated with porcine alveolar macrophages in the presence of normal pig serum over time.

a) Control macrophages incubated in the absence of bacteria.
b) HK 361 incubated with normal pig serum and alveolar macrophages at time 0.
c) HK 361 incubated with normal pig serum and alveolar macrophages at time 2 hours.

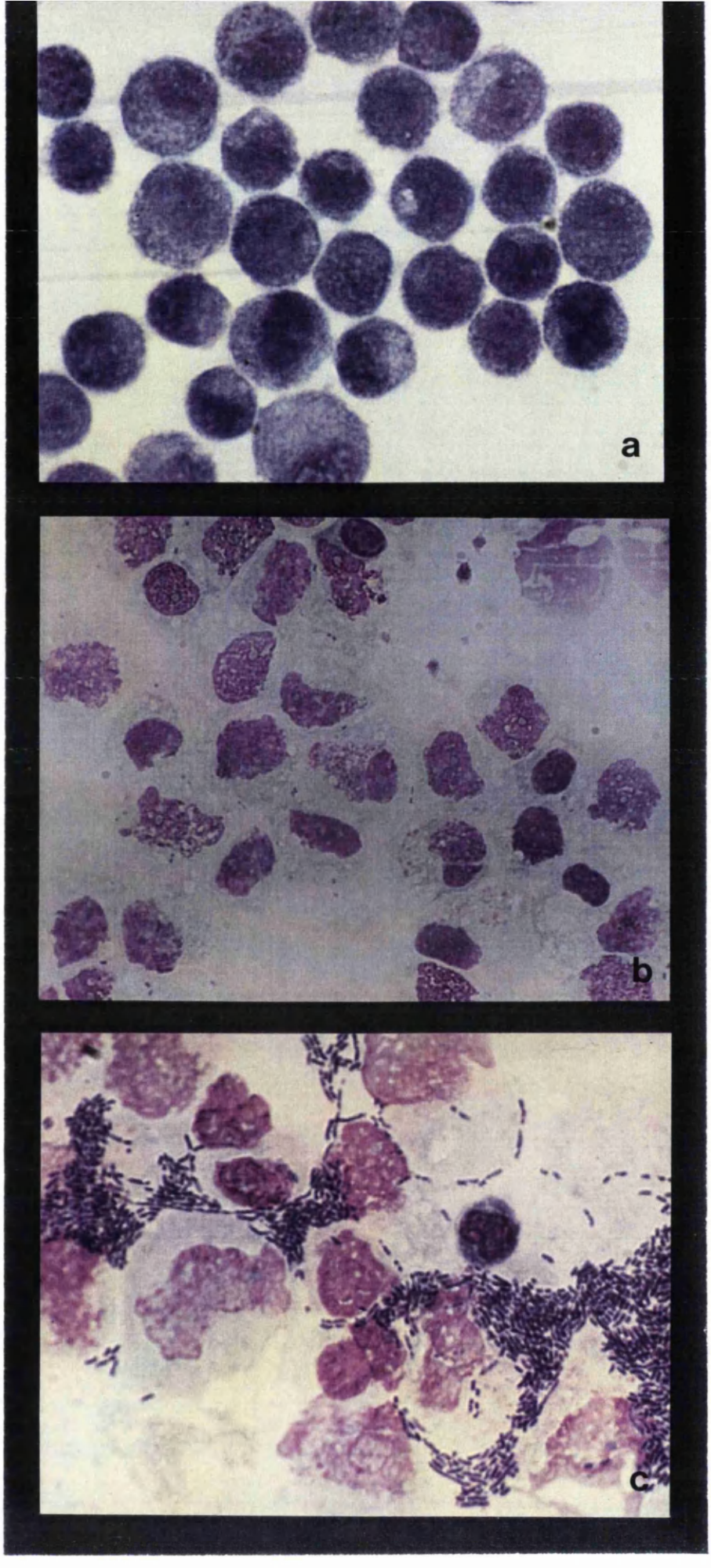


Fig. 6.2

phagocytosed or adhering to the macrophages in the absence of serum. After further incubation for 1 and 2 hours, all macrophages displayed extensive damage both in the presence and absence of serum (Fig. 6.2c). Bacteria appeared to be located both around and within the dead macrophages, again only in the presence of serum. The numbers of bacteria observed in the cytospin preparations increased greatly with continued incubation (Fig. 6.2c).

Control macrophage samples, incubated either in the absence of bacteria (Fig. 6.2a), or with *E. coli* RY22 (Fig. 6.3), showed no signs of damage at any stage of incubation. Large numbers of *E. coli* were seen to be phagocytosed after 1 and 2 hours incubation in the presence of serum (Fig. 6.3c,d).

6.3.2 Phagocytosis of *A. pleuropneumoniae* haemolysin and cytotoxin negative mutants

A. pleuropneumoniae HK 361 mutants e and h were evaluated for phagocytosis in the presence of normal pig serum.

The haemolysin deficient mutant, HK 361e, which possessed only the cytotoxin (Ptx), was found to behave in an identical fashion to the parent strain HK 361 (Fig. 6.4). Incubation of this strain with the macrophages resulted in the rapid death of macrophages both in the presence and absence of serum. The increase in bacterial numbers over time, seen associated with the degenerating macrophages in the presence but not the absence of normal pig serum in the cytospin preparations, appeared to be similar to the parent strain, HK 361 (Fig.6.4c).

Incubation of macrophages with the cytotoxin and haemolysin negative mutant h, resulted in a large

Fig. 6.3 Giemsa stained cytospin preparations of control *E. coli* incubated with porcine alveolar macrophages in the presence of heated normal pig serum over time.

Time samples taken at:
a) 0 hours
b) 1 hour
c) 2 hours
d) 3 hours

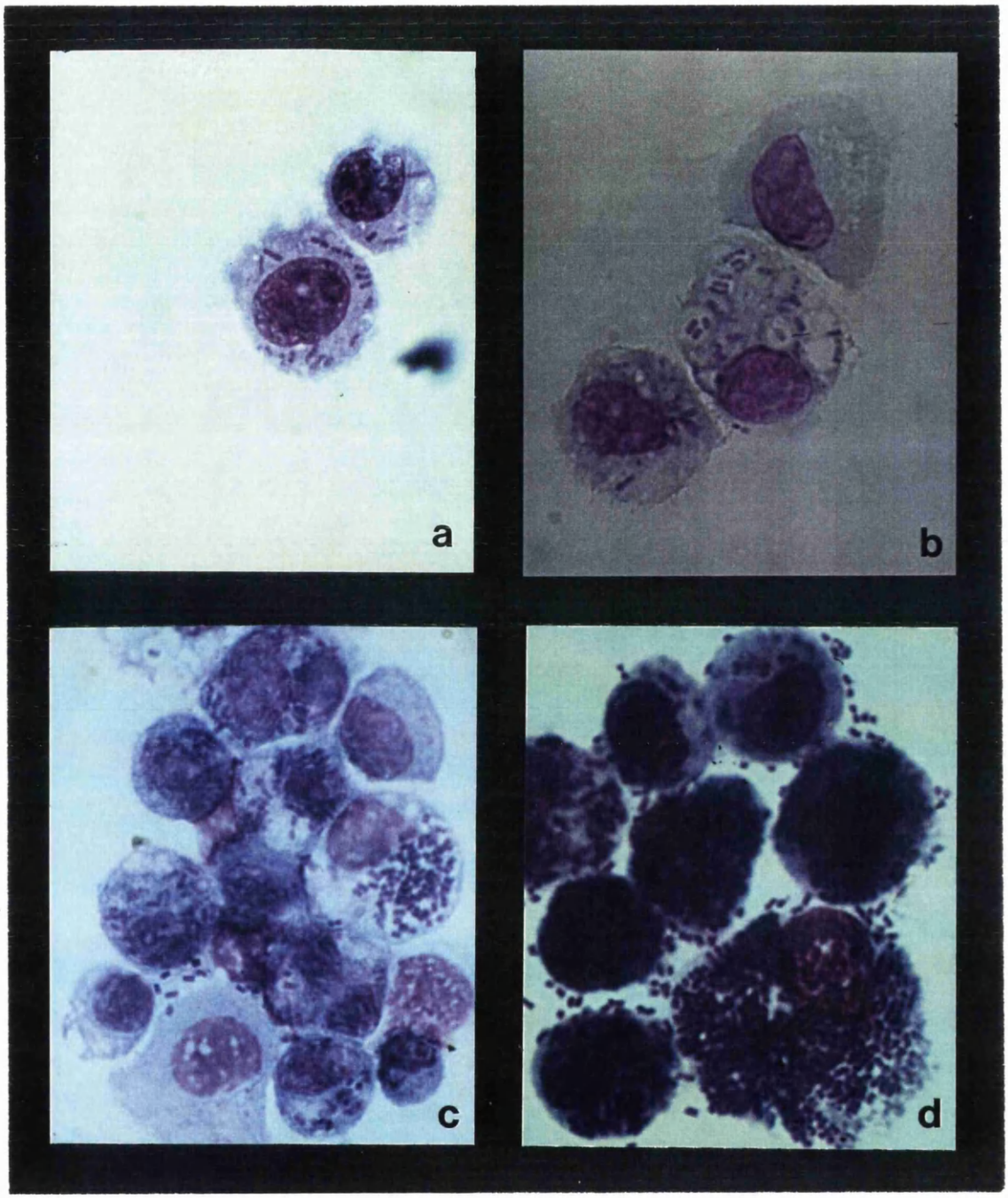


Fig. 6.3

Fig. 6.4 Giemsa stained cytospin preparations of HK 361 mutant e incubated with porcine alveolar macrophages in the presence of normal pig serum over time.

a) Control macrophages incubated in the absence of bacteria.
b) HK 361 mutant e incubated with normal pig serum and alveolar macrophages at time 0.
c) HK 361 mutant e incubated with normal pig serum and alveolar macrophages at time 2 hours.

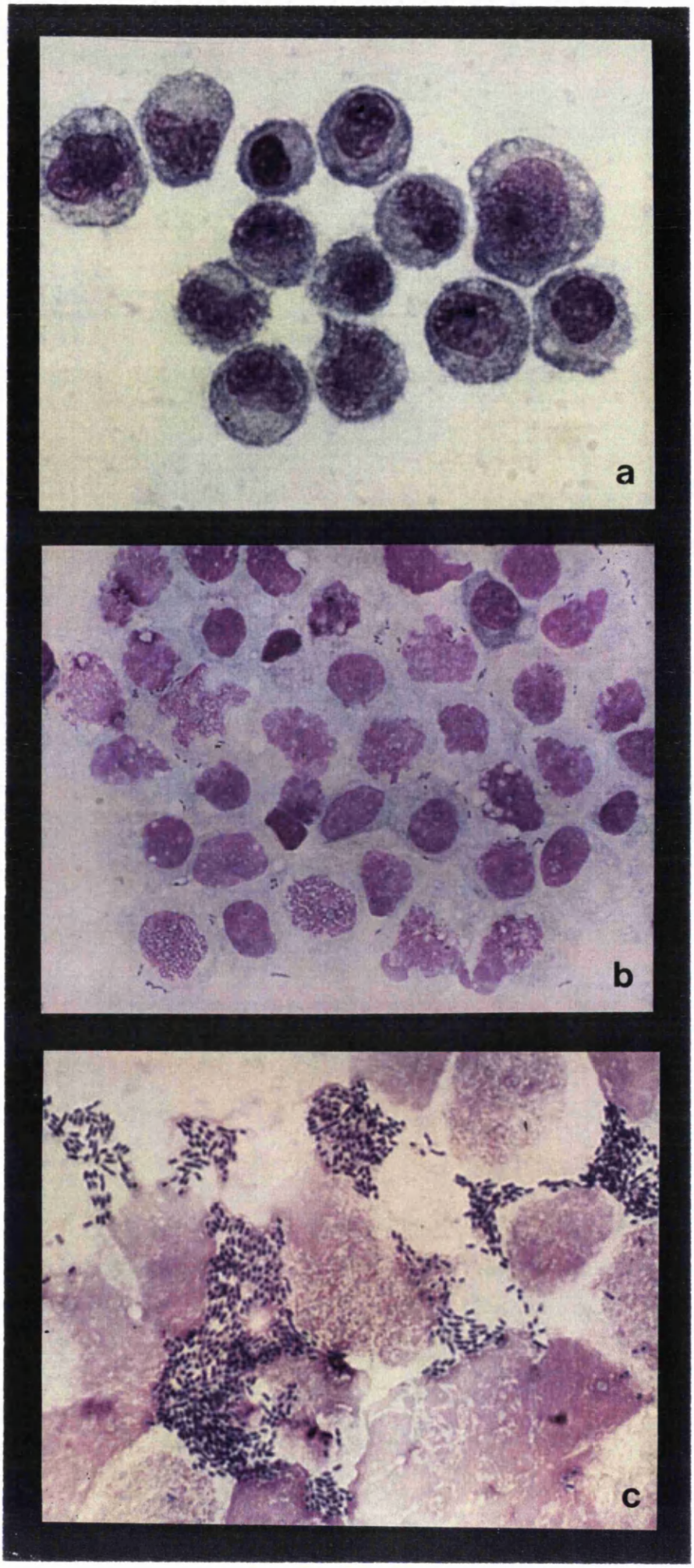


Fig. 6.4

proportion of the macrophages remaining alive in the time 0 sample (Fig. 6.5b) Bacteria were observed both adhering to, and within, the macrophages in the presence of serum (Fig. 6.5b). After 1 and 2 hours incubation a proportion of macrophages still appeared healthy however, a similar number showed signs of degeneration (Fig. 6.5c). Phagocytosed bacteria could still be seen, although numbers did not appear to increase with prolonged incubation (Fig. 6.5c).

6.3.3 Effects of immune sera on macrophage survival and ability to phagocytose

Phagocytosis assays were also carried out in the presence of immune sera, which contained antibodies against Hly II and Ptx. This was to assess firstly, any increase in survival of macrophages incubated with *A. pleuropneumoniae* due to the presence of neutralising antibodies against the Hly II and Ptx and secondly, any increase in the number of phagocytosed *A. pleuropneumoniae* when incubated with immune sera. HK 361 and mutants e and h were assessed for phagocytosis by alveolar macrophages incubated with both convalescent pig serum and hyperimmune rabbit serum.

No difference in the degree of damage caused to the macrophages incubated with HK 361 and mutant e was seen when incubated with either of the immune sera. Macrophages incubated with HK 361 and mutant e continued to sustain extensive damage. Preopsonisation of HK 361 bacteria for 30 minutes at 37^{o}C prior to incubation with the macrophages, did not reduce the level of damage to macrophages.

Incubation of mutant h with immune sera resulted in the macrophages initially appearing healthy. Continued incubation in the presence of hyperimmune rabbit serum resulted in the killing of a proportion of macrophages

Fig. 6.5 Giemsa stained cytospin preparations of HK 361 mutant h incubated with porcine alveolar macrophages in the presence of normal pig serum over time.

a) Control macrophages incubated in the absence of bacteria.
b) HK 361 mutant e incubated with normal pig serum and alveolar macrophages at time 0.
c) HK 361 mutant e incubated with normal pig serum and alveolar macrophages at time 2 hours.

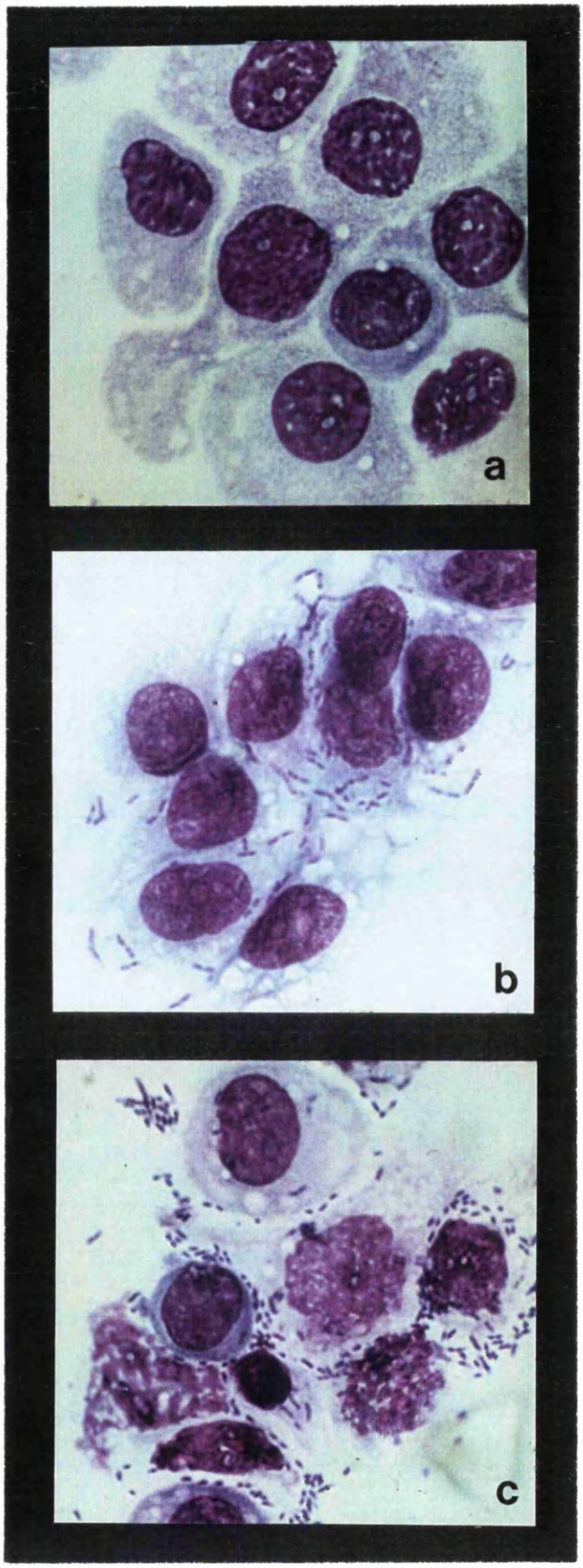


Fig. 6.5

comparable to that seen with normal pig serum (Fig. 6.5c). The presence of convalescent pig serum however did appear to reduce damage to the macrophages, resulting in most of the cells remaining healthy following continued incubation (Fig. 6.6). Bacteria could be clearly seen adhering to macrophages, and within phagolysosomes (Fig. 6.6b,c,d). The numbers of phagocytosed bacteria also appeared to increase with prolonged incubation (Fig. 6.6 c,d). Phagocytosis of mutant h was confirmed following electron microscopy. Bacteria could be seen within phagolysosomes (Fig. 6.7a). A double membrane could be clearly seen surrounding several of the bacteria (Fig. 6.7b).

In control samples where mutant h was incubated with macrophages in the absence of serum, a slight reduction in the level of toxicity was observed after 1 hours incubation compared to all other serum conditions excluding the convalescent serum. This difference could only be seen in the 1 hour sample. After 2 hours incubation the macrophages were at similar levels of degeneration in all the other serum conditions except the convalescent serum. Controls of macrophages incubated in the presence and absence of *E. coli* bacteria, showed no signs of damage as previously described (Fig. 6.2a, 6.3).

6.3.4 Phagocytosis of inactivated *A. pleuropneumoniae*

Both toxins produced by HK 361 are heat-labile (Rycroft *et al.*, 1991). Other researchers have documented a heat stable toxin(s) that possesses both haemolytic and cytotoxic properties, and was polysaccharide in nature from a different serotype 2 strain (Kume *et al.*, 1986). To assess whether the damage seen to macrophages incubated with mutant h in the presence of normal pig serum was related to such a factor, bacteria, inactivated by heat or formaldehyde, were assessed for

Fig. 6.6 Giemsa stained cytospin preparations of HK 361 mutant h incubated with porcine alveolar macrophages in the presence of immune pig serum over time.

Time samples taken at:

a) 0 hours
b) 1 hour
c) 2 hours
d) 3 hours

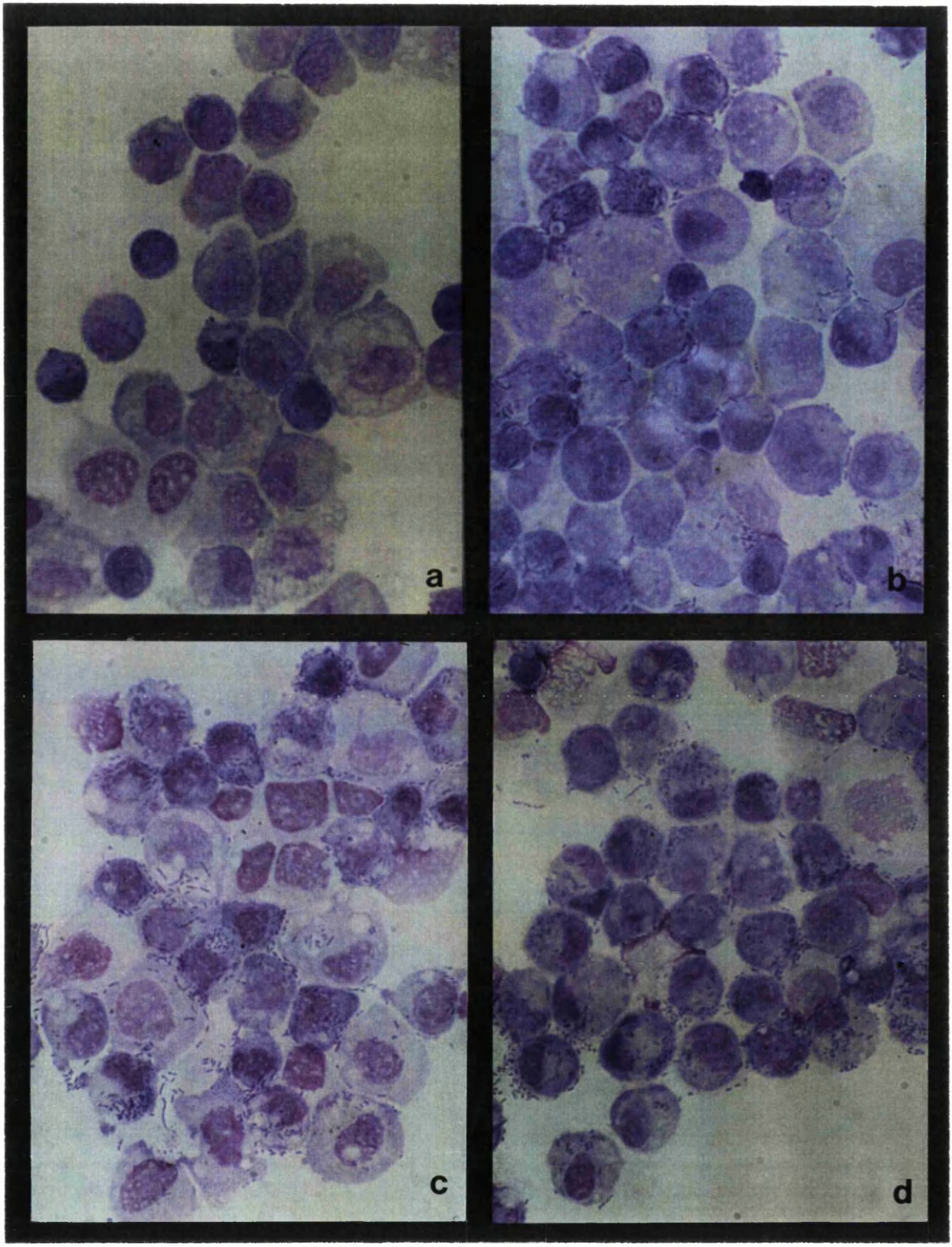


Fig. 6.6

Fig. 6.7 Electron micrographs of HK 361 mutant h incubated in the presence of immune pig serum within phagolysosomes of porcine alveolar macrophages.

a) Time 3 hour sample x 14,000 magnification
b) Time 3 hour sample x 24,000 magnification

Bar represents 1μm.

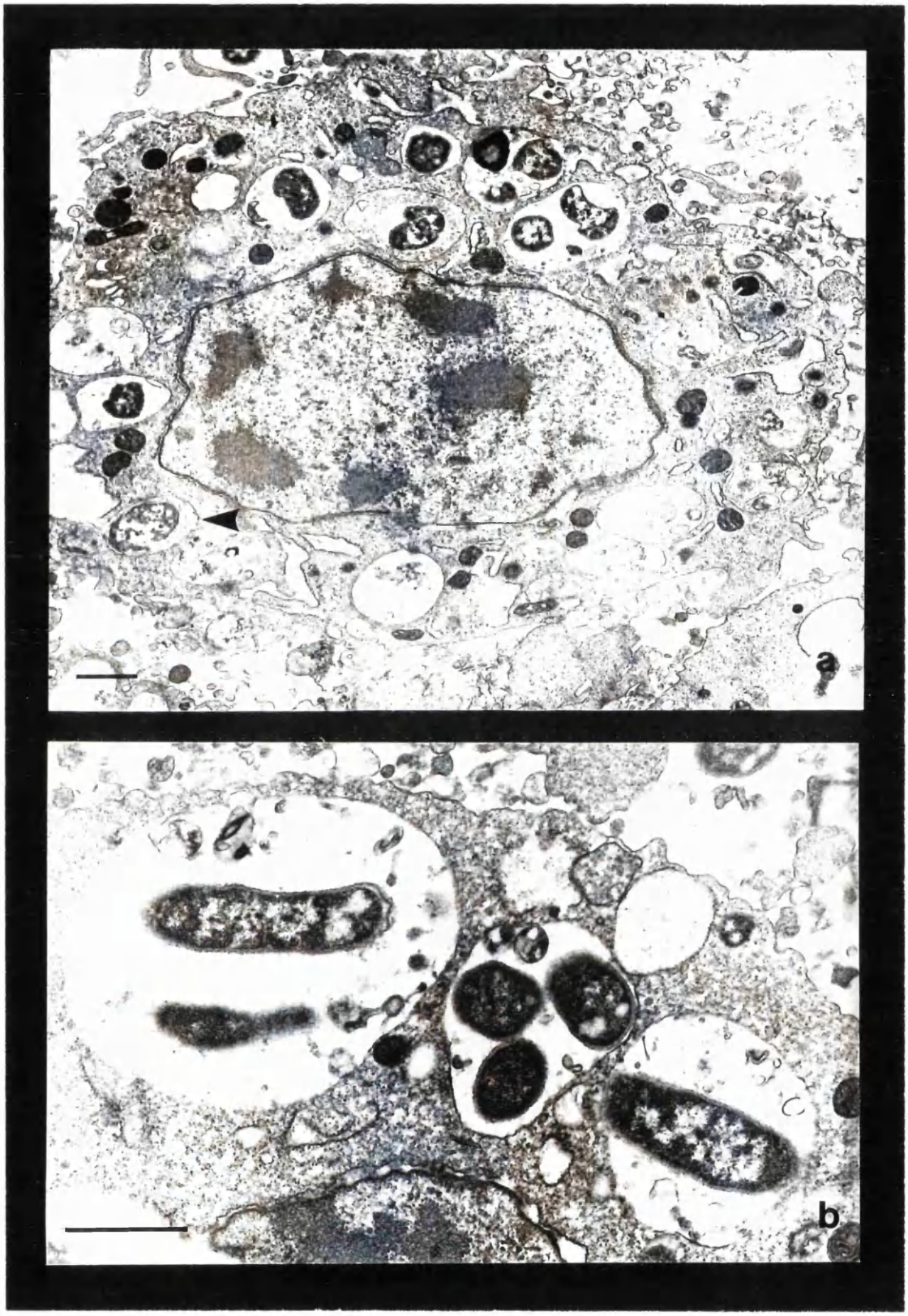


Fig. 6.7

phagocytosis. Killed HK 361 and mutant h appeared to be readily phagocytosed by the macrophages in the presence of each serum used, independent of the method used to inactivate them. In the time 1 samples, mutant h bacteria could already be clearly seen inside the macrophages (Fig. 6.8). After incubation for 2 hours the number of phagocytosed bacteria had increased substantially compared to the first sample (Fig. 6.8c). No damage to the macrophages was apparent in any of the later samples (Fig. 6.8d). No difference in the degree of phagocytosis of *A. pleuropneumoniae* was detected when incubated in the presence of either normal or immune pig serum. All macrophage controls were as previously described.

6.3.5 Determination of extracellular viable bacterial counts

Reproducible extracellular bacterial counts could not be determined for *A. pleuropneumoniae* HK 361 or mutants e and h. The ability of *A. pleuropneumoniae* to form chains and the adherence of large numbers of bacteria to the degenerate macrophages, even after 3 slow speed washes to remove extracellular bacteria, may contribute to the observed irregularities. Reproducible extracellular bacterial counts were obtained for the *E. coli* controls. Bacterial counts decreased with increased incubation time in the presence of both macrophages and serum. Increases in extracellular bacterial counts over time were observed for the serum free control, and also in the macrophage free control both in the presence and absence of serum. The increase in bacterial counts was very similar for the above three latter controls.

6.3.6 Intracellular survival

Attempts to assess the viability of phagocytosed mutant h were inconclusive. The use of the detergent Triton X-

Fig. 6.8 Giemsa stained cytospin preparations of killed HK 361 mutant h incubated with porcine alveolar macrophages in the presence of normal pig serum over time.

Time samples taken at:

a) 0 hours
b) 1 hour
c) 2 hours
d) 3 hours

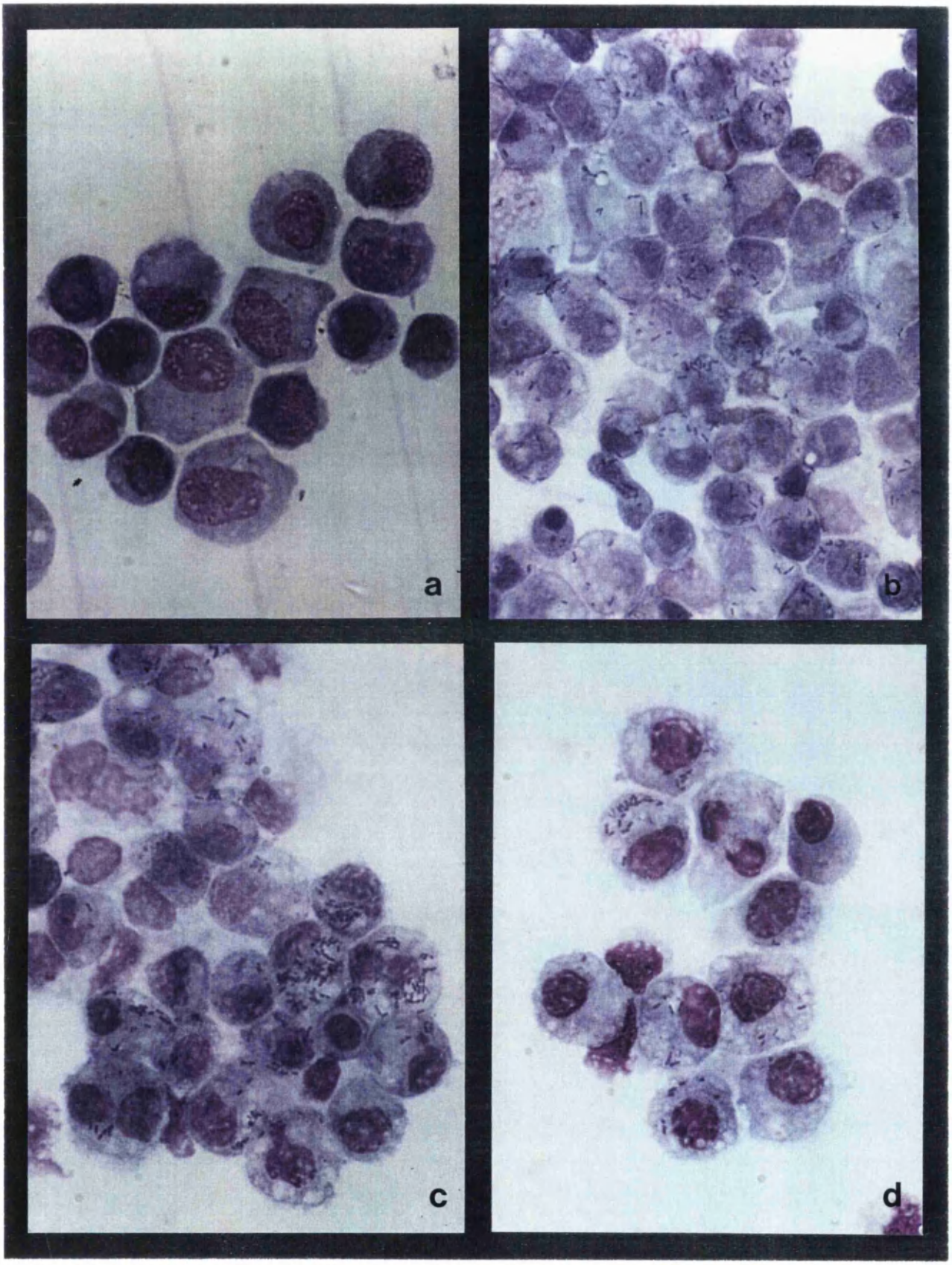


Fig. 6.8

100 at a concentration which lysed the macrophages (0.1%), was found to be toxic for the bacteria. Lower concentrations found to be non-toxic for *A. pleuropneumoniae*, did not lyse the bacteria. Freeze, thawing techniques using liquid nitrogen and a 37°C water-bath, in the presence of bovine serum albumin, also killed the bacteria.

6.4 DISCUSSION

The results obtained to date regarding phagocytosis of *A. pleuropneumoniae* strains have been documented with strains that produce both the Hly I and Hly II (Inzana *et al.*, 1988; Udeze & Kadis, 1988; Thwaits & Kadis, 1991; Udeze & Kadis, 1992). Serotypes 2,3,4,6 and 8 also produce a 120 kDa protein (Chapter 7, Cullen & Rycroft, 1992a; MacDonald & Rycroft, 1992), which is associated with cytotoxicity (Rycroft *et al.*, 1991). The aim of this work was to assess whether a serotype type 2 strain of *A. pleuropneumoniae* is phagocytosed by alveolar macrophages in the presence or the absence of toxic activities, Hly II and Ptx.

Phagocytosis of HK 361 or mutant e could not be assessed due to the rapid toxic effect of these organisms on the macrophages (Fig. 6.2, 6.4). The addition of immune serum, with or without preopsonisation for 30 minutes at 37°C, did not remove this toxic effect. The difference observed in the neutralising ability of the immune serum compared to that previously assessed (Rycroft *et al.*, 1991) may be related to the continual production of toxic activity by the growing bacteria during the phagocytic assay and the fixed amount of toxin which would have been present in the culture supernatant used in the previous study. This continued production of toxin may allow it to overcome the neutralising ability of serum antibody. The lack of toxin neutralisation also did not appear to be related to the heterologous nature

of the immune serum, since incubation of a serotype 3 strain, which also produces both Hly II and Ptx (Rycroft *et al.*, 1991), with its corresponding homologous immune serum, did not neutralise the toxic activities, resulting in damaged macrophages.

The absence of Hly II in mutant e appeared not to reduce the level of macrophage damage suggesting it was not an important cause of the observed toxicity. This is in agreement with work of others, who have found the Hly II to be weakly haemolytic and the least cytotoxic of the 3 known cytotoxins (haemolysin I & II and pleurotoxin respectively) (Frey & Nicolet, 1990; Rycroft *et al.*, 1991, Smits *et al.*, 1991; Kamp *et al.*, 1991).

The limited macrophage damage observed after 1 and 2 hours incubation with mutant h and normal pig serum may be due to the retention by this mutant of a level of toxic activity (Fig. 6.5). Culture supernatant from mutant h has previously been found to be non-toxic for alveolar macrophages, and neither Hly II nor Ptx were detected in the culture supernatant by immunoblotting (Rycroft *et al.*, 1991). This is in contrast to both HK 361 and mutant e supernatant, which is extremely cytocidal for these cells (Rycroft *et al.*, 1991). It may be speculated that the reduced toxic activity observed in *A. pleuropneumoniae* mutant h represents a cell-associated toxic component.

Only in the presence of convalescent pig serum was the toxicity of mutant h for macrophages neutralised (Fig. 6.6). The lack of neutralisation of toxic activity by the hyperimmune rabbit serum, which contained cross-reacting but not neutralising antibodies against both haemolysin II and pleurotoxin (personal observations), may also imply the involvement of toxic activities in the macrophage damage by mutant h. The increase in phagocytosed mutant h following prolonged incubation in

the presence of the immune pig serum suggests the toxin neutralising abilities of the convalescent pig serum are required to enhance the phagocytosis of this organism (Fig. 6.6). Whether this is a result of the neutralising abilities of the immune serum alone, or in combination with the presence of specific antibodies, which have been reported to enhance opsonisation of *A. pleuropneumoniae*, is not known (Thwaits and Kadis, 1991).

The rapid phagocytosis of the killed bacteria and the lack of damage to the alveolar macrophages incubated in normal pig serum suggest that *A. pleuropneumoniae* is phagocytosed in the absence of any heat or formaldehyde sensitive extracellular toxins (Fig. 6.8). The phagocytosis of killed mutant h also suggests that the toxic activity of mutant h is heat labile and therefore not LPS-associated or the heat stable toxin described by Kume *et al.*, (1986b). The similar numbers of phagocytosed killed mutant h in normal pig serum compared to viable mutant h in immune pig serum, suggested the presence of specific antibody may not be necessary for phagocytosis of *A. pleuropneumoniae* by alveolar macrophages.

Attempts were made to assess whether phagocytosed mutant h was killed by macrophages. However, the use of the detergent Triton X at a concentration which lysed the macrophages (0.1%), was found to be toxic for the bacteria. Freeze thawing techniques using liquid nitrogen and a 37^{o}C water-bath, in the presence of bovine serum albumin, also killed the bacteria. It is of interest in Fig. 6.7, as detailed by an arrow, the bacterium within the phagolysosome appears to have undergone a degree of degeneration. Whether this demonstrates killing of mutant h within alveolar macrophages, or is merely an artifact of the electron microscopy, requires further investigation.

A. pleuropneumoniae HK 361 and its mutants were found to remain attached to both the viable and the degenerate macrophages after washing to remove extracellular bacteria. This occurred only in the presence of serum irrespective of whether it had been heated or contained specific antibodies. Both Thwaits and Kadis (1991) and Inzana *et al.* (1988) recovered high percentages of total bacteria in the supernatant following 3 slow speed washes to remove extracellular bacteria in a procedure identical to that used here. Both these researchers used PMN and perhaps the differences can be attributed to this factor. This problem of adherence was not detected using the *E. coli* control.

Phagocytosis of live *A. pleuropneumoniae* by PMN has been described for strains 4074 (serotype 1) and K17 (serotype 5a) that produced Hly I and II (Inzana *et al.*, 1988; Thwaits & Kadis, 1991). Hly I is reported to possess strong cytocidal activity and Hly II a weaker activity (Kamp *et al.*, 1991). However, toxicity of alveolar macrophages has also been caused by the same strains (Pijoan, 1986). These differences may have been related to these 2 strains being toxic for porcine alveolar macrophages but not for PMN. A third study used porcine alveolar macrophages to study phagocytosis of serotype 1 strain, 4074 (Udeze and Kadis, 1988). They did report toxicity of the purified haemolysin for alveolar macrophages and PMN alike, however there was no discussion on whether toxicity was observed in the phagocytosis assays with whole bacteria. Recent studies with the same strain, 4074, have since found that this strain was in fact toxic for PMN (Udeze & Kadis, 1992). These researchers found that substantial phagocytosis could only be achieved when the assay was carried out in the presence of serum containing antibodies to the haemolysins I and II. It would therefore appear that strains producing Hly I and II are toxic for both PMN

and macrophages alike, however the presence of immune serum neutralises the toxic effect of the bacteria and phagocytosis can therefore be assessed. The above explanation would therefore explain why there have been contradictory reports of damage to phagocytic cells by both culture supernatant and live bacteria, which were assessed in the absence of immune serum, and phagocytosis of the same strains which were assessed in the presence of immune serum. In the case of viable strains producing the Ptx, it would appear that even the presence of immune serum is not sufficient to neutralise all the toxic effects of Ptx for pig alveolar macrophages.

The delayed death of the macrophages in the serum-free control incubated with mutant h, compared to all other serum conditions excluding convalescent serum, may suggest that attachment of the organism to the macrophages enhances the action of the toxic components. It has been suggested that the toxin operates by diffusion mechanism (Udeze & Kadis, 1988), which would be in agreement with the observed results. It is of interest that *A. pleuropneumoniae* activates complement component C3 (Chapter 4, Cullen & Rycroft, 1990; Udeze and Kadis, 1992), and has been found to possess C3b on its surface (Udeze & Kadis, 1992) although it is resistant to immune pig serum (Chapter 3, Rycroft & Cullen, 1990a). C3b acts as an opsonin to attract macrophages to phagocytose organisms coated with C3b. It is possible that *A. pleuropneumoniae* has become adapted using opsonisation to increase the speed of macrophage attraction resulting in enhanced toxic activity of the organisms against these host defence cells.

In summary the results of this chapter suggest that phagocytosis of *A. pleuropneumoniae* serotype 2 strain by alveolar macrophages can only occur in the absence of both Ptx and Hly II and in the presence of immune serum.

When Ptx is present either alone (mutant e) or in combination with Hly II (HK 361), phagocytosis cannot be assessed. This is due to the extremely toxic activity of the Ptx, which cannot be entirely neutralised by immune serum when live bacteria are used. The absence of the Hly II was not found to reduce the toxicity of the serotype 2 strain in the presence of Ptx. Therefore, it could be suggested that the Ptx of this serotype is both a potent cytotoxic and an anti-phagocytic factor. It is therefore suggested that *A. pleuropneumoniae* would be phagocytosed except for the fact it produces toxins active against phagocytic cells.

CHAPTER 7

IMMUNOLOGICAL ASSESSMENT OF THE HAEMOLYTIC AND CYTOTOXIC PROTEINS OF *A. PLEUROPNEUMONIAE*

7.1 INTRODUCTION

Over the last decade one of the major virulence factors of *A. pleuropneumoniae* to emerge has been the haemolytic and cytotoxic activities of both viable bacteria and their secreted products for a range of porcine cells including alveolar macrophages, neutrophils, PMN and erthrocytes (Bendixen *et al.*, 1981; Pijoan, 1986; Udeze & Kadis, 1988; Rosendal *et al.*, 1988; van Leengoed *et al.*, 1989).

The haemolysin of *A. pleuropneumoniae* was first partially characterised from a serotype 1 strain, as a protein of 105 kDa (Frey & Nicolet, 1988a). Further studies demonstrated a requirement for Ca^{++} as an inductor for biosynthesis of haemolysin. However, Ca^{++} was found not to be required for the biological activity of the haemolysin (Frey *et al.*, 1988; Frey & Nicolet, 1988b). The opposite result was found with the haemolysin of a serotype 2 strain. This haemolysin was not induced by the addition of Ca^{++} to the growth medium, however Ca^{++} was found to be required for biological activity in erthrocyte lysis. This discovery led these researchers to propose that the haemolysin of serotype 2 and its regulation was different from that produced by serotype 1. The haemolysin of serotype 1 was designated haemolysin type I (Hly I) and the haemolysin of serotype 2, haemolysin type 2 (Hly II) (Frey & Nicolet, 1988b).

All 12 serotype reference strains were then assayed for haemolytic activity both in the presence and absence of Ca^{++} in both the growth medium and assay titration buffer. Four distinct haemolytic patterns were found, serotypes 3 and 6 showed very weak haemolytic activity which could not be further quantified, serotype 1 required Ca^{++} for the biosynthesis of haemolytic activity but not for its activity, serotypes 2, 4, 7 and

8 possessed weak haemolytic activity that required Ca^{++} for activity but not for biosynthesis, and lastly serotypes 5a, 5b, 9, 10, 11 and 12 showed both haemolysins, one requiring Ca^{++} for biosynthesis and another for activity (Frey & Nicolet, 1990). This information suggested that *A. pleuropneumoniae* strains possessed at least 2 different haemolysins.

Other researchers studying the same serotype 1 strain found that the haemolysin possessed both haemolytic and cytotoxic activities associated with a 130 kDa band and 3 protein bands of smaller molecular weight (Udeze & Kadis, 1988). The purified haemolysin was found to be toxic for porcine alveolar macrophages, PMN and neutrophils.

Attempts were made to isolate these toxic activities in order to purify and characterise them. However it was found that the cytotoxic and haemolytic activities produced in the bacterial culture supernatant of serotype 1 co-purified (Udeze & Kadis 1988) and a substantial loss of activity was experienced both on purification and in the crude form (Frey & Nicolet, 1988a; Devenish & Rosendal, 1989). Further examination of representative strains from the 12 serotypes revealed the presence of a 104 kDa protein in all strains that reacted with a polyclonal antiserum raised against a serotype 1 strain (Devenish *et al.*, 1989). In addition, this serum also detected proteins of a similar size in related pathogens *Pasteurella haemolytica*, *Escherichia coli* and *Actinobacillus suis*. The haemolytic activity of the serotype 1 strain was neutralised by some, but not all, of the sera raised against the strains from the 12 serotypes of *A. pleuropneumoniae*. This led these researchers to propose that a similar, but not identical, 104 kDa protein was produced by all the serotypes of *A. pleuropneumoniae*, and suggested it may be related to the cytolysins produced by other Gram-

negative bacteria (Bhakdi *et al.*, 1986; Lo *et al.*, 1985). Examination of culture supernatants of the 12 serotypes reference strains for haemolytic and cytotoxic activity by another research group also revealed both similarities and differences in the activities reported (Kamp & van Leengoed, 1989). They found the haemolytic activity in all serotypes were serologically homogeneous, but that 4 distinct cytotoxic activities could be detected.

The situation was therefore very confusing regarding whether the haemolytic activities were the same or related, or if the haemolytic and cytotoxic activities, observed in some but not all of the serotypes, was in fact the Hly I or II or indeed another protein. The similarity in molecular weights between Hly I and II also added to the confusion.

Several serotype 2 and 3 strains under study in this laboratory were found to secrete both cytotoxic and haemolytic activities into their culture supernatant and initially a protein of 109 kDa was identified in the culture supernatants of these strains. This activity was found to require Ca^{++} for the expression of haemolytic but not cytotoxic activity (denoted haemolysin II, Hly II). In order to study these proteins in greater detail, Mabs were developed against the 109 kDa protein, found in serotype 2 and 3 strains under investigation within this laboratory, that appeared to have both cytotoxic and haemolytic properties associated with a 109 kDa protein and which required Ca^{++} for the activity of the haemolytic but not for the cytotoxic activity.

Further analysis of culture supernatant from these serotype 2 and 3 strains revealed the presence of another secreted polypeptide that was shown to be a 120 kDa protein. Mutant strains had also been produced within this laboratory from a serotype 2 strain HK 361

minutes at 200V). Standard and mini gels were fixed and stained in 40% (v/v) methanol, 10% (v/v) glacial acetic acid and 0.25% (w/v) Coomassie Brilliant Blue R250 for 30 and 15 minutes respectively. Gels were destained in a mixture of 40% (v/v) methanol and 10% (v/v) glacial acetic acid overnight.

7.2.2 Bacterial culture supernatant

Bacteria were grown to logarithmic phase as previously described (2.3). Subcultures were then made (1 in 25) into fresh TSB/NAD and grown for a further for 5 hours. Bacteria were removed by centrifugation for 10 minutes at 10,000 x g and culture supernatants stored at -20°C.

7.2.3 Isolation and purification of the pleurotoxin of *A. pleuropneumoniae* (120 kDa protein)

A. pleuropneumoniae Hly II⁻ mutant e, which secretes only the pleurotoxin (120 kDa protein), was used to prepare the immunising antigen sample for Mab production against the 120 kDa cytotoxic protein of serotype 2 strain HK 361. Bacteria-free culture supernatants of mutant e were freshly prepared and used without prior freezing. The culture supernatants (30ml) were held in an ice-cold container and ethanol added to give a final concentration of 23% (v/v). The supernatant was held on ice for 60 minutes and centrifuged at 12,000 x g for 15 minutes at 4°C. The resultant pellet was gently redissolved in 3ml of saline. 1.5ml was retained and Triton X-100 added to the other 1.5ml to make a final concentration of 0.2% (v/v) Triton X-100. Triton X-100 treated supernatant was processed through a Sephacryl S-300 column (Pharmacia) column which had been previously washed through with Tris-Triton X-100 buffer. 1ml fractions were collected and refrigerated until tested. Eluate fractions were tested for 120 kDa protein content by SDS-PAGE. 30μl of each sample was assessed and those

fraction(s) containing the maximum amount of 120 kDa protein were saved.

7.2.4 Protein A preparation

Staphylococcus aureus Cowan strain was used to prepare protein A and was obtained from Dr. Rick Randell, St Andrews University, Scotland. 3g of dry weight *S. aureus* was resuspended in 30ml of phosphate buffered saline (PBS) containing azide (0.1%). The bacteria were centrifuged at 8,000 x g for 10 minutes and washed twice in the same buffer. The pellet was resuspended in 10% w/v PBS/azide and 1.5% formalin and stirred at room temprerature for 90 minutes. Following the centrifugation and washing procedure as described before, the pellet was resuspended in 10% w/v of PBS/azide in a flask, keeping the depth of liquid below 2.5cm. This was heated to 80^{o}C for 5 minutes shaking occasionally and then cooled in an ice-bath. The solution was centrifuged at 8,000 x g for 10 minutes and washed twice in PBS/azide (pH 7.2). The pellet was dried, reweighed and resuspended at 10% w/v in PBS/azide. *S. aureus* protein A was aliquoted and stored frozen at -70^{o}C.

7.2.5 Preparation and isolation of 109 kDa protein (haemolysin II)

A Mab had been previously produced against the culture supernatant of serotype 2 strain HK 353, which contained only haemolysin II (109 kDa protein). This Mab did not produce a strong reaction when tested against culture supernatant from HK 353 by both ELISA and immunoblotting. It was decided to use this Mab as part of an immunising complex and conjugate it to protein A, which would act as a carrier, and also to the 109 kDa protein. This immunising complex would hopefully elicit

a stronger response to the 109 kDa protein due to its attached carrier protein A.

200μl of protein A was centrifuged 8,000 x g for 10 minutes and the supernatant discarded. The protein A pellet was resuspended in 200μl of tissue culture supernatant containing Mab against the 109 kDa protein and incubated overnight at 4^{o}C with rotation. The mixture was centrifuged at 8,000 x g for 10 minutes and washed 4 times with PBS.

The protein A-antibody complex was incubated with 0.5ml culture supernatant from HK 361 containing 109 kDa protein overnight at 4^{o}C with rotation. This allowed the Mab to bind with the 109 kDa haemolysin protein to complete the complex. The mixture was centrifuged at 8,000 x g for 10 minutes and the pellet resuspended in immunoprecipitate buffer. Following centrifugation at 8,000 x g for 10 minutes, the pellet was washed a further three times in immunoprecipitation buffer and once in PBS. The mixture was resuspended in sterile PBS to 10x the original volume of antibody solution (final volume 2ml) and stored at -70^{o}C.

7.2.6 Immunisation protocol

7.2.6.1 120 kDa protein

150μl of the purified 120 kDa protein was diluted with PBS to 800μl and thoroughly emulsified with 800μl of Freund's complete adjuvant. 0.2ml of the immunising mixture was injected i.p. into 6 week old female Balb C mice. Further immunisations were given using Freund's incomplete adjuvant at day 21. The third and fourth immunisations were administered at a minimum of 4 weeks between each injection. All injections were given i.p. except the final, which was i.v.

7.2.6.2 Protein A-Ab-Ag

0.2ml of the protein A-Ab-Ag immunising antigen was administered i.p. for all immunisations. No adjuvant was used in any of the immunisations.

Blood samples were taken around 10 days after an injection from the mouse tail vein. Blood was allowed to clot and serum recovered as previously described (2.4.1).

7.2.7 Enzyme linked immunosorbent assay (ELISA)

A double antibody indirect ELISA was constructed to screen Mabs for reactivity against the immunising antigens of either haemolysin II (109 kDa) or pleurotoxin (120 kDa).

7.2.7.1 Materials used and their preparation

Normal mouse serum and serum recovered from the fusion mouse were used as negative and positive controls respectively and was obtained as described in 7.2.6. Hyperimmune rabbit serum raised against a serotype 2 strain (HK 353) and immune pig serum from a pig infected with a serotype 3 strain (see Chapter 2) were also used as positive controls. Test samples consisted of hybridoma fluid taken from each well containing growing cells.

Bacteria-free culture supernatant from either serotype 2 strain HK 361 or its mutant, e, was used as the antigenic material bound to the ELISA plates and was prepared as described in 7.2.2.

Conjugated antiserum used included goat anti-mouse IgG and goat anti-rabbit IgG alkaline phosphatase conjugates (Bio-rad) and rabbit anti-pig HRP (Sigma Ltd.). All

conjugates were raised against both the heavy and light chain IgG.

Substrates used were alkaline phosphatase substrate kit (Bio-rad). The reaction was stopped by adding 100μl/well of 0.4M NaOH and OD were read at 405nm. TMB peroxidase EIA substrate (Bio-rad) kit was used to develop HRP colour reaction. The reaction was stopped with 100μl/well of 1N H_2SO_4 and the OD read at 655nm.

ELISA Maxisorp plates (Nunc) were used with an ELISA reader (Micro-ELISA Autoreader MR 580, Dynatech Laboratories Inc., Virginia, USA).

7.2.7.2 Construction and standardisation of ELISA

Optimum working dilution of antigens, control serum and conjugated antiserum was determined by chequerboard titration. Firstly the relative dilution of conjugate was assessed. A fixed concentration of antigen was coated on the plates and fixed dilution of positive and negative mouse serum was added to wells and then double dilutions were made across horizontal rows. Conjugate was then double diluted down vertical columns. Substrate was added for the manufacture's recommended time and once the reaction was stopped, the plates were read at 405nm. The well with the maximum readable absorbance, the lowest concentration of conjugate and the highest concentration of serum was assessed from the ELISA reader printout. This dilution of conjugate was regarded as the relative dilution and was used until the optimum dilution was calculated. Chequerboard titrations were repeated for each antigen and antiserum used. Next the relative antibody dilution was found by using a fixed relative conjugate dilution and titrating both the antigen and serum to detect the well that produced the maximim readable absorbance with the lowest concentration of serum and the highest concentration of

antigen. Once the relative dilutions for the control serum and conjugate were found, the ELISA was repeated under different temperature conditions and incubation periods for each step in the ELISA (Burrells & Dawson, 1982). Once the optimum conditions had been assessed, the ELISA was repeated by titrating each of the reagents to assess the optimum working dilution. The optimum working dilution for each reagent was taken to be the concentration that gave the maximum readable absorbance at the end of the plateau of the curve. Following completion of standardisation, the ELISA was carried out as described below.

1. Optimum concentrations of bacterial culture supernatant from HK 361 and mutant e were found to have batch to batch variations and ranged from 1/2 to 1/8 dilutions. Samples (50μl) of antigen were mixed with coating buffer (50μl) to give the final optimum dilution. 100μl were added to each well of the 96 well plate and incubated overnight at 4^{o}C. During all incubations the plates were covered with cling film to prevent loss of liquid and drying of the wells.

2. Plates were washed 3 times with washing buffer and blocked for 1 hour at 37^{o}C with 200μl/well of blocking solution.

3. Following incubation, plates were washed twice and 100μl of the appropriate serum (test serum or hybridoma supernatant) was added and incubated for 2 hour at 37^{o}C. Initial concentrations of control positive and negative serum used were 1/100 and double diluted down.

4. Plates were washed 3 times and 100μl of the appropriate conjugated second antibody added to each well and incubated at 37^{o}C for 1 hour. All conjugates were used at a working dilution of 1/500.

5. Wells were washed a final 4 times and 100μl of the relevant substrate was added to each well. For the alkaline phosphate substrate, the reaction was allowed to continue for 30 minutes at room temprerature. The reaction was stopped by adding 100μl/well of 0.4M NaOH and the OD read at 405nm. TMB peroxidase EIA substrate kit was added and the reaction was continued until a dark blue product colour had developed. The reaction was stopped with 100μl/well of 1N H_2SO_4 and the OD read at 655nm.

7.2.7.3 ELISA controls

Positive and negative controls included the use of positive mouse serum recovered from the fusion mouse and preimmune serum taken from the same mouse was used as the negative mouse serum. Rabbit serum raised against a serotype 2 bacteria-free culture supernatant and immune pig serum were also used as positive controls for the detection of 109 and 120 kDa proteins. Hybridoma fluid from unrelated clones was also used as a negative control to assess the possible interference of bovine serum used as a growth supplement. Intra-assay controls included wells containing all reagents except one to assess possible background from each reagent.

The lower cut off point was determined to be 1.5 times the background from a well containing the known negative sample of preimmune mouse serum. The end point for the assay was chosen to be 0.2 OD units above the background.

7.2.8 Myeloma fusion partner cells (NSO)

Myeloma fusion partner cells, NSO (Galfre & Milstein, 1982) were recovered from liquid nitrogen stocks approximately 1 week prior to the fusion. The NSO were grown in $RPMI^{++}$ containing 10% FCS. NSO cells were re-

fed daily to ensure the cells were growing rapidly in a logarithmic fashion until the day of the fusion. Cells were recovered using a cell scraper and collected in $RPMI^{+++}$. NSO cells were then centrifuged at 900 x g for 5 minutes and resuspended in 20ml $RPMI^{+++}$.

7.2.9 Splenocyte feeder cultures

Splenocyte feeder cultures were prepared from the spleen of a sacrificed female Balb C mouse. The spleen was removed aseptically and transferred to a tissue culture dish containing RPMI. Excess tissue was trimmed off and the spleen transferred to fresh medium and teased apart to release the splenocytes. Cell clumps were disrupted by pipetting and the cells and medium were transferred to a container. The cell suspension was held for 5 minutes to allow large cell clumps to settle to the bottom. Following this, the cell suspension was transferred to a centrifuge tube taking care to avoid the sediment. Cells were centrifuged at 900 x g for 5 minutes and resuspended in 2ml $RPMI^{+++}$. The cell suspension was irradiated for 9 minutes at 3000 RADS (^{60}Cobalt). The suspension was washed a further twice in $RPMI^{+++}$ and finally resuspended in 60ml of $RPMI^{+++}$HAT. 100μl of splenocyte suspension was added to each well in the 96 well tissue culture plates (Microtest III, Falcon). Plates were wrapped in a plastic bag and incubated at 37^{o}C in a 5% humid CO_2 incubator for 1 day prior to use.

7.2.10 Mouse fusion

3 days after the final immunisation, the mouse was sacrificed, the spleen removed aseptically and transferred to a tissue culture dish containing prewarmed RPMI. Excess tissue was trimmed off and the spleen transferred to fresh medium where it was teased apart to release the splenocytes. Cells clumps were

disrupted by pipetting and the cells and medium were transferred to a universal bottle. The cell suspension was allowed to sit for 5 minutes to allow any large cell clumps to settle to the bottom. The cell suspension was then transferred to another centrifuge tube taking care to avoid the sediment. The cells were centrifuged at 900 x g for 5 minutes and resuspended 5ml of $RPMI^{+++}$. Spleen cells were underlayed in ficoll-paque (Pharmacia) and centrifuged at 900 x g for 10 minutes. The interphase, which contains the B and T lymphocytes, was removed carefully, resuspended in 20ml $RPMI^{+++}$ and centrifuged for 5 minutes at 900 x g. The cell pellet was resuspended in 20ml of $RPMI^{+++}$. Both NSO and spleen cells were counted using a haemocytometer and adjusted to a concentration containing 3 spleen:1 NSO. The cell mixture was centrifuged, the supernatant discarded and the pellet tapped to break up the cells. 0.5ml of prewarmed PEG-1500 (Boehringer) was slowly added dropwise to the cells with continual shaking of the cells to ensure constant mixing. The cells were incubated at $37^{o}C$ for 2-3 minutes and 20ml of RPMI added very slowly, gently swirling the cells to ensure continual mixing. Following centrifugation at 900 x g for 5 minutes, the cells were resuspended in 60ml of $RMPI^{+++}$HAT. 100μl of the cell suspension was pipetted into each well which already contained 100μl of irradiated spleen cells prepared 24 hours previously. Plates were wrapped in individual plastic bags to minimise contamination and incubated in a 5% humid CO_2 incubator.

Mouse fusions were carried out using mice immunised with either the 120 kDa protein or the protein A complex containing the 109 kDa protein.

7.2.11 Hybridoma screening

Plates were screened 10-14 days after each fusion for developing clones. Clones were tested for antibody secretion at a size just visible to the naked eye. At this stage sufficient antibody should have been produced by the cells to give a positive reaction by ELISA. 100μl of culture supernatant was carefully removed and tested by ELISA.

7.2.12 Hybridoma cell cloning by limiting dilution

Clones deemed positive by ELISA were selected and dilution cloned using the method below to give 0.3, 1 and 3 cells per well. This procedure will select individual cell secreters from which "all positive" clones can be selected.

Cells were diluted to 1 x 10^5 cells/ml. The following dilutions were made:-

1. 10μl of 1 x 10^5 cells/ml added to 1ml medium (1 x 10^3 cells/ml)
2. 150μl of 1 x 10^3 cells/ml added to 5ml of medium (3 x 10^1 cells/ml)
3. 2ml of 3 x 10^1 celsl/ml added to 4ml of medium (1 x 10^1 cells/ml)
4. 2ml of 1 x 10^1 cells/ml added to 4ml of medium (3.3 x 10^0 cells/ ml)

100μl of the above dilutions were added to wells already containing 100μl of irradiated splenocytes. Plates were wrapped in plastic bags and incubated in a humid 5% CO_2 37^{o}C incubator for 10-14 days.

7.2.13 Expanding positive clones

Clones secreting antibody against the immunising antigen, determined by a positive reaction of their

supernatants by ELISA, were transferred to a 24 well tissue culture plate (Cluster^{24}, Costar) supplemented with splenocyte feeders and RPMI^{+++} HAT and incubated at 37^{o}C in a 5% CO_2 humid incubator until growth became dense. The cells were then transferred into 5ml of RPMI^{+++}HAT in a 50ml tissue culture flask (Nunclon, Nunc) and a further 5ml of RPMI^{+++}HAT added when cell concentration reached around 10^6 cells/ml. Drug selection was removed at this stage by switching from RPMI^{+++}HAT to RPMI^{+++}HT and then decreasing the concentration of HT by half each time the cells were fed. Cells were transferred to 260ml and then to 600ml flasks following their increase in numbers. At this stage a maximum of 300ml of hybridoma culture supernatant can be obtained from each 600ml flask. The positive clones were continued to be screened at each growth phase to verify the continued secretion of positive antibody. Only those clones which continued to secret positive antibody were further grown and purified.

7.2.14 Freezing positive clones

Cells were frozen in a solution of 90% FCS plus 10% dimethylsulfoxide (DMSO) (Sigma Ltd.) at various stages of growth. Cells were centrifuged at 900 x g for 5 minutes and the supernatant discarded. The cells were suspended in cold freezing solution and transferred to freezing tubes (Cryotubes, Nunc). Cells were stored overnight at -70^{o}C and transferred to liquid nitrogen the following day.

7.2.15 Purification of monoclonal antibodies

Cells were grown up to 100ml in 600ml flasks. Further additions of medium were added to a volume of 300ml using RPMI^{++} containing decreasing concentrations of FCS to give a final concentration of 2% FCS in 300ml. Cells

were allowed to overgrow the medium and die. Increased amount of antibody are produced as clones die. Culture supernatant was centrifuged for 10 minutes at 12,000 x g. Supernatants were filter through a 500ml bottle filter (0.2μm) (Costar) under pressure. IgG antibody was purified from the culture supernatant using a Protein G Sepharose 4 fast flow column (MAbTrap G, Pharmacia LKB). Purified IgG antibodies were precipitated overnight in an equal volume of 50% saturated ammonium sulphate in the cold. The precipitation mixture was centrifuged at 12,000 x g for 10 minutes, the supernatant discarded and the pellet carefully resuspended in 3ml saline. The amount of purified antibody present in the sample was evaluated by calculating the total amount of protein. This was determined by measuring the absorbance at 280nm using a spectrophotometer. An OD_{280} equivalent to 1.0 is approximately equal to 0.75 mg/ml of purified immunoglobulin. Purified Mabs were stored at -70^{o}C in a standard phosphate buffered solution.

7.2.16 Isotyping monoclonal antibodies

A commercial kit was used to determine the isotype of the mouse Mabs (Inno-Lia Mouse Mab Isotyping (Innogenetics N.V., Antwerp, Belgium). The kit is based on a line immunoassay and consists of strips coated with rat anti-mouse Mabs against the different mouse antibody isotypes. Following incubation with test hybridoma supernatant, the strip was developed and compared to a control strip. The isotype was determined by comparing the test line to the known control lines.

7.2.17 Screening reactivity of monoclonal antibodies raised against the 109 and 120 kDa protein by Western blotting

A panel of Mabs deemed positive by ELISA were screened to assess their specificity against the immunising antigen visually by Western blotting.

Due to the work involved in dilution cloning and after care only a handful of possible clones were assessed at any one time. All remaining clones were held frozen for future assessment. A panel of 6 positive clones that remained stable following cloning, were chosen to be examined. Hybridoma fluid from the 6 clones was assessed for the possession of antibody against the immunising antigens of the haemolysin II (109 kDa) and pleurotoxin (120 kDa). Bacteria-free culture supernatant from HK 361 and mutant e were used as the screening antigens, which possessed both the 109 and 120 kDa proteins and only the 120 kDa protein respectively.

The Mabs were next screened against all the serotype reference strains of *A. pleuropneumoniae*, together with related pathogens, to assess the presence of similar proteins that may cross-react with the Mabs raised against the haemolysin II (109 kDa) and pleurotoxin (120 kDa) of the serotype 2 strain HK 361. HK 361 and mutant e together with the *A. pleuropneumoniae* reference serotype strains and related pathogens including *E. coli, A. suis, A. equuli, and P. haemolytica* were grown with the addition of 10mM $CaCl_2$, and culture supernatant obtained as described in 7.2.2. The culture supernatants (5ml) were held in an ice-cold container and ethanol added to give a final concentration of 23% (v/v). The supernatant was held on ice for 60 minutes and centrifuged at 12,000 x g for 15 minutes at 4°C. The resultant pellet was gently redissolved in 60μl roH_20 and 30μl of x3 sample buffer. 90μl of each sample was

added to each well and electrophoresed using SDS-PAGE. Gels were then Western blotted.

7.2.18 Western blotting

Both standard and mini Trans-Blot Cell systems were used (Bio-rad). Prior to blotting gels were washed in NT buffer for 30 minutes. Proteins were electrophoretically transferred onto nitrocellulose membrane filters (Bio-rad) using both the standard (overnight blotting) and the mini (1 hour) Trans-Blot systems in the above buffer. Membranes were incubated overnight in TNT blocking solution. Proteins were then probed overnight with the appropriate antiserum diluted in 5% (w/v) skimmed milk in TNT. Antibody-antigen complexes were probed with HRP conjugated antibody for 1 hour and developed with HRP substrate. Samples were probed with Mabs against both the pleurotoxin (120 kDa protein) and the haemolysin II (109 kDa protein) and immune pig serum from a pig recovering from infection with a *A. pleuropneumoniae* serotype 3 strain, 6664.

7.2.19 Neutralisation assays

Mabs were tested with active pleurotoxin (120 kDa protein) and haemolysin II (109 kDa protein) to assess their neutralising capabilities against these toxic activities.

7.2.19.1 Haemolysin determination and neutralisation

Production and measurement of *A. pleuropneumoniae* culture supernatant containing haemolytic activity has been described in 5.1.4 and 5.1.5. Haemolysin-neutralising activity of the Mabs was determined (Rycroft *et al.*, 1991). A sample (10μl) of hybridoma fluid or diluted hybridoma fluid was incubated with 90μl of haemolytically active cell-free culture supernatant

at 0°C for 60 minutes. Samples were then assayed for haemolytic activity as described in 5.2.5 and compared to controls treated identically with immune or preimmune pig serum. The neutralising titre was expressed as the reciprocal of the dilution of serum required to achieve at least 50% reduction in haemolytic activity of a standard fresh haemolysin preparation.

7.2.19.2 Cytotoxin preparation and neutralisation

Cytotoxin was prepared by growing the *A. pleuropneumoniae* as previously described in 7.2.2. Subcultures were then made (1 in 25) into fresh TSB/NAD and grown for a further 4 hours. Bacteria were removed by centrifugation for 10 minutes at 10,000 x g at 4°C. Culture supernatants were filtered (0.22μm), held on ice and used immediately.

Pig alveolar macrophages (6.2.4) were recovered from liquid nitrogen and diluted in Minimum Essential Medium (MEM) (Gibco, BRL) with 10% foetal calf serum to 10^6/ml cells. A sample (100μl) was added to each well in a 96 well culture plate (Nunc) and incubated for a few hours at 37°C in a 5% CO_2 humid incubator. The supernatant was replaced with fresh medium after 2 hours.

Cytotoxic-neutralising activity of the Mabs was determined (Rycroft *et al.*, 1991). 50μl of the cytotoxic culture supernatant was mixed with an equal volume of hybridoma fluid or diluted hybridoma fluid at 0°C for 60 minutes. The medium from the macrophages was removed and a sample (10μl) of the previously incubated cytotoxic culture supernatant and hybridoma fluid was added to 90μl MEM in a well containing alveolar macrophages and incubated at 37°C for 1 hour. The wells were examined microscopically following the addition of the vital stain, Trypan blue, and macrophage viability was determined. The neutralisation titre was the reciprocal

dilution of hybridoma fluid or control immune or preimmune pig serum required to prevent completely the cytotoxic effect of the test sample.

7.3 RESULTS

7.3.1 120 kDa cytotoxin protein purification

Analysis of the SDS gel demonstrated that well 3 contained the largest amount of 120 kDa protein (Fig. 7.1). The remaining sample from the corresponding eluate fraction was used as the immunising antigen.

7.3.2 ELISA

The number of wells containing hybridoma cells which secreted antibody reactive against the immunising antigen of 120 kDa pleurotoxin were determined by screening against mutant e culture supernatant which only possessed the 120 kDa protein. 68 wells from a total of 6 x 96 well plates were positive for reacting antibody. Of those wells, the hybridoma fluid obtained from 7 wells gave readings over 3.0 OD units, 42 wells gave OD readings of between 2.0 and 3.0 and 19 wells readings of between 1.0 and 2.0 OD. Due to the large number of wells containing positive secreting cells, those wells with an OD below 1.0 were not further assessed. The contents of all other wells with an OD above 1.0 were either further grown or stored in liquid nitrogen for future assessment.

The number of wells secreting positive antibody against the staph A-Mab-109 kDa immunising antigen were screened against HK 361 culture supernatant which contained both the 109 and 120 kDa proteins. Only 2 wells were deemed positive from a total of 6 x 96 well plates. The cut off point for this assay was the normal background plus an OD of 0.2 units.

Fig. 7.1 Detection of 120 kDa pleurotoxin in column eluates by SDS-PAGE followed by Comassie blue staining.

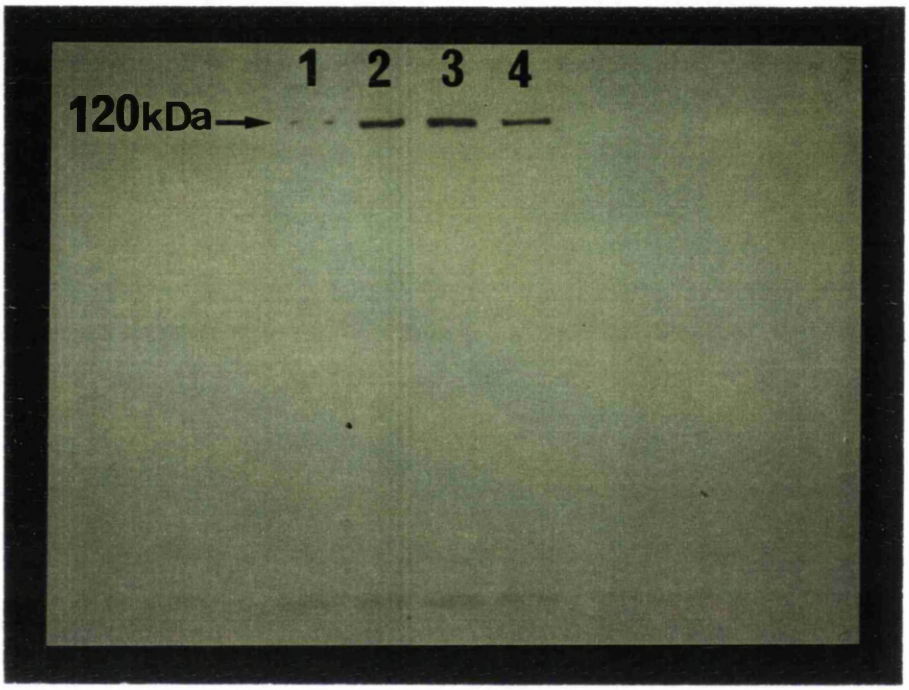


Fig. 7.1

The final 6 Mabs, 5 of which were from the mouse fusion immunised with 120 kDa pleurotoxin (GJMC-1 to 5) and 1 from 109 kDa complex (GJMC-6), were selected due to their continued secretion of specific antibody as determined by ELISA.

Following the observation that the Mabs raised against the 120 kDa pleurotoxin were also cross-reacting with the 109 kDa, all ELISAs employed HK 361 culture supernatant as the screening antigen.

7.3.3 Isotyping monoclonal antibodies

The final selection of 6 Mabs (GJMC-1 to 6) were assessed for isotype. Mabs raised against the 120 kDa protein were found to be of both isotype IgG2a and IgG2b (Fig. 7.2). The 1 Mab raised against the 109 kDa protein was isotype IgG1 (Fig. 7.2). The faint reaction seen with GJMC-5 was correlated with a decrease in antibody levels produced by these clones as detected by ELISA. This clone was frozen down in order to be recloned and assessed at a later stage.

7.3.4 Reactivity of monoclonal antibodies by Western blotting

Reactivity of Mabs raised against the 120 kDa protein (GJMC-1 to 5) was assessed by screening against antigen containing both the 109 kDa and 120 kDa proteins obtained from HK 361 culture supernatant. The Mabs raised against the 120 kDa protein reacted with the 120 kDa protein in the concentrated culture supernatant of the parent strain HK 361 (Fig. 7.3). The Mabs also reacted with a protein present in the culture supernatant of the parent strain, HK 361, corresponding to around 109 kDa, a similar size to the haemolysin II protein (Fig. 7.3). It had been determined within this laboratory that cloned pleurotoxin (120 kDa) produced

Fig. 7.2 Isotypes of monoclonal antibodies raised against either the 109 or 120 kDa polypeptides secreted by *A. pleuropneumoniae*.

Mabs raised against 120 kDa protein:

1) Mab GJMC-1, isotype IgG2b
2) Mab GJMC-2, isotype IgG2b
3) Mab GJMC-3, isotype IgG2b
4) Mab GJMC-4, isotype IgG1
5) Mab GJMC-5, isotype IgG2b

Mab raised against 109 kDa protein:

6) Mab GJMC-6, isotype IgG2a

7) Control illustrating all isotype locations

1 2 3 4 5 6 7
positive control Kappa
IgG1
IgG2a
IgG2b
IgG3
IgM
IgA
IgE

Fig.7.2

Fig. 7.3 Western blot reactions of Mabs raised against the 120 kDa protein probed against HK 361 bacteria-free supernatant antigen preparation.

1) Mab GJMC-1 hybridoma supernatant (neat)
2) Mab GJMC-2 " " "
3) Mab GJMC-3 " " "
4) Mab GJMC-4 " " "
5) Mab GJMC-5 " " "
6) Positive control immune pig serum (1/200)
7) Negative control preimmune pig serum (1/200)

Fig. 7.4 Western blot reactions of Mabs raised against the 120 kDa protein probed against HK 361 and mutant e bacteria-free supernatant antigen preparation.

1) Mab GJMC-1 reacted with HK 361 antigen
2) Mab GJMC-1 reacted with mutant e antigen
3) Mab GJMC-2 reacted with HK 361 antigen
4) Mab GJMC-2 reacted with mutant e antigen
5) Mab GJMC-3 reacted with HK 361 antigen
6) Mab GJMC-3 reacted with mutant e antigen
7) Mab GJMC-4 reacted with HK 361 antigen
8) Mab GJMC-4 reacted with mutant e antigen
9) Mab GJMC-5 reacted with HK 361 antigen
10) Mab GJMC-5 reacted with mutant e antigen

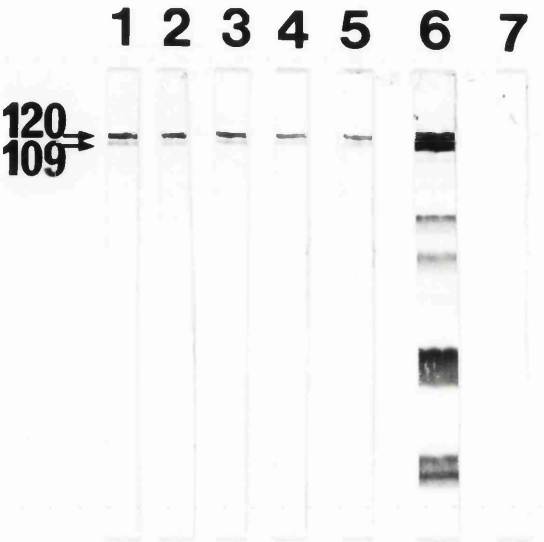


Fig.7.3

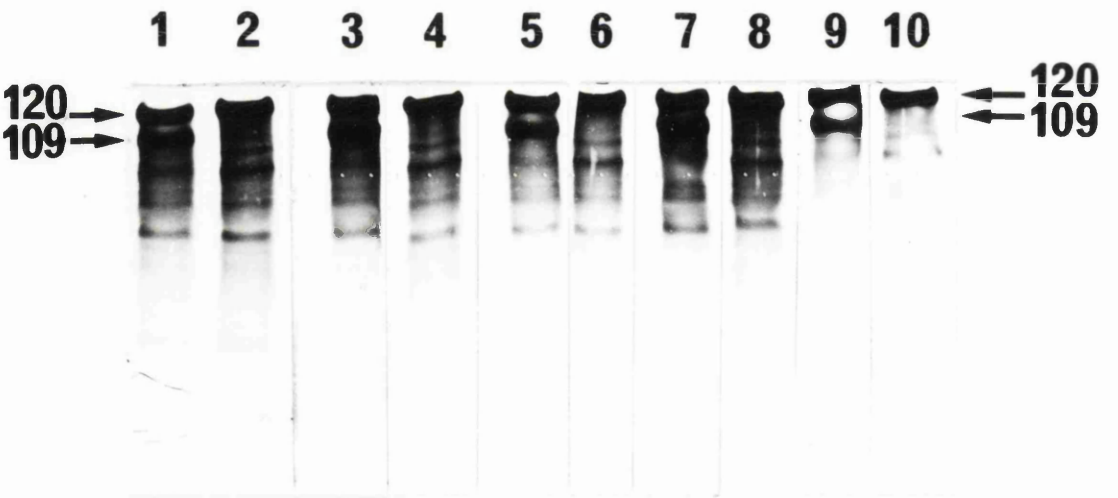


Fig.7.4

breakdown products, one of which was of a similar molecular weight to the 109 kDa haemolysin II.

The Mabs were then rescreened against bacteria-free culture supernatants from mutant e which only produced the 120 kDa protein. It was hoped to compare any breakdown product that was in a similar size position to that seen in the HK 361 antigen preparation previously.

All 5 Mabs reacted in a similar fashion. The Mabs reacted strongly with the 120 kDa protein present in both mutant e and HK 361 antigen preparations (Fig. 7.4). The Mabs also reacted with several protein bands of various molecular weights, however none of these appeared to be the same size (109 kDa) as that seen with the HK 361 antigen.

To assess whether indeed the Mabs raised against the 120 kDa pleurotoxin were also cross-reacting with the haemolysin II (109 kDa), the Mabs were screened against antigen material from an *E. coli* strain in which the haemolysin II had been cloned (available within the laboratory). All 5 Mabs (GJMC-1 to 5) were found to react with the cloned 109 kDa haemolysin II (Fig. 7.5). The negative controls, which consisted of the *E. coli* antigen which did not possess the cloned Hly II, did not show a reaction when screened against one of the GJMC-1 to 5 Mabs raised against the 120 kDa pleurotoxin. Other controls included screening both the cloned Hly II and the host *E. coli* antigen with immune pig serum which resulted in a positive and negative reaction respectively.

The Mab (GJMC-6) raised against the 109 kDa haemolysin II protein was tested in the same way with cloned 109 kDa Hly II and was found to react with it (Fig. 7.5). The Mab GJMC-6 was then tested against bacteria-free culture supernatant from both HK 361 and mutant e to

Fig. 7.5 Western blot reactions of Mabs with cloned 109 kDa haemolytic protein.

E. coli host producing cloned 109 kDa haemolytic protein from *A. pleuropneumoniae*:

1) Mab GJMC-1
2) Mab GJMC-2
3) Mab GJMC-3
4) Mab GJMC-4
5) Mab GJMC-5
6) Mab GJMC-6
7) Positive control immune pig serum (1/200)
8) Negative control preimmune pig serum (1/200)

E. coli host antigen, negative control

9) Mab GJMC-2
10) Mab GJMC-6
11) Positive control immune pig serum (1/200)
12) Negative control preimmune pig serum (1/200)

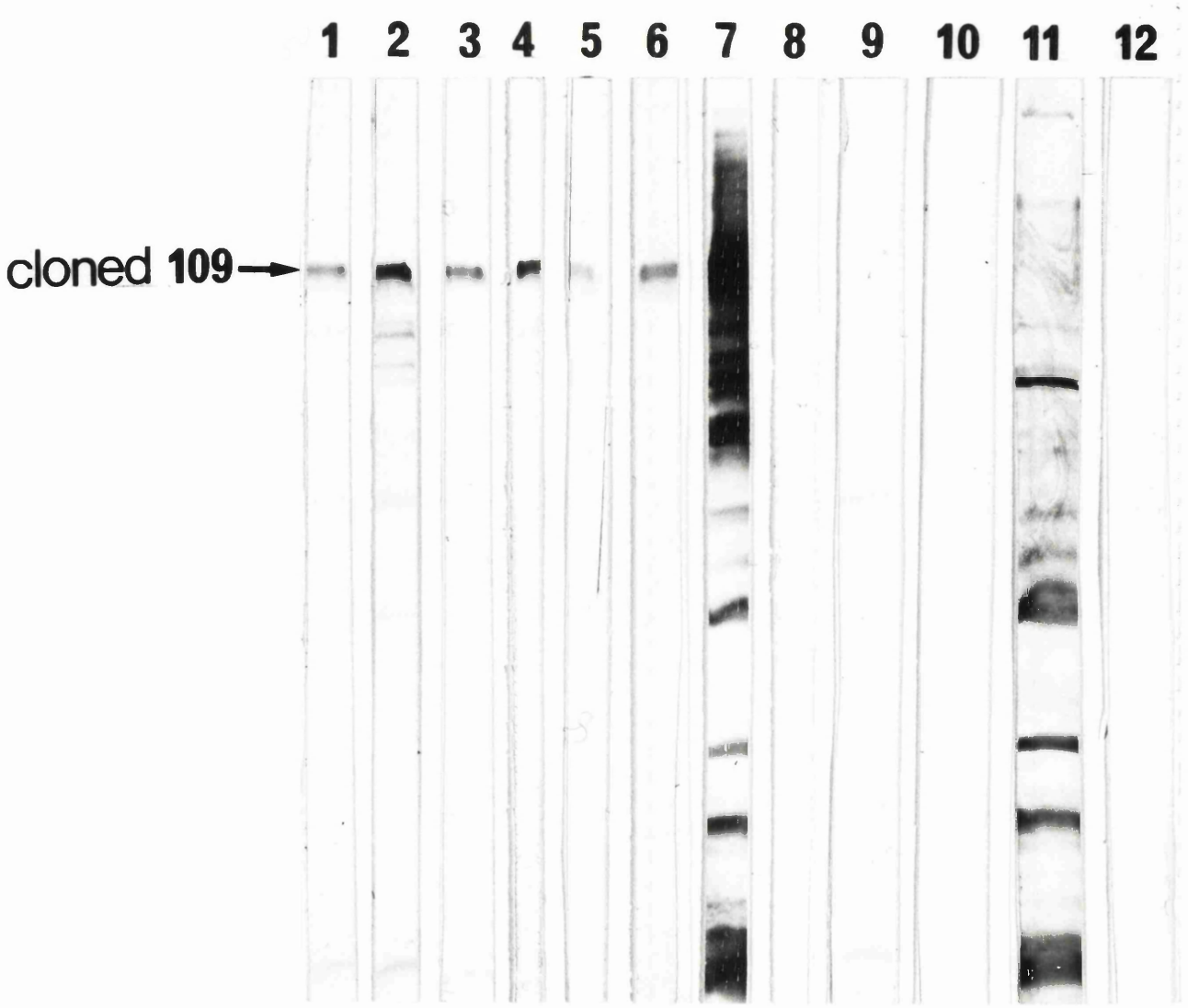


Fig.7.5

assess any cross reactivity of GJMC-6 with the 120 kDa pleurotoxin present in both these antigen preparations. Culture supernatant from mutant h was used as a negative control. This Mab raised against the 109 kDa haemolytic protein was also found to cross-react with a protein of 120 kDa molecular weight present in both HK 361 and mutant e antigen preparations (Fig. 7.6).

The 120 kDa pleurotoxin protein had also been sucessfully cloned within this laboratory (available within the laboratory) and this was used to screen for conclusive reactivity of the GJMC-6 Mab (Hly II) against 120 kDa pleurotoxin. The GJMC-6 Mab was found also to react with the cloned 120 kDa pleurotoxin (Fig. 7.7).

Mabs raised against both the 109 kDa haemolysin II protein, (GJMC-1, GJMC-2, GJMC-3, GJMC-4 and GJMC-5), and the 120 kDa pleurotoxin (GJMC-6), were used to screen for the possible presence of similar cross-reacting haemolytic and cytotoxic activities in culture supernatant from the 12 reference serotypes together with related pathogens from which toxic activities had been reported.

The Mab GJMC-2, raised against the 120 kDa pleurotoxin, cross-reacted with proteins of a similar size in serotypes 2,3,4,6 and 8 (Fig. 7.8). This Mab also reacted with protein in the 109 kDa size range in all serotypes except 2 (Fig. 7.8). In serotype 1, 5 and 9 there was a thick band around the 109 kDa location in certain blots which appeared to demonstrate 2 closely related bands. Cross-reacting proteins were also detected in related pathogens *E. coli*, *A. suis*, *A. equuli* and *P. haemolytica* but not *H. parasuis*. Mab GJMC-2 reacted with proteins in the molecular weight range of 102 (*P. haemolytica*) to 107-109 kDa (*E. coli*, *A. suis* and *A. equuli*).

Fig. 7.6 Western blot analysis of the cross-reactions of Mabs GJMC-2 to GJMC-6 with HK 361, mutant e and mutant h antigens.

Mab GJMC-2 (raised against 120 kDa protein):

1) HK 361 antigen
2) Mutant e antigen
3) Mutant h antigen

Mab GJMC-6 (raised against 109 kDa protein):

4) HK 361 antigen
5) Mutant e antigen
6) Mutant h antigen

Fig.7.6

Fig. 7.7 Western blot reaction of Mab GJMC-6 (raised against 109 kDa protein) with cloned 109 and 120 kDa proteins.

1) Mab GJMC-6 reaction with cloned 120 kDa cytotoxic protein from *A. pleuropneumoniae*
2) Mab GJMC-6 reaction with cloned 109 kDa haemolytic protein from *A. pleuropneumoniae*

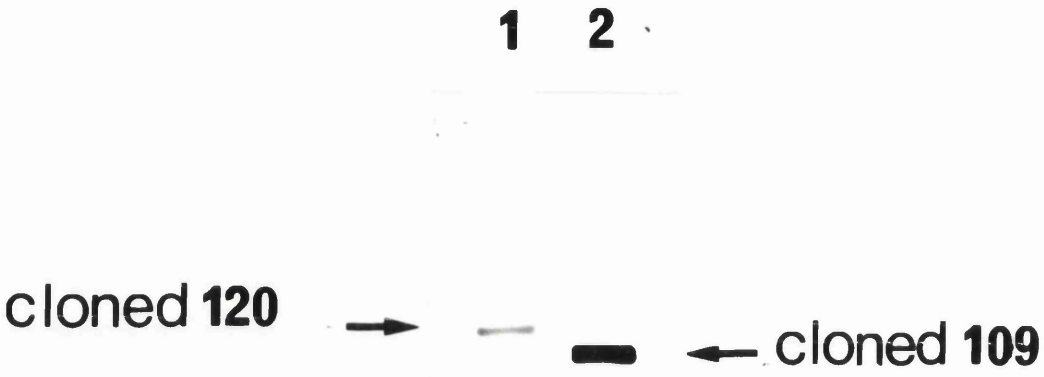


Fig.7.7

Fig. 7.8 Western blot assessment of proteins present in the bacteria-free culture supernatants of *A. pleuropneumoniae* reference serotype strains and related pathogens reacting with a Mab GJMC-2 raised against the 120 kDa cytotoxic protein from *A. pleuropneumoniae* serotype 2 strain HK 361.

Numbers 1-12 represent the serotype reference stains of *A. pleuropneumoniae*. Related pathogens are denoted by their name. The control was HK 361.

120
109
102
Control
A. equuli
P. haemolytica
H. Parasuis
A. suis
E. coli
120
109
1
2
3
4
5a
5b
6
7
8
9
10
11
12

Fig.7.8

Similarly Mab GJMC-6, raised against the 109 kDa haemolysin II protein, reacted with proteins of the same molecular weight in all serotype except 2 (Fig. 7.9). As with Mab GJMC-2, Mab GJMC-6 also detected what appeared to be 2 bands closely related in serotypes 1, 5 and 9. It also reacted with proteins in the 120 kDa weight range in serotypes 2,3,4,6 and 8 (Fig. 7.9).

Mab GJMC-6 also reacted with proteins in the molecular weight range of 102-109 kDa in related pathogens *E. coli*, *A. suis*, *A. equuli* and *P. haemolytica* (Fig. 7.9).

To ensure that the Mabs were reacting with all the 109 and 120 kDa protein present, the bacterial culture supernatant of the 12 serotype reference strains and related pathogens were assessed by both the silver stain method for detection of total protein and Western blot assessment followed by probing with immune pig serum (against serotype 3 strain, 6664). Both the silver staining and screening via immune pig serum gave identical results for the presence of 109 and 120 kDa protein bands. The reactions of immune pig serum was comparable to that with Mabs GJMC-2 and GJMC-6 in that proteins in the 109 kDa range were present in all serotype except 2 and 120 kDa protein was present in serotype 2,3,4,6 and 8 (Fig. 7.10). The reaction of immune pig serum with all the proteins present in the silver stain gel demonstrated cross-reactivity of the immune pig serum with all the the haemolytic and cytotoxic proteins present in the *A. pleuropneumoniae* serotype specific strains. Cross reaction was also seen in the 102-109 kDa range with all the related pathogens except *H. parasuis* (Fig. 7.10). The presence of similar cross-reacting proteins in the 12 serotype reference strains is summarised in Table 7.1.

Fig. 7.9 Western blot assessment of proteins present in the bacteria-free culture supernatants of *A. pleuropneumoniae* reference serotype strains and related pathogens reacting with a Mab GJMC-6 raised against the 109 kDa haemolytic protein from *A. pleuropneumoniae* serotype 2 strain HK 361.

Numbers 1-12 represent the serotype reference stains of *A. pleuropneumoniae*. Related pathogens are denoted by their name. The control was HK 361.

120
109
1
2
3
4
5
5
6
7
8
9
10
11
12
E. coli
A. suis
H. Parasuis
P. haemolytica
A. equuli
Control
120
109
102

Fig.7.9

Fig. 7.10 Western blot assessment of proteins present in the bacteria-free culture supernatants of *A. pleuropneumoniae* reference serotype strains and related pathogens reacting with immune pig serum from *A. pleuropneumoniae* serotype 3 strain 6664.

Numbers 1-12 represent the serotype reference stains of *A. pleuropneumoniae*. Related pathogens are denoted by their name. The control was HK 361.

120
109
E. coli
A. suis
H. parasuis
P. haemolytica
A. equuli
Control
120
109
1 2 3 4 5a 5b 6 7 8 10 9 11 12
120
109

Fig.7.10

Table. 7.1 Distribution of the 109 and 120 kDa proteins in the 12 serotype reference strains of *A. pleuropneumoniae*

Serotypes	1	2	3	4	5	6	7	8	9	10	11	12
M.W. kDa												
109	*	*		*	*	*	*	*	*	*	*	*
120		*	*	*		*		*				

7.3.5 Toxin neutralisation tests

All 6 Mabs were found not to neutralise either the haemolytic or cytotoxic activity even when neat hybridoma supernatant was used (Table 7.2). This was in contrast to immune pig serum which was found to possess strong neutralising activity against both haemolytic and cytotoxic activities (Table. 7.2).

7.4 DISCUSSION

In the 3 year period between the beginning and end of the experimental work described in this thesis, there had been an enormous amount of literature reported on the haemolytic and cytotoxic activities of *A. pleuropneumoniae*. The information available at the beginning of the work described here was that a protein of 105 kDa had been identified as the haemolysin of *A. pleuropneumoniae*. On the basis of different requirement for Ca^{++}, 2 different haemolysins were designated, Hly I and Hly II (Frey & Nicolet, 1990). These 2 haemolysins were shown to be immunologically related but possessed distinct activities following the observation that polyclonal rabbit serum raised against Hly I from a serotype 1 strain cross-reacted with the Hly II from a serotype 2 strain as shown by Western blotting. However, the Hly II activity could not be neutralised by polyclonal rabbit serum raised against Hly I, but could be neutralised by convalscent pig serum from a serotype 1 strain. In contrast immune pig serum from a serotype 2 infection however could not neutralise the Hly I activity.

Other studies using the same serotype 1 strain reported both haemolytic and cytotoxic activity for a range of pig cells that appeared to be associated with a 130 kDa protein along with several smaller molecular weight proteins (Udeze & Kadis, 1988). Haemolytic activities

Pig Serum	Anti-haemolytic activity	Anti-cytotoxic activity
Convalescent	256	128
Normal	8	8
Monoclonal Antibody		
GJMC-1	<2	<2
GJMC-2	<2	<2
GJMC-3	<2	<2
GJMC-4	<2	<2
GJMC-5	<2	<2
GJMC-6	<2	<2

Table 7.2 - Reciprocal dilution of antibody source required for neutralisation of haemolytic and cytotoxic activity.

were found in strains from 12 serotypes associated with a 104 kDa protein and were showed to be immunologically related but possessed differences in activity (Devenish *et al.*, 1989). In this laboratory both 109 and 120 kDa proteins were identified in strains from serotype 2,3 and 4 that were associated with both haemolytic and cytotoxic activity. The isolation of a serotype 2 mutant that secreted the 120 kDa protein only, and possessed cytotoxic but not haemolytic activity, and a mutant, h, which was negative in both the 109 and 120 kDa proteins and did not secrete cytotoxic or haemolytic activity, suggested the cytotoxin of this strain was associated with a 120 kDa protein. This together with the observation that polyclonal serum raised against the 120 kDa protein neutralised cytotoxic but not haemolytic activity, suggested that the cytotoxic activity, associated with the 120 kDa protein, was distinct from the haemolysin in these strains.

Although similar proteins to the 109 kDa haemolysin had been reported by other researchers (104 and 105 kDa; Devenish *et al.*, 1989; Frey & Nicolet, 1990), the appearance of the non-haemolytic 120 kDa cytotoxic protein had not.

The cross-reactivity of the Mabs produced individually against the 109 kDa Hly II and the 120 kDa Pleurotoxin with both the 109 and 120 kDa proteins demonstrated that in fact these 2 proteins appeared to be immunologically related (Fig.7.6). The intensity of reaction of each Mab was stronger for the immunising antigen than for the cross-reacting antigen (Fig. 7.6). This was in contrast to that seen with the polyclonal rabbit serum described by co-workers who did not detect cross-reaction with the 109 kDa protein. However the concentration of antigen described in this chapter was much greater that that used in the previous study, and further assessment of the polyclonal serum used in that study (Rycroft *et al.*,

1991) with the antigen preparation used here, demonstrated that in fact the polyclonal rabbit serum raised against the 120 kDa protein did cross-react with the 109 kDa protein. This suggested that although the 2 proteins appeared to be distinct in activity, they were immunologically related. Similarly the previous reaction seen with the original Mab produced against the 109 kDa protein, which was used as part of the immunising complex in an attempt to elicit a stronger reaction, also appeared to react only with the 109 kDa protein. However this reaction was weak and in hindsight the antigen concentration used in those previous examinations would not have been high enough to detect any possible cross reaction.

Around this time other researchers had also noted the presence of a 120 kDa protein in the culture supernatant from a different strain of serotype 2 of *A. pleuropneumoniae*. They proposed that the 120 kDa protein was the cytotoxin and that the 105 kDa was its inactive form (Kamp *et al.*, 1990). Following the successful cloning of both the 109 and 120 kDa proteins within this laboratory by co-workers, it was conclusively shown that the the 109 kDa protein was not a breakdown product of the 120 kDa, and that, in fact, they were 2 distinct proteins. They did however share some degree of immunological relatedness as demonstrated by the cross-reactivity of the Mab raised against the 109 Hly II protein, GJMC-6, to both the cloned 109 and 120 kDa proteins, and similarly the cross reaction of Mab GJMC-1-5 raised against the 120 kDa pleurotoxin, to both the cloned Hly II and Ptx (Fig. 7.5, 7.7).

The presence of a 120 kDa protein had been previously reported within this laboratory in serotypes 2, 3, and 4. Following the analysis of all the serotypes by Mabs, immmune pig serum and silver staining, the 120 kDa protein was found in serotypes 2, 3, 4, together with 6

and 8 (Figs. 7.8, 7.9, 7.10). Other groups also had recently reported the presence of a 120 kDa protein in serotypes 2, 3, 4 and 8 but not 6 (Kamp *et al.*, 1991). The presence of a 120 kDa protein in these serotypes, including 6, was confirmed by DNA hybridisation experiments within this laboratory that showed the gene encoding for this protein to be in serotypes 2,3,4,6 and 8. The presence of a potent cytotoxin in serotype 6 was unexpected because it had been found to produce little or no cytotoxic activity *in vitro* (Pijoan, 1986; Kamp & van Leengoed, 1989). This highlighted the differences that were being experienced by all researchers in this field who found a varying range of activities from the same strains. The method of culture appeared to be important in the expression of both haemolytic and cytotoxic proteins due to their fast biodegradability once secreted by the organism.

The Mabs and immune pig serum detected the presence of a 109 kDa protein in all serotypes (Fig. 7.8, 7.9, 7.10). This had previously been reported by others who found the corresponding 104 or 105 kDa protein in all serotypes (Devenish *et al.*, 1989, Frey & Nicolet, 1990). It had also been previously reported that Hly I and Hly II were immunologically related and therefore following the detection of Hly II 109 kDa protein, in all serotypes that possessed either Hly I or Hly II or both, it was shown that the Mabs against Hly II also cross-reacted with Hly I.

Other researchers had also raised Mabs against the culture supernatant from a serotype 2 strain and also against a serotype 9 strain (Kamp *et al.*, 1991). They found the presence of 3 different proteins in the 12 serotypes. A 103, 105 and 120 kDa protein. The 103 kDa protein was proposed to be Hly II, the 105 kDa to be Hly I and the 120 kDa protein, to be the equivalent of the pleurotoxin reported here. These researchers found the

Hly I (105 kDa) to be present in serotypes 1, 5, 9, 10 and 11, Hly II (103 kDa) in all serotypes except 10 and the equivalent of Ptx (120 kDa) in serotypes 2,3,4 and 8. The location of the closely related 103 and 105 kDa proteins in serotypes 1,5,9 and 11 correlates at least in part to the thick band that appeared in certain blots to be two closely related proteins in serotypes 1,5 and 9 (Fig. 7.10). The closeness of these bands explains why it is very difficult to distinguish them.

The panel of Mabs from the study by Kamp *et al.* (1991) reacted with either the Hly I, or Hly II or the Ptx. Only one Mab reacted with both the Hly I and Hly II. This suggested that only Hly I and II possessed cross reactive epitopes. However here it has been shown that all 3 protein possess similar cross reactive epitopes. The Mabs in that study were found to possess neutralising abilities against the reacting antigen. The Mabs described in this chapter did not possess any neutralising abilities and therefore it would appear that these Mabs have been raised against a common, but not neutralising, epitope.

The cross-reaction of both Mabs with related pathogens confirm the relatedness of the toxins of *A. pleuropneumoniae* with other Gram-negative bacteria. DNA studies have also confirmed this recently and found the toxins of *A. pleuropneumoniae* (Hly I and II) to be members of the RTX (repeat in structural toxin) toxin family (Chang *et al.*, 1989; Frey *et al.*, 1991) along with alpha-haemolytic *E. coli*, *A. suis*, *A. equuli* and *P. haemolytica*. *H. parasuis* has not been reported to be a member of the RTX secreters and non-detection of any cross-reactive proteins is therefore in agreement with this (Fig. 7.8, 7.9, 7.10).

The reduction in the antibody levels of Mab GJMC-5 was perhaps due to non-secreting cells overgrowing the

secreting cells. This appearance of non-secreting clones can be due to these clones having a genetic rearrangement and the vital genetic material for specific antibody production being thrown out. The solution to this is to continually test clones and to perform dilution cloning to detect any non-secreting sub-groups.

In summary the results reported here are in agreement with those reported by others with a few minor differences. Kamp *et al.* (1991) reported the presence of a weak 103 kDa protein together with a 120 kDa protein in the serotype 2 reference strain. The haemolytic activity of the strain has previously been found to be very weak (Frey & Nicolet, 1990). The presence of a strong 109 kDa (equivalent to 103 kDa) band has been found in other serotype 2 strains at lesser concentrations of antigen within this laboratory and therefore the difference between these strains and the serotype 2 reference strain may be due to the quantity produced by individual strains. The presence of a 120 kDa protein in serotype 6 also contrasted with the results reported here and those of Kamp *et al.* (1991). However it was confirmed by DNA hybridisation experiments within this laboratory (MacDonald & Rycroft, 1992). It therefore appears that each serotype of *A. pleuropneumoniae* possesses at least 1 of the 3 toxic proteins. The activities of the 3 toxins have been further analysed by Kamp *et al.* (1991). The Hly I (105 kDa) is strongly haemolytic and cytotoxic, the Hly II (103 kDa, 109 kDa in this laboratory) is weakly haemolytic with moderate cytotoxic activity, and the equivalent of the 120 kDa Ptx reported here, was strongly cytotoxic with no haemolytic activity. The presence of these toxins in all serotypes of *A. pleuropneumoniae* helps to explain why the 12 serotypes are all capable of producing the same pattern of disease (Nielsen, 1986a; Rosendal *et al.*, 1985; Sebunya and

Saunders, 1983). The differences in virulence between the serotypes may be due to the differences in the type and/or amount of toxin produced.

The cross-reactivity of the Mabs described here for related pathogens is similar to that seen by others using a polyclonal serum against the haemolysin of serotype 1 (Devenish *et al.*, 1989). The lack of specificity of these Mabs for *A. pleuropneumoniae*, rules out the potential use of these Mabs to screen specifically for *A. pleuropneumoniae* infection.

GENERAL DISCUSSION

The specific aims of this study were to examine the interaction of the pig's host defences against *A. pleuropneumoniae*. Clinical signs can occur within 4 hours post infection. The rapid onset of clinical signs, together with the severity of the disease, suggest that the natural defences of the non-immune pig have little or no effect against *A. pleuropneumoniae*. Two of the major host defences available to fight against infection are the complement and phagocytic systems. The role of these 2 host defence systems in the control of *A. pleuropneumoniae* was therefore assessed. The first aim was to examine the effect of the complement system against *A. pleuropneumoniae*, which had been previously unexplored.

The results in Chapter 3 describe how *A. pleuropneumoniae* was found to be resistant to both normal and immune pig serum. This suggested the presence of specific antibody did not sensitise *A. pleuropneumoniae* to the bactericidal effects of complement. During this period other researchers also found different strains of *A. pleuropneumoniae* to be resistant to immune pig serum (Inzana *et al.*, 1988; Udeze & Kadis, 1992). One of the reasons proposed for the serum resistance of *A. pleuropneumoniae* was the possession of a capsule (Inzana *et al.*, 1988). Capsules have been described to aid the resistance of bacteria to complement by inhibiting complement activation (Kasper, 1966).

The results presented in this chapter also suggest that the outer membrane may be important in the resistance of *A. pleuropneumoniae* to complement bactericidal activity since *A. pleuropneumoniae* could not be sensitised to complement following treatment with a membrane disorganising chemical, polymyxin B. It therefore appears that both the capsule and the outer membrane are perhaps involved in the serum resistance.

The work described in Chapters 4 and 5 describes attempts to define the exact mechanism used, by which *A. pleuropneumoniae* avoids complement bactericidal activity. Bacteria employ a range of mechanisms to avoid the detrimental activities of complement (Joiner, 1988). One of these methods includes non-activation of complement. The results presented in Chapter 4 suggest that this is not the case with *A. pleuropneumoniae*. High percentages of complement activity were consumed via both the classical and the alternative pathways by all strains of *A. pleuropneumoniae* tested. This is in agreement with other researchers who have since found that different strains of *A. pleuropneumoniae* also consume complement activity (Udeze & Kadis, 1992). The possession of a capsule by *A. pleuropneumoniae* therefore did not appear to prevent complement activation. It has been shown in other bacteria that possession of a capsule does not always block subcapsular activation by LPS (van Dijk *et al.*, 1979). Udeze and Kadis (1992) found that the amount of complement activity consumed increased when increased amounts of immune pig serum were added to pig serum previously absorbed with *A. pleuropneumoniae*. Up to 100% complement consumption was seen in the presence of 2.5% immune serum. Normal pig serum was also found to increase the amount of complement consumed but to a lesser extent than immune serum. This suggested both specific and non-specific antibodies function to increase complement consumption. It is reported in this thesis that high complement consumption rates occurred in the presence of normal pig serum which did not contain any detectable specific antibodies to *A. pleuropneumoniae*. This may suggest the presence of specific antibody against *A. pleuropneumoniae* is not a prerequisite for high complement consumption.

Consumption of complement activity does not always result in activation of complement as described in

Chapter 4. However it was shown that complement component C3 was in fact activated. This demonstrated that both consumption and activation of complement was occurring at least up to the C3 stage. Other researchers have demonstrated bound C3 on the surface of a serotype 1 strain 4074 suggesting that C3 was also activated (Udeze & Kadis, 1992).

Knowing that the lack of complement activation was not the mechanism used by *A. pleuropneumoniae* to avoid complement-mediated activity, other mechanisms were investigated. Serum resistant bacteria can also avoid complement damage by releasing molecules that can activate, deplete or destroy complement components (Joiner, 1988). These mechanisms may or may not depend on bacterial viability. In Chapter 5 the ability of viable and non-viable bacteria to consume complement was assessed and only viable bacteria were found to consume complement activity. This suggests the factor(s) responsible for consumption of complement was produced only by viable *A. pleuropneumoniae*. The secreted products of metabolism, which include toxins produced by *A. pleuropneumoniae*, were next assessed for consumption of complement activity. However, it was found that bacteria-free culture supernatant, which possessed haemolytic activity, did not consume complement activity. At that time only the haemolytic activity associated with a 109 kDa protein (haemolysin II) had been identified from these strains. Later, a potent cytotoxin (pleurotoxin, Ptx), that possessed no haemolytic activity, was also found to be secreted by these same serotypes used in Chapter 5. The preparations used in Chapter 5 would have also contained cytotoxic activity as determined by later experiments using identical growth conditions. It can therefore be concluded that Hly II and Ptx, are not responsible for the consumption of complement activity.

There are numerous other ways in which complement bactericidal activity can be avoided and these include incorrect formation or insertion of the complement membrane attack complex (MAC). Alternatively, both completion of the complement cascade and correct formation of the MAC may occur, but are prevented from causing lethal damage by perhaps the capsule or outer membrane of *A. pleuropneumoniae*. These other options of complement resistance can only be studied when sufficient pig complement components have been purified and antisera raised in order to study the complement cascade in detail.

Although the specific mechanisms utilised by *A. pleuropneumoniae* to avoid complement damage are still not known, it has been shown by this study that the lack of complement damage is not due to non-activation of complement activity at least to component C3. It has also been demonstrated that a component(s) of viable *A. pleuropneumoniae* is required to consume complement activity and the component(s) responsible is not one of the secreted toxins of *A. pleuropneumoniae*.

Other researchers have proposed that the mechanism of complement resistance in *A. pleuropneumoniae* involves non-specific antibody present in both non-immune and immune pig serum (Udeze & Kadis, 1992). This has been suggested to block the bactericidal activity of complement. In agreement with the results shown here, it was found that *A. pleuropneumoniae* was resistant to both normal and immune pig serum and consumed up to 100% of the complement activity. They also found that non-immune pig serum absorbed with *A. pleuropneumoniae* was lethal for *A. pleuropneumoniae* when purified specific antibody was added into the system. The non-immune serum used in that study reacted with almost all of the proteins found in *A. pleuropneumoniae* as determined by Western blotting. In contrast the pre-immune serum described in

this thesis did not possess any reactive antibody against *A. pleuropneumoniae* and did not kill *A. pleuropneumoniae*. Although the preimmune serum described in this thesis did not possess reacting antibody against *A. pleuropneumoniae*, and therefore so called "blocking antibody" would not be present, the presence of purified specific antibody against *A. pleuropneumoniae* is perhaps required to detect killing. Only one serotype strain was studied by Udeze and Kadis (1992) and until more serotypes are analysed, and the strains used in this study are assessed by the same method, this mechanism of serum resistance for the strains used here cannot be confirmed.

The second aim of the work was to define the role of phagocytic cells in the presence of toxins produced by *A. pleuropneumoniae*. Chapter 6 detailed the effect of the secreted toxins of *A. pleuropneumoniae* on the ability of pig alveolar macrophages to phagocytose. Only mutant h, which was deficient in both Hly II and Ptx, was found not to cause damage to alveolar macrophages and to be phagocytosed in large numbers, but only in the presence of immune pig serum. Heat or formaldehyde-treated mutant h was phagocytosed readily in the presence of normal pig serum demonstrating that specific antibody was not required for the phagocytosis of *A. pleuropneumoniae* as previously thought (Udeze & Kadis, 1988; Thwaits & Kadis, 1991). Immune serum was required however to neutralise the toxic activity to allow the survival of the phagocytic cells and to enable them to phagocytose. The results reported in the thesis therefore demonstrate the mechanism used by *A. pleuropneumoniae* to avoid phagocytosis is by secreting toxins which kill alveolar macrophages.

The final chapter investigated the relationship between the 2 toxic activities associated with a serotype 2 strain (Hly II and Ptx) and to assess the presence of

similar proteins in the serotype reference strains of *A. pleuropneumoniae*. The presence of 1 or more cross-reactive proteins in all 12 serotypes demonstrated a common property of *A. pleuropneumoniae* strains. Reactivity of Mabs with proteins that were later found to correspond to Hly I, Hly II and Ptx, demonstrated all toxins were in fact immunologically related. This result appears to differ from those of other researchers who found that the majority of Mabs reacted only with either the Hly I or Hly II or Ptx and only 1 Mab reacted with both Hly I and Hly II (Kamp *et al.*, 1991). A more recent report using Mabs raised against either the haemolysin from serotype 1 or serotype 2, found that they reacted with only one of the 3 toxins produced by *A. pleuropneumoniae* (Frey *et al.*, 1992). Polyclonal rabbit serum also reacted only with the haemolysins used as the immunising antigen. The Mabs described in Chapter 7 do not neutralise haemolytic or cytotoxic activity in contrast to those reported by Kamp *et al.* (1991) and perhaps this could explain the differences in cross reaction. However, Mabs produced by Frey *et al.*, (1992) also did not neutralise any toxic activity and did not cross-react with the other 2 toxins.

The results presented here show that all 3 toxins do possess cross-reactive epitopes and the reasons for the lack of detection by others may be due to either the Mabs being raised against different epitopes or the concentration of antigen preparation used in Western blotting, which may not have been sufficient to allow a positive reaction. It has also been shown in this thesis that immune serum raised against a serotype 3 infection cross-reacts with all 3 toxins. This serum was raised against a strain producing both Hly II and Ptx and therefore suggests that these antibodies are also able to cross-react with the third toxin not produced by that strain. A degree of relatedness between the 3 distinct toxin patterns has been confirmed by DNA sequencing and

hybridisation studies. These results have recently been summarised by Frey *et al.*, (1993). The 3 toxins have now been characterised in terms of their activities and the serotypes which possess them. Due to several groups working simultaneously on the characterisation, several different designations were used, however this has now been standardised (Frey *et al.*, 1993). The presence of the 120 kDa protein in serotype 6 described in this thesis has now been documented by others (Jansen *et al.*, 1992). The most recent published consensus of the distribution of the 3 RTX toxins in the 12 serotypes of *A. pleuropneumoniae* is summarised in Table 8.1.

The 3 toxins produced by *A. pleuropneumoniae* all belong to the RTX toxin family (Frey *et al.*, 1993). The haemolysins I, II and pleurotoxin described in this thesis are now designated Apx I, II and III respectively. Apx I is a 105 kDa protein and possesses strong haemolytic and cytotoxic activity and requires Ca^{++} for induction but not the expression of these activities; Apx II (103 kDa) is weakly haemolytic with moderate cytotoxicity and requires Ca^{++} for expression but not induction of activity; Apx III (120 kDa) is a strongly cytotoxic protein and possesses no haemolytic activity (Frey *et al.*, 1993).

Although the toxins are likely to be important factors in pathogenicity and have been shown to be important immunogens (Devenish *et al.*, 1990a,b; Ma & Inzana, 1990; Frey & Nicolet, 1991; Rycroft *et al.*, 1991), other virulence factor are also likely to be involved since vaccination with these toxins alone does not always prevent lung pathology, although they do prevent death and reduce the severity of the disease (Devenish *et al.*, 1990a; van den Bosch *et al.*, 1992).

Recent vaccine studies using a combination of an outer membrane protein of 42 kDa, which was not further

Table 8.1 Distribution of RTX toxins, Apx I, Apx II and Apx III, in the 12 serotypes of *A. pleuropneumoniae*

Serotypes	1	2	3	4	5	6	7	8	9	10	11	12
Apx I	*				*				*	*	*	
Apx II	*	*	*	*	*	*	*	*	*		*	*
Apx III		*	*	*		*		*				

characterised, and both Hly I (Apx I) and Ptx (Apx III), was found to prevent death (van den Bosch *et al.*, 1992). In certain studies no lung lesions developed, however when a different challenge serotype was used, small lung lesions were detected. It seems that a multi-component vaccine may be the way forward, providing it possesses all the important immunogens.

It has been demonstrated that specific antibody is sufficient to allow full protection against disease. This has been shown in pigs receiving colostrum from infected sows or pigs passively receiving immune sera, being protected until the decline of the antibody. This suggests stimulation of antibody is sufficient to provide full cross protection. One way to assess which components of the bacteria are important in stimulating a protective immune response is to screen all antibodies produced following infection to determine their antigen specificity.

Certain antigens have been shown to be produced only under conditions that mimic those *in vivo*. These include those directed against iron repressible proteins (Deneer & Potter, 1989a; Niven *et al.*, 1989; Gonzalez *et al.*, 1990; Ricard *et al.*, 1991; personal observations). If antibody detection systems used to determine the reacting antigen following infection do not contain antigens that are only produced *in vivo*, then potentially important immunogens will not be assessed. It has been found that all 12 serotypes of *A. pleuropneumoniae* produce iron repressible outer membrane proteins, many of which are of similar molecular weight (personal observations). Iron is one of the essential requirements of growing bacteria and many have developed systems to sequester iron from the host. A vaccine containing a common iron repressible protein may therefore prevent the continued growth of bacteria. Pigs can also become carriers of *A. pleuropneumoniae* although

they do not appear to suffer from the disease. These bacteria are able to remain in contact with the tonsils of healthy carrier pigs. Certain strains of *A. pleuropneumoniae* are reported to possess pili (Utrera & Pijoan, 1990). Another important immunogen may also contain pili which may block attachment of *A. pleuropneumoniae*. Stress and changes in climate, among other things, have been shown to promote outbreaks of disease perhaps originating from the carrier pigs. Isolation of carrier from the non-carrier pigs would also promote reduction in disease.

The prevention of disease due *to A. pleuropneumoniae* could therefore be angled in 2 ways. Firstly an effective screening system which can be used in the field prior to introdution of new pig stock into a clean herd. The test would ideally be fast and simple to use and be able to screen against all serotypes of *A. pleuropneumoniae* without cross-reaction from related organisms. Secondly, an effective vaccine might include a mixture of the 3 toxins and a common iron repressible protein together with an adhesion factor. This would hopefully neutralise toxic activity, prevent any significant growth and reduce colonisation by the bacteria.

In summary the results presented in this thesis demonstrate that *A. pleuropneumoniae* is capable of avoiding the anti-bacterial effects of 2 major non-specific humoral defences, the complement and phagocytic systems. This may help to explain why the disease can occur so quickly and violently in naive animals.

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APPENDIX 1

Chapter 3 buffers and solutions

Calcium chloride (30mM)
6.573g $CaCl_2.6H_2O$
to 1L reverse osmosis (ro) H_2O. Filter sterilise and store at 4°C.

Magnesium chloride (100mM)
20.330g $MgCl_2.6H_2O$
to 1L roH_2O. Filter sterilise and store at 4°C.

Gelatin solution (10%)
1g gelatin
to 10ml roH_2O. Heat to 56°C to dissolve and filter sterilise. Store at 4°C.

Buffer M
8g NaCl
0.2g KCL
2.9g $Na_2HPO_4.12H_20$
0.2g KH_2PO_4
to 900ml roH_2O and heat sterilise. Once cool add in:
10ml gelatin
5ml 30mM $CaCl_2$
1ml 100mM $MgCl_2$
to 1L roH_2O, pH7.4. Store at 4°C.

Chapter 4 buffers and solutions

Reagents were kept cold unless otherwise stated. All experiments were performed using glassware except for centrifugation procedures.

<u>Functional haemolytic complement assay stock solutions</u>

Veronal-buffered saline x5 (VBS)
Solution A
85.0g NaCl
3.75g $NaC_8H_{11}N_2O_3$
to 1L ro H_2O
Solution B
5.75g $C_8H_{12}N_2O_3$
to 600ml roH_2O by vigorous stirring on a heated plate. Mix solutions A and B and adjust to 2L roH_2O. pH 7.4-7.6. Store at 4°C for 1 month.

Isotonic EDTA (86mM/l)
Solution A
89.338g Na_2H_2 EDTA
to 1.5L roH2O, then to 2L. pH 4.5.
Solution B
24g NaOH
to 1.5L roH2O, then to 2L. Add solution A to B until pH between 7.2-7.6.
Calculate final molarity of the EDTA by:

starting volume EDTA divided by starting volume EDTA plus volume NaOH and this figure multiplied by 0.12. Store at 4°C for 1 month.

EGTA (100mM)
38.04g EDTA
to 500ml roH^{2}0. pH 7.4 with 10M/L until EDTA dissolved. Adjust to 1L roH$_2$O. Store at 4°C for 1 month.

10% Gelatin
10g gelatin
to 100ml roH$_2$O on heated plate. Store at 4°C. Redissolve in boiling water.

Calcium chloride (30mM/L)
3.286g $CaCl_2.6H_2O$
to 500ml roH$_2$O. Store at 4°C.

Magnesium chloride (100mM/L)
10.165g $MgCl_2.6H_2O$
to 500ml roH$_2$O. Store at 4°C.

Isotonic veronal-buffered saline containing gelatin and cations (GVB^{2+})
200ml 5 x VBS
10ml $MgCL_2$ (100mM/l)
5ml $CaCl_2$ (30mM/l)
10ml 10% gelatin
Add gelatin to 100ml roH$_2$O at room temprerature. Add other reagents and roH$_2$O to 1L. Store at 4°C, prepare fresh twice weekly.

Isotonic veronal-buffered saline containing gelatin but without cations (GVB$^=$).
200ml 5 x VBS
10ml 10% gelatin
Melt gelatin in boiling water and add to 100ml roH$_2$O prior to adding to 5 x VBS. Make to 1L. Store at 4°C, prepare fresh twice weekly.

VBS-EGTA
20ml 5 x VBS
10ml EGTA (100mM/L)
7ml $MgCl_2$ (100 mM/L)
to 100ml roH$_2$O. pH 7.4-7.6. Store at 4°C, prepare fresh daily.

Isotonic dextrose without cations (D5W$^=$)
50g D-glucose
to 1L roH$_2$O. Store at
4°C, prepare twice weekly.

Mg-EGTA
10ml EGTA (100mM/L)
7ml $MgCl_2$ (100mM/L)
83ml DGVB$^=$ (3 parts D5W$^=$:1 part GVB$^=$)
pH 7.4-7.6. Store at 4°C, prepare fresh daily.

EDTA (10mM/l) $GVB^{=}$)
58ml 0.086 M/L EDTA
442ml $GVB^{=}$
Store 2-4°C, prepare twice weekly.

Alsever's solution
41g D-glucose
16g $Na_3C_6H_5O_7.2H_2O$
8.4g NaCl
0.8g citric acid (anhydrous)
to 1L roH_2O. pH 6.0-6.5. Store at 4°C for several weeks.

Immunoelectrophoresis stock solutions

Tank buffer
17.0g $NaC_8H_{11}N_2O_3$
23.5ml HCL (1M)
to 1L roH_2O, pH 8.4. Dilute 1:2 with roH_2O before use.

Slide buffer
4.5g $NaC_8H_{11}N_2O_3$
11.65ml EDTA (86mM)
32.5ml NaOH (100mM)
to 500ml roH_2O, pH 8.4.

Agar solution
A 1.2% agar (High EEO, Sigma) solution was prepared by dissolving 1.2g of agar in 100ml of slide buffer. The solution was heated in a microwave until molten.

Chapter 5 buffers and solutions

Haemolysin assay buffer
6g tris/HCL
8.8g NaCl
2.5g $CaCl_2$
To 1L roH_2O. pH 7.2.

150mM NaCl
8.8g NaCl
To 1L roH_2O.

Chapter 6 buffers and solutions

10% Gelatin
10g gelatin
to 100ml roH_2O. Warm until dissolved. Filter (0.2μm) sterilise, aliquot and store refridgerated. Heat to melt before use.

0.5% Gel-HBSS
25ml Gelatin (10%)
500ml HBSS
pH to 7.3 with 7.5% sterile Na_2CO_3. Store 4°C.

Chapter 7 buffers and solutions

1M $CaCl_2$
11.1g $CaCl_2$
to 100mls roH_2O. Dilute 1/100 to achieve 10mM solution.

SDS-PAGE Stock solutions

1.5M Tris-HCL
27.23 g tris
to 80ml roH_2O. Adjust to pH 8.8 with 1N HCL. Make to 150ml roH_2O and store at 4°C.

0.5M Tris-HCL
6g tris
to 60ml roH_2O. Adjust to pH 6.8 with 1N HCL. Make to 100ml with roH_2O and store at 4°C.

20% SDS
20g SDS
to 100ml roH_2O. Heat gently to dissolve.

Sample buffer
1.8ml 1M tris-HCL pH 6.8
3ml 20% SDS
3ml glycerol
1.5ml mercaptoethanol
0.2ml 0.05% Bromophenol blue
to 10ml roH_2O. Store at 4°C.

Electrode running buffer x5
9.0g tris
43.2g glycine
3.0g SDS
to 600ml roH_2O. Store at 4°C. Dilute 1:5 with roH_2O before use.

Mini-Protean II Cell system

Acrylamide/PDA (30% T, 4% C)
87.6g acrylamide
2.4g piperazine di-acrylamide
to 300ml roH_2O. Filter (0.44μm) and store at 4°C in the dark for a maximum of 1 month.

Stacking gel - 4%
2.5ml 0.5M tris-HCL, pH6.8
100μl 10% SDS
1.3ml acrylamide/PDA solution
6.1ml roH_2O

Mix and degas under vacuum in a desiccator for 15 minutes. Immediately before pouring add 50μl 10% (w/v) ammonium persulfate (fresh daily) and 10μl N,N,N','N'-Tetramethylethylene-diamine (TEMED).

Separating gel - 10%
2.5ml 1.5M tris-HCL
100μl 10% SDS
3.3ml acrylamide/PDA solution
4.1ml roH_2O.
Mix and degas under vacuum in a desiccator for 15 minutes. Immediately before pouring add 50μl 10% ammonium persulfate and 5μl TEMED.

10% SDS
10g SDS
to 100ml roH_2O. Heat gently to dissolve.

Standard Gel System

Separating gel
18ml acrylamide/PDA solution
16.8ml 1M tris-HCL, pH 8.7
225μl 20% SDS
10ml roH_2O
Mix and degas under vacuum in a desiccator for 15 minutes. Immediately before pouring add 150μl 10% ammonium persulfate and 38μl TEMED.

Stacking gel
2.4ml acrylamide/PDA solution
1.25ml tris-HCL, pH 6.8
50μl 20% SDS
6.35ml roH_2O
Mix and degas under vacuum in a desiccator for 15 minutes. Immediately before pouring add 50μl 10% ammonium persulfate and 15μl TEMED.

ELISA Stock solutions

Coating buffer
1.59g Na_2CO_3
2.93g $NaHCO_3$
to 1L roH_2O, pH 9.6.

Tris buffered saline x10
42.075g NaCl
15.14g tris/HCL
0.475g $MgCl_2$
to 500ml roH_2O, pH 8.0.

Wash buffer
Tris buffered saline x 1 plus 0.1% Tween 20.

Blocking solution
Wash buffer plus 2% marvel dried skimmed milk. Store at 4°C, keep for 1 day.

Western blotting, Stock solutions

NT
1.21g tris/HCL
8.7g NaCl
to 1L dH_2O, pH 8.0.

TNT
Nt plus 0.5ml tween 20

TNT Blocking solution
TNT plus 10% skimmed milk. Store at 4°C, keep for 1 day.

Horseradish peroxidase substrate
Solution A
30mg 4-chloro-1-napthol
20ml cold methanol
Prepare fresh daily, store on ice protected from light.
Solution B
30µl cold H_2O_2
50ml NT (TNT-Tween 20)
Prepare immediately before use at room temprerature. Mix solution A and B and use immediately.

Antiserum
Goat anti-rabbit (Bio-rad) 1:3000
Rabbit anti-pig (Sigma Ltd.) 1:3000
Sheep anti-mouse (Sapu) 1:50

Monoclonal antibody production stock solutions

Tris-Triton X-100 buffer
2.4g tris
8.2g NaCl
0.2% triton X-100
To 1L roH_20.

Immunoprecipitation buffer
18.99g NaCl
1.436g tris
0.186g EDTA
2.5ml NP_4O[check]
0.5g azide
0.5g SDS
to 500ml roH_2O.

Phosphate buffered saline
8.0g NaCl
0.2g KCl
1.15g Na_2HPO_4
0.2g KH_2PO_4
to 1L dH_2O, pH7.3.

White blood cell fluid
50ml glacial acetic acid
20ml absolute alcohol
to 1L dH_2O.

Aminopterin x 1000
0.016g aminopterin (Sigma Ltd.)
to 50ml roH_2O. Heat 45°C and add 0.1M NaOH to dissolve. Make to 100ml roH_2O. Filter sterilise (0.2μm) and store at -20°C.

HAT x 50
0.340g hypoxanthine (Sigma Ltd.)
0.097g thymidine (Sigma Ltd.)
25ml aminopterin (X1000)
to 500ml PBS. Filter sterilise (0.2μm) and store at -20°C.

HT x 50
0.340g hypoxanthine
0.097g thymidine
to 500ml PBS. Filter sterilise(0.2μm) and store at -20°C

Penicillin/Streptomycin solution
10,000 U penicillin (Gibco)
10mg streptomycin (Gibco)
to 100ml roH_20. Aliquot and store at -20°C.

L-Glutamine solution
30ml L-glutamine (Gibco)
to 100ml roH_2O. Aliquot and store at -20°C.

$RPMI^{++}$
5ml penicillin/streptomycin solution
5ml L-glutamine solution
490ml RPMI 1640 (Gibco)
Incubate 37°C for 24-48 hours prior to use to ensure contamination free. Store at 37°C for a maximum of 2 weeks.

$RPMI^{+++}$
5ml penicillin/streptomycin solution
5ml L-glutamine solution
100ml foetal calf serum
390ml RPMI 1640
Store as above.

$RPMI^{+++}$HAT
2ml HAT X50
to 100ml of $RPMI^{+++}$. Store as above.

$RPMI^{+++}$HT
2ml of HT X50
to 100ml $RPMI^{+++}$. Store as above.

APPENDIX 2

Fig. 4.1a Immunoelectrophoretic patterns of human serum incubated in the absence of bacteria over time and developed with anti-human factor B.

Factor B activation fragments are denoted by Ba and Bb.

Slides

1a. Human serum incubated for 5 minutes
2a. " " " " 10 "
3a. " " " " 20 "
4a. " " " " 30 "
5a. " " " with zymosan (positive control)
6a. Addition of EDTA to control human serum prior to immunoelectrophoresis (negative control)
1-6b. Control human serum incubated without bacteria (negative control)

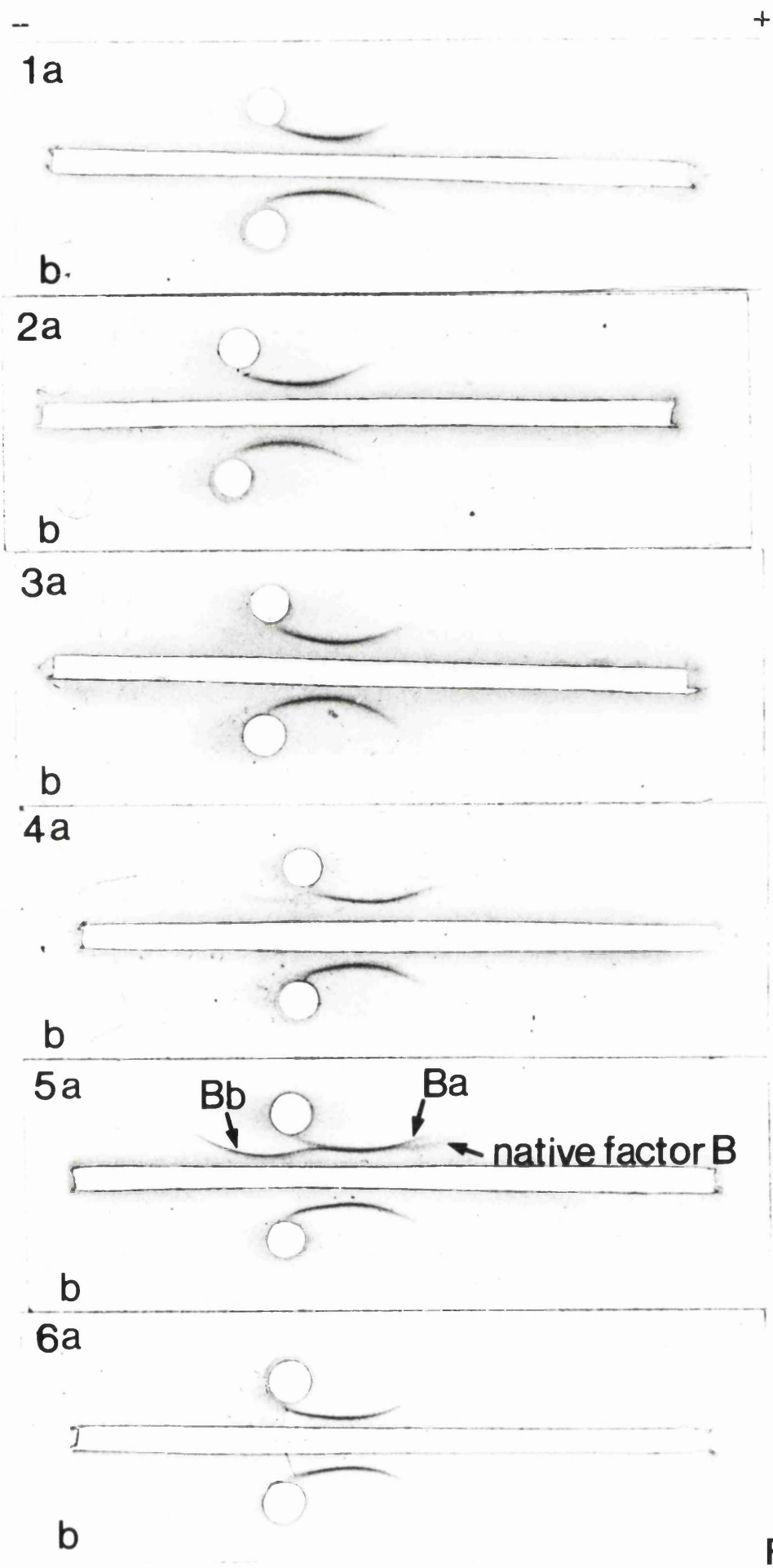

Fig. 4.1a

Factor B depleted serum

Serum was incubated at 50°C in a water bath for 20 minutes. The serum was cooled in ice, aliquoted and stored at -70°C until used.

Classical pathway depleted serum

Serum was incubated at 37°C for 15 minutes in an equal volume of Mg-EGTA buffer. EGTA chelates Ca^{2+} ions which are required for C1 activation of the CCP. The Mg^{2+} ions provided by the buffer allow activation by the ACP.